ASSESSMENT OF METHODS TO MINIMIZE TRANSMISSION OF BOVINE

HERPESVIRUS ASSOCIATED WITH EMBRYOS

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ASSESSMENT OF METHODS TO MINIMIZE TRANSMISSION OF BOVINE HERPESVIRUS ASSOCIATED WITH EMBRYOS

Mylissa Shonda Divina Marley

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ASSESSMENT OF METHODS TO MINIMIZE TRANSMISSION OF BOVINE HERPESVIRUS ASSOCIATED WITH EMBRYOS

Mylissa Shonda Divina Marley

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VITA

Mylissa Shonda Divina Marley, daughter of William Henry and Lynda Kay Edens, was born September 23, 1972, in Lakeland, Florida. She graduated from Niceville High School in 1990. Subsequently, she was awarded the degrees of Bachelor of Science (cum laude) in Animal Science and Doctor of Veterinary Medicine from Mississippi State University in May 1994 and May 1997, respectively. After receipt of her DVM, she completed a one and a half year Equine Medicine and Surgery Internship at Kansas State University in June 1999 and arrived at Auburn University in July 1999 where she enrolled in graduate school and entered the Theriogenology Residency program. Diplomate status in the American College of Theriogenologists was conferred to her on July 13, 2001. She obtained her Masters of Science degree in Biomedical Science from Auburn University in May 2002. She subsequently entered the Doctor of Philosophy program for Biomedical Science in November 2002. She married James Edward Marley Jr. on September 27, 2003

DISSERTATION ABSTRACT

ASSESSMENT OF METHODS TO MINIMIZE TRANSMISSION OF BOVINE HERPESVIRUS ASSOCIATED WITH EMBRYOS

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Doctor of Philosophy, December 17, 2007
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Herpes simplex virus and cytomegalovirus are significant causes of human neonatal morbidity and mortality. In addition, they can cause genital infections, oral lesions, keratoconjunctivitis, skin infections, and encephalitis. It is possible for an individual to transmit these viruses yet be seronegative and show no clinical signs of disease. Further, they can be shed in semen. Therefore, contaminated gametes and serum are potential sources for herpesvirus to enter an in vitro fertilization (IVF) system. If this were to occur, the virus could become associated with the embryo which could transmit herpesvirus to the recipient and result in early embryonic death or fetal anomalies. Addition of an antiviral agent into the in vitro fertilization and in vitro culture

(IVC) media could reduce the level of exposure of the embryo to herpesvirus, thus protecting the conceptus and the recipient.

Animal herpesviruses are similar to human herpesviruses, and for that reason, they are used as models. Bovine herpesvirus 1 (BHV-1) is an alphaherpesvirus that is responsible for abortion, infertility, genital disease and respiratory infections in cattle. This virus has been shown to be associated with gametes, serum, and co-culture cells which are used for IVF. Transferable embryos can be produced from infected oocytes making it possible to transmit virus to the recipient cow. Clearly, there are similarities between bovine and human herpesviruses and their association with natural and assisted reproduction. Thus, the study of anti-herpesvirus agents in IVF could provide disease control options for use in both bovine and human IVF.

The research hypothesis is that select antiviral agents added to a bovine IVF system can effectively inhibit the replication of BHV-1 without impeding development of blastocysts.

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The author gratefully acknowledges the technical support and laboratory instruction of Pat K. Galik and Kay P. Riddell. David A. Stringfellow and M. Daniel Givens are greatly appreciated for sharing their knowledge in the research design, instruction, and writing of this project. Special recognition is given to my husband, Jimmy Marley, for his support and commitment. To God, I am thankful for the abilities He has given me and for teaching me to persevere so that I may become mature and complete, not lacking anything.

Style manual or journal used
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INTRODUCTION

Herpes simplex viruses (HSV) and cytomegalovirus (CMV) are sexually transmissible human viruses that are present in the genital tract and can cause fetal and neonatal morbidity and mortality [167,224,340]. Herpes simplex viruses include HSV-1 and HSV-2. These alpha herpesviruses can cause oropharyngeal disease, genital disease, keratoconjunctivitis, skin infections, and encephalitis [333]. Neonatal infections can occur following in utero, intrapartum, or postnatal transmission [13,333]. Clinical signs in the neonate include skin vesicles, chorioretinitis, microcephaly, hydranencephaly, microphthalmia, disseminated infection, and death [13,131,154]. Cytomegalovirus is a beta herpesvirus transmitted through direct contact with infected urine, saliva, blood, oropharyngeal secretions, milk, tears, semen and cervical secretions from infected individuals [226,340]. Clinical disease associated with CMV infection will be influenced by age at exposure (e.g., fetal, neonatal, or adult) and status of the host immune system [226]. Neonatal infections can occur following transmission in utero, intrapartum, or via nursing [226,292]. Neonates might have petechiae, jaundice, periventricular calcification, microcephaly, hepatosplenomegaly, mental retardation, cerebral palsy, intrauterine growth retardation, chorioretinitis, and impaired hearing, or they might die [162,226,292,340].

Assisted reproductive techniques, including in vitro fertilization (IVF), have become common practice. Although they can be of benefit to infertile couples, there is the risk of viral transmission. It is possible for virus to be associated with gametes and serum used in IVF [10,224]. Although filtration can decrease the transfer of herpesvirus in the blood, it is still a possible source of contamination [292]. Both HSV and CMV can be present in fresh and cryopreserved semen [115,159,174,260,340]. The British Andrology Society requires that men donating semen be CMV seronegative [180], and The American Society for Reproductive Medicine states that semen from CMV seropositive men be used only for CMV seropositive women [174]. However, it has been shown that men with virus present in semen can be asymptomatic and seronegative for HSV and CMV [10,174,203,340]. Moreover, semen quality as demonstrated by percent motility, sperm concentration, and round cell concentration is not adversely affected when associated with HSV or CMV [115,185,224]. Therefore, it is possible that herpesvirus contaminated semen could unknowingly be used for IVF [10]. In addition, it is possible that virally contaminated oocytes might be used for IVF. Cytomegalovirus has been demonstrated in the ovaries of an individual with generalized CMV infection [134] although Witz showed that CMV was not detected in unfertilized oocytes and nonviable embryos of seropositive women [340]. It is possible for women to have CMV present in cervical mucus yet be asymptomatic and seronegative [222,340]. Consequently, it might be possible for an in vitro fertilized embryo to be contaminated with herpesvirus.

A herpesvirus contaminated embryo is a potential source of infection to the recipient; in addition, the embryo could undergo early embryonic death, or develop into a

viable fetus with or without organ dysfunction [13]. Antiviral agents are available which are effective against HSV and CMV, however, as stated above, gamete donors are often unaware that they are infected due to seronegative status and a lack of clinical signs. Also, viruses that are resistant to antiviral agents are emerging [239] and, CMV patients have a need for less toxic and improved antiviral agents [328]. Thus, a new antiviral agent that could be added to the IVF system would be beneficial. Ideally, the antiviral agent would be effective against HSV and CMV as well as allow the development of a viable embryo.

Animal models are required in order to evaluate new treatments for humans [292,339]. Although not exactly the same, bovine embryos can serve as a model for human embryos [186,187]. Furthermore, bovine herpesviruses (BHV) are similar to their human counterparts and have served as model viruses [248,258,297]. Like other herpesviruses, BHV-1 establishes latency and is characterized by persistent infection with intermittent or continuous shedding [209]. Bovine herpesvirus 1 is an alphaherpesvirus which causes infertility, abortion, genital disease, and respiratory disease [234,315]. It is the etiologic agent of infectious pustular vulvovaginitis, infectious balanoposthitis, and infectious bovine rhinotracheitis [211]. Bovine herpesvirus 1 transmission occurs through close contact with nasal, oral or genital secretions [211]. The genital form of the disease is characterized by small pustules on the vestibular, preputial and penile mucosa [190,211,307]. Seminal plasma from affected bulls contains BHV-1, but the virus is not within the spermatozoa itself [307,319]. Infectious bovine rhinotracheitis is characterized by fever, anorexia, depression, dyspnea, nasal discharge, and hyperemic nasal mucosa [137,211]. Bovine herpesvirus 1 is the most frequently diagnosed cause of viral abortion

in North America [17]. The aborting cattle may or may not show clinical disease [17]. If seen, the manifested clinical signs are generally respiratory. Abortions are rarely seen with the genital form of the disease [17,315]. Bovine herpesvirus 1 infection can also lead to infertility. Following intrauterine infection, endometritis and severe necrotizing oophoritis can occur [190,315]. In vitro fertilized embryos are at risk for contamination with BHV-1. This can occur via exposed oocytes and spermatozoa or from contaminated products of animal origin that are used in media for washing, fertilization, and culture [24,315,317]. Oocytes of heifers experimentally inoculated with BHV-1 were matured, fertilized and cultured in vitro [24]. Embryos, oviductal cells and uterine fluid were positive for BHV-1 demonstrating that apparently normal transferable IVF embryos can have BHV-1 associated with them. Such embryos would provide the potential to spread BHV-1 to the recipient.

An antiviral agent that proved effective against BHV-1 in a bovine IVF system as well as allowed development of viable embryos would serve as a model for elimination of HSV and CMV in a human IVF system. Because all herpesviruses share mechanisms of replication and assembly, using homologous viral enzymes and structural proteins, it is feasible that an antiviral agent would demonstrate efficacy against all three viruses. Following the demonstration of a broad spectrum of effect, the antiviral agent could be added during the in vitro fertilization and in vitro culture (IVC) phases to evaluate its effect on embryo development and viability. Finally, the antiviral agent could be added to IVF and IVC phases which have been inoculated with BHV-1 to simultaneously determine antiviral activity and embryo development.

In addition to BHV-1, bovine viral diarrhea virus (BVDV) is a common reproductive pathogen in cattle. Bovine viral diarrhea virus is a member of the family *Flaviviridae* and genus *Pestivirus* [208]. Affected animals might develop a fever, leukopenia, diarrhea, nasal and ocular discharge, and erosive stomatitis. In addition, the virus can be spread transplacentally. Cows infected early in gestation often resorb the embryo. Cows infected at Day 80 to 125 of gestation often result in birth of a persistently infected calf. This calf may show growth retardation and congenital defects. These calves will remain infected for life and serve as a reservoir for spread of infection to susceptible herd mates. Cows infected after Day 125 of gestation often give birth to a calf that is able to produce neutralizing antibody to eliminate the virus. In addition, mucosal disease is seen when susceptible animals are infected with both a noncytopathic and cytopathic strain of BVDV. The animals can demonstrate a fever, anorexia, watery diarrhea, nasal discharge, erosive or ulcerative stomatitis, and death [208]. As well, BVDV can be associated with gametes. The virus has been shown to be associated with semen [26,102,109] and oocytes [49]. The virus can also be associated with in vitro produced embryos [31,275].

An assay that could simultaneously detect both BHV-1 and BVDV would be a beneficial tool. It could be used to screen materials of animal origin prior to using them in an in vitro embryo production or cloning system. This would prevent not only the decreased blastocyst production that can be seen when these viruses are present in an in vitro embryo production system but also prevent the potential spread of disease.

LITERATURE REVIEW

Herpesvirus

Morphology. Nine herpesviruses have been isolated from humans: herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), human cytomegalovirus (CMV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), and *Human herpesviruses 6A, 6B, 7, and 8* [247]. Seven herpesviruses have been isolated from bovines: bovine herpesvirus 1 (BHV-1), bovine herpesvirus 2 (BHV-2), bovine herpesvirus 4 (BHV-4), and bovine herpesvirus 5 (BHV-5), as well as alcelaphine herpesvirus 1, ovine herpesvirus 2, and porcine herpesvirus 1 [209]. In addition, each domestic animal species except ovine is able to contract at least one major disease that is caused by herpesvirus. Herpesviruses consist of a core containing a linear double-stranded DNA in the form of a torus (Figure 1) [247]. The core seems to be suspended by fibrils that are anchored to the inner side of the surrounding capsid and pass through the hole of the torus [209]. The icosahedral capsid is 100 to 110 nm in diameter and contains 162 capsomeres, 150 hexameres and 12 pentameres [246]. A channel 4 nm in diameter runs down the long axis. The tegument is an amorphous material between the capsid and the envelope. It consists of a layer of globular material enclosed by a

lipoproteinaceous envelope with small glycoprotein peplomers [209]. Herpesviruses vary in size from 120 to 300 nm with the variation being due to the thickness of the tegument [246]. The envelope has a trilaminar appearance and consists of lipids and contains numerous glycoprotein spikes.

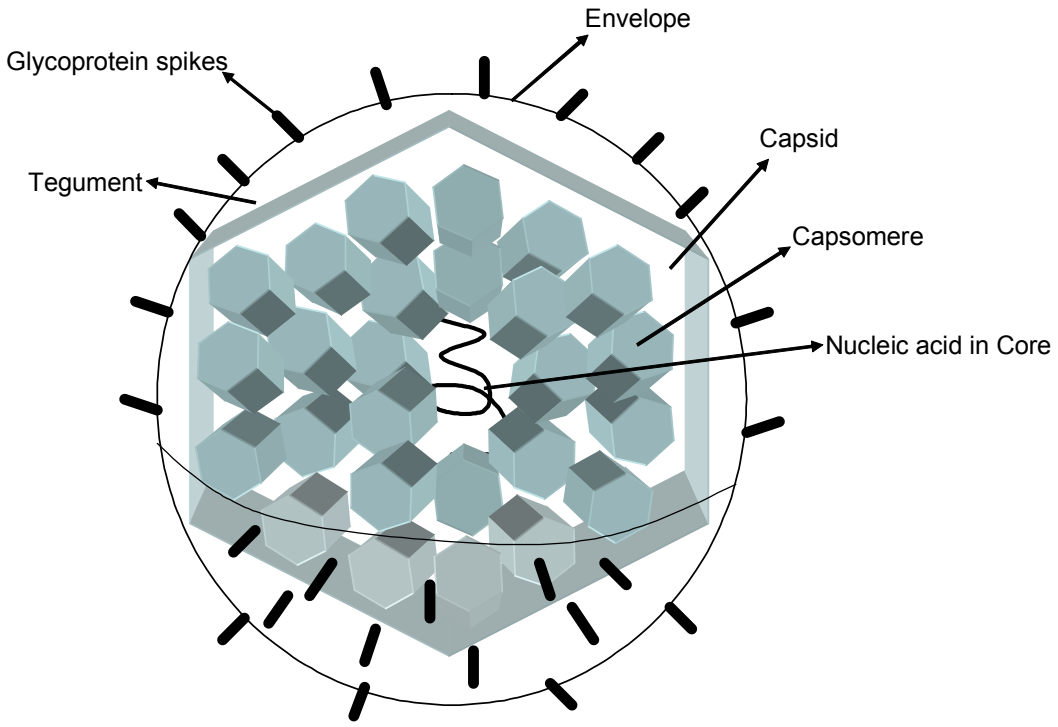


Figure 1. Cutaway diagram of a herpesvirus [1].

Herpesvirus DNA varies in length from 120 to 250 kbp [247]. The genome contains terminal and internal reiterated sequences that can vary in copy number. The base composition varies from 31 to 75 % total guanine and cytosine content. Herpesviruses can be divided into 6 groups (A through F) based on the sequence arrangement of reiterations of terminal sequences greater than 100 bp. Group A is represented by channel catfish herpesvirus, Group B by herpesvirus saimiri, Group C by

Epstein-Barr virus, and Group D by Varicella-zoster virus. Group E is exemplified by HSV and CMV (Figure 2). In Group E, the sequences from both termini are repeated in an inverted orientation and internally they are juxtaposed. The genome is thus divided into 2 compartments each of which consists of unique sequences flanked by inverted repeats. The unique sequences are divided into a long (U_L) and a short (U_S) domain [247]. Both components can invert relative to each other. Therefore, DNA extracted from virions consists of 4 equimolar isomers differing in the relative orientation of the 2 components [247]. Group F is represented by tupaia herpesvirus [247]. All herpesvirus genomes contain signals for packaging the DNA into capsids.

Figure 2. The termini of group E. One terminus contains sequence a and b. The other terminus has sequence a and c. The terminal ab and ca are in inverted orientation (denoted by primers) separating the unique long (U_L) and short (U_S) domains [211].

There are 4 biological properties that all herpesviruses share [247]. The first is the large array of enzymes involved in nucleic acid metabolism (thymidine kinase), DNA synthesis (DNA polymerase), and processing of proteins (protein kinases). Second, the synthesis of viral DNA and the assembly of the capsid occur in the nucleus. The capsid is enveloped as it passes through the nuclear membrane. Third, the production of infectious progeny virus is accompanied by the destruction of the infected cell. Finally, the herpesvirus remains latent in the natural host. Latent virus is able to replicate and cause disease upon reactivation.

Taxonomy. The family *Herpesviridae* is divided into 4 subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, *Gammaherpesvirinae*, and an unnamed subfamily of aquatic hepesviruses (ictalurid herpesvirus 1, cyprinid herpesvirus) [210].

Alphaherpesviruses have a variable host range, grow rapidly, lyse infected cells and establish latent infections in sensory ganglia [210,246]. The prototypic viruses are herpes simplex virus 1 and human herpesvirus 3 (Varicella-zoster virus) [210]. Bovine herpesvirus 1 is a member of the subfamily *Alphaherpesvirinae* and genus *Varicellovirus*. The BHV-1 genome resembles HSV and VZV [157,342].

Betaherpesviruses have a narrow host range, replicate more slowly than alphaherpesviruses and produce enlarged cells, thus the term cytomegalovirus [199,210]. Cell lysis does not occur until several days after infection [210]. The prototype is human herpesvirus 5 (cytomegalovirus). Mammals each have their own characteristic CMV [247]. In latent infections, the viral DNA is thought to be sequestered in cells of secretory glands, lymphoreticular organs, and the kidney [210]. Betaherpesviruses are often associated with continual virus excretion rather than periodic reactivation.

Gammaherpesviruses replicate in lymphoblastoid cells [210]. Infection is arrested in the prelytic stage in lymphocytes. The viral genome persists but is minimally expressed. The virus can also enter the lytic stage where it causes cell death without production of virions. Latent infection can be seen in lymphoid tissue. Epstein-Barr virus is the prototypic virus in this subfamily [247].

There are 9 herpesvirus groups in livestock and 9 groups in humans (Table 1) [84,247].

Table 1. Herpesviruses of ruminants and humans [210,247].

Virus Group	Synonym	Subfamily	Natural Host
Bovine Herpesvirus 1	Infectious bovine rhinotracheitis, Infectious pustular vulvovaginitis, Infectious balanoposthitis	α	Cattle
Bovine Herpesvirus 2	Bovine mammilitis, Pseudo-lumpy skin disease	α	Cattle
Bovine Herpesvirus 4	Bovine cytomegalovirus, Movar herpesvirus	γ	Cattle
Bovine Herpesvirus 5	Bovine encephalitis herpesvirus	α	Cattle
Ovine Herpesvirus 1	Sheep pulmonary adenomatosis-associated herpesvirus	α	Sheep
Ovine Herpesvirus 2	Sheep-associated malignant catarrhal fever of cattle	γ	Sheep- healthy virus carrier; Cattle and deer-diseased dead end host
Caprine herpesvirus 1	Goat herpesvirus	α	Goat
Alcelaphine herpesvirus 1	Bovine malignant catarrhal fever	γ	Wildebeest- healthy virus carrier; Cattle, deer, buffalo, and antelope- diseased dead end host
Porcine herpesvirus 1	Aujeszky's disease, Pseudorabies virus, Suid herpesvirus 1	α	Swine- primary host; Cattle, horses, sheep, goats, dogs, and cats- secondary host
Human Herpesvirus 1	Herpes simplex virus 1	α	Human
Human Herpesvirus 2	Herpes simplex virus 2	α	Human
Human Herpesvirus 3	Varicella-zoster virus	α	Human
Human Herpesvirus 4	Epstein-Barr virus	γ	Human
Human Herpesvirus 5	Cytomegalovirus	β	Human
Human Herpesvirus 6A		β	Human
Human Herpesvirus 6B		β	Human
Human Herpesvirus 7		β	Human
Human Herpesvirus 8	Kaposi's sarcoma-associated herpesvirus	γ	Human

Viral Replication. Viral infection of a host cell can result in several endpoints. It could result in a full replication cycle with production of progeny virus [1]. It might result in an abortive infection in which only a portion of the viral genome is expressed. A restrictive type of infection could occur where a fraction of the cell population produces viral progeny. Finally, a latent infection could occur. During latency, the viral genomes persist within the cell and some viral genes are expressed but infectious viral progeny are not produced and the cell is not destroyed.

The cellular environment in which the virus replicates is unfriendly to the infected cell [246]. During infection, cellular chromosomes are marginated to the nuclear membrane and degraded, the nucleolus disintegrates, the Golgi is fragmented, and the microtubules are rearranged. In addition, the virus must block the induction of programmed cell death (apoptosis) and the activation of the interferon pathway. This occurs by activation of protein kinase R which leads to shutoff of protein synthesis. Depending on which cells the virus replicates in, the virus must block the presentation of antigenic peptides on the surfaces of antigenic cells or it might have to encode proteins to perform some functions of immunomodulators. This all causes a delay in the elimination of the infected cell. This delay is necessary in order to allow the virus to replicate, colonize the host, and become available for transmission to another individual.

The viral replication cycle is divided into 6 steps: attachment, penetration, uncoating, replication, assembly, and release (Figure 3) [1].

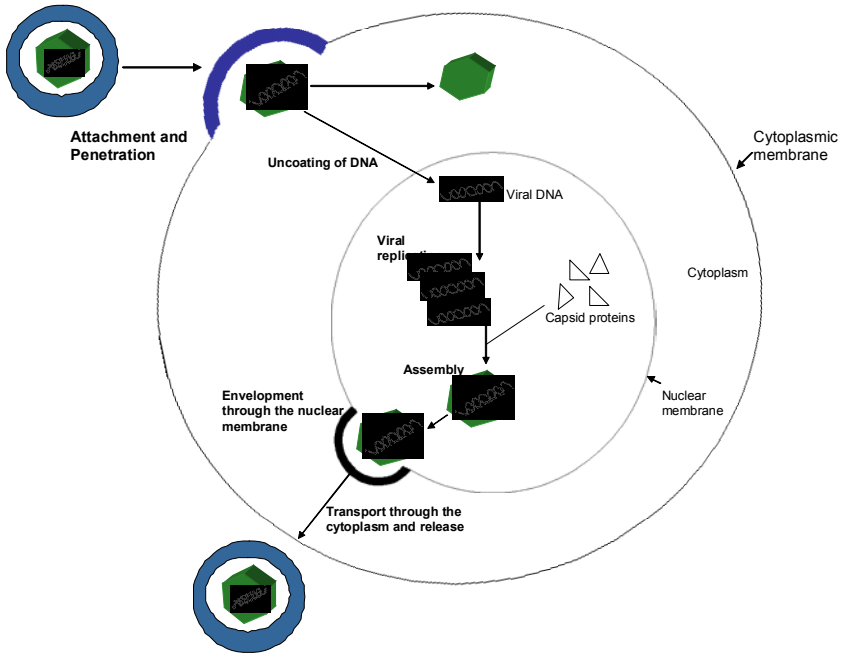


Figure 3. Replication of herpesvirus. The virus attaches to the cell surface. The viral envelope fuses with the cell membrane and the nucleocapsid is released into the cytoplasm. Viral DNA is uncoated and transported to the nucleus. Viral replication occurs in the nucleus. The DNA is assembled into capsids. The capsid is then enveloped as it buds through the nuclear membrane. The virion is then transported through the cytoplasm and released [1].

Attachment- First, attachment occurs when the virion glycoprotein binds to a receptor on the target cell. This is mediated by the viral surface glycoproteins gC and gB [337]. One of the host cell receptors is heparan sulfate proteoglycan [210].

The viral envelope of HSV contains at least 11 membrane glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM) [64]. A twelfth glycoprotein is predicted (gN) [338]. Four of these glycoproteins (gD, gH, gL, and gB) are essential for entry of the virus into cells. Initially, gC and/or gB binds to cell surface heparan sulfate proteoglycans [55,64,246]. The attachment to heparan sulfate enhances infection but is not required because cells devoid of heparan sulfate but containing chondroitin sulfate can be infected [246]. Cells lacking both of these glycosaminoglycans can also be

infected but at a reduced rate. Following initial attachment, gD then binds to a cell surface receptor [55,64,268]. There are several HSV receptors. These include: herpesvirus entry mediator A (HveA/HVEM), nectin-1 (HveC), nectin-2 (HveB), and a modified form of heparan sulfate (3-O-sulfated heparan sulfate). Herpesvirus entry mediator A is a member of the tumor necrosis factor receptor protein family. It binds directly to gD and mediates entry of most HSV-1 and HSV-2 strains. Nectin-1 and -2 are members of the immunoglobulin family. Nectin-1 binds gD and facilitates entry of α herpesviruses, including HSV-1, HSV-2, BHV-1, and pseudorabies virus. Nectin-2 mediates entry of pseudorabies virus and mutant strains of HSV-1 such as rid1. While HSV is able to use multiple entry receptors, the significance of each of these during infection is unknown. Nectin-1 is proposed to be the primary HSV receptor because it is expressed on neurons and is a receptor for many α herpesviruses. On the other hand, HveA mRNA is found in a variety of tissues and mediates infection of human fibroblasts that lack nectin-1. Herpesvirus entry mediator A expression is found in lymphoid tissue where it may facilitate immune system invasion.

Penetration- Second, penetration takes place when the virus enters the cell (Figure 4) [1]. This is an energy-dependent step and takes place rapidly after attachment. Glycoprotein D (gD) is required for entry of the virus into the cell [337]. In addition gE and gI form an Fc (fragment crystallizable) receptor that is required for transmission of the virus into the cell. The viral lipid envelope fuses with the cellular membrane [1]. Fusion is also facilitated by gH, gL, and gB [64]. It is suggested that plasma membrane microdomains (lipid rafts) are enriched with cholesterol and sphingolipids that are important for the lateral organization of the plasma membrane [22,158]. Bender verified

that gB is associated with rafts after virus attachment and during entry [22]. However, gD, gC, gH, HVEM, and nectin-1 were not associated with rafts. Thus, gB might mobilize lipid rafts to serve as a platform for virus entry and cell signaling. Fusion also causes the release of vhs (virion host shutoff) to shut off protein synthesis, and α–TIF which is an α gene *trans*-inducing factor. The nucleocapsid can then enter the cytoplasm of the host.

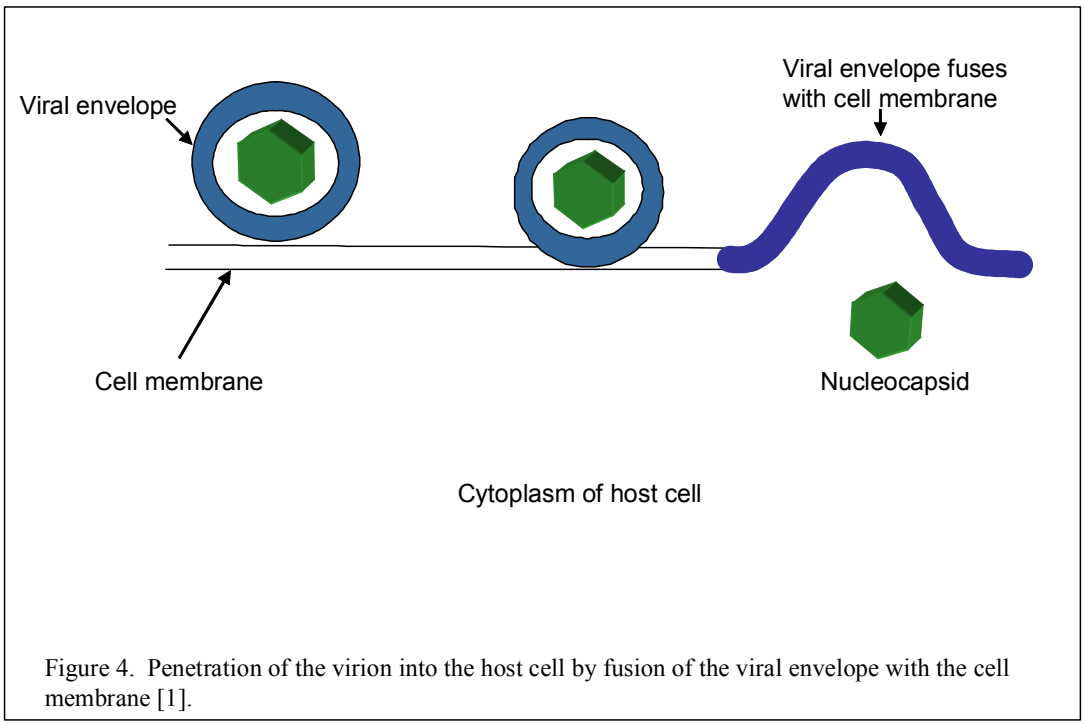


Figure 4. Penetration of the virion into the host cell by fusion of the viral envelope with the cell membrane [1].

Uncoating- Third, uncoating transpires. This involves the release of the nucleocapsid from the envelope, the disintegration of the nucleocapsid, and the exposure of the viral genomic material.

The tegument of HSV contains the rest of the virion proteins that are not in the viral envelope [246]. The most prominent being α-TIF, vhs, the product of the U_S11 gene, and a large protein (VP1-2) associated with a complex that binds to the terminal *a* sequence of viral genome. After fusion, tegument proteins remain in the cytoplasm (vhs,

U_S11), are transported to the nucleus (VP16), or remain associated with the capsid (VP1-2) [246]. The capsid is transported to the nuclear pore. It is proposed that capsids bind to microtubules and use dynein to propel them to the cell nucleus. The nucleocapsids accumulate at the nuclear envelope and associate with nuclear pore complexes. At the pore, the nucleocapsid releases its DNA into the nucleus and leaves the empty capsid in the cytoplasm side of the complex. It is possible that VP1/2 tegument protein plays a role in DNA release at the nuclear pore.

Replication- Fourth, replication can then begin. This occurs in the nucleus of the cell and is via a rolling-circle mechanism yielding concatemers of viral DNA.

During lytic infections, HSV inhibits host transcription, RNA splicing and transport, and protein synthesis [246]. Viral and cellular gene expression is controlled through a set of transcriptional and posttranscriptional controls [83]. One of the posttranscriptional mechanisms is the ability of vhs to destabilize both the host and viral mRNAs. This destabilization is necessary to redirect the cell from host to viral gene expression and facilitate the sequential expression of different classes of viral genes. Thus, HSV-1 and HSV-2 are able to suppress the host cell protein synthesis without viral gene expression [179]. The vhs is a functional tegument protein and is not necessary for virus replication although, mutations that inactivate vhs cause a reduction of virus growth in cell cultures [83,179]. This protein stimulates disruption of polysomes and rapid degradation of all host and viral mRNAs, blocks a reporter gene expression, and interacts with α-TIF [179]. The interaction of vhs with α-TIF down regulates the U_L41 (vhs) gene activity during lytic infection. In addition, vhs interacts with eIF4H, the cellular translation initiation factor [83]. The translation initiation factor, eIF4H, is responsible

during translation initiation for the unwinding of mRNA secondary structures and facilitating ribosome scanning. Thus, the binding of vhs with eIF4H is possibly important for targeting vhs to mRNAs and regions of translation initiation.

Transcription of the viral DNA occurs in the nucleus with the translation of viral proteins occurring in the cytoplasm (Figure 5) [246]. Host RNA polymerase II is responsible for transcription of viral genes on the viral DNA. More than 80 HSV genes are expressed, and their expression is coordinated and ordered in a sequence cascade [145,207,246]. Herpesvirus genes are divided into 3 categories: those that encode proteins for viral replication such as enzymes and DNA-binding proteins (immediate early (IE) and early genes), those encoding structural proteins (late genes), and those encoding proteins not essential for replication [210]. Viral genes form 4 groups. The immediate-early (α) genes are expressed in cells upon infection and require no prior viral protein synthesis for their expression [2,207,210]. Their products enable the expression of early (β) and late (γ) genes [2,210]. The expression of early genes is independent of viral DNA synthesis. The late genes are classified as γ_1 (partial-late, early-late or leaky-late) or γ_2 (true-late) genes. The γ_2 gene expression requires viral DNA synthesis whereas the γ_1 gene expression is enhanced but not dependent on viral DNA synthesis. The importance of late gene expression is to produce large amounts of viral structural proteins for assembly of the progeny viral particles [246].

Selective movement of proteins into and out of the nucleus is essential for proper cell function [207]. Nuclear localization signals, rich in basic amino acids, induce nuclear import. Whereas nuclear export signals (NES), rich in leucine residues, induce nuclear export. The cellular chromosome region maintenance 1 protein (Crm1) is a

nuclear export receptor for proteins possessing an NES. The Crm1 protein binds to these proteins and exports them to the cytoplasm through the nuclear pore. The transport is dependent on Ran-GTP (guanosine triphosphate). Ras-related nuclear protein (Ran) is responsible for directing the direction of the transport process.

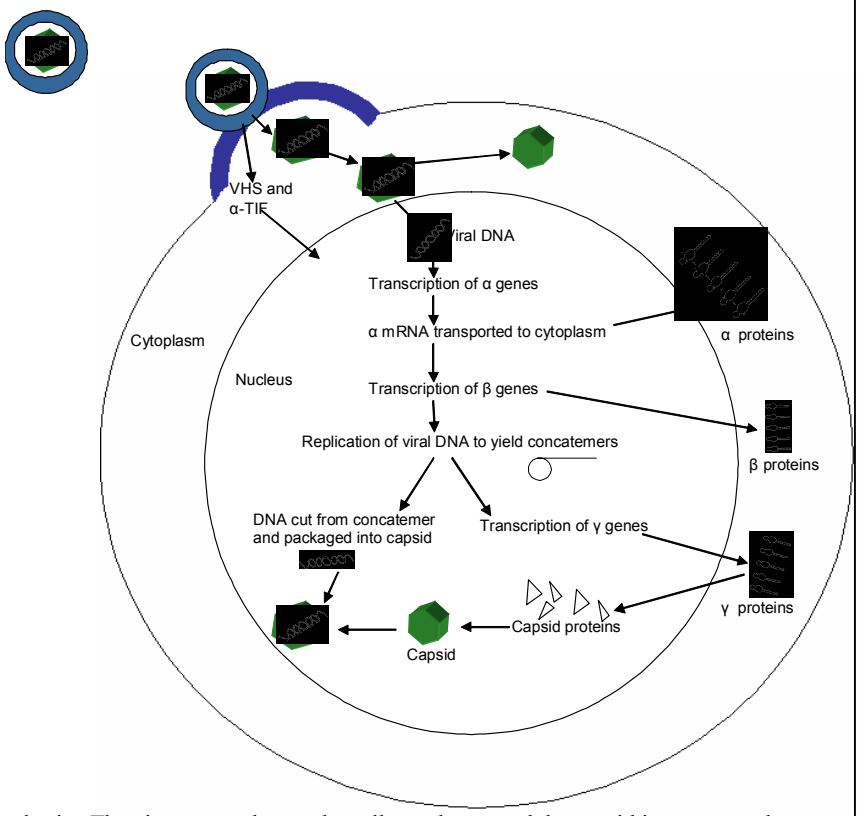


Figure 5. Viral protein synthesis. The viruses attaches to the cell membrane and the capsid is transported to a nuclear pore. Transcription of α genes is induced by α-TIF. α mRNA is transported to the cytoplasm and translated. A new round of transcription results in β proteins. Viral DNA is replicated by a rolling-circle mechanism that results in head-to-tail concatemers of viral DNA. Another round of transcription yields γ proteins which are structural proteins of the virus. Viral DNA is cut from concatemers and packaged into capsids. The capsid is then ready to be enveloped and leave the cell. VHS, virion host shutoff; α-TIF, α-trans-inducing factor

Herpes simplex virus replication begins with the transcription of α genes which is induced by α-TIF (trans-inducing factor) also called VP16 (virion protein number 16) [1,246,337,346]. This protein is packaged in the viral tegument [346]. Following release

from the tegument, VP16 binds to a cellular protein (host cell factor (hcf) or C1) that carries VP16 into the nucleus of the cell [246]. Virion protein 16 does not bind DNA itself but mediates transcriptional activation through a consensus DNA motif of TAATGARAT by forming a complex with cellular factors [346]. The VP16-hcf complex binds to Oct-1 (ocatmer binding protein) which is the POU-domain transcription factor (pituitary-specific transcription factor Pit-1, Oct-1 and Oct-2, and the Caenorhabditis elegans unc-86 cell lineage gene product) [246,346]. Several domains of VP16 are required [246]. Residues 378 to 389 are needed for interaction with Oct-1, residues 173 to 241 are necessary for binding of the complex to DNA, and residues 411 to 490 are essential for transactivation. The C-terminal region is possibly responsible for activating transcription by interaction with host transcription factors (TFII-B and TF-IID).

Six infected cell proteins (ICP0, ICP4, ICP22, ICP27, ICP47, and $U_S1.5$) are made from α gene expression [246,337]. One protein (ICP47) blocks the presentation of antigenic peptides on infected cell surfaces. It is an inhibitor of TAP (transporter associated with antigen presentation) and prevents the peptides from binding to the transporter complex, leads to peptide depletion, and causes instability of MHC class I [157]. The other five α mRNAs are transported to the cytoplasm where they are translated and regulate the expression of the rest of the viral genes [1,207,337]. Infected cell protein 27/IE63 (immediate early) is a phosphoprotein essential for lytic infection. It is the only α protein conserved among all herpesviruses [207]. Infected cell protein 27 moves between the nuclear compartment and the cytoplasm. It binds RNA via a binding motif to enhance 3′ RNA processing, stabilizes the labile 3′ end of mRNA, inhibits splicing of viral and cellular transcripts, stimulates the retention of intron-containing

transcripts in the nucleus, and interacts with ICP0/IE110 and ICP4/IE175 to regulate viral gene expression. Infected cell protein 27 might also suppress apoptotic cell death.

The mechanism of the shutoff of α gene expression is not precisely known [246]. Infected cell protein 4 might play a role by repressing its own gene by binding to its similar DNA binding site across the transcription initiation site. In addition, β gene products down regulate α gene expression. Infected cell protein 8 might be one of the responsible genes, as might be vhs.

The next round of transcription yields β proteins which are responsible for nucleic acid metabolism and viral DNA replication [1,337]. The β1 genes (U_L29 encoding ICP8 and U_L39 encoding the large subunit of ribonucleotide reductase) are expressed almost concurrently with the synthesis of α proteins [246]. The β2 genes (U_L30 encoding DNA polymerase and U_L23 encoding thymidine kinase) are expressed after α protein expression. The difference in the β gene expression might be due to some of these genes requiring ICP27 for their expression.

At this time, chromatin is degraded and moved toward the nuclear membrane and nucleoli disaggregate [1,337]. Once DNA enters the nucleus it circularizes rapidly and localizes in prereplicative sites near cellular ND10 (nuclear domain 10) structures [246]. Viral DNA is replicated by a rolling-circle mechanism to produce head-to-tail concatemers of viral DNA [1]. At least 7 viral gene products (DNA polymerase, U_L42, U_L9, ICP8, U_L5, U_L8, and U_L52) are essential for viral DNA replication [246,337]. Host enzymes (DNA polymerase α-primase, DNA ligase and topoisomerase II) are likely required as well [246]. Viral protein product of U_L9 gene binds to origin sequences to cause a bend in the DNA and form a single-stranded stem-loop structure. Infected cell

protein 8 binds to ssDNA. This binding is required for viral DNA replication. Infected cell protein 8 also interacts with other replication proteins (U_L9, U_L5, U_L8, U_L52 and DNA polymerase) and stimulates helicase activity. Viral protein products (U_L5, U_L8, and U_L52 genes) form a DNA helicase-primase complex. This complex unwinds short oligonucleotides, has ATP-binding and DNA helicase pattern, and divalent metal-binding motifs. Deoxyribonucleic acid polymerase is a holoenzyme that is a heterodimer of U_L30 protein complexed with U_L42 protein. This interaction is essential for viral DNA replication. Based on what is known about the proteins, the following process has been suggested. Viral DNA replication begins with the binding of U_L9 to the origin sequences followed by the looping and distortion of the origin sequences. Infected cell protein 8 then binds to U_L9 or ssDNA regions and the U_L9 helicase unwinds the DNA. Next, the helicase-primase complex is brought to the origin by its interactions with U_L9 or ICP8. The complex synthesizes a primer from which leading-strand synthesis can be accomplished by the pol-U_L42 holoenzyme. Synthesis initiates in the theta form but is converted by an unknown mechanism to a rolling-circle replication.

The third round of transcription yields γ proteins which are structural proteins of the virus [1]. The transition from β to γ gene transcription involves a change in the site of transcription from near ND10 domains to replication compartments [246]. The γ_1 genes encode ICP5, gB, gD, and ICP34.5. The γ_2 genes encode gC, U_L41, U_L36, U_L38, and U_S11. These structural capsid proteins are used to form empty capsids [1]. In the cytoplasm, the major capsid protein VP5 associates with the precursor proteins U_L26 and $U_L26.5$. This complex is transported to the nucleus where the capsid proteins assemble into B capsids. The B capsids consist of a large core of scaffolding protein. The

proteolytic cleavage of U_L26 and $U_L26.5$ leads to a B capsid with a small core. This core is removed when DNA enters.

Assembly- Fifth, assembly involves the formation of mature virus particles and the acquisition of infectivity. Nucleic acids are packaged into preformed capsids within the nucleus. The viral envelope proteins are inserted into the nuclear membrane and the nucleocapsids can then acquire their primary envelopes by budding through the inner lamella of the nuclear membrane.

Following synthesis of γ capsid proteins, capsid assembly occurs in the nucleus [246]. Assembly most likely occurs in nuclear structures called assemblons or within replicative compartments. Empty shells contain internal scaffolding which is lost when viral DNA is inserted into the capsid. Three types of HSV capsids have been described, A-, B-, and C-capsids. All three are 120 nm in diameter, with an outer shell consisting of hexons and pentons made of VP5. The capsomeres are linked by a complex consisting of 2 molecules of VP23 and 1 molecule of VP19C. A-capsids do not contain an internal toroidal structure. They are believed to be failed attempts to package DNA. A-capsids consist of 4 proteins: VP5, VP19C, VP23 and VP26 encoded by genes U_L19, U_L38, U_L18 and U_L35, respectively [111]. B-capsids consist of internal scaffolding proteins but do not contain DNA. B-capsids contain 3 proteins: VP21, VP22a, and VP24 encoded by genes $U_L26.5$ and 26 [111,246]. The U_L26 gene encodes 2 coterminal proteins [111]. The larger protein contains proteinase activity and cleaves itself and the smaller protein is encoded by $U_L26.5$. This results in the 3 capsid assembly proteins, VP21 and VP22a (scaffolding proteins) and VP24 (proteinase). C-capsids contain DNA and can mature

into infectious virions by budding through the nuclear membrane [246]. C-capsids contain VP22. These proteins have a common function in BHV-1 as well.

Subsequently, encapsidation occurs [1,246]. The internal scaffolding proteins VP21 and VP22a are removed from the capsid and the DNA is inserted. Proteins in the capsid recognize the packaging sequence in the DNA genome. They bind to this site and pull the DNA into the capsid. When they recognize the packaging sequence in the next genome of the concatamer they cleave the DNA into unit-length monomers. This packages one full linear genome into the capsid. Cleavage occurs within the DR 1 (direct repeat) sequence of the *a* sequence. Encapsidation requires U_L6, U_L15, U_L25, U_L28, U_L32, U_L33, U_L36, and U_L37 gene products.

Release- Sixth, release involves the acquisition of a definitive envelope from the Golgi network, the release from the cell by exocytosis, and the death of the cell. In fully permissive tissue culture cells, the process takes approximately 18 to 20 hours [246].

The viral glycoproteins and tegument proteins accumulate in the cellular membranes [1]. After encapsidation, envelopment occurs as the nucleocapsids bud through the inner nuclear membrane [246]. The tegument is acquired through this budding process. Virion protein 16 and U_L11 are required for envelopment. There are 2 possible mechanisms of virion egress, the re-envelopment pathway and the lumenal pathway. In the re-envelopment pathway, the enveloped particle fuses with the outer nuclear membrane. This results in the de-envelopment of the nucleocapsid and its entry into the cytoplasm. The nucleocapsid then buds into the trans-Golgi network and the enveloped particle is released through secretory vesicles. The lumenal pathway describes the enveloped particle moving through the cytoplasm in the lumen of the endoplasmic

reticulum or in vesicles to the trans-Golgi network where maturation of the virion glycoproteins can occur.

Inhibition of Apoptosis. Apoptosis is used to describe programmed cell death. Many viruses possess the ability to inhibit apoptosis of the infected cell in order to prolong the life of the cell and thus maximize the production of virus [246,324]. Infected cells undergo structural and biochemical alterations that lead to their demise [246]. One of the earliest changes following infection is the enlargement of the nucleolus and its displacement toward the nuclear membrane. The nucleolus will eventually disaggregate. Moreover, host chromosomes become marginated and the nucleus becomes distorted. Microtubules at the junction of the plasma membrane also rearrange to form parallel bundles around the nucleus. Changes also occur in the cellular membranes. Thickened patches develop that coalesce and fold upon themselves. In addition, fragmentation of the Golgi vesicles can occur. In cells in which this occurs, transport of virions from the nuclear membrane to the extracellular space requires U_S20 gene product. Furthermore, HSV infection causes cells to round up and adhere to each other. Replication proteins accumulate in the nucleus and enlarge to form globular nuclear structures called replication compartments. It is thought that viral DNA synthesis, late gene transcription, capsid assembly, and DNA encapsidation all occur here. It is possible that the accumulation of these replication proteins, progeny viral DNA, and nucleocapsid components is what causes the nuclear cytopathic effect termed nuclear inclusion bodies.

Because viruses replicate in cells it is important that the cells remain alive for viral replication to occur. Thus, viruses have developed a means of inhibiting apoptosis. Inhibition of apoptosis might also help the virus to avoid the cell-mediated immune

response. Herpes simplex virus has several genes that might be responsible for inhibiting apoptosis.

In the mitochondrial-dependent pathway of apoptosis, induction of apoptosis is by Bad (*B*cl-2/Bcl-X_L-*a*ntagonist, causing cell *d*eath) [57,110,220]. Bad is a BH3 domain-only (Bcl-2 homology (BH) domains) proapoptotic member of the Bcl-2 family [57]. When Bad is unphosphorylated it is able to bind with and inactivate Bcl-2 (B-cell lymphoma/ leukemia-2) and Bcl-X_L (*B*cl-2-like Protein, *L*ong isoform) which are anti-apoptotic proteins. The binding of Bad with Bcl-2 releases Bax (*B*cl-2-*a*ssociated *X* Protein) from the Bax/Bcl-2 dimers. Bax is then able to activate the mitochondrial pathway by causing pores in the mitochondria. This in turn causes the release of cytochrome *c* from the mitochondria into the cytoplasm, which then activates the caspase cascade. Caspases are cellular proteases which break down cellular components. Cytochrome *c* binds with Apaf-1 (*A*poptosis *P*rotease-*a*ctivating *F*actor 1) and caspase-9 to from an apoptosome which activates caspases downstream, such as caspase-3 [110]. Caspase-3 might then act by a positive feedback loop to further activate Bad [220].

In the cytotoxic T lymphocyte-mediated apoptosis pathway, U_S3 expression prevents Granzyme-B-dependent activation of Bid [57]. Bid is a pro-apoptotic member of the Bcl-2 family. Bid (*B*H3-*i*nteracting domain *d*eath agonist) and Bad control the activity of Bax and Bak (*B*cl-2 homologous *a*ntagonist/*k*iller), respectively. When Bid and Bad are proteolytically cleaved they are able to stimulate Bax and Bak. Bax and Bak directly control the release of cytochrome *c* from the mitochondria. Also, the binding of Bid to Bax causes a conformational change in Bax which stimulates Bax-mediated cell killing. In addition, 2 other HSV-1 proteins, gD and gJ have been reported to inhibit apoptosis

induced by various stimuli. All of these events lead up to cell death (Figure 6). The dying cell is characterized by cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation [57,110,220].

However, there are survival signal-activated kinases which phosphorylate Bad [57,220]. Phosphorylation releases Bad from Bcl-2 and Bcl-X_L heterodimers and inactivates it by binding it to the cytosolic protein 14-3-3. Bcl-2 is then free to act at the mitochondria to block the release of cytochrome *c* and to prevent the activation of caspase and thus inhibit apoptosis.

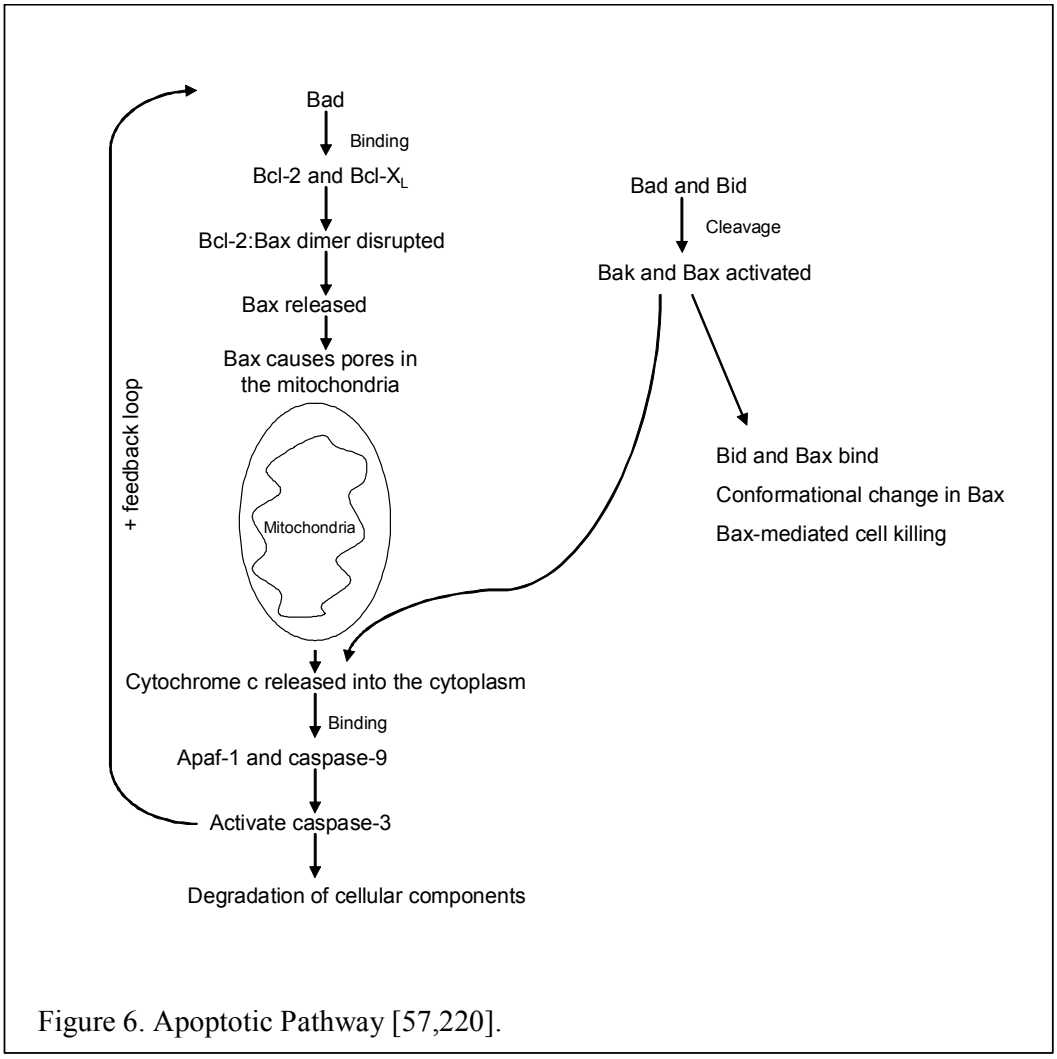


Figure 6. Apoptotic Pathway [57,220].

HSV genes have been suggested to inhibit apoptosis. One such gene is the α herpesvirus conserved gene, Us3. The Us3 proteins are important during envelopment of the progeny virions at the inner nuclear membrane [220]. Cartier et al. showed that Us3 inhibits apoptosis by preventing the activation of caspase-9 and caspase-3 [57]. The Us3 gene encodes a serine/threonine protein kinase which is able to autophosphorylate. Also, they showed that cells which contain high concentrations of Us3 from a viral infection rapidly phosphorylate Bad. Thus, Bad is a direct target of Us3. They also demonstrated that Us3 inhibits the activation of Bid. Based on studies with U_S3-deficient mutants of HSV-1, U_S3 may enhance virus replication and thus promote invasiveness and help with immunoevasion. Cartier et al. showed that U_S3 expression blocks DNA fragmentation induced by HSV-1 infection, Fas ligand triggering, and osmotic shock. In contrast, other researchers have shown that the anti-apoptotic activity of Us3 is not associated with the phosphorylation of Bad but rather with the inhibition of proteolytic cleavage of Bad. Thus, Us3 might act on caspases upstream of Bad. In addition, Us3 is able to inhibit apoptosis induced by overexpression of Bad. However, Ogg et al. demonstrated that Us3 was able to inhibit apoptosis that was induced by overexpression of Bid and Bax [220]. This suggests that the activity of Us3 is downstream from that of Bad and mitochondrial activity. The Us3 gene might inhibit the activity of caspase-3, an event downstream of Bad. The activity of Us3 seen upstream of Bad by other researchers might actually be due to Us3 inhibiting the positive feedback loop of caspase-3 to Bad. Thus, the inhibition appears to occur upstream of Bad but actually occurs downstream of Bad. Ogg et al. also confirmed that inhibition of apoptosis is dependent on the catalytic activity of Us3. In addition, they showed that Us3's inhibition of apoptosis is not due to degradation of

Bax, Bid or Bad. Another theory shown by Benetti and Roizman is that Us3 activates protein kinase A (PKA) to inhibit apoptosis [23]. Protein kinase A phosphorylates one of the regulatory residues of Bad. However, Benetti and Roizman demonstrated that PKA was able to prevent apoptosis in trials where Bad had mutated phosphorylation sites. These results suggest that inhibition of apoptosis may be due to several mechanisms.

Another gene shown to inhibit apoptosis is U_L14 [345]. It has been shown to have functions similar to heat shock protein. Heat shock proteins inhibit apoptosis by inhibiting the caspase pathway, preventing the release of cytochrome *c* and disrupting formation of the apoptosome.

Nuclear factor-kappa B (NF-κB) is also associated with inhibiting apoptosis [104]. Nuclear factor-kappa B is a transcriptional regulatory factor. Goodkin et al. demonstrated that during HSV-1 infections there was a translocation of NF-κB from the cytoplasm to the nucleus of the cells. In the nucleus NF-κB may activate anti-apoptotic proteins. In contrast, reducing the amount of NF-κB in the nucleus increased cell death.

Besides, Wang et al. showed that the bovine herpesvirus 4 BORFE2 gene encodes a protein that can block Fas- and TNFR1-induced apoptosis [324]. The exact mechanism of action is not known. They theorized that BHV-4 BORFE2 interacts with the prodomain of caspase-8 which may prevent its binding to FADD (Fas-associating protein with death domain) and thus activation of caspase. Another theory is that BHV-4 BORFE2 interacts with the caspase-8 prodomain to alter its conformation and thus prevent its activation. The binding of Fas ligand or TNF-α to their respective receptors initiates a cascade of events that ends in apoptosis. The binding of Fas is important for cytotoxic T-cell killing of virus-infected cells whereas tumor necrosis factor (TNF) kills

virus-infected cells directly. Ligand binding to Fas or TNFR1 (tumor necrosis factor receptor 1) results in FADD binding to the intracellular death domain of Fas or TNFR1-associated death domain protein. The death effector domain (DED) of FADD binds to the DED of pro-caspase-8. Pro-caspase-8 is the proenzyme of a cysteine protease and its binding to FADD activates the protease. Additional proteases are activated and the cell undergoes apoptosis.

Inhibition of apoptosis is a strategy used by viruses to support virus replication. Further studies are needed to confirm the interactions of herpesvirus genes with the apoptotic pathway [324]. In addition, these events are being evaluated for potential targets for antiviral therapies. If the virus is unable to inhibit apoptosis, the cell will die and virus replication will cease.

Latency. Herpesviruses can enter latency in which the viral genome is present but gene expression is limited and infectious virus is not produced [340]. In healthy individuals, monocytes contain persistent cytomegalovirus viral DNA. Herpes simplex virus establishes latency in dorsal root ganglia [246,337]. For both HSV-1 and HSV-2, the virus translocates from nerve endings to the nuclei of sensory ganglia by retrograde transport [337]. In BHV-1, multiple copies of viral DNA may be integrated into the chromosomal DNA of latently infected neurons of the trigeminal and sacral ganglia [16,209]. The virus proceeds through attachment, penetration and uncoating, but then the viral genome persists in the cell in a latent state [1]. The latent genome produces a latency-associated transcript that is believed to be responsible for down-regulating lytic gene expression [246]. The viral genome undergoes a symbiotic relationship with the host cell and is replicated with the host DNA [1]. The latent state persists until an

inducing agent (stress, fever, exposure to ultraviolet light, immune suppression) stimulates the viral genome to enter a normal replication cycle [1,246]. In cattle, reactivation is associated with stress from infections, parturition, shipping, cold, crowding or corticosteroid treatment [209]. Following reactivation, the virus again replicates causing lysis of the cells and is moved by anterograde transport to a site at or near the portal of entry [246,337]. Here they may be transmitted to other hosts to cause primary infections in susceptible hosts [82,342]. Clinical signs during reactivation may be mild or nonexistent [342]. Recurrences occur in the presence of both cell-mediated and humoral immunity [337].

Whetstone in 1989 showed that two bovine herpesvirus subtypes can establish latency in the same tissue in the same animal [332]. Three heifers were inoculated intravenously with strain BHV-1.2b (K22). They were then treated with dexamethasone five months later and infected intranasally and intravaginally with BHV-1.1 (Cooper). A second dexamethasone injection was given in four months and both virus subtypes were reactivated, but it is not known if both viruses colonized the same ganglion.

Herpes Simplex Virus

Introduction. Herpes simplex virus (HSV) has been recognized since ancient Greek times [333,337]. Hippocrates used the word "herpes" (meaning to creep or crawl) to describe a spreading skin lesion [337]. Herodotus associated fever with mouth ulcers and lip vesicles and called it "herpes febrilis". Galen surmised that the body was trying

to rid itself of evil humors and described the disease as "herpes excretins". In the 18th century an association of herpetic lesions with genital infections was made by Astruc, physician to King Louis XIV. By the early 19th century, it was known that herpetic infections were associated with vesicular lesions. It was not until 1893 that Vidal recognized person-to-person transmission of HSV infections. By the beginning of the 20th century, multinucleated giant cells were shown to be associated with infection. In 1919, Lowenstein demonstrated that virus retrieved from human HSV keratitis or HSV labialis could produce lesions on the cornea of rabbits [333].

Transmission and Pathogenesis. Herpes simplex virus spreads by contact with infected secretions [42]. This is generally through mucosal contact or aerosol droplets [209]. Herpes simplex virus is divided into 2 subtypes, type 1 and type 2 [13]. Type 1 generally causes oropharyngeal signs and type 2 is usually associated with genital disease although they can each affect either site. Transmission depends on intimate contact of a susceptible seronegative individual with one who is excreting HSV [333]. Following exposure to either type 1 or type 2 HSV, a primary infection often develops without clinical signs [13]. The virus replicates at the portal of entry, usually oral or genital mucosal tissue, and infects the sensory nerve endings [333]. After viral replication, the virus is transported by retrograde axonal flow and becomes latent in the sensory dorsal root ganglia [13]. Recurrent infections occur at varying times and frequencies. In the United States, 30 to 50 % of the population has had an HSV-1 infection by early adulthood and 80 % by late adulthood. Herpes simplex virus 2 affects 25 to 50 % of the population in the United States.

There are 4 types of infection. Individuals without pre-existing HSV antibodies (susceptible individuals) develop a primary infection after the first exposure to either HSV-1 or HSV-2 [337]. A recurrent infection is any infection after the primary infection. Initial infection occurs when an individual with pre-existing antibodies to one type of HSV experiences a first infection with the opposite virus type. Lastly, an exogenous reinfection is a reinfection with a different strain of HSV.

Clinical Signs. There are several forms of disease that can be seen with HSV-1 and HSV-2 infection.

Oropharyngeal Disease- Herpes simplex virus 1 can cause oropharyngeal infection [334]. This most commonly occurs in children between 1 and 3 years of age and is generally asymptomatic [262,334]. The incubation period is 2 to 12 days with an average of 4 days [334]. Symptomatic disease includes fever up to 104 °F, oral lesions, sore throat, halitosis, anorexia, cervical adenopathy, and mucosal edema. Oral lesions are present on the hard palate, cranial portion of the tongue, along the gingival, and around the lips and they can extend down the chin and neck from drooling. The lesions are vesicular but rupture leaving 1 to 3 mm grey-white ulcers on erythematous bases [334,337]. The infection lasts 10 to 21 days. Primary infection in young adults is associated with pharyngitis and a mononucleosis-like syndrome. Ulcerative lesions are frequently seen on the tonsils [334].

Viral shedding occurs in oral secretions for 7 to 10 days but can last as long as 23 days [334,337]. Virus can also be shed in the feces [334]. In addition, virus can be detected in the saliva of asymptomatic children and adults.

Recurrent HSV-1 infection of the oropharynx occurs in about 10 to 20 % of infected individuals [13]. The recurrent infection is asymptomatic in 1 % of children and 1 to 5 % of immunocompetent adults [337]. Approximately 1 % of pregnant women and nursery personnel excrete HSV which provides a source of virus to the newborn. Recurrent lesions are preceded by pain, burning, tingling, or itching for about 6 hours [334,337]. Vesicles appear within 24 to 48 hours at the vermillion border of the lip [334]. Lesions generally crust in 3 to 4 days and heal within 8 to 10 days. Immunocompetent individuals rarely have recurrences of vesicles on the face. A topical formulation of penciclovir is licensed for the treatment of herpes labialis in adults and results in a 20 % reduction in the duration of disease. Docosanol is a topical agent that is available without a prescription. It has been shown to reduce the duration of disease from 4.8 days to 4.1 days [252].

Genital Disease- Genital HSV infection, most commonly HSV-2, occurs by sexual contact with an infected partner [334]. The incidence of genital herpes is 1/100-1/1000 depending on the socioeconomic group evaluated [74]. Incubation is 2 to 12 days with lesions persisting for 21 days [334]. Viral excretion may persist for 3 weeks and is associated with large quantities of virus (10^6 viral particles per 0.2 mL of inoculum) replicating in the genital tract [333,337]. The first episode is frequently associated with systemic illness [227]. True primary disease occurs when there are no preexisting antibodies against HSV-1 or HSV-2. The disease is severe and prolonged in these cases. If antibodies are present against HSV-1, then first episodes of HSV-2 are milder and defined as nonprimary. In 70 % of primarily infected patients, fever, malaise, myalgias, and inguinal adenopathy are seen [334]. Complications seen are extragenital lesions,

aseptic meningitis, and sacral autonomic nervous system dysfunction associated with urinary retention [13,334]. Also, genital HSV is associated with an increased risk of acquiring human immunodeficiency virus (HIV) infection [334].

The disease is manifested by macules and papules followed by vesicles, pustules, and ulcers [337]. In males, primary infection leads to a cluster of vesicular lesions on erythematous bases on the glans or shaft of the penis [334]. In females, painful lesions occur bilaterally on the vulva, and cervicitis occurs in 90 % of women. The lesions ulcerate and become covered with grey-white exudate. The lesions scab and then re-epithelialize [227]. Antiviral treatment is highly effective in first episode infections. Supportive treatment is important to prevent complications. Analgesics, antipyretics and maintenance of hydration are necessary. Saline bathing of the lesions should minimize the risk of superinfection and reduce the formation of adhesions. Topical steroid and antibiotic ointments are not recommended. Topical antivirals are not recommended for treatment of first episode genital herpes. In immunocompetent individuals, acyclovir can be administered topically, orally, or intravenously for the treatment of primary genital herpes [334]. Treatment decreases the duration of symptoms, viral shedding, and time to healing. Treatment does not however decrease the frequency or severity of recurrences. Although, daily administration of oral acyclovir will suppress recurrences of genital herpes in 60 to 90 % of patients. Treatment is interrupted every 12 months to determine if there is a continued need for suppression. Valaciclovir and famciclovir are licensed for the treatment and suppression of recurrent herpes.

In a patient with a prior diagnosis of genital herpes, the appearance of new vesicular genital lesions is indicative of reactivation [227]. Genital infections caused by

HSV-1 recur less frequently than those caused by HSV-2 [337]. Recurrent genital infections can be symptomatic or asymptomatic [334]. Prior to signs occurring, itching, burning, tingling or tenderness might be noted. During recurrence fewer lesions are present and the duration of disease is shorter (7 to 10 days) [227,334]. However, recurrence is characterized by high levels of infectivity whether it is symptomatic or asymptomatic [227]. Approximately 10^2 to 10^3 viral particles per 0.2 mL of inoculum are shed [333]. In males, the lesions are on the glans or shaft of the penis [334]. In females, lesions are on the labia minora, labia majora, and perineum. In 10 % of women with recurrent infections, cervical excretion of HSV occurs. Generally, systemic signs are not seen with recurrent infections.

Herpes simplex virus can also affect male fertility [159]. In males with herpetic lesions, 86 % had HSV-2 in the semen. In infertile patients, 24 % had HSV DNA in their semen. Using in situ hybridization, Kotronias was able to demonstrate HSV DNA in the nuclei of spermatozoa. He showed in a study of 80 infertile men that 46 % had HSV DNA in the nuclei of spermatozoa. Semen with a sperm count lower than 20 X 10^6/mL had a 70 % HSV infection compared with 25.5 % for a sperm count greater than 20 X 10^6/ mL. Of the HSV positive samples, HSV-2 was detected in 56 % of the samples and HSV-1 was present in 44 % of the samples. As the number of infected spermatozoa increased, the sperm count and motility decreased. In samples which contained both HSV-1 and HSV-2, the sperm count and motility were exceptionally low. In contrast, Pallier demonstrated that regardless of viral concentration or washing procedure, the presence of HSV-2 or CMV in sperm does not inhibit sperm motility [224]. They also showed that sperm do not bind to HSV-2 in the presence of seminal fluid however, viral

particles were detected free in the seminal fluid. This was in comparison to the absence of seminal fluid in which HSV-2 adhered to sperm. Therefore, sperm motility is not a good parameter on which to base sperm selection for use in intracytoplasmic sperm injection.

In 8 males with HSV positive semen, acyclovir was administered for 2 weeks after which pregnancy resulted from 3 men [159]. A case report by Moore demonstrates transmission via artificial insemination of HSV-2 in the semen of an asymptomatic donor to the recipient [203]. Over 70 % of HSV-2 seropositive individuals have no history of genital lesions. One third of new cases of genital herpes are acquired from asymptomatic sources. Aynaud demonstrated that 40 % of HSV-positive individuals were asymptomatic for a genital infection [10]. Herpes simplex virus has been isolated from the urethra of 30 % of men with acute primary genital herpes [203]. In 1988, the American Fertility Society no longer allowed the use of fresh semen for artificial insemination but rather required that frozen semen be quarantined for 180 days until the donor was shown to be seronegative for HIV. Similar rules have been put into effect for HSV as seroconversion should occur by 3 months after infection. This seroconversion could be either HSV-1 or HSV-2 in a seronegative individual or seroconversion to HSV-2 in the HSV-1 seropositive individual. Cryopreservation does not destroy HSV-2 [260]. Therefore, there is the likelihood that the recipient could be infected and through her, the neonate could be infected.

Because primary genital HSV infection is associated with a greater risk to the fetus than a recurrent infection, seronegative woman are at risk of developing an infection from their sexual partner [336]. Seronegative women who are sexually involved with a

male with an HSV genital infection have a 10 to 20 % risk of developing an infection. An association has been shown between spontaneous abortion and primary maternal infection before 20 weeks of gestation [333]. Thirty to 50 % of pregnant women who experience an initial or primary infection during the last trimester of gestation are likely to transmit the infection to their fetuses. This is compared with the 3 % transmission rate in recurrent maternal infections. Abstinence is the most effective preventative measure as condoms are not fail-safe [336]. Treatment of the male with acyclovir can be used to decrease the symptomatic and asymptomatic recurrences.

Treatment of recurrent HSV includes episodic and suppressive antiviral therapy [227]. Acyclovir, valacyclovir, and famciclovir are approved for episodic treatment of genital herpes. Acyclovir is administered 5 times a day while valacyclovir and famciclovir are administered twice a day with similar healing and symptom resolution rates. It is important to begin treatment early in the course of recurrence to have improved results. Viral shedding is reduced to 2 or 3 days with antiviral treatment compared with 4 or 5 days in placebo-treated patients. A benefit of valacyclovir is the 44 % increased likelihood of lesions aborting. An aborted lesion is one that does not progress beyond the papule stage. When valacyclovir treatment commences within 6 hours of onset of symptoms, the chance of genital herpes lesions aborting increases 2-fold. However, episodic treatment is not beneficial to patients with severe prodromal symptoms, those unable to recognize the early signs of a pending herpes outbreak, and in those who have established psychosexual problems.

Suppressive antiviral treatment is highly effective for controlling clinical features of the disease and reducing the frequency of recurrences [227]. However, viral shedding

is not completely eliminated, and patients should be warned that the risk of transmission does remain. Acyclovir, valacyclovir, and famciclovir have comparable efficacy and prevent at least 70 % of symptomatic recurrences over 1 year. Treatment for greater than 5 days is required before a reliable suppressive effect is seen. Acyclovir is administered 2 or 3 times a day, and famciclovir is administered once a day. Valacyclovir at once daily administration can effectively control genital herpes. Valacyclovir also has a cost advantage. The United States wholesale prices of 2001 indicate that the cost for 1 year suppressive therapy with valacyclovir, famciclovir, and acyclovir would be $1235, $2683, and $1377, respectively [269]. Some patients may have breakthrough herpes episodes during suppressive therapy [227]. Episodic antiviral treatment can be instigated if needed during these times. If frequent breakthrough continues, the lesions should be cultured to confirm diagnosis of HSV. Herpesvirus resistance to antiviral agents is rare in immunocompent patients, thus an alternative diagnosis should by pursued. Long-term suppressive treatment has been shown to be safe. Patients have remained on acyclovir for up to 10 years with no evidence of clinically significant adverse effects. Stopping suppressive therapy does not appear to influence HSV infection in that recurrence frequency does not change compared with that prior to commencing suppression (Table 2).

Table 2. Treatment for genital and mucocutaneous herpes simplex virus [334].

Type of infection	Drug	Comments
Genital HSV Initial episode	Acyclovir Valaciclovir Famciclovir	Intravenous for severe cases
Recurrent episode	Valaciclovir Famciclovir	
Suppression	Acyclovir Valaciclovir Famciclovir	
Mucocutaneous HSV Normal host	Acyclovir	
Immunocompromised host	Acyclovir Valaciclovir Famciclovir	Intravenous for severe cases
HSV encephalitis	Acyclovir	Serially assess renal function
Neonatal HSV	Acyclovir	
Herpetic conjunctivitis	Trifluridine	Titrate dose Alternate- Vidarabine ointment

Dermatologic Disease- An alteration in the skin, such as occurs with atopic dermatitis, can lead to localized HSV skin infections (eczema herpeticum) [334]. If the lesions occur after trauma they are known as herpes gladitorium (wrestler's herpes or traumatic herpes). Infection of the digits is known as herpetic whitlow. This can occur in neonates sucking their fingers or in dental personnel [262,334]. Most lesions resolve in 7 to 9 days [334]. Although not approved, acyclovir, valaciclovir, or famciclovir are frequently used off-label for these conditions.

Ocular Disease- Ocular HSV infections result in blepharitis or a follicular conjunctivitis [334]. Branching dendritic lesions develop as the disease progresses. Signs which are seen include severe photophobia, tearing, chemosis, blurred vision, and preauricular lymphadenopathy. The treatment of choice is trifluridine because it is the

most efficacious and easiest to administer. Other treatment options include idoxuridine or vidarabine ophthalmic drops. Even with treatment, healing of the cornea can take 1 month [337].

Central Nervous System Disease- Herpes simplex virus infection of the central nervous system (CNS) can occur suddenly or after a 1 to 7 day period of influenza-like symptoms, and this situation can be life-threatening [334]. Signs include headache, fever, behavioral disturbances, speech disorders, altered consciousness and focal seizures. Analyses of cerebrospinal fluid (CSF) are variable but include a moderate pleocytosis with a predominance of mononuclear cells, increased protein concentration, and normal or slightly decreased glucose. Initially, computed tomography shows no abnormalities, but with time involvement of the temporal lobe is seen. Recovery is more favorable when treatment is administered early in the course of the disease. Treatment involves administration of acyclovir. Mortality decreases to 25 % after one year of treatment and 25 % of survivors return to normal function.

Immunocompromised Individuals- Herpes simplex virus can cause severe infection in immunocompromised individuals [334]. The immunocompromised immune system could be due to immunosuppressive therapy, underlying disease, or malnutrition. Disseminated disease can occur and involve the skin, mucosa, and viscera. The disease can also remain localized but persist for longer periods of time than an immunocompetent host. Acyclovir administered orally, topically, or intravenously will diminish the duration of viral shedding, decrease duration of pain, decrease the time of healing and reduce the incidence of symptomatic HSV infection. Valaciclovir and famciclovir can also be used. Foscarnet is the treatment of choice if resistance develops.

Respiratory Disease- Herpes simplex virus has been isolated from the respiratory tract of adults with adult respiratory distress syndrome and acute-onset bronchospasm [333].

Neonatal Infection. Annually, in the United States, 1500 to 2000 cases of neonatal herpes simplex virus infection occur [154]. The incidence of neonatal HSV infection is 1/2,000 to 1/5,000 deliveries [13,47,347]. The risk of neonatal death or serious sequelae is 50 % [154]. Neonatal herpesvirus infection can result from HSV-1 or HSV-2 [48].

Host response of the newborn to HSV differs from that of adults [337]. The immune system is still impaired in the fetus and newborn. Humoral antibodies do not prevent recurrences or exogenous infection and transplacentally acquired antibodies are not completely protective against newborn infection [333,337]. Infected newborns produce IgM antibodies specific for HSV within the first 3 weeks of infection, and these remain detectable for as long as 1 year after infection [337]. Newborns with HSV infection have a delayed T-lymphocyte response compared with adults. Newborns also have decreased interferon-α (IFN) production in comparison with adults, and the lymphocytes from infected newborns have decreased response to interferon-γ.

Human herpes simplex virus can cause neonatal disease from intrauterine, intrapartum, and postpartum transmission [13,131,270].

Intrauterine Infection- Intrauterine infection is the cause of neonatal HSV infections in 5 % of the cases in the United States [13,347] although one report presented a 14 % prevalence[270]. This results in 1 child out of 300,000 deliveries being infected intrauterine [13]. Intrauterine infection can occur transplacentally or from an ascending

cervicogenital infection [13,202,245]. Transplacental infection can lead to spontaneous abortion or a viable fetus with or without organ dysfunction [13]. Transplacental infection might occur from a maternal viremia, although a viremia is rare even during a primary infection [13,202,245,270]. Other routes of transplacental infection might be transneural migration of the virus from persistently infected dorsal root ganglia to the endometrium or from a latently infected endometrium becoming active during pregnancy [245]. Robb hypothesized that a latent endometrial epithelial HSV-2 infection can occur through the following routes: maternal primary infection during gestation, ascending infection, transneural, or postnatal primary infection during childhood or adulthood [244]. Sporadic infections can then be activated once this latent endometrial infection is established. Herpes simplex virus antigen has been found in the endometrium of nonpregnant women and in material from spontaneous abortion [243]. The infection was termed latent because no morphologic or infectious virus was detected to define the infection as persistent. The latent infections occur sequentially and are dependent upon the stage preceding it. Thus, the endometrium is affected first, the amniochorion is affected next, and finally the chorionic villus is affected although infection of the embryo does not always occur. Amniochorionic infection is responsible for about 20 to 30 % of spontaneous abortions. However, in 75 % of normal neonates amniochorionic infection occurred, but chorionic villus infection did not occur. The latent infections are not dependent upon previous activation, in that an infection in a previous pregnancy does not increase the risk for infection in subsequent pregnancies. The latent intrauterine infection produces a latent HSV-2 infection in 40 % of surviving neonates.

Intrauterine infection can also result from chorioamniotic membrane infection from an ascending infection [13,202,270]. It is possible for the membranes to become infected even if they do not rupture [270]. The membranes might reseal following a leak or the virus might be able to traverse intact membranes. It is not uncommon for the mother to be asymptomatic [131].

In vitro studies have shown that the villous trophoblasts support HSV-1 and HSV-2 replication [217,347]. The fetal membranes consist of the amnion and chorion [248]. The amnion is the layer closest to the fetus and contains cuboidal epithelial cells attached to avascular connective tissue. The outer layer of the amnion is adjacent to the chorion. The chorion consists of connective tissue and fetal blood vessels, and the outer layer of the chorion is attached to the uterus. During delivery, the chorion is detached from the decidua, but parts of decidual cells remain adhered to the chorion. Peripheral blood cells infected with virus can penetrate the fetal membranes to transmit disease to the fetus. In an experiment to study cell-free virus penetration of human fetal membranes, HSV-1 and HSV-2 passed through the fetal membrane within 20 minutes of incubation with peak virus transport observed at 1 to 2 hours. However, less than 1 % of the virus in the maternal compartment migrated to the fetal side in 24 hours. During the 24 hour period, 0.02 to 1.0 % HSV-1, 0.03 to 0.2 % HSV-2 and 0.07 % BHV-1 migrated through the fetal membranes. Herpes simplex virus 1 and HSV-2 were transported equally from maternal to fetal side and vice versa. Infectivity of HSV-2 remained stable at 37° C for the entire 24 hour period. Herpes simplex virus 1 was stable for 4 hours and decreased to 58 % infectivity at 6 hours and 3 % at 24 hours. Bovine herpesvirus 1 decreased to 75 % infectivity at 6 hours and to 30 % at 24 hours. Virus penetration was thought to be due to

binding with cellular receptors involved in transcytosis of internalized virus particles. The cell surface receptors were not species specific as BHV-1 penetrated the fetal membranes at rates comparable to HSV. Thermal inactivation of the viruses was not responsible for the diminished migration.

In contrast, Koi et al. demonstrated that the placental structure limits transmission of HSV [154]. Herpes simplex virus 1 and HSV-2 attach to target cells by glycoprotein C binding with heparan sulfate. Entrance is gained into the cells by herpesvirus entry mediators (HveA, HveB, and HveC) interacting with glycoprotein D of HSV. Even with enzymatic removal of heparan sulfate from cells, HSV is able to attach and infect 15 % of cells. Herpesvirus entry mediator A is in the tumor necrosis factor/ nerve growth factor-receptor family. It is the main receptor for entry of HSV into human lymphoid cells. Herpesvirus entry mediator B is a poliovirus receptor-related protein 2. It mediates HSV-2 entrance into cells and the entrance of mutant HSV-1 which cannot use HveA. Herpesvirus entry mediator C is a member of the immunoglobulin superfamily and is also a poliovirus receptor-related protein. It allows the entrance of HSV-1 and HSV-2 into human cells of eptithelial and neuronal origin. Koi et al. showed that extravillous trophoblast cells express all three Hve mediators and are therefore susceptible to infection with HSV-1. Infection can result in impaired placental invasion and miscarriage. It might be that infection of the extravillous trophoblast cells allows the fetus to be rejected because infection blocks the expression of cell surface HLA-G (major histocompatibility complex is referred to as human leukocyte antigen in humans) system molecules which prevent immune rejection. The villous syncytiotrophoblast is in direct contact with maternal blood, and lateral intercellular spaces do not exist within this layer so organisms

must traverse this layer prior to reaching fetal circulation. However, the villous syncytiotrophoblasts do not express the Hve mediators. Therefore, the villous trophoblast cells are resistant to infection by HSV-1 and form an effective barrier to HSV fetal infection.

Herpes simplex virus infection can cause localized genital or disseminated infection when it occurs during pregnancy [13]. Localized genital infection can be primary or recurrent. The risk of transmitting virus to the fetus is greater with primary infections because of the lack of maternal antibodies and a longer duration of viral shedding compared to recurrent infections [74]. Primary infection during the first 20 weeks of gestation has been associated with spontaneous abortion, stillbirth, and congenital malformations with the incidence of spontaneous abortion being 25 % during the first trimester [13]. Other complications of maternal infection are preterm labor and fetal growth retardation [217]. Less than 25 % of women infected with HSV-2 are symptomatic [270]. The virus can be transmitted by asymptomatic women. Asymptomatic cervical shedding of virus occurs in a high rate of women when primary genital HSV infection occurs during gestation [13]. Primary symptomatic infection with HSV-2 during the third trimester resulted in a 35 % incidence of preterm labor and 40 % incidence of fetal growth retardation. Should primary infection occur during pregnancy, treatment with acyclovir is recommended [74].

Recurrent genital infections are more common than primary infections [13]. Recurrent infections occur in 85 % of pregnant women. Of recurrent infections, 15 % are asymptomatic. Recurrent infections are not as likely as primary infections to adversely affect the pregnancy [13,47,131]. If a fetal scalp monitor is used in woman with

recurrent infection the risk of fetal infection is greater [336]. Herpes simplex virus was transmitted to the neonate in 33 % of primary infections compared with a transmission rate of 3 % in recurrent infections [47,336]. In another study, the vertical transmission rate was 34 to 80 % for primary infections compared with 1 to 5 % for recurrent infections [74].

Disseminated infection is the second form of HSV infection that can occur during pregnancy [13]. This form is life-threatening to the mother and the fetus. The fetus generally dies due to maternal complications and not due to the direct effects of the virus. Fetuses that survive do not have evidence of disease.

Clinical signs are seen within 48 hours of birth in intrauterine infections [13,202]. Neonates can have cutaneous disease, ophthalmologic abnormalities, CNS disease, or disseminated infection [13,334]. Of infected neonates, mucocutaneous disease is present in 5 to 10 % of the cases, cutaneous and ophthalmologic in 15 % of the cases, encephalitis with skin and/or eye involvement in 50 % of the cases, and 25 to 30 % neonates have disseminated infection with or without CNS disease [13]. Neonates with disseminated, cutaneous, or ophthalmic abnormalities generally present within the first 2 weeks of life whereas those with CNS disease present between the second and third week of life [334].

Cutaneous lesions that might be seen include bullous, vesicular, or erythematous maculopapules that became vesicles [13]. Other skin abnormalities that might be seen are aplastic skin or denudation scars, and these generally occur at the site of trauma. Skin lesions recur for months or years. Central nervous system lesions seen were microcephaly, hydranencephaly, intracranial calcification, hydrocephalus, porencephalic

cysts, subependymal cysts, blindness, deafness, and mental retardation. Common symptoms encountered include irritability, focal and generalized seizures, lethargy, tremors, poor feeding, unstable temperature, bulging fontanelle, respiratory distress, jaundice, disseminated intravascular coagulopathy, shock, and cutaneous vesicles [334]. Microcephaly and hydranencephaly are caused by damage from the virus rather than defective organogenesis induced by the virus [13]. Clinical signs which can be evident in the baby are lethargy, abnormalities in tone, poor suckling reflex, irritability, tremors and seizures. Ophthalmologic lesions present as chorioretinitis, keratoconjunctivitis, corneal ulceration, anterior uveitis, cataracts, vitritits, optic atrophy, nystagmus, microphthalmia, retinal dysplasia, and strabismus. These lesions most likely result from direct contact of HSV with the eye. Ocular lesions were seen in 60 % of intrapartum infections and 20 % of postpartum infections. Disseminated infection occurs when the infection spreads beyond the dorsal root ganglia and most commonly involves the liver and adrenal glands [333,337]. Other organs which can be affected include the lungs, brain, larynx, trachea, esophagus, stomach, small intestine, large intestine, spleen, kidneys, pancreas, and heart [13,334]. Following disseminated infection, stillbirth or spontaneous abortion usually results [13]. Clinical signs that can be seen are fever, acidosis, apnea, lethargy, and poor suckling reflex. Mortality is high due to respiratory failure, hepatic failure, and/or cardiovascular collapse. It is unclear if the respiratory failure is due to the HSV infection or to prematurity. Neonates can also have latent infections in their liver, pancreas, gastrointestinal tract, bile ducts, and bronchi [243].

Similar clinical signs are seen in babies with congenital toxoplasmosis, rubella, cytomegalovirus, or syphilis [131]. Definitive diagnosis of intrauterine HSV infection is

therefore based on clinical signs within 48 hours of birth as well as histopathologic and virologic examination of the placenta [13]. Intranuclear inclusion bodies are characteristic of herpes simplex virus, but they are not definitive [202]. Robb et al. showed that maternal floor infarct of placenta and calcifying funisitis are manifestations of intrauterine HSV infection [245]. In addition, excretions from the eye, throat, nasopharynx, and CSF can be used for virus isolation [13]. The virus can be identified by growth in cell culture, detection by transmission electron microscopy, and by immunohistochemistry [245]. Serology is not of value during the acute disease [13].

Herpes simplex virus might also be responsible for early embryonic death that is characterized as female infertility [346]. Yueh et al. demonstrated that expression of VP16 is toxic to pre-implantation mouse embryos. The embryos were unable to develop from the 2-cell to the 4-cell stage when a high concentration of VP16 was expressed in the early preimplantation embryos. The toxicity was dose-dependent as embryos with a low concentration of VP16 expression did develop into blastocysts. In addition, VP16 is tolerated at later developmental stages. Embryos are able to develop when VP16 is expressed later in development in the caudal region of the embryo or when expressed in the notochord, lung, intestine, kidney, salivary gland, skin, CNS, skeletal muscle or bone. Other animal models have demonstrated early embryonic death when herpes virus infection occurs before fixation. Therefore, in humans, early loss of pre-implantation embryos might be misdiagnosed as infertility. Other reports have shown that infertile couples treated with acyclovir were able to achieve pregnancy. This further supports the theory that embryos exposed to high concentrations of VP16 would spontaneously abort.

Neonates born to mothers with extensive recurrent genital disease or primary infection should receive prophylactic treatment because the risk of them contacting the disease is 50 % [336]. Therapy for intrauterine infection consists of acyclovir and vidarabine [13]. Even with antiviral treatment, 40 % of neonates with disseminated or CNS disease die [270]. Irreversible lesions such as microcephaly and hydranencephaly are not affected by the antiviral agents [13]. In addition, the infant should be isolated from other neonates. Treatment of the neonate consists of acyclovir at 10 mg/kg intravenously every 8 hours for 14 to 21 days although this drug is not licensed for neonatal disease [334,336]. Mortality due to encephalitis is decreased to 5 % and disseminated disease to 25 % following treatment of the neonate [334]. Neonates with ocular infections should also receive trifluridine. Morbidity however remains high even with treatment. Although cesarean delivery is important for preventing intrapartum transmission, it is not useful for preventing infection by intrauterine transmission [270].

Of treated neonates, 5 to 10 % will develop a life-threatening recurrence of infection requiring treatment in the first month of life [336]. Fifty percent will have recurrent skin lesions. Those with recurrent lesions do not require treatment unless systemic disease occurs. If the neonate acquires 3 or more skin recurrences in the first 6 months of life, oral acyclovir therapy may be of benefit to prevent neurologic sequelae. Insidious infection of the CNS can occur with resultant low-grade encephalitis.

Intrapartum Infection- Intrapartum or perinatal transmission of HSV-1 or HSV-2 is the most common method of transfer to the neonate (75 to 80 %) [48,131,337]. The infant's skin and mucosa contact the virus in the maternal blood and vaginal secretions during transit through the birth canal [47,48,248]. The mother may be symptomatic or

asymptomatic [47,48]. Neonates are more likely to develop congenital malformations if infected intrauterine compared with intrapartum infection [347]. If infected intrapartum, the neonate can develop systemic disease days to weeks following delivery [68]. The virus is present in the mother's birth canal due to reactivation of the disease or primary infection during pregnancy [48]. Brown et al. showed that 2 % of women acquired HSV during pregnancy. Symptoms were seen in 36 % of the women who became seropositive. Women who were seropositive for HSV-1 or HSV-2 before labor had no cases of neonatal herpes, even though the mothers were subclinically shedding virus at the time of labor. However, of 9 women who became seropositive at or near the time of labor, neonatal HSV developed in 4 infants. Infection is uniform throughout gestation. Brown demonstrated that infection of the mother occurred in the first trimester of 30 % of women, 30 % of women in the second semester, and 40 % of women in the third trimester.

It is very important to prevent HSV in the neonate because even following prompt initiation of therapy, many neonates die or have severe neurologic disability [47]. Prevention can involve abstinence from sex during the latter half of pregnancy for women who are HSV-seronegative with seropositive partners. Suppressive acyclovir treatment can be initiated to reduce the clinical recurrence rate and the need for caesarean section [227]. However, antiviral drugs are not licensed for use in pregnancy. In addition, cesarean section has not been proved efficacious if performed when the membranes have been ruptured for longer than 4 hours [337]. It is important that individuals with herpetic whitlow not care for newborns as gloves do not prevent transmission of infection [333]. Hospital personnel should wear masks when they have

active lesions present. The importance of hand washing needs to be emphasized to health care workers.

Postpartum Infection- Postpartum transmission is less common than intrapartum infection, 15 to 20 % versus 75 to 80 %, respectively [333,336]. Postpartum transmission of herpes simplex virus occurs via breast feeding, nosocomial, or contact with infected individuals such as relatives or hospital personnel [248,336].

Diagnosis. Definitive diagnosis of disease is by isolation of HSV in cell culture [334]. Sites which can be sampled are skin lesions, cerebrospinal fluid, urine, throat swabs, nasopharynx secretions, conjunctivae, and duodenal samples. The samples should be shipped on ice and upon arrival at the laboratory are inoculated onto cell cultures of human foreskin fibroblasts or Vero cells [337]. Cytopathic effect (CPE) develops within 24 to 48 hours. Cytologic examination of intranuclear inclusion bodies and multinucleated giant cells are indicative but not diagnostic for HSV [334]. The diagnostic choice for confirming CNS infection is through the detection of HSV DNA in the CSF by PCR (polymerase chain reaction). The test uses primers for glycoprotein B or the DNA polymerase gene [333]. Polymerase chain reaction can also be used to detect HSV DNA in genital lesions. Serology is useful for prognosis of genital and neonatal infections [334]. If no genital lesions are evident but there is a history of recurrent lesions, serological tests can be performed [67]. Serum can be evaluated for the presence or absence of antibodies for HSV-1 (g)G1 and HSV-2 (g)G1. The type 2-specific POCkit-HSV-2 test (Diagnology) is approved by the United States Food and Drug Administration for use in adults [227]. An ELISA (enzyme linked immunosorbent assay) and immunoblot (Focus Technologies) have also been approved by the FDA and can be

used in pregnant women. For primary infections, cultures should be performed because antibodies can take 2 to 12 weeks to develop [67,227]. Other antibody detection tests include complement fixation, passive hemagglutination, neutralization and immunofluorescence [337]. In addition, antigen tests such as EIA (enzyme immunoassay) can be used [227].

Histopathology. Viral infection causes ballooning of cells, condensed chromatin within the nuclei, and degeneration of nuclei [337]. Cells lose their intact plasma membrane and form multinucleated giant cells [333,337]. With cell lysis, vesicular fluid containing large quantities of virus accumulates between the epidermis and dermal layer [337]. The vesicular fluid contains cell debris, inflammatory cells, and multinucleated giant cells. With healing, the vesicular fluid becomes pustular. Vesicles are replaced by shallow ulcers when mucous membranes are involved. With disseminated neonatal HSV infection, perivascular cuffing and areas of hemorrhagic necrosis occur in the areas of infection [333].

Control. Vaccination is the ideal method for prevention of viral infection [337]. Protection against HSV has been demonstrated in animals with avirulent, inactivated, and subunit glycoprotein vaccines. Few studies in humans have been placebo controlled. Killed viruses might have an initial benefit for patients with recurrent infection but the long-term benefit was not evaluated. Newer techniques have made these vaccines obsolete [333]. Subunit vaccines that do not contain viral DNA might allow the amelioration and prevention of primary disease and be of benefit in decreasing the frequency of recurrences [337]. A genetically engineered HSV is also being evaluated for use as a vaccine. The genome was deleted in the domain of viral thymidine kinase

gene and in the junction of U_L and U_S. In its place, an HSV-2 fragment encoding gD, gG, and gI were inserted. Other approaches for vaccine development are underway.

Cytomegalovirus

Introduction. Cytomegalovirus (CMV) is a large beta herpesvirus (230 kb) with the capabilities of encoding over 200 viral proteins [14,173,218]. It was not grown and identified in cell culture until the early 1950s [14]. The virus is species specific, only infecting humans and human cell lines. Human cytomegalovirus is a ubiquitous organism [107,336]. It is present in 30 to 60 % of healthy asymptomatic adults [285]. Disease caused by CMV is the exception not the rule [336]. Disease occurs most commonly in immunocompromised individuals and neonates.

Human cytomegalovirus is the most common viral cause of congenital malformations [34,162,292,293]. Between 2 and 10 % of infants are infected by 12 months of age [107]. Cytomegalovirus is present in 60 to 100 % of adults over 40 years of age [14,292]. Adults acquire CMV through organ transplantation, blood transfusion, and direct contact with young children or affected adults. Generally, the disease is asymptomatic, however, if the individual is immunocompromised the risk of disease is higher [14]. Signs seen are pneumonitis, gastroenteritis, retinitis, hepatitis, and infectious mononucleosis [14,107].

Transmission. Entry of cytomegalovirus into the host occurs when infectious virus contacts a mucosal surface, generally the genitourinary tract, upper alimentary tract, or respiratory tract [226]. In addition, the virus can be transmitted hematogenously and

by transplanted organs. Transmission to adults can occur sexually, through blood transfusions, through organ transplants, and spread from children [107,292]. A virus-positive organ transmits virus 60 to 80 % of the time [107]. Conversely, transmission by blood transfusion is rare, occurring in only 1 to 5 % of seronegative recipients receiving virus-positive blood. When transmission occurs, the virus is in the leukocyte fraction. [226,292]. In healthy individuals, the virus persists in monocytes and replicates in polymorphonuclear cells [218,292]. Three to twelve CMV infections occur per 100 units of transfused blood [292]. When virus-positive blood is given to premature infants, seroconversion occurs in 10 to 30 % of infants. Clinical signs are less severe compared with congenitally infected infants. Leukodepletion by filtration can be used to prevent transmission of CMV by blood transfusion [226,292]. This is more effective than washing of blood [292]. Also, gamma irradiation can be used in combination with filtration to prevent transmission of CMV. However, a study using filterered-irradiated and non-filtered-irradiated blood for premature infants showed that 9 % of infants receiving filtered blood and 5 % of infants receiving non-filtered blood were infected with CMV. In addition, freezing blood might eliminate post-transfusion infection with CMV [115].

Cytomegalovirus infection occurs in 0.2 to 2.2 % of all newborns in the United States, some sources stating up to 4 % [86,172,335] with 2 to 6 % of the infected children dying as a consequence of the infection [112,162,292]. Congenital CMV infection can result from primary or recurrent maternal infection or reinfection with different viral strains [168,172,292]. The incidence of primary infection in susceptible pregnant women is 0.8 % [336]. Following primary maternal CMV infections, 15 to 50 % of individuals

develop intrauterine infection with 5 to 10 % of neonates being symptomatic [34,162,292]. Spontaneous abortion occurs in 15 % of women with a primary CMV infection occurring during early pregnancy [86]. When a primary infection occurs during late pregnancy, 25 % of affected neonates have intrauterine growth retardation.

Once primary infection occurs, the virus remains latent until the individual becomes immunocompromised at which time it is reactivated [292,340]. Reactivation leads to asymptomatic viral excretion [174]. In immunocompetent individuals, the primary infection is generally subclinical [340]. In recurrent infections virus is excreted intermittently from multiple sites whereas in primary infections and infections of immunocompromised patients prolonged excretion of virus is seen. Recurrent maternal infections cause 3.2 % of all congenital CMV infections with 17 % of neonates being symptomatic [292]. Neonatal CMV infection can occur throughout gestation. The fetal infection rate is about 50 % for each trimester with the period of greatest fetal risk for disease and neurologic impairment being the first 22 weeks of gestation [336].

Women, who possess CMV antibodies before conception and subsequently develop congenital infection, have children who are asymptomatic at birth or present with later sequelae [167]. Later sequelae occurred in 25 % of the congenitally infected children of mothers with primary infection occurring during pregnancy. Minor sequelae only occurred in 8 % of the children when their mothers were immune before pregnancy. When primary infection occurs during pregnancy, the rate of transmission of virus to the fetus is 30 to 40 % whereas, when immunity has occurred prior to pregnancy, the rate of transmission is 1 to 2 %. It has also been shown that when primary infection occurs during the first half of pregnancy the outcome is worse than infection occurring later in

gestation. Therefore, women who are not immune to CMV are at the greatest risk because transmission of the virus could occur at the time of insemination.

Cytomegalovirus transmission from mother to fetus not only occurs but is common and is an important role in maintaining CMV infection in the population [226]. The virus can be transmitted to the neonate in 3 ways: intrauterine, intrapartum, and postnatal [107]. Intrauterine infection follows maternal viremia and associated placental infection. Approximately 40 % of pregnant women with primary infection transmit the virus by the intrauterine route [107,226]. Of seropositive pregnant women, 1 % transmit CMV trans utero [107].

Intrapartum or perinatal infection can occur through contact with infected maternal genital secretions during parturition [107]. Infection occurs up to 1 month after birth [292]. This is more common than intrauterine infection. Of infants infected, 90 % are asymptomatic.

Postnatal infection occurs up to 12 months after birth [292]. Postnatal infection occurs through contact with the virus from breast milk, saliva, fomites, contact with other children, and blood transfusions [107,292]. Virus in breast milk can be detected as soon as 1 week postpartum [292]. However, transmission to children more commonly occurs via saliva using toys as fomites in day-care centers [107]. The infectious child can then transmit the virus to adults [107,336]. If the adult is pregnant there is a risk of fetal infection especially if the mother is seronegative [107]. This is the most common source of infection for the pregnant woman [336]. As many as 50 % of children in group child care shed CMV in their urine [14]. The resulting infection is generally asymptomatic [336]. The risk of disease is greatest in low birth weight (<1,500 g) premature infants

[226]. Abnormalities seen are thrombocytopenia, neutropenia, apnea, and sepsis syndrome. Although intrapartum and postnatal transmission are not associated with the morbidity seen in intrauterine infection, they are important for maintaining the virus in the community. Young children who acquire the virus from a maternal source shed the virus for years and are a source of transmission to other children and adults.

Pathogenesis. The placenta is not an effective barrier to CMV [86]. The virus is transmitted in leukocytes through the placenta to the fetus [86,292]. Humans have a hemochorial placenta [75]. A single trophoblast layer separates the fetal from the maternal circulation [86]. Implantation begins with trophoblast differentiation into 2 cell types: cytotrophoblasts and syncytiotrophoblasts [50]. As implantation continues, cytotrophoblasts and syncytiotrophoblasts further differentiate to form the chorion (Figure 7). Cytotrophoblasts are specialized epithelial stem cells which, during placentation, differentiate into the floating and anchoring chorionic villi [155,172]. In the floating villi, cytotrophoblasts differentiate by fusing into the multinucleated syncytiotrophoblasts. Lacunae develop in the syncytiotrophoblasts [50]. Projections of the chorionic villi extend into the lacunae and maternal blood fills these spaces, now called intervillus spaces [50]. Syncytiotrophoblasts cover the villous surface and are in direct contact with maternal blood [172]. They transfer substances from maternal blood across the placenta to the conceptus, such as immunoglobulin G (IgG) Fc receptor which binds and transports maternal IgG to the conceptus. In the anchoring villi, cytotrophoblasts remain as single cells that collect into columns and invade the endometrium and the first third of the myometrium [155,172]. They also invade portions of the maternal arterioles becoming endovascular trophoblast [75,172]. By midgestation,

cytotrophoblasts have completely replaced the endothelial lining and most of the smooth muscle wall of the arteries [155,172]. They express adhesion molecules and proteinases required for invasion and molecules for maternal immune tolerance [172]. They also transform their adhesion receptor phenotype to resemble that of the endothelial cells in which they replace.

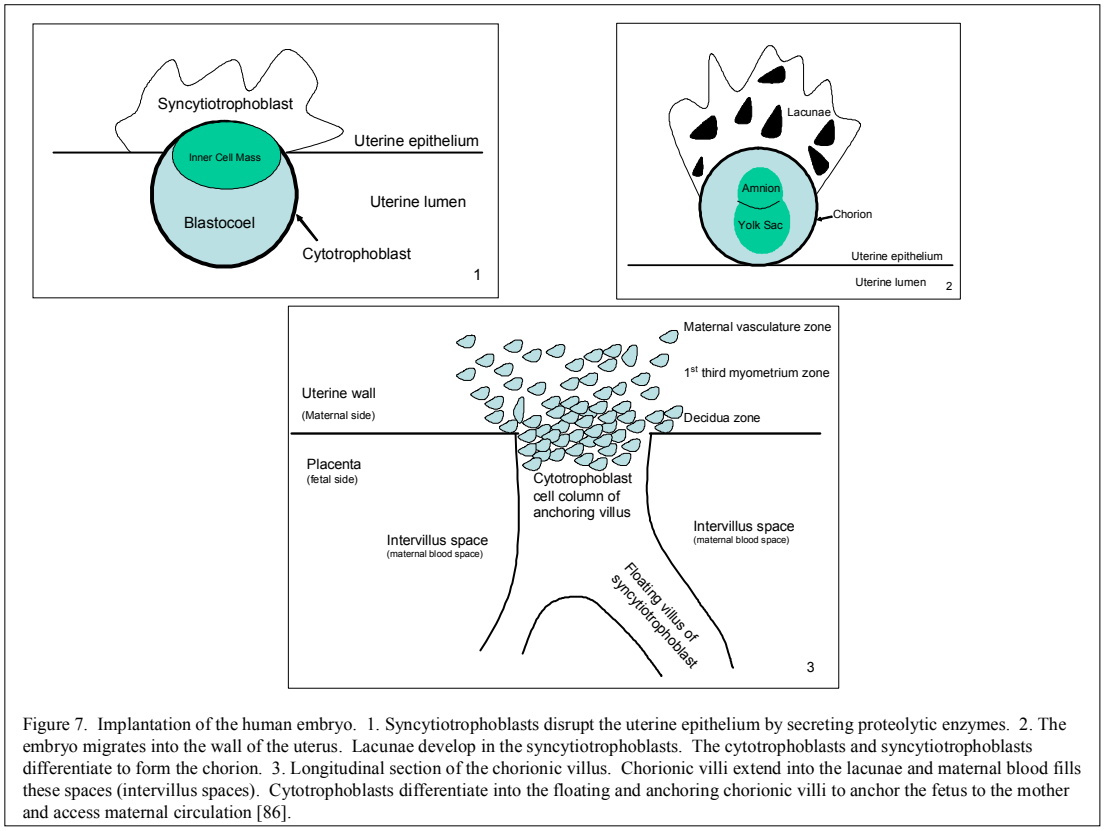


Figure 7. Implantation of the human embryo. 1. Syncytiotrophoblasts disrupt the uterine epithelium by secreting proteolytic enzymes. 2. The embryo migrates into the wall of the uterus. Lacunae develop in the syncytiotrophoblasts. The cytotrophoblasts and syncytiotrophoblasts differentiate to form the chorion. 3. Longitudinal section of the chorionic villus. Chorionic villi extend into the lacunae and maternal blood fills these spaces (intervillus spaces). Cytotrophoblasts differentiate into the floating and anchoring chorionic villi to anchor the fetus to the mother and access maternal circulation [86].

The vrus replicates within the placental fibroblasts, syncytiotrophoblast and cytotrophoblast cells from which it can enter the fetal circulation via the umbilical cord [172,292]. There are 3 suggested sites of transmission: from maternal blood cells to cytotrophoblasts in floating villi, from infected macrophages to differentiating or invasive cytotrophoblasts in the decidua or from infectious cells in maternal blood to endothelial cells and endovascular cytotrophoblasts [172]. Neutrophils do not directly infect

cytotrophoblasts, but rather infect uterine endothelial cells which then in turn infect endovascular cytotrophoblasts of the anchoring villi. Following hematogenous infection, CMV virions could be coated by antibodies to virion envelope glycoproteins and undergo transcytosis to cytotrophoblast stem cells via the neonatal Fc receptor or differentiating cytotrophoblasts underneath the syncytiotrophoblast surface. Following ascending infection, virus could replicate in the cervical epithelium and reach the uterus and infect susceptible cells in the deciduas and glands. The placenta could be infected when invasive cytotrophoblasts meet infected maternal blood cells in the uterine wall or when endovascular cytotrophoblasts form intimate contact with infected endothelial cells. The virus is then transmitted retrograde to the placenta via adjacent cytotrophoblasts. In guinea pigs, when CMV infected the placenta, 27 % of fetuses contained virus [86]. Therefore, the virus replicates in the placenta prior to reaching the conceptus. In humans, infection of the trophoblasts soon after implantation could compromise the embryos ability for interstitial implantation which buries the embryo deep into the uterine wall. This could result in early embryonic loss. Infection later in gestation could impair formation of floating and anchoring villi.

Once infected, latency is established and the virus can be reactivated with immunosuppression or pregnancy [162,285]. The virus can be shed from the cervix, vagina, urinary tract, saliva, tears, feces, blood and breast milk [162,285,340]. The rate of shedding increases as gestation progresses [162]. If infection occurs within the first 16 weeks of pregnancy there is an increased risk of fetal damage compared with infection later in pregnancy [292]. Cytomegalovirus DNA has been detected in the cervical

mucous of seronegative women [340]. It is not known if these samples were able to cause infection as cultures were not performed.

Clinical Signs. Cytomegalovirus infection is the leading cause of sensorineural deafness and infectious cause of brain damage in children [226]. Congenital CMV infection occurs up to 3 weeks after birth [292]. Clinical signs in the neonate include petechiae, hepatosplenomegaly, microcephaly, intrauterine growth retardation, intracranial calcifications, strabismus, thrombocytopenia, chorioretinitis, spastic diplegia, brain calcification, deafness, and mental retardation [112,162,340]. In symptomatic neonates, mortality rates are 11 to 20 % (Figure 8) [86,167,340]. Also, up to 80 % of symptomatic neonates have long-term neurologic abnormalities, most commonly deafness [340]. Although 90 % of infants are asymptomatic at birth, 10 to 15 % are at risk for neurologic sequelae later in life, including deafness and mental retardation [34,162,167]. Progressive hearing loss appears to be mediated by immune complex deposition in the inner cochlea of the ear [336].

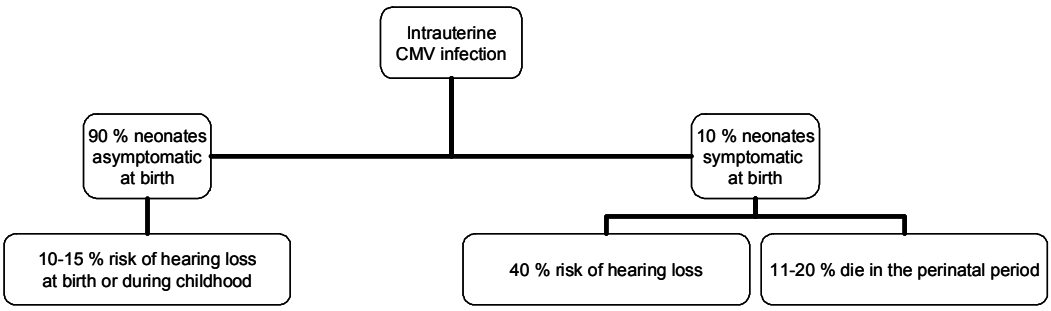


Figure 8. Clinical manifestations of intrauterine CMV infection [34,162,340].

Fertility. Cytomegalovirus might potentially affect female fertility by causing oophoritis [134]. In one case of a female affected with generalized CMV infection,

cortical necrosis of the ovaries was seen. Virus had infected the granulosa, thecal, stromal, and endothelial cells, and the oocytes were necrotic. A murine model demonstrated the presence of murine CMV in the ovarian stromal cells surrounding the follicular region of newborn mice [44]. It is possible that the ovary might act as a reservoir for latent virus later in life of adult mice.

Cytomegalovirus has been detected in the semen of patients seropositive and seronegative for CMV, infertility patients, healthy donors, and patients with mononucleosis caused by CMV [185,285,340]. The virus has also been cultured in semen of individuals in which the urine, saliva, or blood have been negative for viral particles [178]. Reactivation of a latent infection or reinfection with a different CMV strain can lead to intermittent excretion of CMV in the semen [167]. Aynaud et al. showed that CMV positive men can be asymptomatic [10]. It has also been demonstrated that CMV can persist in the urogenital tract of asymptomatic men [178]. In addition, 2 % of the men in a study by Aynaud et al. had both HSV-2 and CMV in their semen [10]. Cryopreservation does not destroy CMV in semen [115,167,340]. A study carried out in 2 French university hospitals demonstrated that CMV was detected by virus culture in frozen semen from 2 % of donors and by PCR in 5.1 % donors to a sperm bank [174].

Most seropositive donors do not excrete CMV in their semen [174]. However, different methods to detect CMV in semen samples have been described [167]. Viral culture illustrates if the virus is able to replicate and potentially infectious. However, viral culture is time consuming, and semen is toxic to the cell monolayer therefore the samples must be diluted. Nucleic acid hybridization and PCR can detect viral DNA but does not demonstrate if the virus is infectious. Moreover, the presence of viral DNA has

been demonstrated for prolonged periods of time after the disappearance of infectious virus from the blood. Therefore, PCR is too analytically sensitive for screening semen. Nucleic acid hybridization is not more sensitive than viral culture and is less specific. Serological tests are not 100 % sensitive [174]. Detection of serum IgG is not a sensitive test in that it only indicates past infection whereas, serum IgM was not predictive of CMV shedding in semen. Therefore, there is the possibility that CMV could be transmitted to the recipient causing maternal and fetal infections [226]. It is logical to take steps to avoid this complication.

A mouse model showed that Leydig cells are likely preferential cells for CMV [285]. Viral particles were not detected in the seminiferous tubules. No virus or CMV DNA was found in the fertilized oocytes, blastocysts, newborn mice, placenta or maternal organs. However, with the use of advanced reproductive techniques such as intracytoplasmic sperm injection, a spermatozoa associated with CMV could be chosen and carried directly to the oocyte [178,285]. Thus, congenital infection could occur even in the presence of maternal antibodies [178].

The American Society for Reproductive Medicine and the British Andrology Society state that sperm donors with IgM antibodies not be used as donors because of suggestive active CMV infection [180,340]. As well, the French legislation only allows CMV seronegative donors to be used for artificial insemination [174]. In addition, the American Society for Reproductive Medicine recommends that the serological status of the donor be determined by a CMV specific IgG assay and that semen from seropositive donors only be used for seropositive recipients. The semen samples are frozen and quarantined for 180 days until the donor is shown to be seronegative. Liesnard et al.

evaluated 86 healthy semen donors and found 44 % to be CMV seropositive [167]. Of 126 women candidates for insemination, 48 % were seronegative for CMV. Thus, without matching for CMV antibodies, the risk of seronegative women being inseminated with CMV seropositive semen is 21 %. Seminal quality is not adversely affected during viral shedding [115]. Thus, cryopreserved semen samples from healthy volunteer donors are a possible source of CMV infection to the recipient through artificial insemination [174].

The American Society for Reproductive Medicine suggests that women receiving donor semen should be tested for antibodies to CMV [340]. There are no similar recommendations for recipients of donated oocytes or embryos [226,340]. Seronegative women who receive contaminated gametes could be at risk for a primary infection [340]. However, Witz et al. was unable to detect CMV DNA in unfertilized oocytes and discarded embryos of seropositive women [340].

Diagnosis. The general public is not aware that CMV is the leading cause of mental retardation second only to Down's syndrome [173]. For that reason, the diagnosis of CMV in pregnant women is important to predict congenital infection with CMV. Because most maternal CMV infections are asymptomatic or accompanied by symptoms not specific for CMV, laboratory methods are needed to diagnose CMV infection. Diagnosis is accomplished by detection of virus in blood, urine or saliva or through serology. It is imperative to determine if the infection is primary or recurrent because intrauterine transmission during a primary infection causes more significant fetal damage than during a recurrent infection (Figure 9). It is also vital to determine if primary

infection is present early in gestation (< 16 weeks) because intrauterine transmission causes severe fetal damage during this period of gestation.

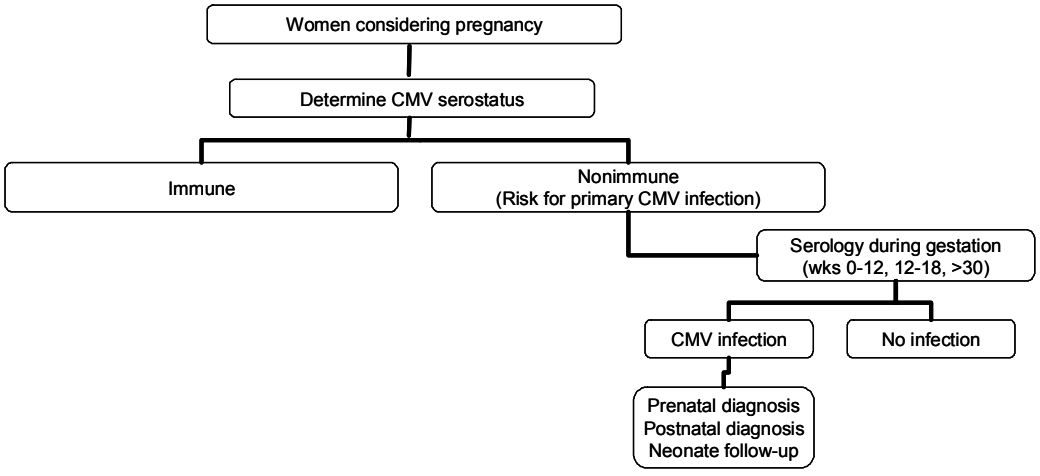


Figure 9. Preventative measures of CMV infection. CMV, cytomegalovirus [173].

Routine screening of women for CMV antibodies has been a challenge [173]. If seroconversion to CMV-specific antibodies is detected than the diagnosis of primary CMV infection is made. However, seroconversion is rare because women are not routinely screened prior to gestation. The detection of CMV IgM is more commonly used as an indicator of active or recent CMV infection. However, it can be produced during a recurrent infection as well [173,292]. Thus the detection of CMV IgM does not indicate that a primary infection is present [173]. Serology is also complicated by Epstein-Barr virus (EBV) which also results in the production of IgM antibodies against CMV. Primary CMV infections can also produce EBV-specific IgM leading to misdiagnosis. Dual infection with both CMV and EBV is also possible. Diagnosis of CMV is of most importance as EBV does not cause congenital infections. In addition, commercial tests which detect CMV-specific IgM range in agreement from 44 to 77 %.

This is due to the different viral preparations used. Both structural (pU_L32, pU_L83, pU_L80a) and nonstructural (pU_L44), PU_L57) viral proteins are required to detect IgM in response to CMV infection. Thus, different stoichiometric compositions of viral antigens are used in the IgM tests. These are based on how the virus is grown and purified. Therefore, no standard for CMV IgM serology exists. Furthermore, there is no standard management of CMV IgM-positive results in pregnant women. Some laboratories screen with 2 or more tests others base their results on one test. Other laboratories used avidity of CMV IgG antibodies, and some patients are not screened at all because of a lack of treatment options during pregnancy. Thus, CMV remains the most common organism for congenital infection.

A need for a standard CMV IgM assay was addressed [173]. A standard could not be based on previous commercial CMV IgM assays because of their lack of concordance. It could also not be based on virus detection because the duration, quantity, and site of viral excretion varies and is intermittent in immunocompetent individuals. However, detection of CMV IgM by a Western blot correlated well with virological data. Therefore, Maine et al. developed 2 versions of a CMV IgM immunoblot that uses both purified virus and recombinant antigens on the same strip [173]. The latest version is a slot blot which consists of a viral section (viral proteins VP150, VP82, VP65, and VP28 purified by preparative gel electrophoresis), a recombinant section (purified recombinant antigens rp150, rp52, rp130, and rp38), and assay validity controls (human μ chain, positive control; E. coli CMP-2-keto-3-deoxyoctulosonic acid synthetase, negative control). A sample was positive for CMV IgM antibodies when both the viral and recombinant sections of the strip contained at least one reactive protein band or when at

least 3 recombinant protein bands were reactive. This test has a high sensitivity (100 %) and specificity (98.6 %) for the detection of CMV-specific IgM and is considered a reference test for CMV IgM serology.

A prototype CMV IgM immunoassay was then developed based on recombinant CMV antigens [173]. The immunoblot was used as a standard to develop the Abbott AxSYM CMV IgM microparticle enzyme immunoassay (MEIA). This test uses 3 purified recombinant antigens coated onto microparticles for detection of CMV-specific IgM. The antigens consist of structural and nonstructural proteins (U_L32, U_L44, U_L83, U_L80a). In the microparticle dilutent are 2 pp150 peptides to alter the detection of low concentrations of CMV IgM antibody as in latently infected individuals. The assay is highly sensitive (96 %) and specific (97 %) and capable of detecting early seroconversion.

Using the immunoblot, a peptide CMV IgM enzyme-linked immunosorbent assay (ELISA) was also developed [173]. Peptides from U_L44 and U_L32 were used in a microtiter ELISA with a sensitivity of 96.9 %. In addition, a Biotest CMV IgM ELISA was also developed. This test uses recombinant antigen from nonstructural proteins of the virus (U_L44 and U_L57) [173]. It has improved detection of acute CMV infection. As of 2001, only the Abbot and Biotest assays are commercially available.

Once pregnant women have been screened with a CMV IgG and IgM assay, IgM positive individuals should be tested with a CMV IgG avidity assay (Figure 10) [173]. The avidity assay measures the binding affinity of IgG in response to infection. Production of IgG antibodies with low avidity occurs during the first few weeks of a primary infection. The avidity of the IgG antibodies increases with time which can then be used to distinguish a primary from a recurrent infection. In a primary infection IgG

avidity is low (< 60 %) compared with reactivation where it is > 80 % [292]. In pregnant women, detection of low avidity CMV IgG indicates that a primary infection occurred in the past 18 to 20 weeks, whereas a high avidity excludes a primary infection [173]. When the avidity assay is performed before 18 weeks of gestation the negative predictive value is 100 %. In a study of women in a prenatal diagnosis program, a high avidity at 6 to 18 weeks of gestation indicated no transmission of virus, whereas, a high avidity at 21 to 23 weeks failed to identify 4 women who transmitted virus. Another study showed that virus was transmitted in < 1 % of cases with high avidity antibodies. Therefore, if avidity testing occurs prior to 18 weeks of gestation it can be used for prenatal diagnosis.

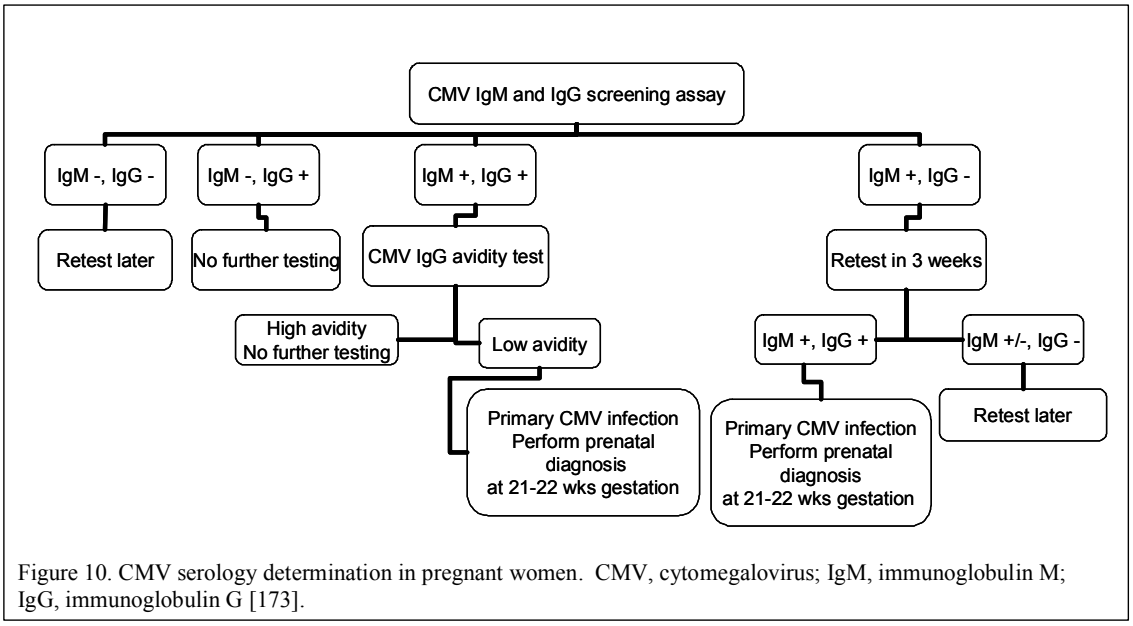


Figure 10. CMV serology determination in pregnant women. CMV, cytomegalovirus; IgM, immunoglobulin M; IgG, immunoglobulin G [173].

Although CMV IgG and IgM titers rise simultaneously in primarily infected immunocompetent individuals, pregnant women have been demonstrated which were only positive for IgM initially and later developed IgG antibodies [173]. Therefore, it is necessary to screen for both CMV IgG and IgM. If CMV IgM is positive then avidity testing is performed. Of the CMV IgM positive specimens from pregnant women,

approximately 90 % will have a high avidity and require no further testing. This indicates that the infection is recurrent.

Avidity testing can only be performed on CMV IgG-positive samples [173]. Therefore, if only CMV IgM is positive, retest in 3 weeks. If the CMV IgG is positive after 3 weeks then perform CMV IgG avidity. If the CMV IgG remains negative then the CMV IgM was a false-positive. False-positive results can occur from EBV cross-reactivity.

Prenatal diagnosis is performed because maternal infection does not mean that fetal infection has occurred [173]. Therefore, once a primary maternal CMV infection has been determined, either through seroconversion or detection of CMV IgM and low avidity CMV IgG, the next step is to verify if intrauterine transmission has occurred. Transmission of CMV to the fetus occurs in 25 to 50 % of primarily infected pregnant women. Virus is excreted during gestation in 5 to 15 % of seropositive women with more virus being excreted in the later stages of gestation. Isolation of virus from the cervix or urine is a poor indicator of intrauterine infection. Also, the viral load in blood shows no correlation with the clinical course of the disease, intrauterine transmission of the virus or severity of the clinical disease in the fetus or neonate. However, it has been shown that the amniotic fluid can be tested to confirm that intrauterine transmission of virus has occurred. This is the specimen of choice for prenatal diagnosis.

Prenatal diagnosis is by virus isolation or nucleic acid testing of amniotic fluid [14,34,292]. The fetus excretes CMV into its urine which enters the amniotic fluid [292]. The detection of CMV in the urine of the fetus is the gold standard because 100 % of congenitally infected neonates excrete CMV in the urine, while, only 70 % are positive

for CMV in the cord vein blood [112]. While time consuming, cell culture is the "gold standard" [107]. Cytomegalovirus is grown on human fibroblasts and the cultures are maintained for 21 days and observed for CPE. Cytopathic effect is demonstrated by prominent cell enlargement, a basophilic nuclear inclusion, and a large, well-defined, eosinophilic perinuclear inclusion [226]. The nuclear inclusion is accumulation of nucleocapsids. The perinuclear inclusion is a complex of nucleocapsids, dense bodies and Golgi apparatus. Progeny virus accumulates in the cytoplasm of infected cells and appears in tissue culture fluids approximately 96 to 120 hours postinfection. Cell culture is the "gold standard" but can be time consuming [107]. The detection of virus by PCR in amniotic fluid does not mean that the fetus will be congenitally infected [173]. Qualitative methods of virus detection have a poor positive predictive value (48.5 %) and are not used to predict the clinical outcome of the neonate. They do have a high negative predictive value (100 %) and can exclude infection when the result is negative. However, in liver and renal transplant recipients, there is a strong correlation between viral load detected by quantitative PCR and CMV disease post-transplant. These quantitative virological methods can also be used in pregnant women and neonates (Figure 11). A quantitative PCR value of 10^3 genome equivalents (GE)/mL has to be reached for CMV infection to be present. On the other hand, a value of 10^5 GE/mL has to be reached for CMV disease to be present. The combination of qualitative and quantitative PCR of amniotic fluid can be used to differentiate no infection, CMV infection, and CMV disease.

Virus might be undetectable until 6 weeks after maternal infection [34]. Others have suggested waiting until 20 weeks of gestation to test the amniotic fluid because false negative results have been reported if the amniocentesis is performed prior to 20 weeks of

gestation [173,292]. Also, severe CMV disease generally occurs in the first 12 weeks of gestation followed by a 6 to 9 week time period from maternal infection until the virus is detectable in the amniotic fluid [173]. Therefore, a quantitative PCR performed at 21 to 22 weeks of gestation can assess the potential clinical outcome of the pregnancy. Nevertheless, the demonstration of an in utero infection is not a prediction of neonatal sequelae [34].

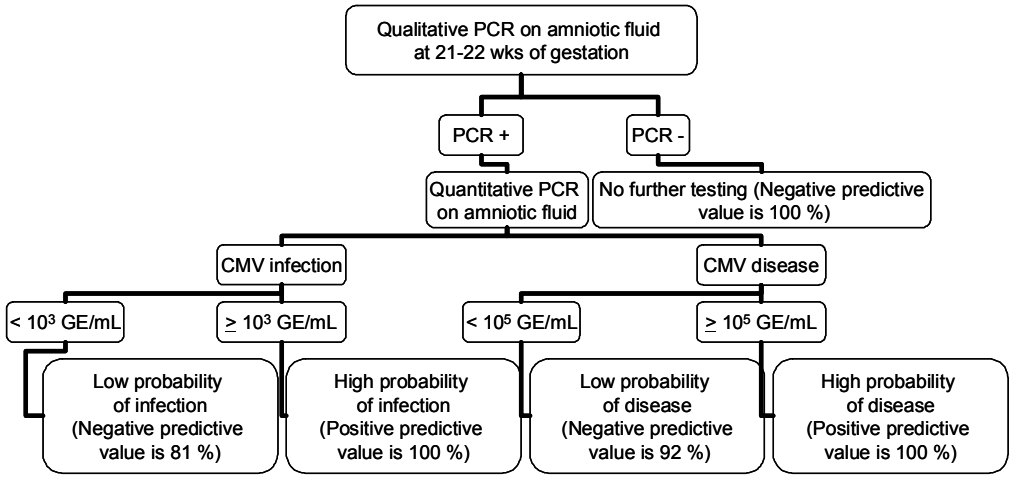


Figure 11. Prenatal diagnosis of CMV in pregnant women. PCR, polymerase chain reaction, CMV, cytomegalovirus, GE, genome equivalents [173].

Postnatal diagnosis of newborns is necessary to confirm transmission of virus to the neonate [173]. This is performed in neonates that are symptomatic at birth as well as in those born to women who had a primary CMV infection during gestation. Confirmation is accomplished by detecting virus in urine or saliva within the first week of life [14,173]. Diagnosis is by virus culture, shell vial assay or PCR [292]. Because the shell vial assay of urine is 100 % sensitive and specific, it is preferred over PCR [14]. A high viral load in peripheral blood leukocytes, as determined by quantitative PCR,

correlates well with symptomatic congenital CMV infection [173]. The median viral load was 3000 GE/10^5 peripheral blood leukocytes in symptomatic neonates contrasted with 30 GE/10^5 peripheral blood leukocytes in asymptomatic neonates. The viral load persisted longer in symptomatic neonates but was reduced by treatment with ganciclovir. Thus, the outcome of the antiviral treatment can be monitored.

Furthermore, since most congenitally infected neonates are asymptomatic at birth and primary maternal infection during gestation can be asymptomatic, failure to test asymptomatically infected neonates can occur [173]. If these neonates are not diagnosed and treated, they will likely develop sensorineural hearing loss. It is, thus, imperative that women be screened for CMV infection during gestation to identify at risk pregnancies (Table 3).

In stillborn children, CMV antigens can be present in almost all organs whereas, in live-born infants the antigen is restricted to the lungs, liver, spleen, bone marrow, and CNS [112].

Table 3. Diagnostic tests for cytomegalovirus. CPE, cytopathic effect; CMV, Cytomegalovirus [173,226].

Test	Sample	Advantages and Disadvantages
Virus culture	Urine, blood, tissue	3-4 weeks for results; Expense; Confusion with adenovirus CPE; specimen contamination
Viral Nucleic Acid Detection Nucleic acid testing	Urine, blood, CSF, tissue	Rapid detection of virus; Quantification of viral load; Virus strain typing; Expense; Contamination
In situ hybridization	Tissue	Definitive demonstration of CMV
Histophathology	Tissue	False negative rate high
Antigen Detection Direct immunofluorescence	Blood, urine	Rapid detection of virus within 48 hours
Antibody Detection IgG-enzyme immunoassay	Serum	Seroconversion takes 2-3 weeks and need 2 samples; False seroconversion with administration of blood products; Detect acute infection
IgM-EIA	Serum	Rare cross reactivity with EBV; 5 % are seropositive 2 years post acute infection; Sensitivity of single cord blood is 70-80 %
Complement fixation	Serum	False negative in 2-5 % of cases

Control. Current antiviral treatment has limited efficacy and significant toxicity [226]. No antiviral treatment has been shown to be safe for pregnant women experiencing a primary CMV infection during gestation [336]. Treatment of neonatal cytomegalovirus can be attempted with ganciclovir [112,336]. Ganciclovir has

significant antiviral activity as well as bone marrow suppression and gonadal toxicity [335]. In one study, 10 symptomatic infants were treated with ganciclovir [112]. Nine of the ten infants did not show clinical signs at 6 months of age. In another study, ganciclovir administered at 12 mg/kg/day in two divided doses led to a significant reduction of virus in the urine [335]. Several children developed neutropenia, thrombocytopenia and elevated liver function tests however, 81 % of the children were able to complete a 6 week course of treatment. Two years after treatment 24 % of the children developed normally.

Preventative measures should be taken in pregnant women to avoid congenital CMV infection [173]. Women contemplating pregnancy should be screened for CMV-specific IgG and IgM prior to conception. Therefore, seronegative women can be advised on prevention of primary CMV infection. They should practice good hygiene especially if they are exposed to young children. This includes washing hands after diaper changes, avoidance of kissing children on the mouth, and not sharing food, drinks and eating utensils [14,173]. In addition, blood to be used for blood transfusions should be from seronegative individuals or should be passed through in-line filters capable of retaining leukocytes [107]. As well, recombinant vaccines against glycoprotein B of CMV are being developed but no vaccine is currently licensed [107,226].

Antiviral Agents

Antiviral agents have been developed for the treatment of HSV over the past 40 years [42]. Treatments are available for primary and recurrent HSV infections. When selecting treatment, it is important to consider the immune status of the host, the site of infection and whether the infection is primary or recurrent.

Because herpesvirus establishes latency and reactivation occurs in the presence of humoral and cell-mediated immunity, vaccines are difficult to develop [42]. A recombinant truncated gD2 (glycoprotein D of HSV-2) combined with alum and the adjuvant MPL (3-de-O-acylated monophosphoryl lipid A) vaccine is in phase III of clinical trials for the prevention of genital herpes in seronegative women. In the interim, improvements need to be made in the treatment of these diseases [173].

Acyclovir is the prototypic antiviral drug for treatment of viral infections [334]. It was first synthesized in 1974 [336]. Acyclovir (9-([2-hydroxyethoxy]methyl) guanine) is a synthetic acyclic purine nucleoside analogue of guanosine [334,336]. Transportation of acyclovir into cells occurs by the nucleoside transporter that also transports guanine [125]. Herpes simplex virus contains thymidine kinase which phosphorylates acyclovir to its monophosphate derivative (Figure 12) [46,334]. This allows acyclovir to become active only in virus infected cells because uninfected cells do not efficiently phosphorylate acyclovir [205]. Cellular kinases convert the monophosphate form to acyclovir di- and triphosphate [334,336]. The triphosphate form competes with deoxyguanosine triphosphate (dGTP). Acyclovir lacks a 3´ hydroxyl group necessary for phosphodiester bond formation with an incoming nucleoside triphospate and is therefore unable to be

incorporated into the elongating DNA molecule [114,125,334]. It therefore inhibits viral DNA polymerase activity and leads to complete and irreversible inhibition of further viral DNA synthesis [46,125,198,334,336]. Thus, acyclovir is also an obligate DNA chain terminator [114,125,334]. Of the herpesviruses, only HSV and VZV encode thymidine kinase capable of phosphorylating acyclovir [334]. Cytomegalovirus metabolizes acyclovir to suppress reactivation of CMV infection. Uninfected cells do not phosphorylate acyclovir to its monophosphate derivative; therefore, the concentration of acyclovir is 40 to 100 times higher in infected cells [125,336]. Also, viral DNA polymerase has a 10 to 30-fold greater affinity for acyclovir triphosphate than does cellular DNA polymerase [336]. Thus, acyclovir is minimally toxic to normal host cells. The oral bioavailability of acyclovir is 15 to 30 % therefore an L-valine ester of acyclovir (valacyclovir) was synthesized to increase bioavailability [214,334].

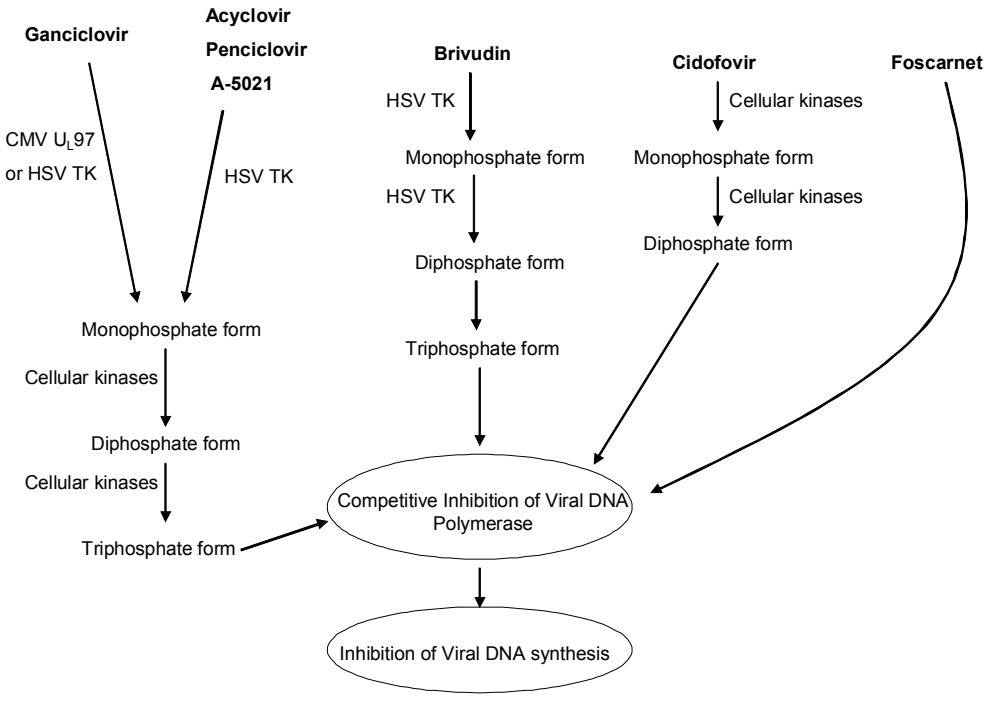


Figure 12. Mechanism of action of antiviral drugs. CMV, cytomegalovirus; HSV, herpes simplex virus; TK, thymidine kinase [125,205,214].

Valacyclovir (2-[2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl-methothy]ethyl-L-valinate hydrochloride) is converted (> 99 %) to acyclovir by valacyclovir hydrolase [46,114]. The remaining metabolism is the same as acyclovir [46,201]. Valacyclovir's bioavailability is 50 % which is 3 to 5 times that of oral acyclovir [239,334]. This permits less frequent dosing than acyclovir (1 to 3 times daily compared with 5 times daily) [239]. There are no pediatric formulations of valacyclovir, whereas, acyclovir is available intravenously or orally for pediatric patients [214,334]. Adverse effects associated with intravenous acyclovir and valacyclovir are inflammation at the site of administration, alterations in renal function if administered too rapidly, and encephalopathic changes if extremely high doses are used [334]. Dehydration and preexisting renal disorders are risk factors for renal toxicity [336]. In patients with renal disturbances, acyclovir can be administered slowly or at a smaller dose [214]. It has not been shown to affect bone marrow, liver or spermatogenesis [114,334]. Orally administered acyclovir has few adverse reactions [334]. When administered orally to neonates following intravenous treatment, neutropenia has been demonstrated [334,336]. The safety of acyclovir administration during pregnancy has not been established [336]. Acyclovir does cross the placenta and can concentrate in the amniotic fluid which can result in potential renal toxicity in the fetus. More than 1000 women who received acyclovir before or during early pregnancy did not have an increased rate of miscarriage or birth defects [46]. However, it is recommended that acyclovir only be given to pregnant women if the expected benefit outweighs the risk to the fetus. Acyclovir has been studied to determine if its use in pregnant woman with recurrent genital HSV will reduce the rate of the need for a cesarean section [336]. Treatment initiated at 38 weeks of gestation did decrease

the rate of the need for a cesarean section from 26 % to 0 %. No neonates developed HSV infection, and no toxicity was reported in the neonates. While acyclovir is excreted in human breast milk, no adverse effects have been noted in breast-fed infants [46].

Ganciclovir (9-(1,3-dihydroxy-propoxymethyl)guanine), a nucleoside analogue, is also a DNA polymerase inhibitor [46,181]. It is structurally similar to acyclovir, differing only by the presence of a hydroxymethyl group [114]. It has good activity against HSV and CMV [214]. It is converted to the triphosphate form by the protein kinase of CMV, U_L97 [214,336]. This enzyme (U_L97) is essential for virus replication [214]. The triphosphate form is then incorporated into the newly synthesized DNA chain and inhibits DNA synthesis [46]. This is accomplished by inhibiting DNA polymerase and by inducing premature chain termination. Ganciclovir was the first licensed antiviral treatment of CMV retinitis [336]. Ganciclovir is mutagenic, teratogenic, and carcinogenic. It causes neutropenia and thrombocytopenia in humans and has been shown to cause testicular atrophy in rodents. Ganciclovir is the drug of choice only for CMV in transplant recipients or AIDS (acquired immunodeficiency syndrome) patients [214]. Preemptive treatment is recommended for CMV-seronegative patients receiving donor material from CMV-positive patients. Treatment in neonates should only be reserved for the most severe cases with a dosage of 5 to 6 mg/kg intravenously [336]. Due to its low oral bioavailability (6 %), therapy requires placement of an indwelling venous catheter for long-term therapy [214,336]. Postnatal treatment will not prevent the neurodevelopmental sequelae of intrauterine CMV infection but it might reduce the severity of the signs [14]. Resistance can occur with ganciclovir through mutations in the U_L97 phosphotransferase gene [114]. Mutations at codons 460, 594, and 595 of the U_L97

gene are the most common. Isolates will remain susceptible to foscarnet and cidofovir. In addition, resistance can occur with mutations in the U_L54 DNA polymerase gene [114,226]. In these cases, cross-resistance with foscarnet and cidofovir can occur [114].

Ganciclovir's prodrug is valganciclovir [328]. Valganciclovir has a bioavailability 10-fold that of oral ganciclovir in CMV-HIV (human immunodeficiency virus) infected patients and liver transplant recipients [239]. If it is demonstrated to be safe and equally efficient, oral valganciclovir will replace intravenous ganciclovir.

Famciclovir (9-(4-hydroxy-3-hydroxymethylbut-1-yl) guanine), a synthetic acyclic guanine derivative, is a diester prodrug of penciclovir (Figure 13) [334]. Famciclovir is rapidly deesterified to penciclovir. Penciclovir (9-(4-hydroxy-3-hydroxy-3-hydroxymethylbut-1-yl) guanine), an acyclic nucleoside analog of guanosine, is phosphorylated to penciclovir monophosphate by HSV and VZV thymidine kinase. Cellular kinases phosphorylate penciclovir monophosphate to the active triphosphate form which is a competitive inhibitor of viral DNA polymerase. Penciclovir has a 3´ hydroxyl group that is capable of being incorporated into the growing DNA radical. In addition, penciclovir is a conditional viral DNA chain terminator [46]. The viral DNA chain continues to extend for a limited time after incorporation [114]. Famciclovir has only been evaluated in adult patients for the treatment of recurrent HSV and VZV [334]. A study showed that semen parameters were not affected in 18 men treated with famciclovir [201]. Famciclovir is administered 2 to 3 times a day [214]. Because of its extremely low bioavailability, penciclovir is only available topically for the treatment of HSV labialis [46,334]. It is not as efficacious as oral therapy with acyclovir or valacyclovir [334].

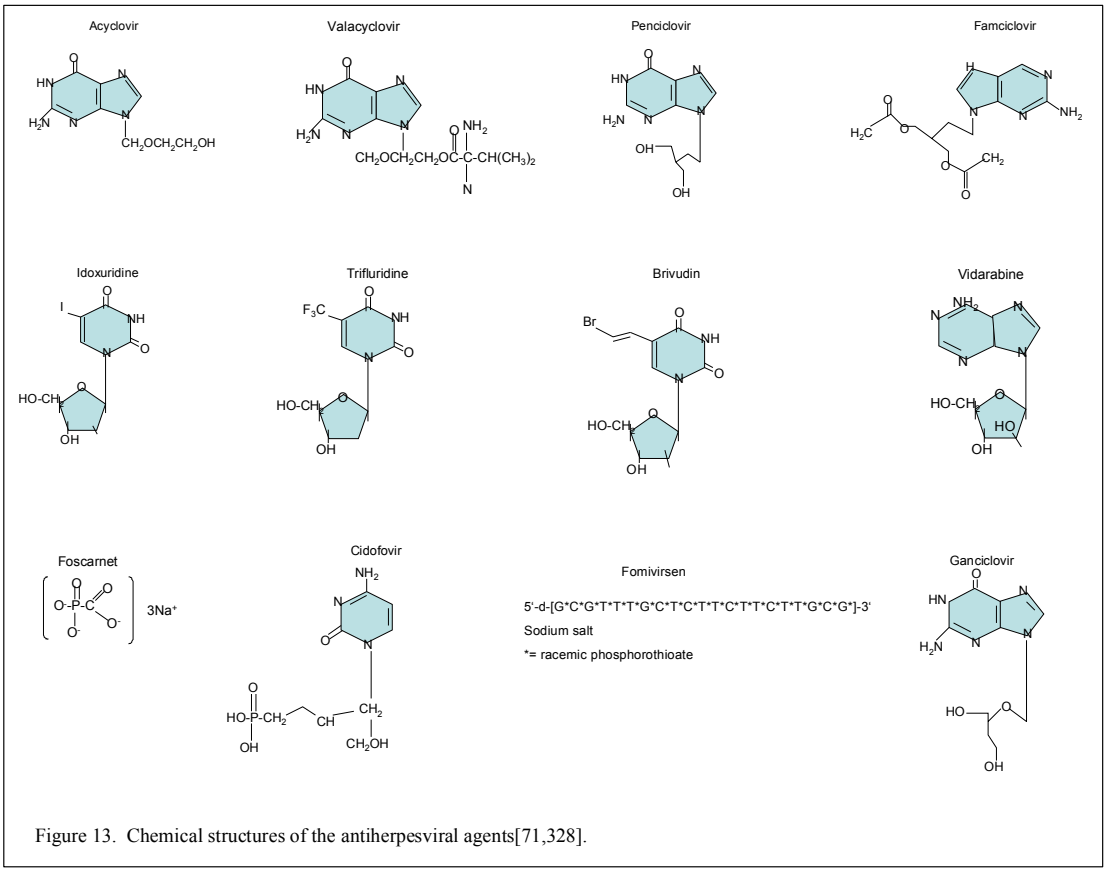


Figure 13. Chemical structures of the antiherpesviral agents[71,328].

Foscarnet (trisodium phosphonoformate and phosphonoformic acid) and fomivirsen are non-nucleoside antivirals [114,328]. Foscarnet is a pyrophosphate analogue [125,334]. It functions by selectively inhibiting virus-specific DNA polymerase at the pyrophosphate-binding site [201,334]. By preventing cleavage of pyrophosphate from deoxynucleotide triphosphate, viral replication is inhibited and thus elongation of DNA [46,125,334]. Phosphorylation is not required as with nucleoside analogues, therefore patients who are resistant to acyclovir, valaciclovir, penciclovir, and ganciclovir can be treated with foscarnet [334]. It is approved for the treatment of CMV retinitis and acyclovir-resistant HSV infections [46,334]. Oral bioavailability is low, thus intravenous administration is the only formulation available [114]. Treatment has been shown to affect renal function and electrolyte balances; therefore it should be administered slowly,

prehydration should be administered, and dose adjustment should be made based on creatinine clearance [214,334]. Symptoms associated with treatment are fever, nausea, anemia, diarrhea, headaches, and seizures [334]. Its safety in pregnancy has not been thoroughly evaluated [114]. A single case report of treatment in a patient at 32 weeks gestation did not indicate any adverse effects in the infant [201]. Resistance to foscarnet involves alteration in binding to viral DNA polymerase [114,125]. Cidofovir is an option for resistant isolates [114].

Fomivirsen inhibits CMV replication by an antisense mechanism [46,214]. It prevents viral replication by binding to complementary CMV mRNA sequences [214]. Translation of CMV immediate early proteins is then inhibited [71,214]. Because the mode of action is independent of viral thymidine and protein kinase, it can be used for strains of CMV that are resistant to ganciclovir, foscarnet, or cidofovir [214]. It is available for intravitreal injection in the salvage treatment of AIDS patients with CMV retinitis.

Cidofovir ((S)-1-[3-hydroxy-2 (phosphonylmethoxy)-propyl] cytosine, HPMPC) is a cytosine derivative [114,328]. It inhibits viral DNA synthesis independently of viral TK [214]. Cidofovir enters the cell by fluid-phase endocytosis [303]. Cidofovir requires a 2-step activation by cellular kinases [214]. Cidofovir is converted to the diphosphate form which then inhibits the viral DNA polymerase by competitive inhibition with dCTP [114,214]. All human herpesviruses are susceptible to cidofovir. However, CMV strains that are highly resistant to ganciclovir (mutations in both U_L97 and DNA polymerase gene) have been shown to be cross-resistant to cidofovir. Oral bioavailability is low making intravenous administration necessary. Intravenous administration is

advantageous in that it can be given biweekly, but the renal toxicity may outweigh the benefits [336]. In addition, oral probenecid will help diminish cidofovir accumulation in the renal tubular cells [214].

Bila et al. showed that cidofovir has embryotoxic effects [32]. They evaluated cidofovir at varying concentrations on diploid human embryo lung cells (HEL) and rat embryo fibroblasts (REF). On HEL cells, cidofovir caused 28 % chromosomal aberrations at a concentration of 3 μg/mL. An increase to 30 μg/mL of cidofovir caused complete cessation of cell division without cytological changes. On REF cells, 5 μg/mL of cidofovir caused 8 % chromosomal aberrations. This percent increased to 100 with 100 μg/mL of cidofovir. Increasing the dose to 300 μg/mL caused complete cessation of cell division without cytological changes. In addition, they showed that cidofovir at concentrations of 0.1 to 1 μg injected into chicken embryos adversely affected the growth of the caudal trunk. They also demonstrated that cidofovir injected into day 2 to 4 chicken embryos resulted in early embryonic death when administered at day 2. The embryos at more advanced stages of development were less sensitive to the effects of cidofovir. Finally, cidofovir was administered intraperitoneally to rats at day 12 of pregnancy. On day 22 the females were euthanized and the fetuses were examined. At 100 mg/kg of cidofovir, no fetuses survived. At 20 mg/kg cidofovir, 1 of 3 litters was completely resorbed.

According to the inserted product information from Gilead Sciences, rats which received cidofovir subcutaneously at 1 mg/kg/day from day 7 of gestation to day 21 postpartum did not have adverse effects on viability, growth, behavior, sexual maturation or reproductive capacity in the offspring [96]. However, rats which received 1.2

mg/kg/day (0.09 times the human recommended dose) cidofovir intravenously up to 6 weeks prior to mating and for 2 weeks post mating had decreased litter sizes and fewer live pups per litter. In addition, early resorptions per litter were increased. Also, rats which received 1.5 mg/kg/day of cidofovir and rabbits which received 1 mg/kg/day intravenously had fetuses with reduced body weights. In the rabbits, the fetuses also had an increased incidence of meningocele, short snout and short maxillary bones. However, no embryonic effect was seen at a dose of 0.5 mg/kg/day in rats and 0.25 mg/kg/day in rabbits. This is 0.04 and 0.05 times, respectively, the clinical dose for humans.

Brivudin is a uracil-containing compound effective against HSV[214]. Sorivudine (1-β-D-arabinofuranosyl-E-5[2-bromovinyl]-uracil) is a close analogue of brivudin [114,214]. Sorivudine has activity against HSV-1 but not HSV-2 or CMV [114]. Thymidine kinase mediates sorivudine's phosphorylation to the mono- and diphosphate forms. Cellular enzymes complete the metabolism to the triphoshpate form which is then a competitive inhibitor of viral DNA polymerase but does not act as a DNA chain terminator. Its development has been halted due to fatal interactions with fluorouracil [214].

Trifluorothymidine inhibits thymidylic phosphorylase and viral DNA polymerase which is required for the incorporation of thymidine into viral DNA [334]. It also has the potential to incorporate into host cell DNA, thus its toxicity is higher than that of nucleoside analogues. It is a topical ophthalmic ointment which is the treatment of choice for HSV keratoconjunctivitis. Trifluorothymidine (1 %) is administered as 1 drop per eye every 2 hours (maximum 9 drops daily per eye) until reepithelization of corneal ulcer occurs and then 1 drop per eye every 4 hours while awake (minimum 5 drops daily

per eye) for 7 days [336]. Maximum duration of treatment is 21 days. Trifluorothymidine has not been evaluated in neonates. Affected neonates should be concomitantly treated with intravenous acyclovir and evaluated for systemic HSV infection.

Trifluridine (5-trifluoromethyl-2′-deoxyuridine) is a fluorinated pyrimidine nucleoside with activity against HSV-1, HSV-2, and CMV [46,114]. It is phosphorylated to the triphosphate form which is a competitive inhibitor of HSV DNA polymerase [46,114]. Trifluridine is a 1 % ophthalmic solution that is FDA-approved for treatment of primary keratoconjuctivitis and recurrent epithelial keratitis caused by HSV-1 and HSV-2. Trifluridine is also useful for treatment of acyclovir-resistant mucocutaneous herpes simplex infections [46].

Idoxuridine (5-iodo-2′-deoxyuridine) is a thymidine analogue which acts by inhibiting viral DNA polymerase and as a chain terminator [114,334]. It also has potential to be incorporated into host cell DNA [334]. It is available topically for the treatment of HSV keratoconjunctivitis.

Vidarabine (9-β-D-ribofuranosyladenine,ara-A, Vira-A), a purine nucleoside analogue of adenine, is phosphorylated to its active 5′ triphosphate derivate [114,334]. This form competitively inhibits DNA-dependent DNA polymerases of DNA viruses. The viral polymerase inhibition is greater than that of the host cell polymerase inhibition, but the therapeutic index is narrow [334]. Vidarabine also is incorporated into the viral DNA chain where it is a chain terminator [114]. It is available as a topical ophthalmic ointment for the treatment of HSV keratoconjunctivitis [334]. Adverse reactions to

ophthalmic preparations of triflurothymidine, idoxuridine, and vidarabine are local irritation, photophobia, corneal edema, punctate keratopathy, and keratitis sicca.

Although not approved, vidarabine has been shown to be effective for treatment of neonatal HSV infection [336]. Vidarabine administered at 15 mg/kg/day as a continuous infusion for 12 hours over 10 to 14 days resulted in a decrease in mortality from 75 % to 40 % in infants with disseminated or CNS disease. Of the survivors, 50 % developed normally. Progression of disease from skin, eye and mouth infection to encephalitis or disseminated form was decreased from 70 % to 42 %. Increasing the dosage to 30 mg/kg/day decreased the progression to 4%. Severe neurologic impairment was decreased from 30 % to 10 % with treatment.

In a comparison between neonatal treatment with acyclovir and vidarabine, no difference was noted in mortality rate [336]. In skin, eye and mouth affected neonates, 90 % and 98 % treated with vidarabine and acyclovir, respectively, developed normally to 2 years of age. For those neonates who survived encephalitis, 43 % and 50 % of those treated with vidarabine and acyclovir, respectively, developed normally. Neonates that survived disseminated infection had a normal development in 62 % and 57 % of vidarabine and acyclovir treatment, respectively. Thus, no difference was noted between mortality or morbidity rates for vidarabine and acyclovir. Because acyclovir has a safer toxicity profile, it is the drug of choice (Table 4) [114].

Table 4. Antiherpesviral agents and their side effects. HSV, herpes simplex virus; CMV, cytomegalovirus; GI, gastrointestinal; CNS, central nervous system [114,334].

Antiherpes viral agents	Indication	Major toxicities
Acyclovir	**Immunocompetent**	Headache, nausea, neurotoxicity, nephrotoxicity
	Genital HSV Primary Recurrent Suppression	
	Herpes simplex encephalitis	
	Neonatal HSV	
	Immunosuppressed	
	Mucocutaneous HSV Primary Recurrent Suppression	
	Invasive HSV	
	CMV prophylaxis in transplant recipients	
Valacyclovir	Recurrent genital HSV	Same as acyclovir
Famciclovir	Recurrent genital HSV	Headache, nausea
Ganciclovir	CMV hepatitis, GI or CNS disease	Neutropenia, thrombocytopenia
	CMV pneumonia in bone marrow transplant recipient	
	Pre-emptive CMV therapy	
Cidofovir	CMV retinitis	Nephrotoxicity, cardiomyopathy, rash, iritis
	Acyclovir and Foscarnet-resistant HSV infection	
Foscarnet	CMV disease	Nephrotoxicity, electrolyte disturbance, neurotoxicity, anemia, neutropenia
	Acyclovir-resistant HSV infection	
Docosanol	Recurrent herpes labialis	Headache, pruritis
Penciclovir	Recurrent herpes labialis	Headache, pruritis
Idoxuridine	Herpes simplex keratitis	Local eye irritation, mutagenic, carcinogenic and teratogenic potential
Vidarabine	Herpes simplex keratitis	Nausea, vomiting, diarrhea, leucopenia, thrombocytopenia, neurotoxicity
Trifluridine	Herpes simplex keratitis	Local eye irritation, mutagenic, carcinogenic and teratogenic potential

Docosanol blocks viral entry into the host cell by inhibiting the fusion between the host cell plasma membrane and the HSV envelope [42]. Docosanol is available as a topical cream for the treatment of herpes labialis.

A-5021 is a new guanosine analogue [214]. It combines the advantages of both acyclovir and penciclovir. It depends on HSV TK for its first activation step. Phase 1 trials have been completed and warrant further clinical development.

Phosphonoacetic acid is an effective inhibitor of HSV growth in tissue culture cells as well as other herpesviruses [126]. It is the related analog of foscarnet [125]. It is a virus-specific DNA polymerase inhibitor [58,126]. Phosphonoacetate inhibits the DNA polymerase by interacting at the pyrophosphate binding site [164]. Phosphonoacetic acid is used to inhibit late gene expression [156]. However, it is toxic to cells, is irritative to skin, accumulates in bone and is toxic to laboratory animals [147,257].

Lactoferrin has been shown to have antibacterial, antiviral, antifungal, and antiprotozoal effects [20,282]. It is produced by mucosal epithelial cells [20]. Lactoferrin is a mammalian iron-binding glycoprotein that is found in milk, saliva, tears, bile, seminal and vaginal fluids, synovial fluid, mucous secretions and secondary granules of neutrophils [20,282,303]. The concentrate on of lactoferrin in bovine milk is 0.02 to 0.2 mg/mL [183]. Lactoferrin is an 80 kDa glycoprotein that is folded into two homologous lobes (N-lobe and C-lobe) [176,261,303]. These two lobes are connected by a 'hinge region' [302]. Each lobe is divided into two structural domains (N1, N2 and C1, C2) [261]. A lactoferrin molecule can bind two iron atoms [282]. This is dependent on the simultaneous binding of anions such as carbonate or bicarbonate which play roles in firmly holding iron. Therefore, lactoferrin has an iron-free (apo) and an iron-bound (holo) state. Its antibacterial effect is due to depriving bacteria of iron required for their survival [20]. It might also bind directly to the cell wall of bacteria and fungi and thus cause membrane damage and leakage of intracellular components [20,302]. Lactoferrin is

actively secreted by neutrophils during an inflammatory response and is a component of colostrum and milk to prevent infections in neonates [20,282]. It also acts indirectly by enhancing the cytotoxic effects of monocytes and natural killer cells [20,302]. Lactoferrin has been shown to inhibit the in vitro replication of HSV-1, HSV-2, human cytomegalovirus, human immunodeficiency virus, rotavirus, respiratory syncytial virus, human hepatitis B virus, human hepatitis C virus, hantavirus and simian SA-11 rotavirus and poliovirus type 1 [20,282,303]. Further, bovine lactoferrin has been shown to be the most effective antiviral among the transferrins [20]. In addition, lactoferrin is readily available, has low cytotoxicity and has low cost.

Lactoferrin expression during murine embryogenesis has been examined [327]. The expression of lactoferrin in the reproductive tract is under the control of estrogen from the uterus of the mouse. Lactoferrin is at high concentrations in the uterine epithelia during the first 2 days of pregnancy and returns to basal concentrations by days 3 to 4 (the time of implantation). This corresponds with the preovulatory surge of estrogen and the subsequent down-regulation as progesterone is released from the corpus luteum. In the mouse embryo, lactoferrin is first expressed in the 2- to 4-cell embryo and continues until the blastocyst stage. At the 16-cell stage, lactoferrin mRNA is produced by the inner cell mass and the protein is taken up the trophectodermal cells. This distribution continues until the blastocyst stage. It is then not expressed again until the last half of gestation when it is localized to the neutrophils after the onset of myelopoiesis (Day 11.5) and the epithelial cells of the respiratory and digestive system.

The antiviral activity of lactoferrin occurs in the early phase of infection at the level of viral attachment without affecting normal host-cell metabolism [20,282].

Glycosaminoglycan (GAG) chains are part of the cell surface [176]. When these chains are attached to core proteins they are called proteoglycans. The GAG chains consist of repeating, sulfated disaccharide units. In heparan sulfate, the disaccharide unit consists of uronic acid and glucosamine. Chondroitin sulfate contains galactosamine as the amino residue. The glycoproteins gC and/or gB of HSV-1 bind to heparan sulfate on the cell surface. If heparan sulfate is not present, the glycoproteins can bind to chondroitin sulphate on the cell surface [7]. The positively charged N-terminus of the lactoferrin binds to GAG [302]. Binding of lactoferrin to the cell surface GAG prevents gC of HSV-1 from attaching to the cell [176].

As well, Tanaka et al. demonstrated that bovine lactoferrin is effective against canine herpesvirus (DFD-6 strain) on Madin Darby canine kidney cells (MDCK) at concentrations ranging from 0.125 to 1 mg/mL [282]. Although, this is higher than the concentrations of lactoferrin found in canine milk (0.05 mg/mL), they showed that bovine lactoferrin is not cytotoxic to the cells. The cells growth was enhanced which shows that the antiviral effect was not due to cytotoxicity. They also evaluated human lactoferrin, bovine transferrin and ovotransferrin. Only bovine and human lactoferrin were effective against canine herpesvirus. This illustrates that the antiviral effect of lactoferrin is not dependent on the species of origin and it is not a general property of the iron transporter protein family. In addition, both apo- and holo-bovine lactoferrin were evaluated. Both forms inhibited canine herpesvirus indicating that the iron complex formation is not involved with the antiviral effect. This has also been shown with HSV-1 and HSV-2 [302]. It is suggested that bovine lactoferrin competes with cell receptors for canine herpesvirus attachment. Lactoferrin might bind to glycosaminoglycans which then

prevent the virus binding to the host cell [282]. Both bovine and human lactoferrin have positive charges at the N-terminal domain which is important for binding to glycosaminoglycans. As well, the N-terminal region of lactoferrin forms a loop which is designated lactoferricin. This structure contains a basic amino acid cluster that is responsible for antibacterial activity.

Also, Beaumont et al. evaluated the effect of lactoferrin from bovine colostrum against feline herpesvirus strain 727 on Crandell-Reese feline kidney cells (CRFK) [20]. Exposure of CRFK cells with 0.5 to 10 mg/mL lactoferrin for 30 minutes prior to virus adsorption inhibited 91 % viral replication. Exposure of virus with lactoferrin prior to virus adsorption inhibited 90 % viral replication. Addition of the lactoferrin after virus adsorption did not cause an inhibition of viral replication relative to the control. As well, they demonstrated that there was no synergistic effect when lactoferrin was added at more than one stage of virus adsorption. When lactoferrin was added pre-adsorption and during adsorption the mean viral replication was 90 %. Lactoferrin exposure during virus adsorption and post adsorption resulted in a mean viral replication of 93 %. And lactoferrin addition during all three stages of virus adsorption resulted in 93 % viral inhibition. The concentration of the lactoferrin in these 6 experiments did not affect viral replication. Also, there was no cytotoxicity seen with the lactoferrin.

Beaumont's study contrasts slightly with Hasegawa's study evaluating the effect of lactoferrin against HSV-1 and CMV [122]. Hasegawa et al. showed that when human embryo lung cells were incubated with lactoferrin for 5 minutes prior to virus adsorption, 93 % of virus replication was inhibited [20,122]. However, when lactoferrin was added

to the virus inoculum prior to virus adsorption only 58 % of virus replication was inhibited [122]. This suggests that lactoferrin inhibits viruses in different ways [20].

Lactoferrin, administered orally, has also been evaluated in vivo [323]. Wakabayashi et al. showed that oral feeding of 1.5 % bovine lactoferrin to mice did not eradicate HSV-1 (strain HF) infections. However, the treatment did prevent both loss of body weight and a decrease in splenocyte number. Also, the serum interleukin-18 (IL-18) concentration and splenocyte production of IFN-γ and IL-12 were increased. A preliminary human trial is now under way in patients with recurrent genital herpes to evaluate the effectiveness of lactoferrin.

In addition, van der Strate et al. demonstrated that bovine lactoferrin works synergistically with cidofovir to inhibit CMV (RC256 strain) on fetal lung fibroblasts [303]. Because resistance to antiviral agents can arise, and long-term treatment can result in undesirable side effects associated with cytotoxicity, combination therapy is an option to overcome these negative effects. Lower concentrations of the toxic antiviral agents can be used to achieve the same result as conventional treatment [302]. Since lactoferrin has been shown to act synergistically with antifungal drugs against *Candida* and with zidovudine against human immunodeficiency virus-1, van der Strate evaluated the combined antiviral effect of bovine lactoferrin with acyclovir, ganciclovir, foscarnet and cidofovir [6,303]. Because the effect of lactoferrin is primarily at the level of virus adsorption or penetration [120], lactoferrin and the antivirals were added to the cells 15 minutes prior to addition of the virus [303]. The combination of lactoferrin with acyclovir and foscarnet resulted in antagonism. The combination of lactoferrin and ganciclovir revealed neither antagonism nor synergy. The combination of lactoferrin (8

μg/mL) with cidofovir (4 μg/mL) resulted in marked synergy. The virus was inhibited 42 % more efficiently than theoretically expected on basis of the individual dose-response curves of both agents. The anti-CMV activity for bovine lactoferrin has been shown to have IC_{50} (inhibitory concentration) values of 7, 15 (RC256 strain) and 36 μg/mL (AD169 strain) depending on the strain of virus [21]. In addition, lactoferrin has been shown to be non-cytotoxic to human fetal lung fibroblasts in concentrations up to 2 mg/mL. Combination therapy uses antiviral compounds that have different mechanisms of action so that simultaneous inhibition occurs at different steps of the viral replication cycle [303]. Another theory is that the glycoprotein serves as a carrier molecule to specifically deliver the antiviral agent into infected cells where the antiviral drug will then inhibit virus replication.

In another combination therapy study, Andersen et al. showed that bovine lactoferrin acts synergistically with acyclovir against HSV-1 (strain MacIntyre) and HSV-2 (strain G) on Vero cells and MRC-5 (human diploid lung fibroblasts) cells [6]. Lactoferricin B was also evaluated in combination with acyclovir. It is a peptide which is produced by pepsin cleavage from the N-terminal of lactoferrin. Lactoferrin was more effective than lactoferricin and less cytotoxic. Also, the bovine form of lactoferrin was more effective against both viruses than the human or goat form of lactoferrin. The addition of fetal bovine serum to the media caused slight inhibition of bovine lactoferrin and lactoferricin B. Synergistic activity was shown with acyclovir and both bovine lactoferrin and lactoferricin B. In addition, 2- to 7-fold reductions in concentrations of acyclovir could be effective when used in combination with bovine lactoferrin or lactoferricin B.

The antiviral activity of lactoferrin would appear to be inconsistent with the transmission of CMV via breast milk [21]. Because concentrations of lactoferrin are high in breast milk (2 mg/mL), this would suggest that transmission of CMV should not occur. However, CMV transmission through breast milk only occurs with high viral loads (> 7 x 10^3 genome equivalents/mL) [21,62,183], indicating that the lactoferrin is not able to prevent the transmission when higher viral concentrations occur[21]. However, the viral load in plasma is lower compared with breast milk and lactoferrin may be able to inhibit virus infections. Also, during inflammation, lactoferrin concentrations increase in the serum which might contribute to the inhibition of viral infections.

Resistance to virus strains that carry alterations in viral thymidine kinase (HSV) or U_L97 protein kinase (CMV) affects 5 to 25 % of immunocompromised patients receiving long-term treatment with acyclovir or ganciclovir [214,334]. In the immunocompetent host the prevalence of resistance is 1 % [334]. Resistance in HSV patients can be due to viruses not producing a functional thymidine kinase gene, producing a thymidine kinase gene with diminished ability to phosphorylate acyclovir, or changes within the viral gene encoding DNA polymerase [125,334]. Resistance due to mutation in the thymidine kinase gene accounts for 95 % of the acyclovir resistant cases [205]. The mutation is an insertion or deletion (codons 92 and 146 of thymidine kinase gene) or substitution (codon 176, 177 and 336 of thymidine kinase gene). This mutation is not detrimental to the virus because it is not essential for viral replication. Resistance to penciclovir has also been documented in HSV [334]. It is most likely due to mutations within the viral thymidine kinase and DNA polymerase gene. Infections which are resistant to thymidine kinase-dependent drugs can be treated with foscarnet or cidofovir

[205]. However, both of these drugs are more toxic than acyclovir. In addition, resistance to foscarnet has been documented in both HSV and CMV [239,334]. Mutations of viral DNA polymerase lead to its resistance [334].

The need to treat pregnant women offers opportunities for antiviral drug development because pregnancy results in a degree of immunosuppression, infections can be transmitted in utero from the woman to the fetus, and infection can be transmitted intrapartum from infected maternal genital secretions [336]. At present there is no antiviral treatment for congenital CMV infection during gestation [173]. The safety of currently available drugs for the treatment of congenital CMV infections during pregnancy and in the neonate is debatable [214]. Trials with valacyclovir and CMV hyperimmunoglobulin will be studied in the future for treatment of primary CMV infection during gestation [173]. Therefore, more antiherpes agents are needed for small children and pregnant women [214].

New drugs and new classes of drugs that are safe, effective, and cost-effective are needed [46,328]. Due to the increasing numbers of people immunosuppressed due to cancer, transplantations, AIDS and aging, there is an increased demand for improved antivirals to treat herpesvirus infections [328]. The only drugs approved for treating CMV infections have toxicities, modest efficacy, and poor drug delivery options (Table 5). In addition, acyclovir and ganciclovir have low oral bioavailability and possible drug-resistant virus mutants [214]. Also, drugs that can used to treat HSV and reduce the number and severity of subsequent outbreaks as well as reduce subclinical shedding are needed [46].

Table 5. FDA approved antiviral drugs [46].

FDA-approved antiviral drugs	
HSV	CMV
Acyclovir (Zovirax) Valacyclovir (Valtrex) Penciclovir (Denavir) Famciclovir (Famvir) Foscarnet (Foscavir) Trifluridine (Viroptic) Vidarabine (Vira-A) n-Docosanol (Abreva)	Ganciclovir (Cytovene, Vitrasert) Valganciclovir (Valcyte) Foscarnet (Foscavir) Cidofovir (Vistide) Fomivirsen (Vitravene)

Interferons have antiviral capabilities [114]. There are five approved interferon preparations: recombinant interferon-α-2A, recombinant interferon-α-2B, leukocyte-derived interferon-α-N3, recombinant interferon-β-1B, and recombinant interferon-γ-1B. Interferon – α and – β have greater antiviral activity than interferon- γ. Interferon – α and – β can be produced in nearly all cell types while interferon- γ is produced by T and natural killer (NK) cells. Interferons have no innate antiviral activity but act by inducing an antiviral state within target cells. The interferons bind to receptors and receptor-associated tyrosine kinases are activated. These in turn phosphorylate specific ctyoplasmic proteins which then move to the nucleus. It is here that they bind to specific cis-acting elements in promoter regions of genes. Transcription of these genes occurs and viral replication is inhibited. General toxicity from interferons include pyrexia, leukopenia, hypotension, fatigue and anorexia [201].

Resiquimod is an immune-response modifier that is in clinical trials for herpes simplex virus infections [46]. It is in the family of imidazoquinolines. It functions by inducing cytokines, such as IFN-alpha, tumor necrosis factor-α, IL-1, IL-6, and IL-12. In a pilot study of individuals with frequent recurrences of genital herpes, resiquimod

significantly reduced the frequency of genital herpes. The drug is in phase III of clinical trials.

A number of new CMV inhibitors have been identified at several pharmaceutical companies [328]. Pharmacia Corp. has identified PNU-183792, a 4-oxo-dihydroquinolone-3-carboxamide, which is a non-nucleoside DNA polymerase inhibitor. It inhibits 6 of the 8 human herpesviruses, the exceptions being HHV-6 and HHV-7. Bayer has discovered a class of phenylenediamine-sulphonamides that inhibit the cleavage and packaging of CMV DNA into capsids. One of these agents, BAY 38-4766, has 5-fold greater antiviral cell culture activity than ganciclovir against CMV. Another class, benzimidazole ribosides, also inhibits CMV DNA cleavage and packaging. In addition, inhibitors of CMV U_L97 protein kinase have been recognized. Maribavir (also known as benzimidavir) has approximately 10 times more potency against CMV than ganciclovir [71,328]. Its inhibition of CMV does not involve DNA polymerase but might include inhibition of CMV U_L97 protein kinase [214].

Other areas of viral inhibition include protease inhibition [328]. Serine protease is essential for capsid maturation. In CMV the active site of protease is contained in a shallow cleft that limits the points of interaction for a small molecule inhibitor. The protease active site is formed by an amino acid triad consisting of Ser132, His63, and His157 [181,328]. A Cys161 residue is in close proximity to the active site [328]. These amino acids provide the opportunity to discover inhibitors that bind covalently to the active site by reacting with the serine hydroxyl group or bonding with cysteine. Several classes of compounds such as thieno[2,3-d]ioxazinones, spirocyclopropyl oxazolones, and benzimidazole sulphoxides inhibit CMV protease. In addition, other agents which

covalently modify the CMV primase component of helicase/primase complex involved in DNA replication have been identified. They have been shown to inhibit CMV in cell culture 30-fold more than ganciclovir. However, many of these agents have been put on hold or development halted due to pharmaceutical mergers and reorganizations. It is therefore not certain whether these agents will reach the marketplace.

Bovine Herpesvirus 1

The clinical disease associated with herpesvirus infections in livestock resembles that for herpesvirus infections in humans [84]. Because animal herpesviruses are very similar to human herpesviruses they are suitable for studying the molecular biology of both [258,297,321]. Most of the alphaherpesviruses are related to BHV-1 [258]. Bovine herpesvirus 1 shares certain biological properties with HSV-1 and HSV-2 [95,124]. Most BHV-1 genes exhibit sequence homology to HSV-1, occur in the same order as the genes in the HSV-1 isomer with inverted U_L, and the proteins have similar nomenclature to those in HSV-1 [258]. Exceptions are the BHV-1 genes $U_L0.5$, $U_L3.5$, *circ*, and $U_S1.5$ that have no counterpart in HSV-1. In addition, BHV-2 is closely related to HSV, and BHV-4 is similar in its genome organization to that of Epstein-Barr virus [210,258].

Introduction. Bovine herpesvirus has been recognized in Europe since the mid-nineteenth century [304]. Infectious pustular vulvovaginitis (IPV) was first reported in 1841 in Europe as coital exanthema or Blaschenausschlag, and infectious bovine rhinotracheitis (IBR) was first seen in feedlots in Colorado in 1950 [138,242,342]. The

IBR virus was initially isolated in the United States in 1956 from cattle in feedlots and dairies with rhinotracheitis [209,304,342]. In 1958, BHV-1 was isolated in the eastern United States from dairy cattle with vulvovaginitis [209,304]. By then, it was clear that the viruses causing IBR and IPV were indistinguishable [209]. The first case of IBR in Europe was reported in 1960 [242,342]. Bovine herpesvirus 1 is now known to cause a variety of diseases in cattle including genital and respiratory forms [209]. The virus occurs worldwide and causes economic loss due specifically to respiratory disease, infertility, abortions, weight loss, and death [233,315]. Economic losses due to bovine respiratory disease complex, to which BHV-1 is a major contributor, have been estimated to be about $500 million annually in the United States (1996) [144].

Pathogenesis. Most alphaherpesviruses replicate rapidly causing lysis of infected cells [82,209]. Many alphaherpesviruses replicate in epithelial cells at the entry site and destroy these cells, producing localized lesions in the skin or mucosa [47,82,297]. Localized infection is followed by generalized infection that occurs via viremia or neural spread [82,342]. Generalized infections in animals less than three months of age without maternal antibody can be characterized by foci of necrosis in any organ [209]. Mononuclear-cell-associated viremia in pregnant animals can result in abortion and focal necrotic lesions throughout the fetus with the liver being the organ most consistently affected. Alphaherpesvirus can become latent in neurons meaning that infectious virus persists in the body but can not be detected in the latently infected cells [82,342]. Following local infection, the virus enters the peripheral nervous system where it is transported by retrograde axonal transport to the trigeminal and sacral ganglia [342]. The

genomes of the virus remain in the nuclei of neural ganglia but do not produce viral antigens [82,342].

Glycoproteins of BHV-1. Glycoproteins have numerous functions [15]. They are responsible for the attachment and penetration of the virus into the host cell, envelopment of the capsid, viral egress, and transmission of infection via cell to cell spread. Bovine herpesvirus 1 consists of three major glycoproteins: gB, gC, and gD, formerly named gI, gIII, and gIV, respectively. They are prominent on the virion envelope and plasma membrane of virus infected cells. Glycoprotein C is a non-essential type I transmembrane glycoprotein involved in viral attachment to the cell [221,242,308]. It is a γ2 protein [15]. Glycoprotein C has a highly hydrophilic N-terminal region that forms long spikes on the virion and contains a region that binds heparan sulfate proteoglycans on the surface of the host cell [82,242]. It also has a region that is homologous to the constant domain of MHC class II antigens [242]. Glycoprotein C and D contain both N- and O-linked oligosaccharides [15]. Following viral attachment by gC, gD is involved in penetration of the virus into cells [82,289]. Glycoprotein B and D are β proteins [342]. Following viral entry, gB causes fusion of the viral envelope with the cell membrane [54,82,319]. Glycoprotein B contains only N-linked oligosaccharides [15].

There are several minor BHV-1 glycoproteins as well (Table 6) [15]. These glycoproteins are less abundant and consist of gH (gp108), gE (gp93), gG, gI, gK, gL, gM and gp42 with gE/ gI and gH/ gL forming complexes [15,319]. Glycoproteins gH, gE, and gp42 are β proteins [15]. Glycoprotein H might be essential for virus replication. Glycoprotein H might also work with gB and gD to control viral penetration and cell to

cell spread. Glycoprotein G contains both N- and O-linked oligosaccharides and is further processed by the addition of glycosaminoglycans. Glycoproteins gE, gG, and gI are non-essential glycoproteins that are not needed for virus replication. They might be important for spread of the viral infection [15,308]. Glycoprotein M might be important for membrane penetration [319]. Glycoprotein K might promote cell surface transport of virions which controls the fusion process as well as plays a role in virus capsid envelopment at the nuclear membrane [15,319]. The gH/ gL complex might be important for intracellular transport [15]. Cell to cell spread of BHV-1 might be mediated by gE/ gI and gH/ gL complex as well as by gM [319]. The gE/gI complex also acts as an Fc receptor to prevent normal action of antibodies [297]. Thus, the glycoproteins have many functions. Continual progress is being made in understanding the functions of the glycoproteins.

Table 6. Bovine herpesvirus 1 glycoproteins. E, essential for viral replication; NE, non-essential for viral replication; N, N-linked oligosaccharides; O, O-linked oligosaccharides [258].

Protein	Gene	Essential/ Non-essential	Glycosylation	Function
gB (gI) Major	U_L 27	E	N	Fusion to the cell membrane Binds to heparan-like factors Attach and enter the cell Cell to cell spread
gC (gIII) Major	U_L 44	NE	N,O	Viral attachment to the cell Binds to heparan-like factors Binds to complement factor C3b
gD (gIV) Major	U_S 6	Conditionally essential	N,O	Penetration into the cell Cell to cell spread
gH (gII) Minor	U_L 22	E	N	Penetration into the cell Cell to cell spread Virus replication Intracellular transport with gL
gE Minor	U_S 8	NE	N	Binds Fc receptor with gI Cell to cell spread
gG Minor	U_S 4	NE	N,O	Cell to cell spread
gK Minor	U_L 53	?	N	Cell surface transport of virion Virus capsid envelopment
gI Minor	U_S 7	NE	?	Binds Fc receptor with gE Cell to cell spread
gM Minor	U_L 10	NE	N	Membrane penetration Spread in CNS Cell to cell spread
gL Minor	U_L 1	E	O	Entry into the cell Cell to cell spread Intracellular transport with gH

Isolates of BHV-1. Bovine herpesvirus 1 has been separated into the subtypes BHV-1.1, BHV-1.2a, and BHV-1.2b [16]. Bovine herpesvirus 1.1 genotype is the 'IBR-like' virus (Cooper-type) and BHV-1.2 is the 'IPV-like' virus (FI and K22-type)

[16,197]. These strains can be differentiated by restriction endonuclease digestion of their DNA and polyacrylamide gel electrophoresis (PAGE) [16,233]. Although each genotype is better adapted to either the genital or respiratory tract, the different clinical syndromes have not been consistently aligned with different genotypes [16,65,242]. Clinical respiratory disease has been associated with the IPV-like subtypes, and abortions have been seen with both BHV-1.1 and BHV-1.2a [16,65]. The molecular mechanisms for the adaptation of the specific genotype with the particular organ are not known [242]. The BHV-1.1 strain Cooper and the BHV-1.2 strain K22 have an estimated sequence identity of 95 % [242]. It is difficult to determine the exact biological differences between the two genotypes. It has been suggested that there is a difference between glycoprotein C between BHV-1.1 and BHV-1.2 [242]. There are two anti-gC monoclonal antibodies (MAbs 71 and 77) that can differentiate between BHV-1.1 and BHV-1.2. They are able to recognize BHV-1.1 gC. Monoclonal antibody (MAb) 71 recognizes the N-terminal half of gC, and MAb 77 recognizes the C-terminal half. The failure of MAb 71 to react with BHV-1.2 was shown to be due to one amino acid difference between glycoprotein C of BHV-1.1 and BHV-1.2.

The Cooper (Colorado, BHV-1.1) virus was isolated from the lungs of a cow with upper respitory disease in 1956 [197]. Iowa, LA, Haberink, and LX537 strains were isolted from cows with upper respiratory disease [40,191]. The FI (fetal Iowa, BHV-1.2a) virus was isolated from the blood of an aborted bovine fetus in 1984 [197]. The K22 (BHV-1.2b) virus was isolated from the vagina of a cow with IPV in 1958 (Table 7).

Table 7. The source of various strains of bovine herpesvirus-1 (BHV-1) and the clinical signs that they can produce. IBR, infectious bovine rhinotracheitis; IPV, infectious pustular vulvovaginitis; IBP, infectious balanoposthitis [121,342].

SUBTYPE	STRAIN	CLINICAL SIGNS	SOURCE
BHV-1.1	Cooper (Colorado)	IBR, Abortions	Nose
	Los Angeles (LA)		Nose
	Iowa		Nose
	3156		Lung
	Jura		Nose
BHV-1.2a	Fetal Iowa (FI)	Abortions, IPV/IBP	Fetus
	Spiel		Nose
	227		Prepuce
	B4		Vagina
BHV-1.2b	K22	IPV,IBP	Vagina
	Wabu		Prepuce
	739		Nose

Physico-chemical Properties. Bovine herpesvirus 1 is fairly resistant to the environment and can remain infective at 4 °C for up to one month [16,331]. It is inactivated more rapidly at higher temperatures [331]. Further, the virus is able to survive in aerosols at high humidity. During cold periods when the humidity is greater than 90 %, BHV-1 was reported to survive for up to 30 days. Fortunately, the virus is susceptible to many common disinfectants, including phenol derivatives, quaternary ammonium bases, and formalin [16,331].

Clinical Signs. There are several forms of disease that can be seen with BHV-1 infection. The clinical signs vary from mild to severe.

Genital Disease- Infectious pustular vulvovaginitis is seen most commonly in dairy cows [209]. It is generally observed 1 to 3 days following natural breeding [342]. Genital infections are usually not seen in conjunction with the respiratory disease

[16,342]. Affected cows might be febrile, depressed, anorexic, and hold their tails away from their vulvas [209,342]. A vulvar discharge is seen, the labia are swollen, and the vestibule is red with small pustules present on the mucosa. Adjacent pustules might coalesce to form a fibrinous pseudomembrane. Temporary infertility is seen due to pain associated with breeding [190]. Vaginal prolapse can be seen as a result of straining from the pain associated with extensive lesions. The acute stage of the disease lasts 4 to 5 days, and lesions usually heal in 10 to 14 days [190,342]. Many cases are subclinical [209]. Cows can conceive from the service in which they acquire the IPV, and pregnant cows that develop IPV rarely abort. Abortions due to 'IPV-like' subtype BHV-1.2a are infrequent but they have been reported [16,309]. Genital infections induce slow, variable, low or no serum antibody response [309].

Infectious balanoposthitis (IBP) is the genital form of the disease in bulls [209]. The preputial and penile mucosa are hyperemic with dark-red areas [309]. These red areas develop into nodules, vesicles, and pustules. The pustules can coalesce to form plaques and become covered with a yellow-white fibrinous membrane. Ulcers can develop and become secondarily infected with bacteria. A purulent preputial discharge can be present, and frequent urination might be observed. The bull will refuse to breed when lesions are extensive and painful [209,309]. Infectious balanoposthitis also can result in fever, depression, and anorexia [309]. Uncomplicated lesions generally heal in 10 to 14 days. Severe infections can result in scarring of the prepuce and penis leading to penile deviations or adhesions [190]. Finally, a decrease in semen motility and an increase in morphologic abnormalities as a consequence of general illness might be observed [309,322].

Semen from affected bulls can be contaminated with BHV-1 from preputial shedding following respiratory or genital infections [140,209,309,329]. It is the seminal plasma not the spermatozoa that contains the virus [309,319]. Antigen of BHV-1 has been associated with the post-nuclear cap area of the sperm head, but antigen has not been shown to be within the spermatozoa [80,309]. Bovine herpesvirus 1 was found in preputial washings for 2 to 10 days after intranasal and genital infections in bulls older than 18 months of age [140,309]. Viral replication after infection is restricted to the penis and preputial mucosa [322]. Virus was also isolated once from the distal part of the urethra [309]. Bovine herpesvirus 1 has not been isolated from the proximal urethra, accessory glands, epididymides, or testes. Intranasal inoculation of calves caused BHV-1 to be isolated from the prepuce but did not result in signs of balanoposthitis. Vogel showed that BHV-1.2 (SV-56/90 strain) inoculated intrapreputially causes a severe balanoposthitis [322]. Virus was shed in preputial secretions up to day 14 post-inoculation (pi) and in semen up to day 13 pi. Reactivation at day 60 pi with dexamethasone led to virus shedding in preputial secretions and/or semen. At day 50 post-reactivation the animals were euthanized, and viral DNA was detected in the dorsal root ganglia of the genito-femoral nerves, obturator nerves, pudendal nerves, sciatic nerves and rectal caudal nerve ganglia.

Bovine herpesvirus 1 is the most common viral contaminant of semen and can persist in frozen semen for over one year [140]. Insemination with contaminated semen can result in IPV, endometritis, and salpingitis.

Respiratory Disease- Bovine herpesvirus 1 infects the nasal cavities and upper respiratory tract to produce a disease called infectious bovine rhinotracheitis [233].

Cattle affected with IBR may show fever, depression, anorexia, dyspnea, open mouth breathing, salivation, coughing, and a nasal discharge [138,209]. Focal areas of epithelial cell necrosis with a severe inflammatory response can be seen in the respiratory tract [144,209]. The nasal mucosa is hyperemic ('red nose') and focal pustular necrosis can be seen [138,209,342]. However, oral lesions are uncommon [342]. The incubation time in experimental cases is 2 to 3 days, and cases which are acute and uncomplicated may last 5 to 10 days [209,342]. However, in immunosuppressed cattle, the damaged necrotic mucosa may become secondarily infected with bacteria, thus contributing to the shipping fever complex (bovine respiratory disease complex) and a prolonged recovery [144,209]. The morbidity rate in dairy cattle affected with IBR is about 8 % while in unvaccinated feedlot cattle it is usually 20 to 30 % and may reach 100 % [233]. The higher morbidity rate in feedlot cattle is likely due to frequent introduction of susceptible animals as well as abundant sources of bacteria for secondary infections. In addition, conjunctivitis with lacrimation can be seen alone or with other signs of IBR [209]. The corneal opacities appear to originate at the corneoscleral junction [138]. This is in contrast to pinkeye, caused by *Moraxella bovis*, in which the corneal opacity originates in the center of the cornea and spreads centrifugally.

Abortion- Following maternal infection, viremia can result in placental infection and subsequent infection and death of the fetus [146]. Bovine herpesvirus 1 is the most frequently diagnosed cause of viral abortion in North America [16]. It represents 3 to 5 % of diagnosed abortions. Exposure of a susceptible herd to BHV-1 can result in abortion storms with between 35 and 60 % of the cows aborting. Sporadic abortions can also be seen in herds with a history of vaccination or previous exposure. Abortions might

also occur in pregnant cows vaccinated with modified-live-virus vaccines. These abortions can occur as late as three months post-vaccination [146]. The placenta may harbor the virus for up to 90 days before transmitting the virus to the fetus [233]. The aborting cattle may or may not show clinical disease [16]. If seen, the manifested clinical signs are generally respiratory disease or conjunctivitis. Abortions are rarely seen with IPV. When respiratory disease is observed, abortions generally will not be observed for 3 to 6 weeks and in some cases, may not occur until up to 100 days following exposure to BHV-1 [16,315]. This long period can be attributed to the virus residing in the placenta for extended periods prior to infecting the fetus [16,146]. Abortions are generally seen in the second half of gestation [16,146]. The virus results in a disseminated fetal infection causing rapid fetal death and abortion [16]. The fetus is generally expelled 4 to 7 days following death [146]. The fetuses are autolyzed and there can be red-tinged serous fluid in the body cavities [16]. The placenta is often retained and edematous. In some cases, nonlethal fetal infections occur [190]. In these situations, the calves are born with IBR antibody but no clinical signs of disease. They are latently infected and can shed the virus when stressed. In other cases, the calves are born with the systemic form of the disease [233]. They have severe inflammation and necrosis of the respiratory and alimentary tracts.

The abortifacient property of three BHV-1 strains was compared by Miller in 1991 [197]. They showed that the Cooper (BHV-1.1) and FI (BHV-1.2a) strain inoculated intravenously in heifers during weeks 25 to 27 after breeding could cause abortion. The K22 (BHV-1.2b) strain did not cause abortion but was isolated from the placentas. All heifers became febrile and viremic 2 to 5 days post-inoculation. The

results of this study were in contrast to another in which BHV-1.2a did not result in abortion [194,197]. Therefore, further studies need to be performed [197].

Infertility- Bovine herpesvirus 1 infection can also lead to infertility. This can result from intrauterine infections from contaminated semen or systemic infections, including those resulting from use of modified-live-virus vaccines. When the virus is placed into the uterus, a severe, necrotizing endometritis occurs [190,191,315]. The lesions, however, are localized to the site of deposition and resolve in one to two weeks [190]. Therefore, only a temporary infertility would result from the use of semen contaminated with BHV-1. Bovine herpesvirus 1 should not be a cause of repeated pregnancy failure because virus could not be reactivated in heifers that had recovered from intrauterine BHV-1 infection. In addition to the endometritis, a severe necrotizing oophoritis involving both ovaries occurred following intrauterine infection. Necrotic follicles were present, yet the most severe damage occurred in the corpus luteum. The lesions varied from focal necrosis to diffuse hemorrhage and necrosis [315]. The corpus luteum is most susceptible during the first 3 to 4 days after ovulation, but in most heifers returns to normal the next cycle [190]. In some heifers, however, normal cycling might not occur for two months. The virus does not appear to be reactivated in the ovarian tissue, and no lesions appear in the ovary even if the virus is reactivated [190,192].

Because the virus is disseminated hematogenously, systemic infection can occur and lead to infertility [190]. When BHV-1 (Iowa strain) was given intravenously to heifers 7 to 14 days after breeding, a mild necrotic oophoritis developed leading to loss of the conceptus [16,193]. In a similar study by van der Maaten in 1984, heifers were inoculated with BHV-1.1 intravenously, intramuscularly, or by aerosol the day after

estrus [300]. On days 11 to 15 after estrus, virus was isolated from the ovaries of the heifers inoculated intravenously and intramuscularly but not from heifers following aerosol exposure [300,301]. The lesions were confined to the ovary containing the corpus luteum [300]. The increased potential for periestrual ovarian infections is possibly due to the increased blood flow to the corpus luteum occurring during estrus and the resulting exposure of the cells to BHV-1 [16,193].

In addition, cows vaccinated with modified-live-virus vaccines just before estrus or 1 to 2 months prior to breeding can have decreased conception [190]. Smith et al., in 1990, demonstrated that heifers inoculated intravenously with modified-live-BHV-1 vaccine during estrus secreted the virus in nasal and vaginal secretions and developed necrotic oophoritis [267]. The ovarian lesions are not permanent however [16]. Also, Chiang in 1990 showed that heifers vaccinated intramuscularly with modified-live-BHV-1 vaccine during estrus had a conception rate of 40 % compared to 78 % for the unvaccinated control group [60]. Therefore, intrauterine and systemic BHV-1 infections or vaccination with modified-live-virus vaccine that occurs during estrus or shortly before or after breeding can cause infertility or early embryonic death [16,60,190,193,195,301].

Transmission. Transmission generally requires close contact with nasal, oral or genital secretions (including semen) but can occur through droplets (aerosol) [209]. During acute infection, 10^{10} $TCID_{50}$/ mL (tissue culture infective dose) of BHV-1 is excreted in nasal secretions [331]. The respiratory form of the disease usually occurs from droplet transmission as a result of coughing and sneezing or direct nose contact with nasal secretions [209,331]. Vaginal secretions can contain up to 10^{11} $TCID_{50}$/ mL

BHV-1 and semen can contain up to 10^8 $TCID_{50}$/ mL BHV-1 [309,331]. The genital form of the disease can occur from coitus or the use of contaminated semen or equipment during artificial insemination [16,209]. It can also result from switching of a contaminated tail and thus touching another cow [331]. Transmission also has been reported to occur by a dog licking cows' vulvas as they lay in stanchions [138].

A cell associated viremia follows infection [209]. Infected cattle shed the virus from respiratory and genital secretions for 8 to 16 days following exposure [16]. The highest serum virus titer is seen on days 4 to 6 after infection [331]. The fetus may also serve as a means of transmission, as virus is present in all fetuses aborted as a result of BHV-1 [16]. Following genital and respiratory disease, the sciatic and trigeminal ganglia, respectively, are the sites of latency [16,209]. After recrudescence in latently infected cattle, up to 10^6 $TCID_{50}$/ mL BHV-1 can be shed in nasal or vaginal secretions [331]. Latently infected cows that recrudesce during parturition can infect their calves which in turn spread the virus to other susceptible cattle.

The minimum infective dose for transmission via the respiratory route has been reported to be 100 $TCID_{50}$ or lower [331]. The exact dose required to cause infection by artificial insemination is not known. A straw of semen with 200 $TCID_{50}$ led to seroconversion in 6 of 25 animals by day 14 post-insemination, while semen with 32 $TCID_{50}$ did not cause antibody development in 44 cows [309,331]. On the other hand, insemination of cows with greater than $10^{5.3}$ $TCID_{50}$ always resulted in infection [331].

The genital and respiratory forms of the disease rarely occur in the same herd at the same time [16,209]. This may be related to differing transmission routes, genital versus intranasal exposure, as well as different herd management practices (feedlot versus

pasture) [16]. Infectious bovine rhinotracheitis is uncommon in range cattle but is significant in feedlots due to the close contact [209,304]. Cattle entering feedlots often have a low level of immunity, are immunosuppressed due to the stressful nature of the event and are exposed to a variety of new viruses and bacteria [304]. Outbreaks of IBR can occur 2 to 3 weeks after the arrival of unvaccinated cattle. Outbreaks might continue for 2 to 4 weeks longer if new cattle are introduced into the group.

Transmission can occur among bulls in artificial insemination centers when common equipment is used without adequate sanitation between bulls [209]. Corticosteroids have been administered to bulls to reactivate the virus and thus, to detect and eliminate carrier bulls. Semen can also be tested for BHV-1 by inoculation of cell cultures and daily examination for cytopathic effects [140]. Undiluted semen is cytotoxic so it must be diluted with 5 to 10 parts trypsin-inhibiting-substance to one part raw semen [140,329]. If this lowers the virus titer below detection, false negative results might be obtained [140]. An alternative approach is to centrifuge the semen at 600X g and use the seminal plasma as the specimen. Polymerase chain reaction of semen is a more sensitive method to detect BHV-1 in semen [72,309]. Results can be obtained in 6 hours compared to 7 days for virus isolation [72,306]. A method to test large numbers of semen samples is the Cornell Semen test [309]. Semen is pooled and administered intranasally and intravenously into seronegative calves. The calves are tested for BHV-1 antibodies at 3 and 5 weeks post-inoculation. It is a simple test to perform. However, it is impossible to determine which specific sample is contaminated, and the test requires the use of animals for experiments. Semen infected with BHV-1 can be treated with

0.3 % trypsin to inactivate the virus [28]. No detrimental effect is seen on fertilization when treated semen is used.

Diagnosis. Diagnosis includes electron microscopy of scrapings or fluid from vesicles, immunofluorescence staining of smears by specific antibodies, virus isolation, serological tests and polymerase chain reaction for detection of viral nucleic acid [209,297]. Definitive diagnosis is performed by viral isolation in cell culture [209,304]. Neutralization and PCR are used to identify the virus [342].

Viral growth is seen in primary cultures of fetal bovine cells (kidney, testicle, and lung), Madin Darby bovine kidney cells, and bovine turbinate cells [342]. There is a rapid cytopathic effect with syncytia and eosinophilic intranuclear inclusion bodies, and a diagnosis can be confirmed in 24 to 48 hours [209,304]. Plaque assays are used to quantify the virus particles [342]. Since maximal virus replication and shedding occur at 3 to 6 days after infection, it is important to obtain ocular and nasal swabs as soon as possible [304]. However, because clinical signs are not seen for the first few days, the virus might be cleared prior to obtaining samples. Cotton and polyester swabs are used for collecting nasal samples that are cooled but not frozen for transport to the diagnostic laboratory [233,304]. These samples should be processed within 24 hours of collection [297].

Detection of virus can also be performed by ELISA, direct and indirect immunofluorescence techniques, immunoperoxidase, and by electron microscopy [233]. The ELISA is very sensitive. Virus isolation can be enhanced by combining ocular and nasal swabs from several animals.

Polymerase chain reaction-based assays have high sensitivity and the results are available within a day [233,297]. It can be used for latent infections and for testing semen, nasal swabs, and fetal serum. Bovine herpesvirus 1 can be detected in nasal swabs up to 14 days post-inoculation with the virus [233]. Polymerase chain reaction with Southern blot hybridization can detect BHV-1 in semen before the animal develops any detectable antibody. Different PCRs have been developed for detecting target regions, such as TK gene or glycoprotein genes [297].

In addition, real-time PCR based on fluorescence resonance energy transfer (FRET) is being used to detect and quantify BHV-1. This technique detects and quantifies a fluorescent signal. The amount of fluorescence increases in proportion to the amount of PCR product in a reaction. A 5′ fluoroscein probe is labeled at its 3′ end with carboxyfluorescein dye [52,143,232]. A 3′ probe is labeled with a fluorescent dye at its 5′ end and also contains a phosphate attached at its 3′ end to block extension by *Taq* polymerase. These probes are located at an internal region of the PCR product (40 base pairs from the 3′ end of the primer that is annealed to the same strand as the probe). During annealing, both probes bind to the target sequence. The 5′ fluoroscein probe transfers energy to the 3’ probe. This is referred to as FRET (fluorescence resonance energy transfer) (Figure 14). During each extension cycle, as the probes melt off at the elongation temperature (72 ºC) or are displaced by *Taq* polymerase, the 3’ fluoroscein probe no longer generates a fluorescent signal. Thus, the amount of fluorescence increases in proportion to the amount of amplicon produced.

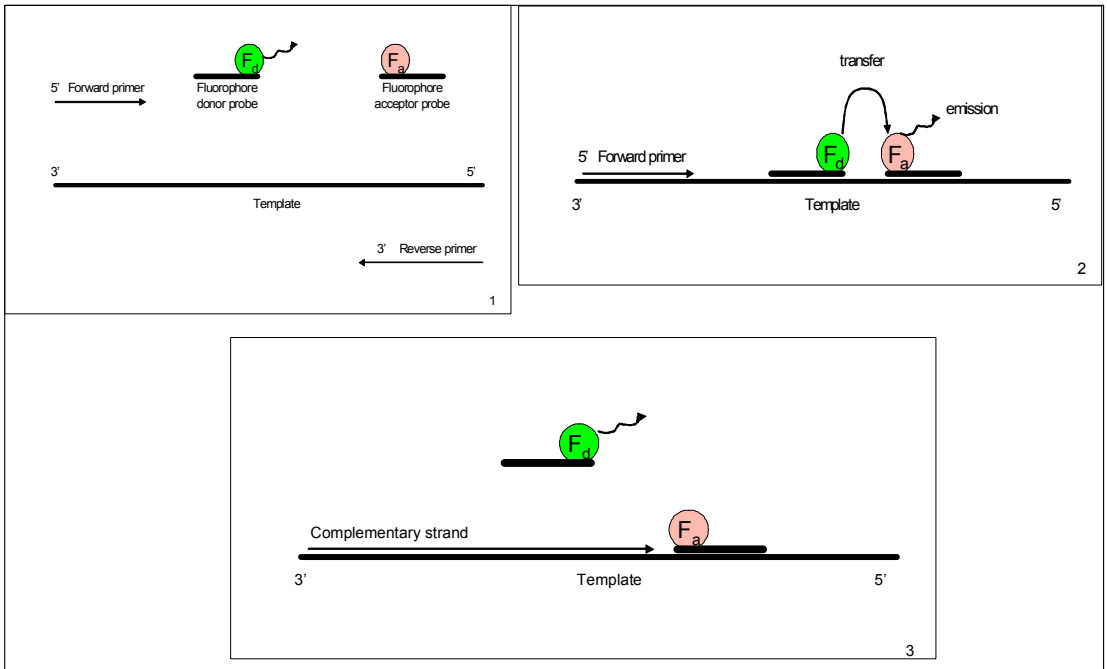


Figure 14. Real-time polymerase chain reaction using fluorescence resonance energy transfer (FRET). 1. When probes are not bound to the template, there is no fluorescent signal from the acceptor fluorophore. 2. During annealing, both probes attach to the sequence, and there is energy transfer between the fluorophores. The fluorescent signal from the acceptor fluorophore can be detected. The amount of signal is proportional to the amount of target. 3. During extension, the probes are displaced and there is no fluorescent signal from the acceptor fluorophore [232].

If samples for virus isolation cannot be obtained, then serum is collected two to three weeks after an outbreak and analyzed for specific isotype serologic responses [304]. A four-fold rise in antibody titers between acute and convalescent phase are seen in positive cases [233]. The serological diagnosis is commonly performed using virus neutralization (VN), indirect hemagglutination (IHA), indirect fluorescent tests, and ELISA [90]. Enzyme linked immunosorbent assay is more sensitive than IHA [90,233]. Virus neutralization tests and ELISA tests have high specificity [160,233]. European Union standard reference sera recognized by the World Organization for Animal Health are available to test the efficacy of serological tests [160]. The reference sera consists of a negative (IBR-EU3), a weak-positive (IBR-EU2) and a positive (IBR-EU1). Tests used in diagnostic laboratories to detect latently infected cattle should be able to score IBR-

EU2 as positive. In one study, the 24 hour neutralization test and home-made blocking ELISA were the most reliable tests used to detect the IBR-EU2 correctly. Half of the commercial ELISAs did not correctly detect the IBR-EU2. Polymerase chain reaction is a sensitive and fast way to analyze samples [204]. A portion of the viral TK gene is amplified. It can detect virus at less than 0.125 $TCID_{50}$. It can be used to detect BHV-1 at a concentration of 1 $TCID_{50}$/50 μL in semen. It can also be used to differentiate the wild type BHV-1 from the TK-negative BHV-1 strains.

The diagnosis of BHV-1 abortions can be confirmed by examining fetal and placental tissues [16]. Because of the rapid death of the fetus, many of the fetuses are autolyzed indicating that they have been dead 48 hours to 1 week prior to being expelled [150]. The tissues are dark red due to imbibition of hemoglobin from hemolyzed erythrocytes. Serosanguineous fluid is present in the body cavities of the fetus, and the quantity of fluid present depends on how long the dead fetus remained in the uterus. The fluid migrates to the fetal body cavities for the first 2 to 4 days after death and then is resorbed through the uterus. There might be clotted blood surrounding the kidneys. The gross lesions seen in the autolyzed fetus are not pathognomonic for BHV-1. Foci of necrosis in several organs including, the liver, spleen, adrenal glands, lung, and kidneys can be seen [16,342]. Herpesvirus intranuclear inclusion bodies can be seen adjacent to the necrotic foci. There is no inflammatory cell reaction indicating rapid fetal death [16].

Use of the fluorescent antibody test on sections of kidney is very reliable [150]. Bovine herpesvirus 1 antibody titers on paired maternal serum are not diagnostic, because most abortions occur several weeks after exposure when rising titers already have

occurred [16]. The maternal antibody titers only indicate exposure which may be from previous vaccination or exposure.

Detection of viral DNA, viral antigen, or virus isolation confirms the diagnosis of abortion [16]. Placental cotyledons can be the best tissue for isolating the virus [16,150]. Because of autolysis of the fetus, virus isolation might be unsuccessful [265]. In one study, BHV-1 could only be isolated from 33 to 50 % of infected fetuses [150]. Bovine herpesvirus 1 viral antigen is detected by fluorescent antibody tests (FA) on acetone-fixed frozen fetal tissues (kidney and adrenal glands) or by immunohistochemistry using monoclonal or polyclonal antibodies on paraffin-embedded formalin fixed tissues (liver, lung, kidney, adrenal glands, and placenta) [16]. An indirect immunoperoxidase (IP) technique using an avidin-biotin-peroxidase complex (ABC) and a specific monoclonal antibody can be used to detect viral antigen in formalin-fixed, paraffin-embedded tissues [265]. The tissues in which the most antigen is detected are liver, lung, and kidney. The IP test has a sensitivity of 94 % in comparison to fluorescent antibody with a sensitivity of 67 %. The ABC increases the sensitivity of the IP test by forming a lattice with the antibody and the complex which amplifies the antibody-antigen reaction. In addition, autolysis does not cause a problem with the IP test as it can with FA. The orange-red fluorescence in autolytic tissues can make diagnosis difficult with FA, whereas the IP staining is easier to detect. In situ hybridization can also be used to detect viral nucleic acid [16].

Pathology. Histopathologic examination of the trachea and nasal passage reveals mild catarrhal inflammation with edema and infiltration of neutrophils [342]. Diffuse hemorrhages can be seen. Following epithelial necrosis, mononuclear cell accumulation

occurs in the lamina propria and submucosa. Histopathology of the pustules on the vulva and vagina show accumulation of inflammatory cells. The nucleus of the epithelial cells shows karyolysis. Eosinophilic intranuclear inclusion bodies can be seen in cells at the periphery of the lesion.

Gross lesions might be seen in the ovary [300]. The corpus luteum might be pale orange to grayish yellow. The follicles can be white to gray in color and contain a cloudy fibrinous fluid. Histopathological examination of the corpus luteum might reveal cavities filled with fibrinous eosinophilic staining material. Around the cavity is a zone of hemorrhagic necrosis bordered by a zone of mononuclear cells.

Managing Clinical Cases. Treatment of the respiratory form of IBR involves isolation of the infected animals as well as treatment with broad-spectrum antibiotics to reduce the impact of secondary bacterial infections [304]. Treatment of an entire group of animals with antibiotics should be considered if greater than 10 % of the animals are sick. Cattle that are exposed to infected animals but not yet showing clinical signs can be vaccinated with an intranasal modified-live-virus IBR vaccine to reduce the number of new cases.

Immunology. There are three types of immunity that can be achieved with BHV-1 infection and vaccination [305]. The first is the induction of non-specific immunity with the activation of cytokines, macrophages, and natural killer cells. Cytokines can directly or indirectly inhibit virus replication [297]. The macrophages and natural killer cells can kill virus-infected cells directly by MHC class I recognition or indirectly by interacting with antibody to induce cell death by antibody-dependent cell cytotoxicity. The second immune response is cell-mediated immunity which limits the infection and

helps the host to recover after infection [297,305]. The cell-mediated response is first seen about 5 days after infection, peaks at Days 8 to 10, and is responsible for recovery from the disease [82,297]. Finally, antibodies (humoral immunity) are formed that neutralize the virus and limit viral spread within the host [297,305]. Neutralizing antibodies prevent attachment and penetration into host cells [297]. Ten days after infection, IgG-1 and IgM are detected and eliminate the free virus [82,297,304]. These antibodies take part in antibody-mediated complement lysis of infected cells and antibody-dependent cell cytotoxicity [297]. Immunoglobulin A can also be detected in nasal and genital secretions a few days after IgM is detected [82,233,297].

The non-specific immune responses are important during primary BHV-1 infections [297,305]. Macrophages respond by producing cytokines and then respond to cytokines produced by T cells to kill infected cells [297]. This is seen within 2 days post-infection in the lung and 3 to 5 days later in the blood leukocytes. During an acute infection the animal is immunosuppressed possibly by down-regulation of MHC class I molecules on the infected cells. The infection may interfere with the CD8 cytotoxic T lymphocytes which are involved in lysis of the BHV-1 infected cells. Both non-specific and specific immune responses are important for protection from secondary infections or reactivation of latent virus [305]. In a secondary infection, IgG-1 and IgG-2 antibody responses are seen [304].

Bovine herpesvirus 1 is able to elude the host immune system and establish latency [157]. Cytosolic viral proteins are degraded into peptides by proteosomes and transported into the endoplasmic reticulum by TAP (Transporter associated with Antigen Processing) (Figure 15). Here the peptides associate with MHC class I heavy chains and

β_2 microglobulin to form stable heterotrimeric complexes. The MHC class I complexes are then transported to the cell surface. On the cell surface, they present the antigenic peptide to cytotoxic T lymphocytes (CTL). Herpesviruses (HSV-1, HSV-2, CMV, human herpesvirus-8, VZV, Epstein-Barr virus, murine gammaherpesvirus-68) are able to interfere with the MHC class I-CTL detection. Pseudorabies virus, BHV-1, and VZV have been shown to downregulate MHC class I expression. The viral gene products specifically responsible for this are not known.

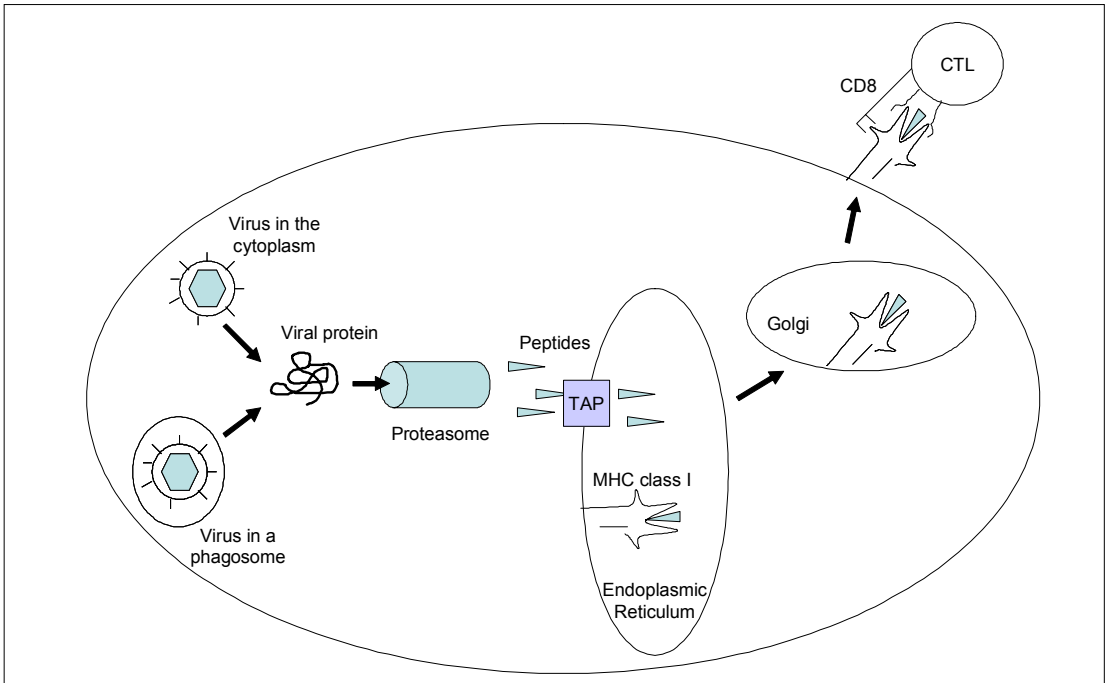


Figure 15. A method of immune evasion by bovine herpesvirus 1. The proteasome degrades the viral proteins into peptides. The transporter associated with antigen processing (TAP) moves the peptides from the cytoplasm into the endoplasmic reticulum. MHC class I are synthesized in the endoplasmic reticulum, and a peptide binds in its cleft. The stable MHC class I complexes move through the Golgi and are expressed on the cell surface. The peptide antigen can then be recognized by $CD8^+$ T cells. TAP, transporter associated with antigen processing, MHC, major histocompatability complex, CTL, cytotoxic lymphocyte, CD8, cluster of differentiation [157].

Bovine herpesvirus 1 decreases the intracellular transport and expression of MHC class I on the infected cell surface [124,156,157]. The virus has 3 possible mechanisms of inhibiting MHC class I expression. One mechanism is partially due to a virion host shutoff (vhs) protein. This protein degrades cellular mRNAs, inhibiting host cell protein synthesis within 3 hours of infection. It is likely associated with the virion and does not

require viral gene expression. It is most likely encoded by U_L41, an alphaherpesvirus conserved gene. This protein decreases the synthesis of new MHC class I molecules. It also decreases the mRNA of the housekeeping genes for actin and glyceraldehyde 6-phosphate dehydrogenase (GADPH) [124]. It is also possible that other early viral proteins bind to the existing MHC class I molecules to keep them in the endoplasmic reticulum and prevent them from being expressed on the cell surface [156,157]. As an aside, Koppers-Lalic et al. showed that the viral tegument proteins that are released in the cell when the virus enters are not responsible for MHC class I downregulation. The second mechanism for downregulation of MHC class I is through the product of $U_L49.5$ [171]. The $U_L49.5$ product interacts with TAP to prevent the conformational change needed in TAP for peptide translocation. The $U_L49.5$ product also causes degradation of TAP. The third mechanism of MHC class I downregulation is to cause the heavy chain-β_2 microglobulin complexes to dissociate and become unstable [156,157]. This instability might be due to the absence of peptides which could be caused by several means. Early in a BHV-1 infection, TAP function is inhibited, and thus, peptide transport is decreased [124]. In HSV-1 and HSV-2 infections, the inhibitor of TAP is the cytosolic immediate-early protein, ICP47 [156,157]. Another potential method is for the viral proteins to interfere with the interaction between MHC class I and TAP, and thus hinder peptide loading of MHC class I. These MHC class I molecules will then degrade in the cytosol. Therefore, without MHC class I, the viral antigen can not be presented to the CTL.

There is also a secondary shutoff of protein synthesis that occurs later in infection [124]. It is independent of vhs. It is directed by the host and requires the expression of

viral genes. The cellular double stranded RNA-dependent protein kinase becomes activated and phosphorylates translation initiation factor eIF2α so that it will not synthesize protein. Conversely, HSV $\gamma_1$34.5 protein binds to the cellular protein phosphatase 1α to direct dephosphorylation of eIF2α. The cell's response to infection is then blocked.

In addition, to the above mechanisms, BHV-1 evades the immune system by spreading without the aid of cells. It does this through intercellular bridges and intra-axonal transport [82]. The humoral response is then limited because the virus is not in cells. In addition, BHV-1 can inhibit the innate immune response. Bovine infected cell protein 0 (BICP0) degrades interferon regulatory factor 3 leading to inhibition of transcription of type I IFN [213]. BHV-1 can change the alternative complement pathway [82]. Glycoprotein C binds to C3 (complement component) and potentially destabilizes C3 convertase. Also, BHV-1 is able to evade the immune system by molecular mimicry. This occurs because gC is antigenically similar to the glycoproteins expressed on macrophages and other leukocytes. As well, gE and gI form a complex with acts as an Fc receptor and can prevent the function of antibodies.

Vaccines. Bovine herpesvirus 1 was first isolated in 1956, and a live attenuated vaccine was available that same year [297]. Modified-live-virus (MLV), killed-virus, genetically engineered, and subunit vaccines are currently available [297,304]. The ideal vaccine should induce both cell-mediated immunity and neutralizing antibodies [305]. Bovine herpesvirus 1 vaccines that reduce the incidence and severity of disease are available, but they may not prevent acute or latent viral infection [16,209].

Modified Live Vaccines- Live vaccines consist of an attenuated strain of BHV-1 that is able to replicate in the host [308]. The RLB 106 strain is used in many live vaccines [297,308]. It was modified by treatment with nitrous acid [308]. Modified-live-virus vaccines are administered intramuscularly or intranasally [304]. They produce a rapid immune response with protection within 72 hours of administration, and the intranasal vaccine induces local mucosal immunity [304,305,308]. For maximal protection, the animal should be vaccinated three weeks before exposure to BHV-1 [304]. The recommendations are that the animal is vaccinated at 4 to 6 months of age, revaccinated at 8 to 12 months of age, and then vaccinated annually thereafter [139]. The intranasal vaccines can be difficult to administer because the head must be restrained and raised which is time-consuming [304]. The intranasal vaccine is safe for pregnant cows [342]. It induces IgA and cell-mediated immunity [305]. Modified live vaccines can result in mild clinical signs, viral shedding with transmission to unvaccinated animals and eventually the establishment of latent infections. Modified live vaccines induce immune suppression and can interfere with the induction of immunity to *Mannheimia haemolytica* vaccines when given simultaneously. The intramuscular vaccines should not be administered to pregnant animals or calves suckling pregnant cows as it might result in abortion [304,305]. Further, the vaccine virus can be shed so vaccinated animals should not come into contact with unvaccinated, pregnant animals. Also, breeding bulls in an artificial insemination center vaccinated with a MLV vaccine shed the vaccine virus in the semen for 2 to 3 months following vaccination [233]. The vaccine can be administered as a combination with vaccines against other viruses [342]. A study by Talens in 1989 showed that a multivalent vaccine consisting of modified live BHV-1 that

was chemically altered provided adequate protection and did not result in vaccine-related abortions when given intramuscular [281].

Killed Vaccines- Killed vaccines use viral strains that have been grown in cell culture and are inactivated by treatment with chemicals such as alkylating agents, formalin, ethanol and betapropiolacetone or by heat or ultraviolet treatment [305,308] Killed vaccines are non-infectious and unable to replicate in the host and therefore include an adjuvant to induce immunity. Killed vaccines are not attenuated [308]. The killed vaccines produce a slow immune response with protection occurring 7 to 10 days after administration of the second dose [304,305]. It is recommended that the animal receives 2 vaccinations 2 to 4 weeks apart beginning when the calf is 4 to 6 months of age [139]. Annual revaccination is required. The killed vaccines are safe for pregnant cows, and the vaccine virus is not shed and does not create a risk of latent infections [304,305]. The killed vaccine does not prevent the reactivation of field strains of BHV-1 or prevent the establishment of latency [304]. It might however, reduce the virus shedding after recrudescence [308]. Vaccinating pregnant cows during an abortion storm is not effective in preventing more abortions [150,304]. The time from infection to abortion may be more than 2.5 months. Therefore, most susceptible cows are infected before the first abortion occurs. Vaccination of these cows will not change the course of the infection, i.e. abortions will continue.

Genetically Engineered Vaccines- Marker vaccines allow differentiation between wild and vaccine viruses. The use of marker vaccines can aid in eradication programs for BHV-1 if they are able to reduce the transmission of the field virus [308]. Because of the location of glycoproteins in the viral envelope and on the infected cell, they are targets

for the host immune response [297]. Recombinant DNA vaccines are produced that have glycoprotein and thymidine kinase genes deleted [209]. Mutant viruses with deletions in genes encoding non-essential glycoproteins (gC, gE, gI, and gG) are used [73]. Serology can be used to detect antibodies against the deleted glycoproteins in cattle vaccinated with the mutant virus and differentiate them from the cattle infected with the wild type virus [73,297]. The vaccinated cattle do not develop clinical disease and are unable to revert to the wild type due to the deletion of the virulence factors [297,305]. In one study, the gC^- deletion mutant stimulated the best cell-mediated immune response in calves [73]. Glycoprotein C is nonessential for viral growth and is the most abundant glycoprotein expressed by BHV-1 [165]. It forms projections on the virion surface that are targets for virus-neutralizing humoral antibody and cytotoxic T cells. The mutant does evoke an efficient immune response. Deletion of gC might partly attenuate the virus. It is able to induce clinical disease, but there is less virus shed and for a shorter period than occurs in naturally infected cattle [73,165]. Because this gC gene deletion virus is still virulent, it might be combined with deletion of other genes, such as TK gene, to result in an attenuated vaccine [165].

In another study, a BHV-1 strain was passaged multiple times through cell culture and found to have a deletion that involved the entire gE gene and a downstream gene, U_S9 [308]. A modified live and a killed (Intervet Inc, Bovilis Rhinobovin) marker vaccine have been produced from this mutant [278,308]. Strube in 1996 performed several studies and showed that the aluminum hydroxide and purified saponin adjuvant for the gE-deleted killed virus marker vaccine was superior to two other adjuvants and the minimum vaccine dose needed was 10^8 $TCID_{50}$ [278]. Cattle were shown to require

two vaccinations subcutaneously at 4-week intervals. Revaccination was required in six months. The vaccination was safe in young calves and pregnant animals. Strube also tested the modified live gE-deleted marker vaccine and showed that a minimum vaccine dose is 10^5 $TCID_{50}$. Two vaccinations 4 weeks apart (intramuscular or intranasal) are required with revaccination in six months. Using this protocol, animals had a 90 % clinical protection. In addition, maternal antibodies do not interfere with vaccination, and the vaccine was safe for breeding bulls and pregnant cattle. The vaccine virus did not revert to virulence. Field trials have shown that both gE-deleted marker vaccines are useful for reducing the spread of BHV-1. It has been shown that calves given a gG- or gE-deletion mutant vaccine were better protected than those given a gI- or gI/gE- double-deletion mutant [308]. The comparison of these marker vaccines should be made cautiously because they were all performed under different experimental conditions.

Another recombinant virus (Gal-TK) vaccine has been constructed in which a chimeric reporter/marker gene has been inserted within the TK gene [61]. This gene codes for bacterial β-galactosidase (β-gal gene) which inactivates the viral TK gene and acts as marker for Gal-TK virus. Colostrum-deprived newborn calves intranasally inoculated with the Gal-TK virus shed less virus and remained healthy compared to the calves intransally inoculated with BHV-1.1 Cooper strain. It is also possible to distinguish the marker gene from the wild type virus infection. However, Miller in 1995 showed that while a thymidine kinase-negative recombinant virus will reduce the abortifacient activity of BHV-1, it will not eliminate it [196].

In addition, Liang et al. showed that a BHV-1 mutant lacking the gene, U_L49h, was impaired in its growth in cell culture as well as in cattle [166]. This gene encodes a

virion tegument protein (VP22 in HSV) and is conserved among different herpseviruses. Cattle receiving this mutant intranasally did not show any clinical signs of disease nor did they shed virus. Deletion of this gene could prove useful for developing recombinant BHV-1 vaccines or vaccine vectors.

Subunit Vaccines- Subunit vaccines are killed vaccines that do not contain the whole virion but may contain selected envelope glycoproteins [297,305,308]. These vaccines contain enough antigen to produce immunity without risk of abortions or latent infections [305]. They also are not immunosuppressive, and serologic tests can be used to differentiate them from the wild type virus. Because gD induces the highest level of immunity against infection in cattle, it was isolated, and a gene coding for the secreted form of gD was engineered [305,308]. Supernatant containing gD was formulated with an adjuvant to produce a vaccine. The immune response is greater when IL-2, a growth enhancer of B cells, natural killer cells and lymphokine-activated killer cells, or IL-1β, a macrophage-derived cytokine, are added to the adjuvants [297].

Antiviral Agents. A number of antiviral agents exhibiting efficacy against human herpesviruses have been evaluated against BHV-1. It has been established that cidofovir is effective against BHV-1 in tissue culture at 4 μg/mL [97]. The drug does reduce clinical signs and viral replication in calves inoculated intranasally with BHV-1 [98]. However, the establishment of latency was not prevented. In addition, Bila states that cidofovir exhibits an embryolethal effect [32].

Phosphonoformic acid and phosphonoacetate are effective against herpesviruses by specifically inhibiting viral DNA polymerase [164,257]. These compounds are

slightly cytotoxic [257]. Phosphonoacetate's use is limited because it is irritative topically, toxic to laboratory animals, and accumulates in bones. Phosphonoformic acid does not irritate the skin or genital mucous membranes and has only minor effects on normal cellular metabolism. Schwers et al. demonstrated that 500 and 1000 μM/mL of phosphonoformic acid significantly reduced the number of plaques produced by BHV-1 (LA strain) on Georgia bovine kidney (GBK) cells. No cytotoxic effect was seen in the uninfected cells [257]. Phosphonoformic acid also inhibits the P8-2 strain of BHV-1 [123,198].

Salvatori et al. showed that N^6-cycloalkyl-2',3'-dideoxyadenosine derivatives are able to reduce the viral titre of BHV-1 (LA strain) by 3 log units on MDBK cells [253]. The compounds had an EC_{50} (effective concentration causing 50 % reduction of CPE) equal to or higher than 50 μM. This was the same efficacy as shown by the reference antiviral agent ganciclovir. The most active derivatives against BHV-1 had an intact purine ring and lacked a hydroxyl group in position 2'. However, most of the derivatives were cytotoxic.

The lipophilic derivative of Bowman-Birk soybean protease inhibitor (BBI) was shown by Larionova to inhibit BHV-1 replication greater than 100-fold in embryonic bovine lung and kidney cell culture [163]. Bowman-Birk soybean protease inhibitor inhibits the maturational proteinase of BHV-1 which hinders the formation of infectious virions. Also, genistein, a soya isoflavone, inhibits BHV-1 replication on MDBK cells [4]. Its activity is due to its ability to inhibit tyrosine kinase activity.

Brivudin is effective against BHV-1 in MDBK cells [70,198]. Misra verified that brivudin can be phosphorylated by BHV-1 (P8-2 strain) thymidine kinase and

incorporated into viral DNA [198]. But, this incorportation did not result in the termination of replicating BHV-1. Brivudin-treated cells still contained numerous viral particles. However, this phosphorylation of brivudin was necessary as a prerequisite for brivudin to interfere with the glycosylation of BHV-1 glycoproteins. Brivudin might interrupt the processing of the immature forms of the viral glycoproteins to the more mature forms. However, the mechanism of action is not fully known.

Smith et al. demonstrated that BHV-1 (Cooper strain) is sensitive to ganciclovir on embryonic bovine trachea cells at a concentration of 8 μg/mL [266]. Whereas, Kit et al. showed that BHV-1 thymidine kinase phosphorylation of thymidine was sensitive to trifluorothymidine and araT (arabinosylthymine) but not to ganciclovir and acyclovir on GBK cells [152]. Also, Rollinson demonstrated that FMAU (1-(2-deoxy-2-fluor-β-D-arabinofuranosyl)-5-methyluracil), FIAU (1-(2-deoxy-2-fluoro- β-D-arabinofuranosyl)-5-iodouracil)and FIAC (1-(2-deoxy-2-fluoro- β-D-arabinofuranosyl)-5-iodocytosine) were more effective against BHV-1 (Oxford, 78/4242, and 78/3883 strain) than ganciclovir on rabbit kidney cells [249]. Ganciclovir, FMAU and FIAU are phosphorylated to the monophosphate by viral thymidine kinase. Phosphorylation to the triphosphate occurs via cellular enzymes. The triphosphate form then inhibits the viral DNA polymerase or becomes incorporated into the viral DNA. FIAC is converted to FIAU by a deoxycytidine-deaminase enzyme. Differences are seen in antiviral efficacy in different cell lines. This might be attributed to the lack of deoxycytidine-deaminase enzyme in certain cell lines.

Thiry et al. also confirmed the lack of efficacy of acyclovir against BHV-1 (LA strain) on GBK cells [287]. The following concentrations of acyclovir were used: 10,

100, 1000, and 10000 μM. There was no loss of titer and only a mild plaque reduction at the highest concentration, 10000 μM of acyclovir. No cytotoxic effect was observed in the uninfected cells. They hypothesize that the virus-cell system affects the efficacy of the compound. Harmenberg noted that the inhibition of herpesvirus by acyclovir in cell culture appears to depend on the type of virus, virus concentration, type of host cell and condition of the cells [119,298].

Control and Eradication. Eradication of BHV-1 has been successful in Switzerland, Denmark, Austria, Finland, Norway, and Sweden [209,341]. Because of viral latency and reactivation, cattle that recover from BHV-1 infections can be a source of future infections [16]. Therefore, eradication was performed in the above countries by controlling cattle movement and test and slaughter of positive animals [209,297,305]. To maintain a negative status all breeding bulls and 10 % of cows in each herd should be tested by ELISA every 6 months and all animals must remain negative [297].

Eradication programs can be implemented by removing all cattle that are detected with BHV-1 from the herd [136]. In areas where BHV-1 has a high prevalence, eradication might not be economically feasible [297]. During the acute phase of the disease, diagnosis can be made by virus isolation [136]. During latency, BHV-1 specific antibodies can be detected in infected cattle with a neutralizing antibody test and BHV-1 glycoprotein specific ELISA. The specific antibodies can be determined up to at least three years following infection. Prohibiting vaccination with conventional vaccines and alternatively, using marker vaccines with their companion diagnostic tests will help to eradicate BHV-1 [308].

Bovine In Vitro Embryo Production

In vitro fertilization in humans was preceeded by another assisted reproductive technique which provided opportunitites for introduction of infectious agents. Artificial insemination in humans was first successful in the United States in 1890 [178]. Because organisms transmitted in the semen are a source of infection to the recipient, it is necessary to ensure that the donor semen is free of infectious organisms. Resulting infections could be local or disseminated and may adversely affect the pregnancy. The risk of spontaneous abortion, prematurity, stillbirth, and teratogenicity are all increased when sexually transmitted diseases occur in a pregnant woman. Also, other advanced reproductive techniques such as intracytoplasmic sperm injection solve fertility problems but increase the risk of transferring viral particles directly into the oocyte [224]. Herpes simplex virus and CMV infections of semen are relevant to assisted reproductive techniques because viral transmission could result in fetal and neonatal infection [10]. Because the risk of transmission of HSV and CMV can not be predicted by history of clinical signs or serology, virus isolation or PCR should be performed on semen prior to use in any advanced reproductive techniques.

Bovine IVF serves as an appropriate model for human IVF [186,187]. Concerning oocyte maturation, the human embryo is more closely related to the bovine embryo than the murine embryo [186]. In the cow and human, the follicle must attain a minimum size in order to complete oocyte maturation in vitro. In addition, bovine and human preimplantation embryos are more similar in biochemical and intrinsic paternal and maternal regulatory processes while the murine embryo has fewer similarities.

Examples of this include: the lack of lactate needed in culture media, similarity in amino acid metabolism, use of co-culture cells, and same targets for paternal factors in bovine and human embryos. Also, the interaction between the embryo and the corpus luteum is similar in cows and humans although the actual signal is different. The human embryo signals with human chorionic gonadotrophin, and the bovine embryo signals with interferon tau (IFN-τ) to maintain pregnancy.

In 1981, the first calf was born through the use of in vitro fertilization [271]. The production of in vitro bovine embryos requires the aspiration of oocytes from ovaries collected at a slaughterhouse or via ovariectomy [315]. Alternatively, oocytes can be collected from the live cow via transvaginal aspiration or laparoscopy. To reduce contamination, the ooctyes should be collected in a clean, sterile manner. Also, contamination between donors should be avoided. Oocytes are collected from follicles which have a diameter of 2 to 7 mm. The oocytes are then matured and fertilized, and presumptive zygotes are cultured in vitro until the blastocyst stage at which time they are transferred to a recipient cow. In an IVF system, the cleavage rate can be as high as 85 % with a blastocyst rate of 30 to 40 % [41,132]. A donor cow can average as high as 4.7 viable embryos per oocyte collection [39].

In Vitro Maturation. In vitro maturation of the bovine oocytes involves nuclear and cytoplasmic changes [315]. Nuclear maturation encompasses resumption of the first meiotic division and progression from prophase I to metaphase II [132,315]. Cytoplasmic maturation involves the molecular and structural changes that will allow the oocyte to be fertilized and undergo normal embryonic development. Oocytes must have a diameter of 100 to 110 μm to undergo meiotic maturation using current procedures. To

determine which oocytes are to be matured, oocytes are evaluated for presence of a homogenous ooplasm and for the quality and quantitiy of cumulus cells surrounding them [41,315]. In vitro maturation of oocytes involves their incubation in a tissue culture medium supplemented with luteinizing hormone (LH) and/or follicle-stimulating hormone for a period of 24 to 26 hours [271,315]. Tissue culture medium 199 (TCM199) is a popular medium. The use of LH in the IVM phase is important because it enhances cumulus expansion and results in increased embryonic development after IVF [41]. Luteinizing hormone acts to increase the energy available for the oocyte to support its subsequent development. It seems to stimulate the tricarboxylic acid cycle and increase glycolysis and mitochondrial glucose oxidation within cumulus-enclosed oocytes; whereas, the oocytes with denuded cumulus cells did not respond to LH.

In Vitro Fertilization. Generally, cryopreserved semen is used for in vitro fertilization [315]. The bull from which the semen was collected should not be shedding IBR or bovine viral diarrhea virus (BVDV) [271]. Several methods are available to select the morphologically normal and progressively motile sperm. Swim-up, migration through a hyaluronic acid preparation, centrifugation through a Percoll gradient, or a glass wool filtration technique can be used. Centrifugation is used to concentrate the sperm in a pellet, but it does not exclude the dead sperm [43]. Swim-up yields a higher percentage of motile sperm because it concentrates the sperm which are able to swim. Percoll gradients filter out the abnormal and less motile sperm to yield more acrosome-intact sperm. Other filtration techniques act to filter out the abnormal sperm through various gradients.

Prior to fertilization the spermatozoa must undergo capacitation [315]. Capacitation of cryopreserved spermatozoa is usually accomplished in 2 to 4 hours [271]. Glycosaminoglycans such as heparin are commonly used for capacitation [315]. The sperm is incubated for 15 minutes with 10 μg heparin/mL [41]. Other methods of capacitation involve the calcium ionophore A23187 with or without caffeine as well as washing the sperm with bovine serum albumin-saline followed by incubation in calcium free Tyrodes. The calcium ionophore A-23187 elevates calcium and cAMP to initiate capacitation, whereas the BSA-saline elevates the sperm pH to promote calcium uptake by the sperm and thereby initiating capacitation. During capacitation, the plasma membrane of the sperm is altered to allow the influx of calcium ions [315]. This influx of calcium initiates the acrosome reaction. Also, penicillamine and hypotaurine have been found by some to increase sperm motility and oocyte penetration whereas, others show no favorable improvement [105].

In vitro fertilization can be performed in 100 μL drops with 20 to 30 oocytes per drop under mineral oil [41]. A sperm concentration of 10^6/ mL is used. In addition, IVF can be performed in smaller drops (42 μL) with 10 to 12 oocytes per drop under mineral oil. A sperm concentration of 50,000 sperm/ drop is used for the smaller drops. The IVF phase is carried out at 39 °C at 5 % CO_2. Approximately 4 to 6 hours are allowed for the capacitated spermatozoa to penetrate the cumulus and zona pellucida and fuse with the oocyte plasma membrane. Following penetration of the oocyte the sperm begins to decondensate in 1 to 2 hours. The sperm head is then reconfigured over a 2 to 6 hour period to form the male pronuclear stage. Commonly used fertilization media are

Tyrode's modified medium (TALP) and Brackett and Oliphant medium (BO). Many variations in media have been reported. For example, the use of glutathione during single oocyte IVF significantly increased the proportion of normal fertilization and decreased polyspermic fertilization compared to controls [89]. Some researchers have shown a higher incidence of polyspermy for oocytes cultured with sperm for 24 hours versus those cultured for 8 hours [105]. The completion of in vitro fertilization generally occurs in 18 to 20 hours [271].

To reduce the risk of disease transmission, the oocytes and presumptive zygotes are washed at least 10 times between the steps of the IVF phase. Each wash should be at least a hundred-fold dilution of the previous wash. Only oocytes and embryos from the same donor should be washed together. In addition, the serum and somatic cells used in the IVF system should be negative for viral and bacterial contamination [271].

In cattle, the use of cumulus cells has been shown to be beneficial to the fertilizing ability of spermatozoa [283]. Tanghe demonstrated that cumulus cells produce a mixture of secretions which improve the fertilizing ability of penetrating spermatozoa and this improvement is not due to the secretion of progesterone or hyaluronic acid. In addition, direct gap junctional communication is needed between the oocyte and corona cells. The cumulus oophorus does play a role in trapping the sperm. Also, when the sperm interact with the cumulus oophorus a higher redox state is encountered which stimulates sperm penetration. Atmospheric O_2 concentrations are needed to create this redox environment. Additionally, human cumulus oophorus cells have been shown to stimulate both sperm velocity and amplitude of lateral head displacement in human IVF [85].

In Vitro Culture. Following fertilization, the presumptive zygote is then cultured to the blastocyst stage over a 5- to 7-day period of time [271,315]. There are several types of culture systems which include co-culture (involving the use of somatic cells), culture in medium previously exposed to somatic cells and culture in defined media which does not use somatic cells [315]. Common co-culture media are Menezo-B2 or TCM199 supplemented with serum. These cells include bovine oviduct epithelial cells (BOEC), cumulus cells or trophoblastic vesicles [41,315]. The bovine embryos begin to metabolize glucose at the 2-cell stage, with increased metabolism at the 8- to 16-cell stage [189]. In the hamster, mouse and pig embryo, glutamine allows them to develop past their in vitro developmental blocks. The effect of glutamine on bovine embryo development has not been determined. Somatic cells are useful for overcoming the 8-cell block seen in cultured bovine embryos [56]. The somatic cells might be beneficial by releasing proteins that bind to the embryos and make them more resistant to proteases [315]. One protein secreted by the oviduct, a tissue inhibitor of metalloproteinases-like protein, is an embryo growth-promoting factor [56]. Alternatively, the somatic cells might produce embryotrophic factors or inactivate embryo toxic substances or both. Because of the potential for viral contamination when supplementing media with animal products the use of defined media is beneficial [315]. It has the advantage of providing a standardized and reproducible system. Synthetic oviduct fluid (SOF) is an example of a defined medium. Carolan et al. showed in 1994 that in vitro produced embryos can develop to the blastocyst stage with SOF in the absence of serum and somatic cells [56]. In addition, established cell lines such as buffalo rat liver (BRL) cells and African green monkey kidney cells (VERO cells) can be used in place of primary cultures of granulosa

and oviductal cells. Serum might also be replaced by a chemically defined surfactant, VF5 [223].

To help reduce the risk of disease transmission to the recipient, the handling of the embryos needs to be aseptic [271]. Also, embryos should be washed according to International Embryo Transfer Society (IETS) guidelines prior to transfer to help reduce disease transmission. The IETS guidelines for washing are briefly outlined as follows: zona pellucida-intact, in vivo embryos that are free of adherent material are washed a minimum of ten times in groups of ten or less [271,277]. The groups must be from the same donor. Each wash is at least a hundred-fold dilution, and separate sterile micropipettes are used between washes. In addition, treatment of the embryos with 0.25 % trypsin at a pH of 7.6 to 7.8 has been shown to be effective in removing several enveloped viruses. When treated with trypsin, embryos are washed 5 times as described above, followed with 2 treatments of trypsin for 60 to 90 seconds, and then washed 5 additional times to remove any residual enzyme.

Assessment of Embryo Viability and Quality. Assisted reproductive techniques are assessed by determining the pregnancy rate. Therefore, viable embryos need to be chosen for the technique to be successful [76]. The best test to determine if an embryo is viable is to see if a normal calf is born following embryo transfer. However in a research setting, this can be time consuming and expensive. Embryos can also be selected for viability using non-invasive or invasive tests. Non-invasive techniques include: evaluation of morphology, timing of development, and metabolic tests. Invasive techniques include a variety of staining procedures.

Morphological evaluation is the most common and simplest method to grade embryos. The age of the embryo, stage of development and morphology of the embryo can all be assessed by visualization with a stereomicroscope [188]. Embryos progress through several stages. The IETS has categorized the embryonic stages of development [271]. Code 1 is an unfertilized oocyte or a 1-cell embryo. Code 2 describes embryos that are 2 to 16-cell (Day 2 to 5). Code 3 is an early morula (Day 5 to 6). Code 4 is a morula (Day 6). Code 5 identifies an early blastocyst (Day 7). Code 6 is a blastocyst (Day 7 to 8). Code 7 describes an expanded blastocyst (Day 8 to 9). Code 8 is a hatched blastocyst (Day 9). Code 9 depicts expanding hatched blastocysts (Day 9 to 10).

The rate of development can also be used to assess embryo viability [188]. Embryos that develop in the appropriate amount of time are more likely to be viable after transfer. Defects in the embryo might present as a slowing of development [188]. Fast cleaving embryos are more likely to develop into morula and blastocysts than slow cleaving embryos [314]. Seventy % of 2 to 4-cell embryos at 30 hours post fertilization will develop to the blastocyst stage [19]. Of embryos that cleave later, only 35 % progress to the blastocyst stage. However, there is no correlation between the timing of cleavage with the timing of blastocyst formation or cell number [314]. Blastocysts that originate from embryos that were 2-cell on Day 2 had fewer cell numbers, decreased blastocyst formation, decreased hatching rate, and less interferon τ production than those originating from 4- or 8-cell embryos on Day 2 [161]. Compaction of the morula occurs at Day 5 to 6 post-fertilization [188]. This is the 32-cell stage of in vivo-derived embryos [310]. The blastomeres shrink, due to increased intercellular adhesions, making it more difficult to visualize individual cells, and the perivitelline space is larger [188]. At Day 6

to 7 post-fertilization, the blastocoel begins to form. For in vivo-derived embryos this is the 64- to 128-cell stage [310]. The timing of blastocyst formation corresponds with cell number in that blastocysts that form at Day 6 and 7 have higher cell numbers than those that form at Day 8 [314]. Two types of cells are seen, the trophoblast cells and the inner cells [188]. The inner cell mass (ICM) will form the embryo proper along with some extraembryonic membranes, and the trophoblast cells will form the placenta. The trophectoderm forms a permeable and selective seal to the inside of the embryo. As the blastocoel cavity grows, the zona pellucida becomes thinner, and the embryo enlarges. During this expanded stage, the trophoblast may collapse. Blastocysts that form at Day 6 or 7 have more ICM and total cells than blastocysts that form at Day 8 [314]. Timing of blastocyst development is more predictive of blastocyst quality than timing of cleavage. Pregnancy rates are higher for blastocysts that form at Day 7 compared with Day 8 [19]. Hatching occurs at Day 9 to 11 post-fertilization. Blastocysts that form later are less likely to hatch [161]. Kubisch et al. showed that 84.6 % of blastocysts that formed on Day 7 hatched, whereas, none hatched that formed on Day 11. Blastocysts can hatch that are largely trophectodermic vesicles and do not contain enough ICM to develop to term [314]. Thus, the number of trophectoderm cells does not indicate whether an embryo will be viable after transfer [311].

Embryos are morphologically evaluated using a stereomicroscope. They are examined for the presence of cellular debris, degree of compaction, blastomere color and texture, density and evenness of cleavage [188]. Poor quality embryos are less likely to result in a pregnancy than higher quality embryos [169]. According to the IETS, embryos are scored with one of 4 codes [271]. Code 1 consists of excellent or good

embryos. They have "symmetrical and spherical embryo mass with individual blastomeres (cells) that are uniform in size, color, and density. This embryo is consistent with its expected stage of development. Irregularities should be relatively minor, and at least 85% of the cellular material should be an intact, viable embryonic mass. This judgment should be based on the percentage of embryonic cells represented by the extruded material in the perivitelline space. The zona pellucida should be smooth and have no concave or flat surfaces that might cause the embryo to adhere to a Petri dish or a straw." Code 2 embryos are fair. They have "moderate irregularities in overall shape of the embryonic mass or in size, color and density of individual cells. At least 50% of the cellular material should be an intact, viable embryonic mass." Poor embryos are code 3. These embryos have "major irregularities in shape of the embryonic mass or in size, color and density of individual cells. At least 25% of the cellular material should be an intact, viable embryonic mass." Code 4 constitutes dead or degenerating embryos, oocytes or 1-cell embryos.

In addition, the diameter of Day 9 expanded blastocysts might be used to determine viability [206]. The diameter is measured using a video micrometer. Mori compared the diameter to the cell count in Day 7, 8, and 9 blastocysts and expanded blastocysts. The correlation coefficient was highest in the Day 9 expanded blastocyst groups. This test might be a good non-invasive technique to evaluate IVF embryos.

An additional test for evaluating pregnancy potential of human embryos is zona pellucida thickness [94]. Embryos were measured Day 1 to 3. There was a correlation between zona thickness and number of blastomeres, embryo grade and fragmentation. Embryos with more blastomeres were able to secrete more lytic factors required for zona

thinning. The thickness of the zona decreased during IVC and correlated with successful pregnancies.

Other assessment factors can affect pregnancy rates. The expertise of the person evaluating the embryos, the method of embryo production, and the quality of the recipient all can influence the pregnancy rate [188]. It is also important to be aware that there are differences between in vivo- and in vitro-derived embryos. In vitro-derived embryos are darker when cultured in the presence of serum. In vitro-derived embryos also have a smaller perivitelline space which might be due to less compaction of the cells. Compared to in vivo-derived embryos, in vitro-derived embryos have an increased rate of aerobic glycolysis and activate glucose metabolism sooner which might lead to higher production of reactive oxygen species. The reactive oxygen species are cytotoxic and can affect the permeability of membranes. Also, in vitro-derived embryos incorporate many fatty acids into triglycerides. These embryos have an increased triglyceride storage which results in lower buoyant density due to a high lipid/protein ratio.

Metabolic tests, which evaluate the potential viability of embryos, are non-invasive tests. These tests might evaluate energy metabolism, uptake and release of amino acids, oxygen uptake, and production of factors for embryo development or recognition of pregnancy [76]. Embryonic metabolism begins after activation of the embryonic genome. Metabolism can indicate the potential for the embryo to transcribe and translate the genome, differentiate into two cell types, regulate blastocyst expansion, and cleave the correct number of cells.

In the early ovine and bovine embryo, pyruvate and lactate are oxidized and used for the TCA cycle followed by oxidative phosphorylation for ATP production (Figure 16)

[288]. Adenosine 5'-triphosphate production is low because the need is low. In addition, glucose is used via the pentose-phosphate pathway for production of ribose and NADPH [76,182,288]. Ribose is a precursor for DNA and RNA synthesis. Due to a block in the glycolytic enzyme, phosphofructokinase, glucose use is limited. However, as the embryo compacts and blastulation occur, ATP demand increases. The phosphofructokinase block is removed, and there is an increase in glucose uptake and glycolysis that occurs at the 8- to 16-cell stage [240,288]. In the mouse embryo, glycolysis has been shown to be higher in the inner cell mass than the trophectoderm cells [141]. Most of the glucose carbon is used for lactate production rather than being oxidized through the TCA cycle [141,288]. As the blastocyst expands, oxidative metabolism increases [76]. Glutamine metabolism varies with the stage of embryo [240]. Metabolism of glutamine is high in the early embryo, decreases at the morula to blastocyst stage, and again increases with expansion and hatching of the blastocyst.

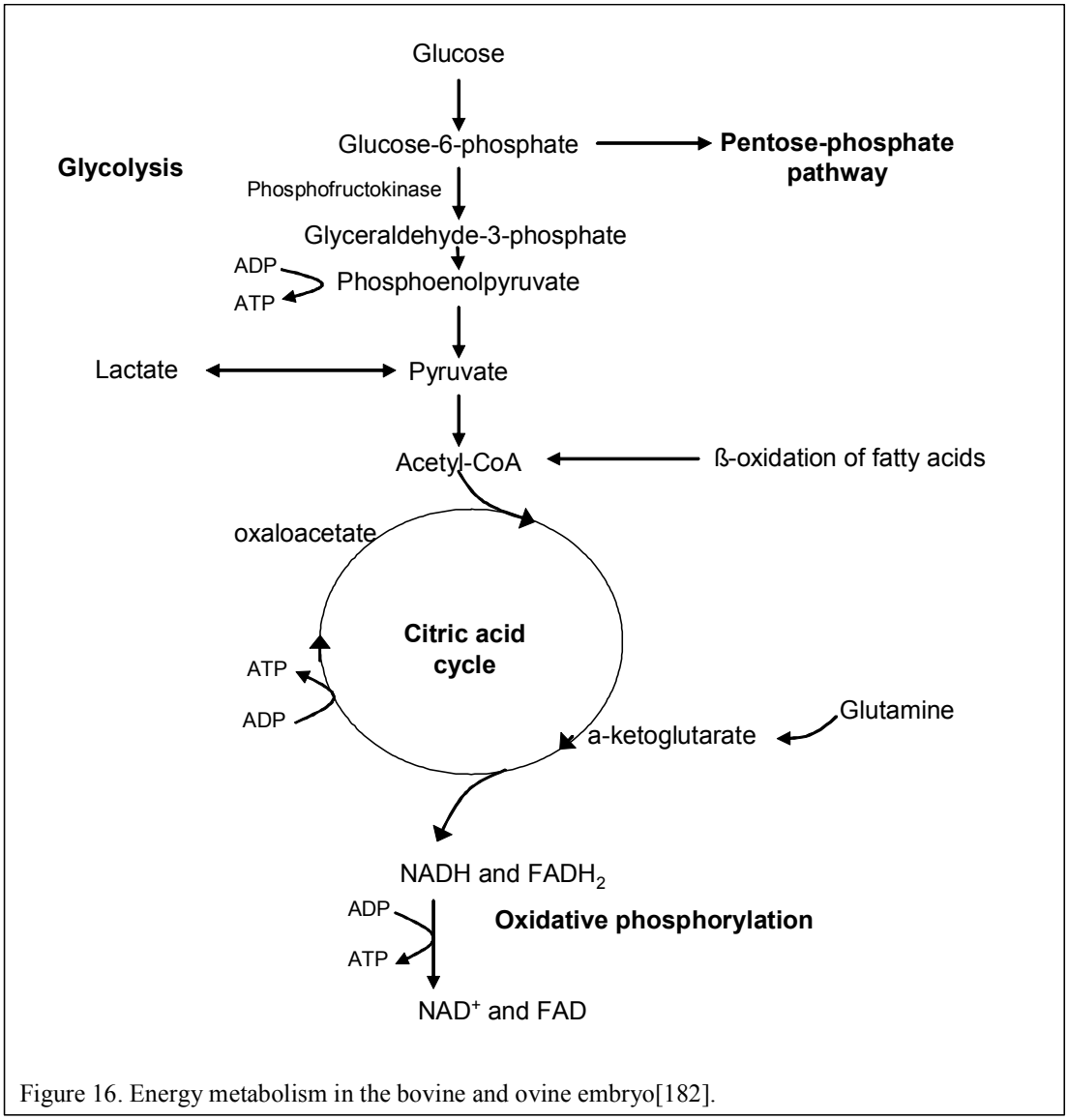


Figure 16. Energy metabolism in the bovine and ovine embryo[182].

There are some differences between in vivo- and in vitro-derived embryos [76]. Exogenous glutamine and pyruvate are important energy sources for in vitro embryos. In vitro embryos convert more glucose to lactate, have increased glycolysis, and decreased respiration than in vivo embryos. The increase in lactate, a membrane permeable weak acid, might cause a change in intracellular pH or membranes, causing the embryo to be more susceptible to cryo-injury [148]. In addition, in vitro-derived embryos cultured in

serum have an increase in accumulation of lipid, while in vitro-derived embryos cultured in serum-free media have 30 % less amino acid uptake than in vivo-derived embryos [76].

There are 2 techniques to evaluate embryo metabolism [76]. One method is to radiolabel glucose, pyruvate, glutamine or acetate and determine the production of $^{3}H_2O$ or $^{14}CO_2$. The other method is to determine changes in the culture medium, such as hormones, growth factors, energy substrates and oxygen via fluorescent-labeled substrates. The first method involves placing embryos in a hanging drop of medium with substrates that are labeled with ^{3}H and ^{14}C [76]. The drop hangs over a tube of Na-bicarbonate. As the labeled substrates are metabolized, radioactive H_2O and CO_2 are produced and trapped in the bicarbonate. They can then be counted. Total glucose metabolism to phosphoenolpyruvate (Embden-Meyerhof pathway) is measured by the production of $^{3}H_2O$ from D-[5-^{3}H]glucose [240]. Glucose metabolized by the pentose-phosphate pathway and the TCA cycle is determined by the production of $^{14}CO_2$ from D-[1-^{14}C] glucose and D-[6-^{14}C] glucose, respectively. The oxidation of glutamine occurs in the mitochondria, thus production of $^{14}CO_2$ from L-[^{14}C(U)]glutamine and of $^{3}H_2O$ from L-[3,4-^{3}H(N)]glutamine assesses TCA cycle activity. The production of $^{14}CO_2$ from [2-^{14}C]pyruvate measures the entire TCA cycle (mitochondrial oxidative metabolism), while the production of $^{3}H_2O$ from L-[3,4-^{3}H(N)]glutamine assesses the α-ketoglutarate to oxaloacetate section [241]. Glucose metabolism is higher in morphologically normal Day 7 embryos as compared with degenerate embryos [76]. However, it is not known if this method is superior to morphological evaluation alone. Khurana and Niemann demonstrated that CO_2 production could not be used prior to cryopreservation to determine post-thaw development [148]. However, embryos that

developed in vitro after thawing maintained at least 58 % of their pre-freeze concentrations of CO_2. Moreover, the use of radioactive substrates is not a clinically applicable technique, and its use could damage embryonic development.

The second method consists of incubating single embryos in small volumes of culture medium and measuring the change in concentration of compounds [76,141]. These results are compared to control drops that do not contain embryos. Pyruvate and oxygen uptake can be determined to evaluate the TCA cycle and oxidative phosphorylation pathways. Prior to compaction, human embryos with the lowest pyruvate and amino acid metabolism are likely to be viable, whereas after Day 3, high glucose uptake is correlated with blastocyst development [93]. This is due to the embryo not being very metabolically active prior to compaction. For example, to assess pyruvate or glucose uptake and lactate production, embryos are incubated for 12 hours in 1 μL droplets or 24 hours in 2 μL droplets [76,141]. The embryos are removed, and 1 to 3 nL samples are incubated under oil containing reactives. A photometer and photomultiplier detects and measures fluorescence. The reduced pyridine nucleotides, NADH and NADPH, fluoresce while the oxidized forms, NAD^+ and $NADP^+$, do not. Glycolytic activity is measured as the ratio of lactate release to glucose uptake. This is based on the assumption that 1 molecule of glucose produces 2 molecules of lactate. One molecule of pyruvate results in 1 molecule of lactate [92]. A high glycolytic activity generally correlates with poor blastocyst quality [76,141]. Gardner et al. showed that glucose and pyruvate uptake and lactate production can not be used prior to cryopreservation to determine which IVF blastocysts would be viable after thawing [92]. However, blastocysts that expanded their blastocoel immediately after thawing had increased

glucose and pyruvate uptake and lactate production compared to those that did not develop further. This could be determined at 5 hours after thawing. In addition, day 10 in vivo-derived embryos with a glucose uptake greater than 5 μg/embryo/hour resulted in a higher pregnancy rate than those with a lower glucose uptake [237]. However, parthenogenetic embryos also have a higher glucose uptake than fertilized embryos [76]. Embryos with high lactate production are associated with impaired oxidative capacity and poor development. Results are varied with regard to pyruvate uptake. It is important to note that comparisons between metabolic studies should be done carefully as there can be differences due to different culture systems.

Also, the culture drops can be evaluated for amino acid uptake and release [76]. Amino acid uptake and release differs between species, embryo quality, and stage of development [129,225]. Amino groups in the samples react with o-phthaldialdehyde and fluoresce [76,129]. The amino acids are separated and quantified by reverse-phase high performance liquid chromatography (HPLC). Houghton demonstrated that human embryos which developed to the blastocyst stage had an increase in uptake of leucine at Day 2 to 3, an increase in serine, arginine, and leucine for compacting 8-cell to morula stage, and an increase in serine, arginine, methionine, valine, and leucine for morula to blastocysts [129]. In bovine embryos, alanine is consistently produced resulting in an increase in concentration throughout development. Houghton et al. showed that human embryos that do not form blastocysts release a high amount of alanine on Day 2 to 3. Threonine is depleted throughout development of bovine embryos [225].

Furthermore, oxygen uptake can be measured using microelectrodes, microspectrophotometry, or microfluorescence [76]. Microfluorescence involves using

pyrene, an oil-soluble fluorescent compound that is quenched in proportion to the oxygen concentration [130,141]. At least 2 embryos are placed in culture medium in a micropipette that contains paraffin oil or mineral oil and pyrene. The fluorescence is measured using a photometer and photomultiplier. As oxygen is taken up by the embryo, fluorescence increases. In murine embryos, oxygen consumption increases at the blastocyst stage [130]. In the early embryo stages, there is no increase in protein content and minimal Na^+K^+ ATPase activity so the need for energy is nominal. Conversely, as the blastocoel forms, there is an increase in protein synthesis and Na^+ pumping. However, there is not yet a correlation between embryo viability and oxygen uptake or amino acid uptake and release [76].

In addition, release of enzymes or hormones can be detected in culture medium [76]. L-lactate dehydrogenase is one of the enzymes that can be measured. There is a higher concentration of enzyme in the medium when embryos fail to develop in vitro. But, this test needs to be accompanied with morphological evaluation because early stage embryos and severely degenerated embryos release very little L-lactate dehydrogenase. Also, IFN-τ can be measured by immunoassay or by antiviral activity [76]. Interferon tau is produced by the trophectoderm of ruminants and serves as the pregnancy recognition factor. Production of IFN-τ begins at Day 8 and peaks at Day 16. There is variability in the amount of IFN-τ produced. Hatched Day 8 embryos produce more IFN-τ than non-hatched embryos. Although blastocysts that form at Day 7 or 8 are more viable, they produce less IFN-τ than those that form at Day 9 or 10. Also, group cultured blastocysts produce more IFN-τ than embryos cultured individually. There is also discrepancy regarding the amount of IFN-τ produced and the quality of the blastocyst. In addition,

plasminogen activators are released by blastocysts during expansion and hatching [76]. Plasminogen activators are serine proteases that are responsible for the transition of plasminogen to plasmin. They are involved in ovulation, hatching, and cell migration during embryogenesis. Production occurs in the endoderm and trophectoderm with secretion into the blastocoel and external environment, respectively. They can be measured by evaluating lysis of casein. Plasminogen activators are associated with the diameter and cell number of in vitro cultured embryos. As well, prostaglandins are produced [76]. Six hours after fertilization, PGE_2 is secreted. It is elevated at 48 hours and then declines at 72 hours. At 48 hours after fertilization, $PGF_{2\alpha}$ is elevated and undetectable at 72 hours. Additionally, tumor necrosis factor alpha is produced 6 hours after fertilization, increases at 48 hours, and is still detected at 72 hours. Furthermore, platelet activating factor (PAF), a phospholipid, is secreted by sheep, mouse, rabbit, pig, and human embryos [251]. Platelet activating factor binds to cell surface receptors on the embryo and increases the intracellular calcium by inducing formation of inositol triphosphate and diacylglycerol. Platelet activating factor can be measured by specific radioimmunoassay (^{125}I). Primary antibodies are added to the culture media samples, samples are incubated, and secondary antibodies and tracer are added. After incubation, samples are centrifuged, blotted, and counted. In mice embryos, exogenous PAF can stimulate early embryonic metabolism and cell division and enhance implantation rates. In sheep embryos, PAF is produced at Day 2, 4, and 6; however, no correlation was seen between secretion of PAF and their development in vitro [18]. In human embryos, PAF is associated with good morphology and pregnancy potential after transfer [251]. An increase in PAF concentration correlates with pregnancy rate. But results are not

consistent [76]. Some embryos do not produce PAF while the patients become pregnant, and some patients do not become pregnant with embryos that did produce PAF.

Therefore, there are several ways to evaluate the metabolic activity of embryos. However, there is not yet much information correlating the results with embryo viability [76]. These tests can be expensive and time consuming. Some of the tests are not sensitive enough to use with individual embryos, and many of the test results are variable with regard to stage of embryo development and culture medium. At some point, these metabolic tests may be combined with other tests or morphological assessment to predict embryo viability.

Invasive techniques can be used to evaluate the chromosomes, nucleus, mitochondria, cytoskeleton, and cell organelles of embryos [313]. Karyotyping can be used to assess chromosomes, and chromosome 6 and 7 can be evaluated using fluorescent in situ hybridization (FISH). The nucleus can be examined by determining the mitotic index, pycnotic or apoptotic index, or total cell number with allocation to the ICM and trophectoderm. The mitochondria are assessed using staining with confocal imaging or 2-photon microscopy. The cytoskeleton is evaluated with staining and confocal imaging.

Within an embryo production system, the higher quality embryos have a higher cell number and pregnancy rate [161,311]. There has to be a correct ratio between the ICM and trophectoderm cells for pregnancy to occur [311]. An ICM/total cell number ratio of 0.36 is observed in early to mid bovine blastocysts. Excessive trophectoderm cells might account for pregnancy anomalies (e.g. hydroallantois) [313]. A lower ratio of ICM cells to trophectoderm cells might indicate that some cells were killed without killing the entire embryo [311]. In a study by Kubisch et al., the total cell number was

not affected by the day at which the blastocyst formed [161]. Blastocysts that formed on Day 7 to 13 had similar cell numbers. However in a study by Mori et al., the cell number was higher in blastocysts that formed on Day 7 compared with those that formed on Day 8 or 9 [206]. Additionally, there are differences in cell numbers between in vivo- and in vitro-derived expanded blastocysts [311]. In vitro-derived embryos have fewer ICM cells than in vivo-derived embryos, but the ratio of ICM/total cell number is not necessarily lower. Also, the grade of morula (excellent, good or fair) does not correlate with the number of ICM cells of the hatched blastocyst [314]. But morulae with a poor grade developed into hatched blastocysts with low numbers of ICM. It is possible that cells are lost during blastocyst formation from apoptosis [311]. In addition to cell number, the size of the ICM can also be determined. The size of the ICM is considered small (less than half of the blastocoel) or large (greater than half of the blastocoel). The embryos with a large ICM had more ICM cells and more total cells than those with a small ICM. However, the ratio of ICM/total cells was greater for the embryos with a small ICM. Thus, in the embryos with a large ICM, the ICM does not contribute proportionally to the total cell number. The trophectoderm contributes more to the total cell number in these embryos. In vitro culture media can also influence timing of allocation to the two cell types and cell number [312]. The ICM is more sensitive to environmental conditions, whereas the trophectoderm is not adversely affected. If assessment is done solely with morphology or total cell numbers, good quality blastocysts may be improperly identified. Porcine embryos cultured in medium with serum have an increased ICM number but the ratio of ICM/total cell number is not increased [311]. Furthermore, porcine in vitro cultured embryos have a reduced ICM number compared to in vivo embryos, but they

can still develop when transferred. Van Soom showed that in vitro-derived embryos might have lower ICM numbers than in vivo-derived embryos because the timing of compaction is shorter and blastulation occurs earlier [310]. Currently, the number of ICM needed for a viable animal to form is not known.

Differential staining can be used to differentiate the inner cell mass from the trophectoderm cells and determine the proportion of ICM/total cell number [311]. Reliable cell counts can be determined in about 85 % of embryos assessed. The procedure involves labeling the outer cells with antibodies and lysing them with complement [313]. The inner cells are protected from lysis by the seal that the outer cells form. The zona pellucida needs to be removed from the embryos so that the trophectodermal cells can by lysed [311,313]. Hatched embryos can be used, or the zona can be removed chemically (acid Tyrode), enzymatically (pronase), or manually. There are four methods to differentially stain embryos. The first method consists of using species-specific antiserum against the cell surface components followed by complement to lyse the trophectoderm cells. The antiserum, produced in rabbits against mouse spleen cells, binds only to the outer cells. Propidium iodide, which stains lysed cells, is present in the guinea pig complement solution and enters the lysed trophectoderm cells to stain them pink. Propidium iodide is DNA intercalating and binds to double stranded DNA with little to no sequence preference [141]. It can enter non-viable cells, but it cannot cross the membrane of viable cells. The ICM is not lysed because the antiserum will not go through the seal formed by the trophectoderm layer [311,313]. Bisbenzimide, which stains lysed or non-lysed cells, is used to stain the ICM blue. Hoechst (bisbenzimide stain) binds to AT-rich regions of DNA in the minor groove, and the helix conformation

at the AT-rich site may affect its binding [141,142]. Hoechst is more lipophilic than DAPI, thus it can penetrate cell membranes [313]. In the lysed cells, bisbenzimide fluorescence is quenched by propidium iodide which absorbs the energy and emits red fluorescence [141]. The second method involves labeling the trophectodermal surface proteins with trinitrobenzenesulphonic acid (TNBS) which cross-reacts with nonspecies-specific antiserum [312]. The antiserum used is anti-dinitrophenyl which cross-reacts with the trinitrophenol groups. This is followed with complement lysis of the antiserum labeled cells and staining as in the first method. All of these products are commercially available. Both of these methods depend on the complement being handled properly. The third method is trophectoderm cell permeabilization with Ca^{2+} ionophore A23187 [311,313]. A red (SYTO 17) and green (SYTOX) nucleic acid stain are used to differentiate the cells. The SYTOX stain is for the permeable cells. It is important to not overexpose the cells as permeabilization of the ICM can occur. The fourth method consists of labeling the trophectoderm cells with FITC (fluorescein-5-isothiocyanate) labeled wheat germ agglutinin. Wheat germ agglutinin binds to saccharide residues on the plasma membrane of trophectoderm cells. After permeabilization and staining with propidium iodide, the trophectoderm cells have green cytoplasm with red nuclei, while the ICM has only red nuclei. While differential staining is a useful tool to assess embryo quality, there are several factors that can alter results. The length of incubation, zona removal, improper handling of complement, wash out of dye, and antiserum difficulties can all affect the staining results. In addition, collapse of the blastocoel in hatched blastocysts interferes with proper staining because the trophectodermal cells are only

partially lysed [310]. Additional culturing to allow the blastocoel to re-expand did not improve the staining.

As well as differential staining, single staining, double staining and triple staining can be used [141]. Single staining involves fixing blastocysts in ethanol and staining with bisbenzimide or Giemsa [142,161,231]. The stain can penetrate the cells, and nuclei appear blue. Double staining is used to assess trophectodermal cell membrane alterations [141]. Kaidi used this procedure to evaluate embryos following cryopreservation and vitrification. Blastocysts are incubated in propidium iodide, fixed in ethanol, and incubated in bisbenzimide. Blue and red nuclei can be visualized with the same excitation wavelength (330 to 380 nm) and barrier filter (420 nm). However, red nuclei are counted more accurately at an excitation of 510 to 560 nm with a barrier filter of 490 nm. Propidium iodide will only enter the damaged cells and will only enter the ICM if there has been damage to the intercellular junctions between the trophectoderm cells. Triple staining is used to further distinguish between cells with damaged membranes, cells with altered bisbenzimide staining, and intact cells. After staining and counting as described in double staining, an additional 20 μL of propidium iodide is injected under the coverslip. The propidium iodide stained nuclei are then counted. The difference between the numbers of nuclei counted after double staining and after triple staining is determined to evaluate the number of cells with intact membranes but showing altered bisbenzimide staining.

Apoptosis also occurs while the embryo is developing [118]. It begins with the activation of the embryonic genome. In the mouse embryo, the trophectodermal cells increase and then reach a plateau as the blastocyst hatches and implants [117]. At this

time, the number of ICM cells decreases, and some of the cells undergo apoptosis. Apoptotic cells are distinguished by cell rounding, separation from surrounding cells, nuclear membrane blebbing, chromatin condensation on the inner nuclear membrane, cytoplasmic vacuoles, and nuclear and cytoplasmic fragments. In contrast to apoptosis, necrosis is caused by injury and results in cellular swelling and membrane rupture. Apoptosis affects single cells while necrosis affects large groups of cells. Apoptosis might be associated with removing ICM cells that have the potential to form trophectodermal cells, elevated glucose concentrations, absence of growth factors, increased concentrations of reactive oxygen species, toxic insults, and nuclear and chromosomal abnormalities. Cell changes include cell rounding due to activation of caspase 8 that cleaves cytoskeleton proteins. Phosphatidlyserine is also redistributed from the inner cytoplasmic leaflet of the cell membrane to the outer leaflet where it might activate phagocytosis. This is an early stage of apoptosis. Initiator caspases cleave and activate execution caspases which are controlled by the bcl-2 family. The bcl-2 family stimulates (bad, bax, bak, bcl-x_s, bik, hrk) and inhibits (A1, bcl-2, bcl-w, bcl-x_L, bfl-1, mcl-1) activation of execution caspases. The caspases cleave proteins responsible for DNA repair, nuclear envelope, cell cycle control and cytoskeleton. They also activate endonucleases that cleave DNA.

There are several techniques available to assess apoptosis. Agarose gel electrophoresis can be used to demonstrate apoptotic cleaved DNA which shows a "ladder" pattern with bands every 180 to 200 bp [118]. Cell necrosis would show DNA cleaved randomly and appearing as a smear on the gel. Annexin V can be used to detect phosphatidlyserine [118]. Annexin V conjugated to FITC labels the apoptotic cells.

This test can also be combined with the fluorochrome propidium iodide to differentiate apoptosis from necrosis. Cells undergoing necrosis will have permeable membranes, thus allowing the propidium iodide as well as annexin V to enter the cells. Apoptotic cells will only be labeled with annexin V. Time lapse cinematography can be used to visualize membrane blebbing in the trophectoderm and ICM [118]. Chromatin condensation and nuclear fragmentation can be detected with transmission electron microscopy, fluorescent microscopy following staining with propidium iodide or Hoechst stain, or by using laser scanning confocal microscopy [118].

In addition, reverse transcription polymerase chain reaction (RT-PCR) can be used to determine expression of mRNA of caspase and Bcl-2, and immunohistochemistry can be used to determine expression of protein of Bcl-2 [118]. To perform RT-PCR, mRNA is lysed from embryos and reverse transcribed to complementary DNA (cDNA). Specific primers are used that amplify products including the intron in order to discriminate between cDNA and genomic DNA. The products are visualized on agarose gel and verified by DNA sequencing. To perform immunohistochemistry, embryos are fixed in paraformaldehyde, embedded in agarose, and blocked in paraffin wax or mounted in antifade mountant. Rabbit and mouse primary antibodies with FITC-labeled anti-rabbit or CY5-labeled anti-mouse secondary antibodies are used with polynucleotide-specific fluorochrome. Also, commercial assays are available that contain caspase inhibitors. These inhibitors bind irreversibly to active caspase that is present in the cells. The samples are fixed in paraformaldehyde and mounted in antifade mountant with DAPI. Apoptotic cells with active caspase will fluoresce.

Also, fragmentation of DNA into oligonucleosomal-length fragments can be detected using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) [118]. Deoxynucleotides (dUTP) are labeled with FITC. Terminal deoxynucleotidyl transferase (TdT) incorporates the labeled nucleotides into the exposed 3'-OH ends of DNA that occurred during apoptosis. Zona-free embryos are fixed in paraformaldehyde to avoid loss of DNA from the permeabilized cells, permeabilized, and then incubated with TdT and fluorescein conjugated dUTP. The embryos are washed and mounted in antifade mountant with DAPI. Positive controls consist of embryos treated with DNase prior to labeling, and negative controls consist of embryos exposed to labeled dUTP but not TdT. In bovine embryos, DNA fragmentation is not seen until the 9 to 16-cell stage, decreases at the morula stage, and increases at the blastocyst stage [53]. Byrne et al. also showed that apoptosis is lower in fast cleaving embryos and embryos cultured in serum-free medium. In order to differentiate apoptosis from necrosis, additional criteria need to be examined. In necrotic cells, the fluorescence is not only in the nuclei but is also seen as diffuse staining in the cytoplasm.

In the mouse embryo, total cell number, ICM cell number and glycolysis were highly correlated with viability, whereas, blastocyst formation and hatching were not well correlated with pregnancy rate [93]. The ideal test to evaluate embryo viability would be simple, fast, affordable, non-invasive, assess multiple embryos, and sensitive enough to evaluate an individual embryo.

Embryo-Pathogen Interaction With Bovine In Vivo-Produced Embryos

Bovine in vitro fertilization and embryo transfer are in high demand to move genetic material between countries and to produce embryos at low cost [77]. These technologies are the basic tools for other technological advances such as embryo sexing, embryo cloning and gene transfer. However, there is still some uncertainty in regards to the potential transmission of pathogens through the embryos.

In theory, viruses could be transmitted to cows via embryos in two ways [315]. The first is through true vertical transmission in which spermatozoa or oocytes are infected. The other method of transmission involves contamination of the oocytes, spermatozoa, or embryos. Contamination of the embryo results from manipulation before or during transfer or from the environment (uterus and culture media). The virus might replicate in the cumulus cells and be carried through the zona pellucida (ZP) by the cytoplasmic processes.

It is not likely that in vivo-produced embryos will carry viral diseases because of the strict handling procedures [315]. Porcine origin trypsin treatment of zona pellucida-intact, in vivo-derived embryos is effective in removing BHV-1 prior to transfer [264,274,316]. Therefore embryos could be collected from donors shedding BHV-1 and transferred to recipients without transmitting the virus [263,316]. It is imperative that proper procedures be followed for embryo washing, trypsin treatment, use of antibiotics in media, quality control of serum used in media, and evaluation of embryos according to the IETS guidelines [316].

In addition, other trypsin products are being evaluated that do not contain animal-origin products. TrypZean™ (Sigma, St. Louis, MO) is a recombinant typsin product [259]. Seidel et al. showed that TrypZean™ treatment of BHV-1 exposed unfertilized ova and in vivo-derived embryos decreased the amount of adhered virus.

Another product, TrypLE™ (Invitrogen, Carlsbad, CA), is a recombinant porcine sequence trypsin-like protease that would be an attractive alternative since it is highly stable at room temperature and does not pose the same threat for contamination as animal-origin trypsin. Preliminary studies have shown that TrypLE™ Select (10X) when used for 10 minutes is effective at removing BHV-1 from day 7 in vivo-derived zona pellucida-intact embryos [177]. TrypLE™ Express (1X) was not effective at removing BHV-1 when used for 1.5 minutes. Pryor et al. has shown that TrypLE™ Express when used for up to 10 minutes does not affect embryonic development of in vitro-derived embryos [230]. However, the safety of TrypLE™ Select (10X) has not been evaluated using embryonic development as the ultimate gauge.

Embryo-Pathogen Interaction With Bovine In Vitro-Produced Embryos

As described, there are differences between in vivo and in vitro-produced embryos (IVP) [316]. In vitro-produced embryos require more manipulation and longer exposure to biological products than in vivo-produced embryos thereby increasing the risk of virus exposure [317]. Differing risks might also be associated with differences in the zona pellucida of IVP embryos compared to in vivo-produced embryos [318].

Embryos used for embryo sexing and cloning might have a damaged ZP, surrogate ZP, or no ZP, thus exposing the embryonic cells to BHV-1 or other infectious agents [316]. In addition, the biological materials used for IVP embryos generally come from cows at the abattoir with an unknown history [315]. These materials (ovaries, follicular fluid, cumulus cells, oocytes, oviducts) might be contaminated with infectious agents. In one report, oocytes collected from ovaries at a slaughterhouse produced IVP embryos of which 2.4 % were positive for BHV-1 [29]. Furthermore, 11.8 % of the follicular fluid and 6.2 % of the oviductal cells assessed in this study were also positive for BHV-1.

There are several factors which can reduce the risk of viral contamination of the embryo [315]. In vitro-produced embryos are transferred on Day 7 therefore, there is a limited amount of time in which the embryo is exposed to virus. Embryos at this stage are smaller than 200 microns and generally contain fewer than 150 cells [277]. In addition, the embyro is limited in its mobility and quantity of media and the spherical surface of the embryo limits the surface:volume ratio [315].

The ZP has epidemiologic significance as well. The ZP is important for sperm binding, blocking polyspermy, preventing the spreading of the blastomeres, aiding the embryo as it migrates through the oviduct, as well as protecting the embryo during early development [318]. Scanning electron microscopy has been used to show that the ZP of immature oocytes has a very irregular exterior surface with numerous pores, crevices, and projections randomly distributed. The ZP of the immature oocyte has wide meshes and deep holes, whereas in vitro matured oocytes have a finer network with meshes and holes which are not as deep. Inseminated oocytes do not have meshes on the ZP, possibly due to fusion of the network. The pores in the ZP are largest at the exterior surface and

diminish in size as channels through the ZP that are associated with these pores progress to the interior surface. Compared to other species, the cow has the smallest pores on the outer surface of the ZP. Most species have elliptical shaped pores that are randomly arranged. In addition, the fibers in the mesh-like network of the bovine ZP are the smallest in size compared to other species. The thickness of the ZP varies among species with the cow having one of the thickest at up to 12 or 13 μm [315]. It also could be that the zona pellucida of IVP embryos is more sticky than in vivo embryos, allowing viruses to attach [275].

To infect an embryo with an intact ZP, BHV-1 must pass through the zona pellucida to the perivitelline space, where it can then invade the embryonic cells and replicate [277]. Vanroose performed experiments to determine the mechanism of the zona pellucida's barrier against BHV-1 infection [318]. In his first experiment, Vanroose used scanning electron microscopy to determine the diameter and the number of the outer pores as well as the pattern of the outer surface of the ZP. The ZP of in vitro-matured oocytes was found to have a rough surface and a spongy pattern with 1511 pores in an area of 5000 μm^2. The diameter of the pores averaged 183 nm. Zygotes had a ZP surface which was smooth and compact with only 1187 pores in an area of 5000 μm^2 with an average diameter of 223 nm. The pores were more shallow than in the oocytes. The smooth, melted appearance on the surface of zygotes might be due to the lytic action of the acrosomal enzymes. The ZP of 8-cell stage embryos had both surface patterns present. The ZP contained 1658 pores over an area of 5000 μm^2 and with an average diameter of 203 nm. The largest pores were in the compact-appearing surface areas. The

rough areas in the ZP may be due to proteins secreted by the oviductal cells. In morulae, the surface of the ZP was rough and spongy. The ZP contained 3259 pores over an area of 5000 μm^2 with an average diameter of 155 nm. Only 19 % of the pores in the morulae were large enough to allow BHV-1 to enter the ZP. In the other three stages, more than 34 % of the pores were large enough to allow the entry of BHV-1. In all four stages, the channel of the pores decreased in diameter closer to the inner ZP. Also, the pore shape was elliptical or circular, and they were randomly distributed.

In his second experiment, Vanroose used confocal laser scanning microscopy to examine the continuity of the meshes of the ZP pregressing inward towards the embryonic cells [318]. The passage through the ZP and localization in the ZP of fluorescent microspheres of different sizes was evaluated. The smallest microspheres (40 nm) were found half-way through the thickness of the ZP. The larger microspheres (200 nm) were found in the outer fourth of the ZP. Therefore BHV-1 (180 to 200 nm), should not be able to penetrate through the entire diameter of the ZP and reach the embryonic cells of IVM oocytes and IVP embryos. Bovine herpesvirus 1 could, however, become trapped in the outer layers of the ZP where it might resist washing and trypsin treatment [273,318].

Vanroose (1997) showed that matured oocytes and zygotes are resistant to infection with BHV-1 even when the zona pellucida is broken [316]. However, embryos beginning at the 4- to 8-cell stage are susceptible to BHV-1 when the zona pellucida has been removed. It has been hypothesized that the earlier embryos are not susceptible because ribosomal RNA synthesis has not begun. Viral proteins can only be produced after the formation of ribosomal RNA. In vitro-derived embryos are able to synthesize

RNA at 36 to 48 hours post insemination and synthesize protein at the 8- to 16-cell stage of development. Another theory about the resistence of very early stage embryos to viral infection is that receptors for the attachment of BHV-1 might not have been expressed [315]. Yet another theory is that specific antibodies are important [316]. In sows, antibodies to suid herpesvirus 1 have been shown to be present in the the cytoplasm of the oocyte [135]. As the cells cleave, the antibodies might be evenly distributed in the cytoplasm of the new cells. This could result in the antibodies being diluted to a concentration that will no longer inhibit viral replication. Vanroose showed that embryonic cells were less susceptible to BHV-1 infection than bovine embryonic lung cells (100 % expression) [316]. Zona pellucida-free hatched blastocysts had 13 % of embryonic cells express viral antigen and ZP-free morulae had 17 % express viral antigen. This might be due to an antiviral component of embryos. It has been shown that embryos produce bovine type 1 interferon (boIFN-τ) which might inhibit viral replication. Its expression is first detected when the blastocoel forms. Antiviral activity increases from 27 % in the early blastocysts to 100 % in hatched blastocysts. Others, however, disagree with this interferon theory.

In zona pellucida-intact IVP embryos, incubation for 1 hour with $10^{6.3}$ $TCID_{50}$/mL BHV-1 did not result in virus replication or embryo degeneration [317]. Bovine herpesvirus 1 was not able to cross the zona pellucida when incubated with the embryos for a short time. It is still possible that undetectable concentrations of BHV-1 are embedded in the ZP, thus trypsin treatment is recommended to avoid contamination of the recipient cow [316]. Trypsin treatment has been shown to not decrease the rate of hatched blastocysts compared to control embryos [30,200]. Although trypsin treatment

does reduce the number of positive embryos it is not as effective at removing BHV-1 from IVP embryos as compared to in vivo-produced embryos [30,273]. Bielanski et al., in 1997, showed that 14 % of single embryos and 54 % of groups of five embryos remained positive for BHV-1 following trypsin treatment [30]. When the virus was present during IVC, trypsin treatment rendered the individual embryos free of virus; however, 57 % of the groups of five embryos remained positive. When the virus was present during IVM, 18 % of the individual embryos and 89 % of the groups of five embryos remained positive. It is not known however if the amount of virus associated with a single embryo would be sufficient to infect the recipient cow via the intrauterine route. In a previous study [79], we showed that trypsin treatment not only rendered individual embryos free of virus but also prevented transmission of the virus from the embryo to BOEC after a 48 hour co-culture. However, 80 % of groups of five embryos were positive after trypsin treatment, and 80 % of BOEC were positive after co-culture with the embryos. This study differed from Bielanski's in that the virus was not present during IVP; instead, Day 7 embryos were incubated in the virus for 1 hr prior to treatment. However, results did suggest that trypsin treatment might prevent infection of recipients if only individual embryos are transferred.

Bovine herpesvirus 1 has been found associated with spermatozoa as well as in media and somatic cells used for in vitro-production of embryos [317]. Vanroose in 1999 performed several experiments to determine the effects of BHV-1 on IVP embryos. In the first experiment, the Cooper strain of BHV-1 at $10^{2.3}$ and $10^{6.3}$ $TCID_{50}$/mL was added to the media used for IVM, IVF, and IVC and the rate of cleavage and development was determined. The percentage of cleavage did not differ from the control group, however

the number of embryos that developed to the blastocyst stage was significantly lower for the BHV-1 exposed embryos compared to the unexposed embryos. Also, the rate of blastocyst formation was 2 % in the embryos exposed to $10^{6.3}$ $TCID_{50}$/mL compared to 8 % in the embryos exposed to $10^{2.3}$ $TCID_{50}$/mL. In a subsequent experiment, BHV-1 was added separately to the IVM, IVF, and IVC phases to determine if BHV-1 had a detrimental effect on embryonic development when added during different phases of IVF. Bovine herpesvirus 1 at $10^{6.3}$ $TCID_{50}$/mL was used. There was a significant difference in the cleavage rate at day 3 post fertilization between the embryos incubated with virus during IVF and the control embryos. Blastocyst development at day 7 after fertilization was significantly lower for each phase of in vitro production compared to the control embryos. Tsuboi showed that oocytes exposed to LA strain prior to maturation developed normally to the 8-cell stage but did not develop into blastocysts [294]. In another experiment, Vanroose cultured embryos with and without oviductal cells to determine if presence of BHV-1 affected embryos indirectly by destruction of somatic cells in co-cultures [317]. Zygotes were cultured in three different culture systems and incubated for 1 hour in culture medium with $10^{6.3}$ $TCID_{50}$/mL BHV-1. Following virus incubation the zygotes were washed 10 times and transferred to virus free culture medium. At day 7 after fertilization, the rate of blastocyst formation was not statistically different between control embryos and the virus exposed embryos cultured in SOF and Menezo-B2. There was a lower rate of blastocyst formation in the embryos cultured in Menezo-B2 + BOEC compared to the control groups. In additon, the percentage of blastocyst development in the control embryos was decreased in the Menezo-B2 group compared to the Menezo-B2 + BOEC and SOF groups. In the final experiment,

monlayers of BOEC were incubated with $10^{6.3}$ $TCID_{50}$/mL BHV-1 to determine the susceptibility of the oviduct cells. Three days after virus incubation all of the oviduct cells expressed viral antigens.

Vanroose (1999) concluded that BHV-1 in an in vitro production system will affect fertilization and embryonic development [317]. Infection of somatic cells during IVM and IVC with BHV-1 might cause the decreased blastocyst rate that was seen. Oviductal cells infected with BHV-1 might not be able to secrete soluble proteins and embryotrophic factors needed to support the embryonic development, and they might be unable to inactivate embryotoxic substances. The destruction of the oviductal cells also can cause the release of embryotoxic substances which changes the pH of the culture medium to affect embryonic development.

When using conventional culture medium, it is necessary to include somatic cells to overcome the 8- to 16-cell block [317]. It is suggested that BHV-1 is strongly attached to the zona pellucida, and the oviductal cells become infected when they contact the ZP resulting in degeneration of the oviductal cells. This causes a decreased cleavage and blastocyst rate. On the other hand, the use of semi-defined medium such as SOF has several advantages. Its use results in a standardized in vitro production system, it allows consistency between laboratories, it eliminates the use of animal products in media which prevents the introduction of pathogens, and the precise requirements of embryo development can be studied. Following exposure to BHV-1, the blastocyst rate of SOF-embryos was not different from control embryos.

Moreover, the adverse effects on cleavage and blastocyst formation are not due to virus replication in the embryonic cells as an intact zona pellucida acts as a protective

barrier against BHV-1 [317]. Because the adverse effects are most obvious when the virus is in the IVF phase of embryo production, these consequences might be due to a direct effect of BHV-1 on the spermatozoa. Bovine herpesvirus 1 attaches to a heparan sulfate receptor which is similar to the heparin receptor on the sperm cell surface that is involved in capacitation. If BHV-1 binds to the sperm cell, it may interfere with zona binding and thus fertilization. Bovine herpesvirus 1 could also indirectly affect spermatozoa by binding to heparin and decreasing in vitro capacitation by decreasing the functionally active heparin.

Vanroose performed several experiments to evaluate the impact of BHV-1 on spermatozoa [319]. In the first experiment, he showed that the fertilization rate of cumulus-free ZP-intact oocytes decreases with the increase of BHV-1 titres (10^{5-7} $TCID_{50}$/ml) and with the decrease of the concentration of spermatozoa. Vanroose demonstrated that the maximal numbers of sperm cells are bound to the ZP between 3 and 6 hours post fertilization. The number of heparinized and non-heparinized sperm cells bound to the ZP is decreased following preincubation of the sperm with BHV-1. A difference in the number of sperm bound to the ZP was not seen in the control group compared to the oocytes preincubated with BHV-1. They also showed that when oocytes were preincubated with BHV-1 there was no difference between the control groups in the number of spermatozoa that bound to the oocytes [284]. However, when spermatozoa were preincubated with BHV-1, there was a significant reduction in the number that bound to oocytes. This demonstrated that BHV-1 interacts with the sperm cells rather than with the ZP. In a follow-up experiment, Vanroose showed that, with a ratio of 10:1 or 100:1 of BHV-1 to sperm cells, the fertilization rates were decreased to 50 % and

75 %, respectively. More than 50 % of the sperm cell-zona binding was inhibited when sperm cells were incubated with BHV-1 at a 10:1 ratio of BHV-1 to sperm cells. This inhibition was due to BHV-1 interacting with the sperm cells not the glycoproteins of the ZP. When BHV-1 was incubated with MAbs against gC, the sperm cells were able to bind to the ZP. Also, MAbs against gD reduced the inhibition of sperm cell-ZP binding. However, gB did not appear to be involved in the attachment of the virus to the sperm cells. Therefore, the mechanism whereby BHV-1 binds to the sperm cell might be similar to the adsorption and penetration of a host cell by BHV-1.

Because the BHV-1 infection status of cows at the slaughterhouse is unknown, oocytes used for in vitro production might have been exposed to BHV-1 [24]. Bielanski and Dubuc in 1994 performed an experiment in which ooctyes were collected from heifers experimentally infected intravenously with BHV-1 (Colorado strain) at estrus and from BHV-1-seropositive heifers treated with dexamethasone. The oocytes were matured, fertilized and cultured in vitro and tested for BHV-1. Embryos, oviductal cells, and uterine fluid were positive for BHV-1. The cleavage rate was 26 % and the blastocyst formation rate was 6 % in the infected donors. The animals treated with dexamethasone had 11 % blastocyst formation which tested negative for BHV-1. Therefore, they showed that transferable embryos can be produced by in vitro production which are associated with the virus and have the potential to spread BHV-1.

Conclusion. In summary, before installing new disease control techniques in human medicine, research in animals is desirable [339]. My studies used bovine embryos as a model for human embryos. It is clear that BHV-1 can be associated with transferable IVP embryos, and that trypsin treatment can reduce the level of viral contamination but

might not be completely effective in producing a pathogen-free embryo. In addition, antiviral agents might be added to in vitro embryo production systems. However, it is not known if the many antiviral agents will allow the embryos to develop normally and if they will effectively remove all virus from the in vitro production system. It is also not known if recombinant trypsin products which are animal-origin-free will effectively remove virus and allow IVP embryos to develop normally. Answers to these simple questions can help to clarify the epidemiologic consequence of producing and transferring embryos from populations of individuals harboring infections with herpesviruses.

III. STATEMENT OF RESEARCH OBJECTIVES

Specific objectives of this research are as follows:

The first objective was to determine the in vitro cell toxicity threshold and viral inhibitory concentration (99 %, IC_{99}) of phosphonoformic acid against bovine herpesvirus 1. In addition, the developmental efficiency of bovine embryos produced and cultured in the presence of phosphonoformic acid was evaluated.

The second objective was to determine the in vitro cell toxicity threshold and viral inhibitory concentration (99 %, IC_{99}) of bovine lactoferrin from milk against bovine herpesvirus 1. Also, the developmental efficiency of bovine embryos produced and cultured in the presence of lactoferrin was evaluated.

The third objective was to evaluate the usefulness of TrypLE™ for the treatment of day 7 in vivo-derived and in vitro-derived, zona pellucida-intact embryos that were artificially exposed to bovine herpesvirus 1.

The fourth objective was to develop a quantitative duplex PCR that could detect bovine herpesvirus 1 and bovine viral diarrhea virus type I and II.

IV. PHOSPHONOFORMIC ACID INCREASES ATTRITION OF BOVINE EMBRYOS DEVELOPING IN VITRO

ABSTRACT

Bovine herpesvirus 1 (BHV-1) is an alphaherpesvirus that is responsible for reproductive and respiratory infections in cattle. The virus can be found in association with gametes, serum, and co-cultured cells that are used for in vitro fertilization (IVF). Transferable embryos produced using oocytes or co-cultured cells that are collected from infected cows provide a mechanism for transmission of virus to embryo-recipient cows. Thus, the use of anti-herpesvirus agents in IVF could provide meaningful disease control options. Phosphonoformic acid has been used to treat human cytomegalovirus retinitis and acyclovir-resistant herpes simplex virus infections. It also has been shown to inhibit BHV-1 (Los Angeles strain) on Georgia bovine kidney cells.

Initially, we evaluated the ability of phosphonoformic acid to inhibit BHV-1 in Madin Darby bovine kidney (MDBK) cells and in cumulus cells that are commonly used in co-culture with bovine in vitro-produced embryos. At 400 μg/mL phosphonoformic acid completely inhibited $6x10^5$ plaque forming units (PFU)/mL, and 200 μg/mL phosphonoformic acid completely inhibited $6x10^4$ PFU/mL of BHV-1. Subsequently, we added phosphonoformic acid (400 μg/mL and 200 μg/mL) to both IVF medium and in

vitro culture (IVC) medium used to culture developing embryos. Unfortunately, the proportion of developed blastocysts decreased, and the number of cells per blastocyst, which is an indication of embryo viability, was lower in the treated embryos. Therefore, phosphonoformic acid can effectively reduce viral titer in co-culture cells but is also likely to decrease the number of viable embryos that are produced.

Mylissa S D Marley, M Daniel Givens, David A Stringfellow, Patricia K Galik, Kay P Riddell. Phosphonoformic acid inhibits development of bovine embryos in vitro. *Veterinary Therapeutics* 2006;7(2):156-166.

INTRODUCTION

Bovine herpesvirus 1 (BHV-1) is a ubiquitous viral pathogen of cattle. Infections with this alphaherpesvirus can result in reproductive and respiratory disease [234,315]. It is the etiologic agent of infectious pustular vulvovaginitis, infectious balanoposthitis, and infectious bovine rhinotracheitis [211]. Transmission of disease caused by BHV-1 occurs through close contact with nasal, oral or genital secretions [211]. The genital form of the disease is characterized by small pustules on the vestibular, preputial and penile mucosa [190,211,307]. Seminal plasma from affected bulls contains BHV-1, but the virus does not enter the spermatozoa [307,319]. Infectious bovine rhinotracheitis is characterized by fever, anorexia, depression, dyspnea, nasal discharge, and hyperemic nasal mucosa [137,211]. Bovine herpesvirus 1 is the most frequently diagnosed cause of viral abortion

in North American cattle [17]. Aborting cattle may or may not show other clinical signs [17]. If seen, the manifested clinical signs are generally respiratory. Abortions are rarely seen with the genital form of the disease [17,315]. Bovine herpesvirus 1 infection can also lead to prolonged infertility due to intrauterine infection, endometritis and severe necrotizing oophoritis [190,315].

Routine application of standard handling procedures for in vivo-produced embryos of cattle has greatly reduced risks of infectious disease transmission via embryo transfer. However, the risks differ between in vivo- and in vitro-produced embryos [316]. In vitro fertilized embryos are at special risk for contamination with BHV-1. This can occur via exposed cumulus-oocyte complexes and spermatozoa or from contaminated products of animal origin that are used in media for washing, fertilization, and culture [24,315,317]. Many of these biological products come from abattoirs where little of the history of the donor animal is known. In vitro-derived embryos require more manipulation and culture than in vivo-derived embryos, thus increasing the risk of viral entry and replication [317]. In addition, the zona pellucida of in vitro-produced embryos is more sticky than that of in vivo-produced embryos, allowing viruses to attach more readily [315], and standard embryo-washing procedures are less efficient for removing virus from in vitro-produced embryos. Bielanski and Dubuc experimentally exposed heifers to BHV-1, and then collected their oocytes and used them for in vitro production of embryos [24]. Embryos, oviductal cells and uterine fluid were all positive for BHV-1, demonstrating that apparently normal transferable IVF embryos can have the virus associated with them. Such embryos provide the potential to spread BHV-1 to the

recipient. Thus, the addition of an appropriate antiviral agent into the in vitro production (IVP) system might deter the virus and allow the embryo to develop normally.

A number of antiviral agents which were shown to be effective against human herpesviruses have been evaluated against BHV-1. Phosphonoformic acid and phosphonoacetate inhibit viral replication by specifically inhibiting viral DNA polymerase through attachment to the pyrophosphate-binding site [164,257,334]. These compounds are slightly cytotoxic [257]. Use of phosphonoacetate is limited because it is topically irritative, toxic to laboratory animals, and accumulates in bones [257]. However, the DNA polymerase in mammalian cells is less susceptible to the action of phosphonoformic acid than the virus-induced enzyme [257]. Consequently, phosphonoformic acid does not irritate the skin or genital mucous membranes and has only minor effects on normal cellular metabolism [257].

An antiviral agent that is effective against BHV-1 and nontoxic to embryos would be beneficial in embryo production. The objectives of this study were to evaluate the anti-BHV-1 activity and cytotoxic effects of phosphonoformic acid in primary bovine cell cultures and the impact on developmental efficiency of bovine embryos when media was supplemented with phosphonoformic acid.

MATERIALS AND METHODS

Media for MDBK cells, oocytes, cumulus cells, in vitro maturation, in vitro fertilization and in vitro culture

Madin Darby bovine kidney cells were grown in 1X Minimum Essential Medium (MEM). This medium was prepared by mixing 90 mL distilled water, 10 mL MEM with Earle's salts (10X, Invitrogen, Carlsbad, CA), 10 mL equine serum (HyClone, Logan, UT), 1mL 1 % l-glutamine (29.2 mg/mL, Invitrogen), 1 mL penicillin/streptomycin/amphotericin B (Invitrogen), and 1 mL sodium bicarbonate (75 mg/mL, Invitrogen).

Cumulus-oocyte complexes were matured in 25 mM HEPES-buffered tissue culture medium 199 (TCM199) with Earle's salts (Invitrogen) supplemented with 10 % (v/v) fetal bovine serum (FBS; HyClone,), 11 μg/mL sodium pyruvate (Sigma, St. Louis, MO), 100 IU/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma), 0.01 U/mL bovine follicle stimulating hormone (Sigma), and 0.01 U/mL bovine luteinizing hormone (Sigma).

Cumulus-oocyte-complexes were washed in Hepes-TALP (Tyrode's salts, albumin, lactate and pyruvate) [106]. This contains modified Tyrode's salts [343], bovine serum albumin (BSA; Fraction V, 3mg/mL, Sigma), 21.6 mM sodium lactate (Sigma), 22 μg/mL sodium pyruvate (Sigma), and 50 μg/mL gentamicin (Sigma).

Cumulus cells were grown in α MEM. This media was prepared by mixing 83 mL α MEM (Cambrex, Walkersville, MD), 1 mL Glutamax-I supplement (Invitrogen),

1 mL penicillin/streptomycin (Invitrogen 10,000U penicillin/10 mg streptomycin/mL), 15 mL FBS (HyClone), and 0.28 mL 2-mercaptoethanol (Invitrogen 1000X).

Plaque assays required mixing equal volumes of 2X MEM and 3 % agarose. Minimum Essential Medium (2X) includes 80 mL distilled water, 20 mL 10X MEM with Earle's salts (10X, Invitrogen), 10 mL equine serum (HyClone), 2 mL l-glutamine (Invitrogen), 2 mL penicillin/streptomycin/amphotericin B (Invitrogen), and 2 mL sodium bicarbonate (Invitrogen). Agarose (3 %, Sigma) consists of 3 grams of type VII low temperature gelling agarose that was dissolved in 97 mL distilled water and autoclaved.

Oocytes were washed prior to in vitro fertilization in TL-Hepes (Cambrex). Matured oocytes were placed in fertilization drops containing Charles Rosenkrans medium (CR2) (10 mL) and BSA 6 mg/mL (Sigma; 0.06 g). This media was also used for in vitro culture drops up to Day 3.5. The CR2 contains 3.143 g sodium chloride (Sigma 108mM), 0.11 g potassium chloride (Sigma 3mM), 1.045 g sodium bicarbonate (Sigma 25 mM), 450 mL water, 0.275 g hemicalcium lactate (Sigma 0.5 mM), 22.2 mg pyruvate (Sigma 0.4 mM), 10 mL Eagle's Basal Medium (BME) amino acids (50X, Sigma), 0.073 g l-glutamine (Sigma 1 mM), 5 mL MEM nonessential amino acids (Sigma), 5 mL penicillin/streptomycin (Invitrogen, 10,000U penicillin/10 mg streptomycin/mL), and 0.5 mL phenol red (Sigma 0.005 µg/mL).

Presumptive zygotes were cultured from Day 3.5 to Day 7.5 in IVC drops containing CR2 and 10 % FBS (HyClone). The equine serum, BSA, and FBS used in this research were determined to be free of BHV-1 and anti-BHV-1 antibodies by virus isolation and virus neutralization.

Cell lines

Madin Darby bovine kidney cells (MDBK), purchased from the American Type Culture Collection (Manassas, VA), were propagated and cultured in Minimum Essential Medium (MEM).

Monolayers of cumulus cell were established from cumulus-oocyte complexes (oocytes surrounded by multiple layers of dense cumulus cells) that were aspirated from ovaries at an abattoir in Omaha, Nebraska, placed in maturation medium in a portable incubator set at 38.5° C and shipped by courier to Auburn, Alabama. The oocytes were matured while they were in transit (20 to 24 hours). Upon arrival of the three hundred oocytes, the expanded cumulus cells were removed by washing and vigorous pipetting in three, 35-mm petri dishes and 3 wells of a 4-well plate containing HEPES-TALP. The media containing the cumulus cells was removed from the three 35-mm dishes and the first well of the 4-well plate. The media was centrifuged for 5 minutes and the supernatant discarded. The pellet was resuspended with 100 µL of α MEM. Cells in 25 µL aliquots were placed in each well of a 4-well plate (2 cm^2) with 1 mL α MEM. The plate was incubated for 24 hours at 38.5° C in an atmosphere of 5 % CO_2 and air. The media on cell cultures was changed after one day. On Day 2, the cells were transferred to a 25 cm^2 flask. The cells were transferred 7 to 8 times before they were cryopreserved at -80° C until needed.

Antiviral Compounds

Phosphonoformic acid trisodium salt hexahydrate (also known as foscarnet and sodium phosphonoformate tribasic hexahydrate) and phosphonoacetic acid were obtained from Sigma. The drugs were supplied as a dry powder and dissolved in 1X MEM. The solutions were filter sterilized (0.2 μm) before use.

Virus

Bovine herpesvirus 1 (Colorado strain) was obtained from American Type Culture Collection (catalog number VR-864, lot number 1222901). It was propagated in MDBK cells. Virus was harvested by freezing and thawing the infected cell cultures and stored in cryovials at -80° C until needed. The PFU/mL of stock virus were determined by quantifying plaques on MDBK cells.

Virus Isolation

Virus isolation procedures were used to determine the effect of the antiviral agent on the presence of BHV-1. One mL of diluted antiviral agent was added to each well of a 24-well plate (2 cm^2 monolayer) or 100 μL was added to each well of a 96-well plate (0.32 cm^2) previously seeded with MDBK cells or cumulus cells. The plate was incubated for 15 minutes at 38.5° C prior to adding BHV-1. Bovine herpesvirus-1 was diluted to provide a multiplicity of infection (MOI) of 0.005 to 0.5. The plate was

incubated for 5 days at 38.5° C in an atmosphere of 5 % CO_2 and air. The cells were examined daily for evidence of cytopathic effect (CPE). At Day 2, a portion of all cultures showing CPE were frozen for later viral quantification using plaque assay. At Day 5, the remainder of all cultures showing CPE were frozen at -80° C.

Plaque Assay

Plaque assays were used to quantify the amount of virus remaining after culture with an antiviral agent. Day 2 samples containing the cell lysate were thawed and assayed. The samples were serially diluted to 10^{-8}. Five hundred μL of dilutions 10^{-3} to 10^{-8} for each sample were inoculated onto a single well of a 6-well plate (9.5 cm^2) previously seeded with MDBK cells. The plate was incubated for 1 hour at 38.5° C in an atmosphere of 5 % CO_2 and air. Three mL of 3 % agarose/2X MEM (50:50) were placed in each well, and the plates were refrigerated for 8 minutes. The plates were then incubated for 5 days at 38.5° C in an atmosphere of 5 % CO_2 and air. On Day 5, the plaques in each well were counted.

Cell Cytotoxicity Assay

The effects of phosphonoformic acid (400 and 200 μg/mL) on MDBK cells were quantified using Cell Counting Kit-8 (Dojindo, Gaithersburg, MD). A water-soluble tetrazolium salt (WST-8) is reduced by dehydrogenases in cells to give a yellow-colored

product (formazan). The amount of formazan dye produced by the dehydrogenase activity in the cells is directly proportional to the number of living cells.

A 96-well plate was seeded with MDBK cells for a final cell count per well of approximately 8 X 10^3. The plate was incubated at 38.5° C for 24 hours. Phosphonoformic acid (10 µL) at concentrations of 400 or 200 µg/mL was added into the culture media. The drug was evaluated in triplicate. The plate was incubated for an additional 48 hours at 38.5° C. Cell Counting Kit-8 solution (10 µL) was added to each well, and the absorbance at 450 nm was measured after 2 hours of incubation.

Mini-prep Percoll-gradient Separation of Spermatozoa

One straw of cryopreserved bull semen previously characterized in the IVF system and confirmed to be free of BHV-1 by virus isolation was used for fertilization. Semen was thawed in a water bath at 37 °C for 30 to 60 seconds. Percoll gradients were prepared as follows. One-half mL of 90 % percoll (Sigma) was placed in the bottom of a 1.5 mL microcentrifuge tube. Then, 0.5 mL of 45 % percoll was placed over this layer, and finally the thawed semen was gently placed on top of the two layers of percoll. Following centrifugation for 7 minutes at 2000 x g, the layers of percoll were removed, being careful not to disturb the sperm pellet at the bottom. The sperm pellet was suspended in 1 mL of TL-Hepes and centrifuged for 1 minute at 2000 x g and resuspended in 60 µL of CR2 and BSA. The supernatant was discarded and the sperm concentration was determined using a hemocytometer. The total number of spermatozoa added to each fertilization drop was approximately 5 x 10^4 in a volume of 2 µL.

In Vitro Fertilization (IVF)

Following arrival of oocytes, cumulus-intact matured oocytes were washed twice in TL-Hepes and once in CR2 and BSA, a modified synthetic oviductal fluid formulation. Ten to twelve oocytes in about 2 μL were placed in 42-μL fertilization drops overlayed with mineral oil. Two μL heparin (0.025 mg/mL) and 2 μL hypotaurine/penicillamine were added to each drop before adding the sperm. Two μL of 1 x 10^6 spermatozoa/mL were added to each drop for a total sperm concentration of 5 x 10^4/50 μL drop. Fertilization plates were incubated for 6 hours at 38.5 °C in an atmosphere of 5 % CO_2 and air (Figure 1). Following the incubation period, the fertilization drops were examined for hyperactivated sperm as an indication of capacitation.

In Vitro Culture (IVC)

Six hours after fertilization, presumptive zygotes were removed from the fertilization drops and washed twice in TL-Hepes and once in CR2 and BSA to remove excess sperm. Ten to twelve presumptive zygotes were placed in each in vitro culture drop containing 30 μL of CR2 and BSA and overlayed with mineral oil. In vitro culture plates were incubated for 3.5 days at 38.5 °C in an atmosphere of 5 % CO_2 and air.

On Day 3.5, presumptive zygotes were cleaned using a sterile stripping tool. This is a glass pipette with a tapered end. Embryos are repeatedly aspirated into the pipette and expelled resulting in removal of the cumulus cells. The nude embryos that had

cleaved to 4-cell or greater were washed 3 times in TL-Hepes and once in CR2 and BSA. Up to 20 to 25 developing embryos were placed in each IVC drop containing 30 µl of CR2 and FBS and overlayed with mineral oil. Also, clusters of cumulus cells were placed in each drop. The stage of embryo development was recorded. In vitro culture plates were incubated for 4 days at 38.5 °C in an atmosphere of 5 % CO_2 and air (Figure 1). To prevent toxic accumulation of ammonia from amino acid degradation, the medium was replaced after 3.5 days of culture [5].

On Day 7.5 of culture, embryonic development and quality were assessed as previously described [231].

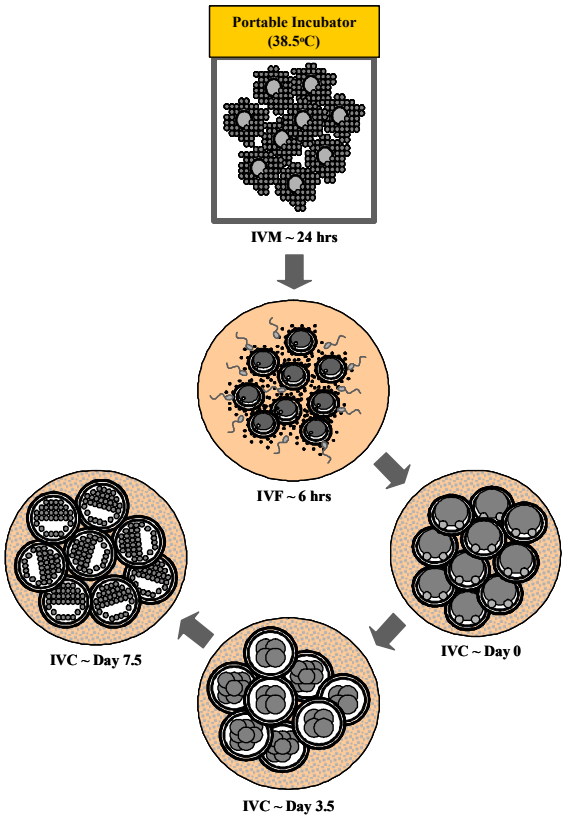


Figure 1. In vitro embryo production. IVM, in vitro maturation of oocytes; IVF, in vitro fertilization; IVC, development of zygotes to blastocyst with cumulus cells.

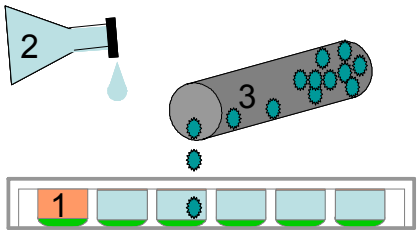
Experimental Design

This project was divided into 4 experiments. The first experiment determined the nontoxic concentration of phosphonoformic acid that was effective against BHV-1 in MDBK cells. The second experiment assessed the efficacy of phosphonoformic acid against four concentrations of BHV-1 in MDBK cells. The third experiment evaluated phosphonoformic acid for efficacy against BHV-1 in primary cell cultures of bovine cumulus cells. The final experiment evaluated the development rate of bovine embryos fertilized and cultured in the presence of phosphonoformic acid.

Experiment 1

Four replicates were performed. Two-fold dilutions of phosphonoformic acid trisodium salt hexahydrate (400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 µg/mL) were tested. The media over actively dividing monolayers of MDBK cells in single wells of a cell culture plate (0.32 cm^2) was supplemented with each of the concentrations to be tested. After 15 minutes, each well was inoculated with $2x10^5$ to $1x10^6$ PFU BHV-1/mL for an MOI of between 0.05 to 0.5, and culture plates were incubated (38.5^oC) in an atmosphere of 5 % CO_2 and air for 5 days (Figure 2). Controls included the following: 1) MDBK cells with phosphonoformic acid but no virus (to evaluate toxicity of agent to cells). 2) MDBK cells with BHV-1 (positive control for viral cytopathic effect). 3) MDBK cells but no test antiviral or virus (negative control). 4) MDBK cells with BHV-1 and phosphonoacetic acid (200 µg/mL) which is known to inhibit BHV-1 in cell culture [126].

During the incubation period, cells were examined daily with an inverted cell culture microscope at a magnification of 400X for presence or absence of cytotoxicity and cytopathic effect (CPE) as an indicator of whether or not the virus was inhibited. At 48 hours, a sample (300 µL) of medium over monolayers exposed to BHV-1 was collected and virus was quantified via plaque assay [79]. The percent of virus inhibited for each test concentration was determined by comparison to equivalent samples from temporal control cultures in which no compound was added before or after inoculation (Percentage of virus inhibited = [Quantity of virus in the control sample lacking the compound-Quantity of virus in the compound sample]/ Quantity of virus in the control sample lacking the compound X 100).



1. MDBK cells established in 24 well plate
2. Added medium containing phosphonoformic acid
3. Inoculated with BHV-1
4. Cultured for 5 days at 38.5 ºC
5. Virus quantification of day 2 cell lysate

Figure 2. Design of experiment one. MDBK, Madin Darby bovine kidney cells; BHV-1, Bovine Herpesvirus-1.

Experiment 2

One trial was performed with 5 replicates. The purpose of this experiment was to determine if the selected concentrations of phosphonoformic acid would completely inhibit BHV-1 in MDBK cells. The following dilutions of phosphonoformic acid were tested: 400, 200, 100, 50, and 25 µg/mL. Subsequently, BHV-1 was added to the cells in

a single well. The following dilutions of virus were used $6x10^5$ PFU/mL, $6x10^4$ PFU/mL, $6x10^3$ PFU/mL, and $6x10^2$ PFU/mL. This allowed for an MOI of 0.2, 0.02, 0.001, and 0.001 respectively. Culture plates were incubated (38.5°C) in an atmosphere of 5 % CO_2 and air for 5 days. During the incubation period, cells were examined daily with an inverted cell culture microscope at a magnification of 400X for presence or absence of CPE as an indicator of whether or not the virus was inhibited. Controls included the following: 1) Monolayer plus BHV-1 but no antiviral. 2) Monolayer of MDBK cells and no chemical or virus. The 50 % end point in virus titration was determined using the Reed-Muench method [236].

Experiment 3

Four replicates were performed to evaluate efficacy against BHV-1 in primary cell cultures (cumulus cells). The purpose of this phase was to identify the minimal inhibitory concentrations for BHV-1 and maximal nontoxic concentrations of phosphonoformic acid in primary cell cultures. This was necessary to determine the therapeutic index (margin of safety between cytotoxic dose and effective antiviral dose).

The following dilutions of phosphonoformic acid were tested: 400, 200, 100, 50, and 25 μg/mL. Subsequently, BHV-1 was added to the cells in a single well at an MOI of 0.005 to 0.5, and culture plates were incubated (38.5°C) in an atmosphere of 5 % CO_2 and air for 5 days. During the incubation period, cells were examined daily with an inverted cell culture microscope at a magnification of 400X for presence or absence of CPE as an indicator of whether or not the virus was inhibited. In addition, the inhibition of BHV-1 in incubating cells was quantified via plaque assay for virus in media (day 2).

Controls included the following: 1) Monolayer of cumulus cells with phosphonoformic acid but no virus (to evaluate toxicity of agent to cells). 2) Monolayer plus BHV-1 but no antiviral. 3) Monolayer of cumulus cells and no chemical or virus. The percent viral inhibition for each treatment was determined.

Experiment 4

Four replicates were performed. Three hundred oocytes that had been matured in transit from Omaha, Nebraska were distributed as follows: 1) 100 oocytes were used as an untreated control. 2) 200 oocytes were used in the treatment, with the specified antiviral concentration placed in both IVF and IVC media. Thus, the IVF and IVC media was supplemented with either 200 or 400 μg/mL of phosphonoformic acid (Figure 3). The use of 300 oocytes with an anticipated minimal blastocyst development rate of 25 % for the control oocytes allowed a difference of 5 % in the treated oocytes to be significant at $p=0.05$ using Pearson's Chi-square statistic.

After embryonic development was assessed on Day 7.5, embryonic quality and viability were evaluated. Embryonic quality was assessed by grading the embryo as described in the Manual of the International Embryo Transfer Society (IETS) [271]. The IETS guidelines grade embryos according to the following codes. Code 1 describes excellent or good embryos. The blastomeres should be uniform, and at least 85 % of the cellular material should be intact. Code 2 describes fair embryos. There are moderate irregularities in the shape of the embryonic mass, and at least 50 % of the cellular material should be intact. Code 3 is used for poor embryos. There are major irregularities in the shape of the embryonic mass, and at least 25 % of the cellular

material is intact. Code 4 is used for dead or degenerating embryos [271]. Embryonic viability also was assessed by counting the nucleated cells of developed blastocysts [231]. The embryos were stained using a rapid whole-mount staining procedure with trypan blue counterstain and Hoeschst 33342. A Nikon Eclipse E800 fluorescence microscope was used to count the cells.

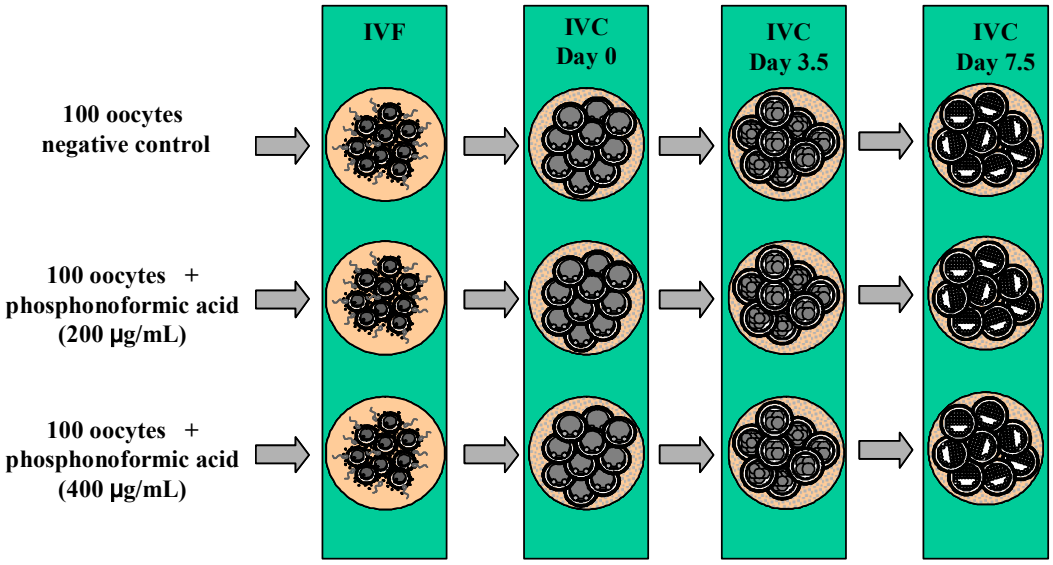


Figure 3. Experimental design for experiment 4. IVF, in vitro fertilization; IVC, culture of embryos to Day 7.5.

Statistical Analysis

The cytotoxicity assay was analyzed using Student's t test (JMP Software, SAS Institute).

For experiments 1 and 3, the percent of virus inhibited for each test antiviral agent at each concentration was determined by comparison to equivalent samples from temporal control cultures in which no compound was added before or after inoculation (Percentage of virus inhibited = [Quantity of virus in the control sample - Quantity of virus in the compound sample]/ Quantity of virus in the control sample X 100).

In experiment 1, the 99 % inhibitory concentration (IC_{99}) was calculated by using the JMP Software, SAS Institute.

For experiment 2, the 50 % end point in virus titration was determined using the Reed-Muench method [236].

In experiment 4, the percent of embryos that developed to blastocysts in each treatment group was compared to those in the control group using a Pearson's Chi Square test. The number of nucleated cells from the embryos in each treatment group were compared to those in the control group using an ANOVA paired T test with JMP Software, SAS Institute. Also, the grades of the embryos from each treatment group were compared to those in the control group using an ANOVA paired T test with JMP Software, SAS Institute.

RESULTS

Cytotoxicity Assay

Phosphonoformic acid (400 and 200 µg/mL) was not cytotoxic to MDBK cells ($P<0.05$). Phosphonoformic acid at a concentration of 200 µg/mL enhanced cell growth

whereas a concentration of 400 μg/mL reduced cell growth; however, these differences were not significant.

Experiment 1

Phosphonoformic acid at a concentration of 200 μg/mL inhibited greater than 10^4 PFU/mL of virus on MDBK cells (Table 1). The IC_{99} was shown to be 46 μg/mL for phosphonoformic acid. Phosphonoformic acid produced no cytotoxicity in the MDBK cells at the concentrations tested. Cell morphology of the treated groups did not appear to differ from cells grown in control medium, and cell detachment was not observed.

Experiment 2

Phosphonoformic acid (400 μg/mL, 1333 μM) was able to inhibit viral replication of all four concentrations tested (6×10^5 PFU/mL, 6×10^4 PFU/mL, 6×10^3 PFU/mL, and 6×10^2 PFU/mL). At 200 μg/mL (666.6 μM) of phosphonoformic acid, complete inhibition of BHV-1 was seen at 6×10^4 PFU/mL, 6×10^3 PFU/mL, and 6×10^2 PFU/mL.

Experiment 3

Phosphonoformic acid was effective at inhibiting 10^7 PFU/mL of virus at 200 μg/mL (Figure 4). Phosphonoformic acid was not cytotoxic to the cumulus cells at the concentrations tested. Cell morphology of the treated groups did not differ from cells grown in control medium, and cell detachment was not observed.

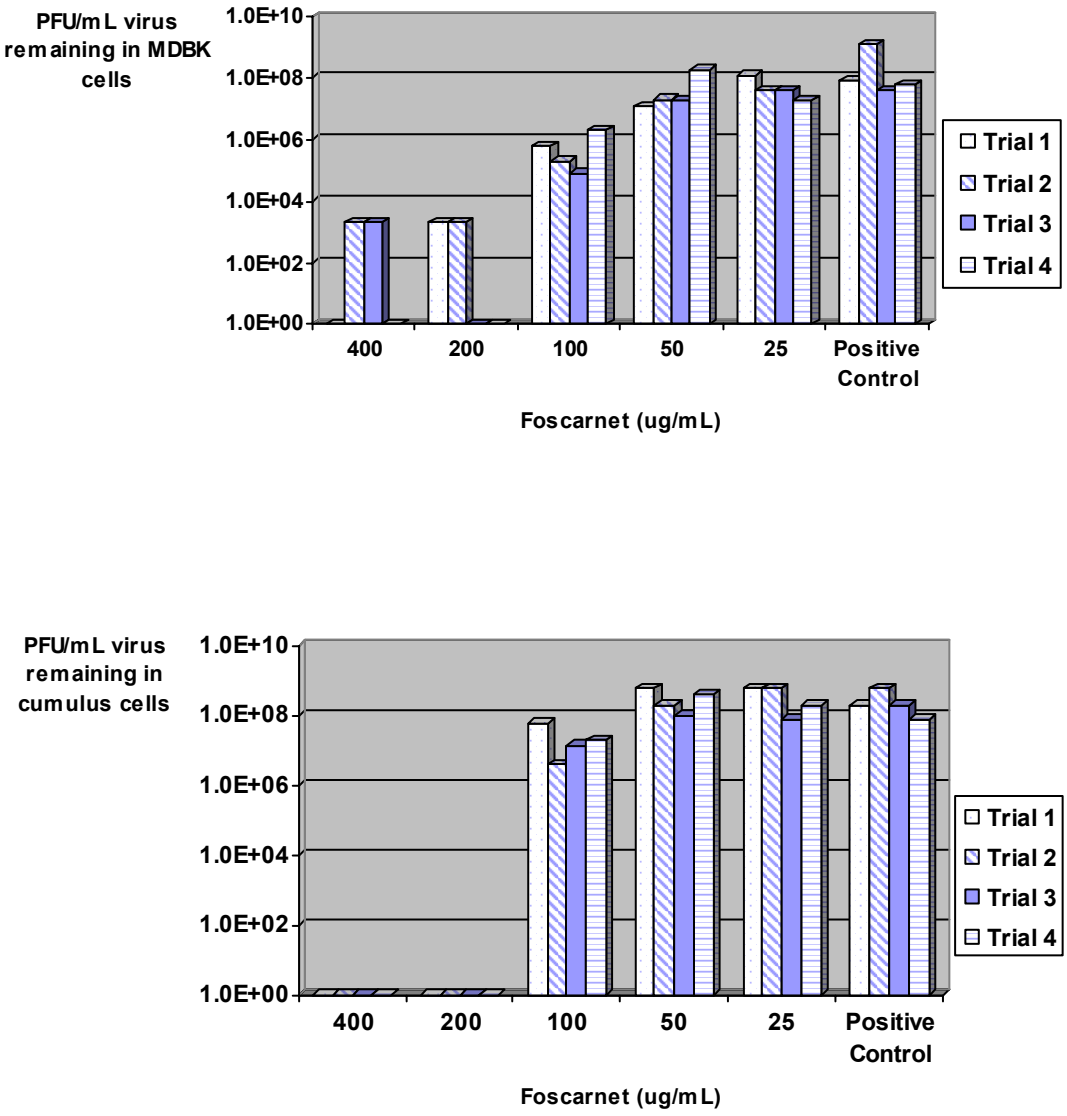


Figure 4. Inhibition of bovine herpesvirus 1 by phosphonoformic acid at concentrations used in experiment 1 (MDBK cells) and experiment 3 (cumulus cells). MDBK, Madin Darby bovine kidney cells.

Experiment 4

Embryos were able to develop in the presence of phosphonoformic acid (Table 1). However, the blastocyst development rates of the treated groups were significantly reduced from that of the control group, 20 % (200 µg/mL phosphonoformic acid) and

3 % (400 µg/mL phosphonoformic acid) compared with 29 % for the control group. In addition, the nucleated cell counts of embryos from the treated groups were lower than that of the control group, 57 (200 µg/mL phosphonoformic acid) and 27 (400 µg/mL phosphonoformic acid) versus 84 (control group). The appearance of viable embryos (embryo grade) was not significantly different among groups.

Table 1. Embryonic development and viability of control embryos and those treated with phosphonoformic acid (200 or 400 µg/mL) from experiment 4.

	Control Group	200 µg/mL Phosphonoformic acid	400 µg/mL Phosphonoformic acid
Day 3.5 development (>4 cell embryos/ oocytes fertilized)	223/374 (60 %)	201/367 (55 %)	104/384 (27 %)[a]
Day 7.5 development (blastocysts/oocytes fertilized)	108/374 (29 %)	74/367 (20 %)[a]	12/384 (3 %)[a]
Nucleated Cell Number (Mean of Day 7.5 blastocysts)	84	57[a]	27[a]
Embryo Grade (Mean of Day 7.5 blastocysts)	1.1	1.1	1.3[a]

[a]Mean differs significantly from control ($P \leq 0.01$).

DISCUSSION

Bovine herpesvirus 1 is a major economic concern in cattle-producing countries [84]. Because the virus has been shown to associate with in vitro fertilized embryos [24,30], it is possible that transfer of these embryos could result in transmission of disease to recipient cattle. Thus, use of an effective antiviral agent in the IVP system could provide a useful deterrent to transmission of disease to recipients. With this as a motivation, the specific aims of this project were to determine the viral inhibitory concentration (99 %, IC_{99}) of phosphonoformic acid against BHV-1 and to determine if bovine IVF embryos would develop normally in the presence of phosphonoformic acid.

There are several circumstances by which a contaminant can enter the IVP system. Virus can be present in the cumulus oophorus cells, follicular fluid and in materials used in fertilization and culture medium [29,317]. Virus can also be associated with the sperm. Vanroose has shown that presence of BHV-1 in all 3 phases of the IVP system (IVM, IVF, and IVC) as well as in each phase separately will decrease blastocyst production [317]. In a worst case scenario, all 3 phases could contain virus. Since IVM was initiated at and oocytes were shipped from another location, it was not feasible to add antivirals to IVM. However, we did elect to test the antiviral agent in both IVF and IVC, simultaneously.

Phosphonoformic acid, also known as foscarnet and trisodium phosphonoformate, has been used to effectively treat human cytomegalovirus infection and acyclovir resistant herpes simplex virus infection. Phosphonoformic acid is a non-nucleoside antiviral and a pyrophosphate analogue [114,125,328,334]. It functions by selectively

inhibiting virus-specific DNA polymerase at the pyrophosphate-binding site [201,334]. By preventing cleavage of pyrophosphate from deoxynucleotide triphosphate, viral replication is inhibited [46,125,334]. Phosphorylation is not required as with nucleoside analogues therefore, patients who are resistant to acyclovir, valaciclovir, penciclovir, and ganciclovir can be treated with phosphonoformic acid [334]. Resistance to phosphonoformic acid involves alteration in binding to viral DNA polymerase [114,125]. The safety of phosphonoformic acid in human pregnancy has not been thoroughly evaluated [114]. However, a single case report of treatment in a patient at 32 weeks gestation did not indicate any adverse effects in the infant [201].

Schwers et al. demonstrated that phosphonoformic acid was effective in reducing the number of plaques produced by BHV-1 (Los Angeles strain) on Georgia bovine kidney cells as well as decreasing the titer of virus [257]. They showed that 500 and 1000 μM of phosphonoformic acid significantly reduced the number of plaques produced by BHV-1 (3 logs) [257]. They also demonstrated a significant reduction in plaque size with 10 μM of phosphonoformic acid, and this reduction increased with the increased concentration of the drug to 1000 μM. In addition, they demonstrated that the drug was safe for uninfected control cells that were grown in MEM with 10 % FBS [257]. Similarly, Helgstrand showed that 500 μM phosphonoformic acid reduced BHV-1 plaque formation on calf kidney cells by 99.9 % [123]. The concentrations that we used are similar to those used by Sarisky et al. to demonstrate that phosphonoformic acid was effective against penciclovir and acyclovir resistant strains of HSV-1 [254]. After our initial testing in MDBK cells, cumulus oophorus cells were used in additional screening assays because they represent somatic cells that are commonly used in co-cultures with

bovine and human in vitro produced embryos. Consistent with the above findings, we demonstrated that phosphonoformic acid was effective at reducing viral concentration by greater than 4 or 5 logs both on MDBK cells and bovine cumulus cells, while exhibiting no signs of cytotoxicity. In addition, virus could be inhibited completely if a lower concentration of virus ($6x10^4$ PFU/mL) or a higher concentration of phosphonoformic acid (400 μg/mL) was used. Inhibition of viral replication and non-toxicity in cumulus cells was encouraging; however, when phosphonoformic acid (400 μg/mL and 200 μg/mL) was placed in the IVF media and IVC media of developing bovine embryos, blastocyst development was reduced compared to the control group. In addition, the number of cells in the treated embryos was lower than in the control embryos. The cell number is an indication of developmental potential and has been shown to be a better indicator of embryo quality then visual morphological assessment [206]. As the cell number decreases, developmental potential of the embryo decreases.

As stated above, the cumulus cells showed no signs of morphological change induced by phosphonoformic acid during the screening assays. Also, during IVF and IVC, no change was seen in the cumulus cell monolayers of the treated groups compared with those of the control groups. Thus, the negative effect on embryonic development was more likely due to a direct effect on the embryonic cells as opposed to an indirect effect via the co-cultured cumulus cells. In addition, hyperactivation of spermatozoa was not affected by the presence of phosphonoformic acid and Day 3.5 development was not significantly different between the control group and the group treated with 200 μg/mL phosphonoformic acid. Thus, the drug, when used at the lower concentration had no effect on fertilization or cleavage rates that was apparent at these stages of observation.

Blastocysts were able to develop; however, they developed at a reduced rate and their developmental potential was lower as determine by the nucleated cell count. Thus, the fertilization and development of bovine embryos were more sensitive indications of the toxic effects of the antiviral agent than were division and growth of either MDBK or cumulus cells. One possibility for this difference lies in the concentrations of serum used in the different media. The antiviral agent was reconstituted in 1X MEM with 10 % equine serum. The MDBK cells were cultured in 1X MEM with 10 % equine serum and the cumulus cells were cultured in α MEM with 15 % FBS. But, the embryos were fertilized and cultured first in CR2 with BSA (day 0 to 3.5) followed by CR2 with 10 % FBS in the second phase of culture (day 3.5 to 7.5). Serum has been shown to have detoxifying effects. Others have shown that the concentration of FBS present in the medium can affect the results of cytotoxicity studies of various agents [35,290]. Tognon et al. showed that cytotoxicity of an anticancer drug decreased (75 % cytotoxicity versus 40 %) as FBS concentration increased from 2.5 % to 10 %, respectively [290]. In comparison, when the drug was diluted with human serum albumin (HSA) cytotoxicity was 100 %. Use of the drug dissolved in HSA and diluted in medium with FBS yielded a lower cytotoxicity but not as low as when the drug was dissolved in FBS. It is believed that FBS provided protection to the cells by preventing complete uptake of the drug by the cells. They showed that the intracellular concentration of the drug was lower in the presence of 10 % FBS compared with HSA. Similarly, Bohets showed that FBS concentration affected the cytotoxicity of nephrotoxic agents [35]. One agent ($HgCl_2$) was less cytotoxic with 5 to 10 % FBS than with 0 to 1 % FBS in the medium. This could be due to FBS binding to Hg. The cytotoxicity of the other agent (paracetamol)

was not affected by the FBS concentrations. Thus, to truly determine the cytotoxicity of an agent, the lowest concentration of FBS required to maintain the cells should be used. The potential cytoprotective effect of FBS on phosphonoformic acid is not known. Based on the above studies, further research using various concentrations of FBS in the presence of phosphonoformic acid should be done to determine if a specific concentration of FBS might mitigate the reduction of embryonic development without reducing the antiviral effects of phosphonoformic acid.

Completion of this research confirmed that phosphonoformic acid is non-toxic to bovine cumulus cells and can be used to inhibit replication of BHV-1in these cells. However, as used in this study the compound was detrimental to embryonic development. Future research should evaluate whether strategic use of FBS might mitigate the negative effects of phosphonoformic acid without compromising its antiviral effect. Also, additional studies should be initiated to evaluate embryonic development when the antiviral is only used in IVC as opposed to its simultaneous use in both IVF and IVC.

V. LACTOFERRIN FROM BOVINE MILK REDUCES BOVINE HERPESVIRUS 1 IN CELL CULTURE BUT INHIBITS DEVELOPMENT OF IN VITRO-DERIVED BOVINE EMBRYOS

ABSTRACT

Lactoferrin is an iron-binding glycoprotein found in milk, saliva, tears and other exocrine secretions. It is known to have in vitro antiviral effects against human, feline and canine herpesviruses. In addition, lactoferrin is known to be safe in cell culture. Bovine herpesvirus 1 (BHV-1) is a likely contaminant of in vitro embryo production. Further, trypsin treatment is not completely effective in removing the virus from these embryos. We hypothesized that a nontoxic concentration of lactoferrin might prevent replication of BHV-1 within in vitro embryo production systems. Thus, the specific objectives of this research were to determine if lactoferrin from bovine milk individually or combined with cidofovir would inhibit BHV-1 in cell culture and to determine if in vitro-derived embryos could develop normally when cultured in lactoferrin.

Lactoferrin (10 mg/mL) inhibited 100 to 25,000 plaque forming units (PFU)/mL of virus. At a concentration of 5 and 2.5 mg/mL, 10 to 1,000 PFU/mL of virus were inhibited, and a concentration of 1.25 and 0.625 mg/mL inhibited 1 to 100 PFU/mL of virus. While lactoferrin did not affect the nucleated cell count of the treated embryos, it

did significantly decrease blastocyst development. Results indicate that lactoferrin from bovine milk can significantly inhibit BHV-1 in cell culture. However, supplementation of in vitro culture with lactoferrin inhibits blastocyst development of in vitro-derived embryos.

M Givens, M Marley, P Galik, K Riddell, D Stringfellow. Lactoferrin inhibits bovine herpesvirus-1 in cell culture and allows normal development of in vitro-produced embryos. *Reproduction, Fertility and Development*. 2006; 18(1,2): 213.

INTRODUCTION

Lactoferrin has been shown to have antibacterial, antiviral, antifungal and antiprotozoal effects [8,122,151,296]. It is a mammalian iron-binding glycoprotein produced by mucosal epithelial cells [20,282,303]. Lactoferrin is found naturally in milk, saliva, tears, bile, seminal and vaginal fluids, synovial fluid, mucous secretions and secondary granules of neutrophils. The antiviral activity of lactoferrin occurs in the early phase of infection, interfering with viral attachment without affecting normal host-cell metabolism [20,282]. Its antibacterial effect is due to depriving bacteria of iron required for their survival [215]. It might also bind directly to the cell wall of bacteria and fungi, causing membrane damage and leakage of intracellular components [81]. Also, lactoferrin is actively secreted by neutrophils during an inflammatory response and is a component of colostrum and milk that prevents infections in neonates [45]. Furthermore,

it acts indirectly by enhancing the cytotoxic effects of monocytes and natural killer cells [184,302]. In addition, lactoferrin is readily available, has low cytotoxicity, and low cost.

Lactoferrin has been shown to have antiviral effects on cell culture against herpes simplex virus, cytomegalovirus, canine herpesvirus, and feline herpesvirus [20,122,282]. Because resistance to antiviral agents can arise and long-term treatment can result in undesirable side effects associated with cytotoxicity, combination therapy has been used to overcome these negative effects. Lower concentrations of the individual toxic antiviral agents can be used in combination to achieve the same result as conventional treatment [302]. van der Strate et al. demonstrated that bovine lactoferrin works synergistically with cidofovir to inhibit CMV (RC256 strain) in fetal lung fibroblasts [303]. This combination was 42 % more efficient than theoretically expected on the basis of dose-response curves of the individual agents. In addition, cidofovir has been shown to be effective against BHV-1 in tissue culture at 4 μg/mL [97]. The drug also reduces clinical signs and viral replication in calves inoculated intranasally with BHV-1 [98].

Animal herpesviruses are similar to human herpesviruses, and for that reason, they are used as models [248,258,297]. Bovine herpesvirus 1 is an alphaherpesvirus that is responsible for abortion, infertility, genital disease and respiratory infections in cattle [234,315]. Bovine herpesvirus 1 has been shown to be associated with gametes, serum, and co-culture cells which are used for in vitro fertilization (IVF) [24,29,309,329]. Further, transferable embryos can be produced from infected cumulus oocyte complexes making it theoretically possible to transmit virus to the recipient cow after transfer of IVF embryos [24]. Thus, the identification of effective anti-herpesvirus agents for use in IVF

could provide disease control options for use in both bovine and human in vitro embryo production.

The aim of this study was to attempt to identify an antiviral agent that might be effective against BHV-1 and allow in vitro-derived bovine embryos to develop normally. The specific objectives were to determine the effective nontoxic concentration of lactoferrin from bovine milk individually and in combination with cidofovir against BHV-1 in MDBK cells. The second objective was to determine the impact on developmental efficiency of bovine embryos cultured with lactoferrin.

MATERIALS AND METHODS

Media for MDBK cells, in vitro maturation, in vitro fertilization and in vitro culture

Madin Darby bovine kidney cells were grown in 1X Minimum Essential Medium (MEM). This medium was prepared by mixing 90 mL distilled water, 10 mL MEM with Earle's salts (10X, Invitrogen, Carlsbad, CA), 10 mL equine serum (HyClone, Logan, UT), 1mL 1 % l-glutamine (29.2 mg/mL, Invitrogen), 1 mL penicillin/streptomycin/amphotericin B (Invitrogen), and 1 mL sodium bicarbonate (75 mg/mL, Invitrogen).

Plaque assays required mixing equal volumes of 2X MEM and 3 % agarose. Minimum Essential Medium (2X) includes 80 mL distilled water, 20 mL 10X MEM with Earle's salts (10X, Invitrogen), 10 mL equine serum (HyClone), 2 mL l-glutamine (Invitrogen), 2 mL penicillin/streptomycin/amphotericin B (Invitrogen), and 2 mL

sodium bicarbonate (Invitrogen). Agarose (3 %, Sigma, St. Louis, MO) consists of 3 grams of type VII low temperature gelling agarose that was dissolved in 97 mL distilled water and autoclaved.

Cumulus-oocyte complexes were matured in 25 mM HEPES-buffered tissue culture medium 199 (TCM199) with Earle's salts (Invitrogen) supplemented with 10 % (v/v) fetal bovine serum (FBS; HyClone,), 11 μg/mL sodium pyruvate (Sigma), 100 IU/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma), 0.01 U/mL bovine follicle stimulating hormone (Sigma), and 0.01 U/mL bovine luteinizing hormone (Sigma).

Oocytes were washed prior to in vitro fertilization in TL-Hepes (Cambrex, Walkersville, MD). Matured oocytes were placed in fertilization drops containing Charles Rosenkrans medium (CR2) (10 mL) and BSA 6 mg/mL (Sigma; 0.06 g). This media was also used for in vitro culture drops up to Day 3.5. The CR2 contains 3.143 g sodium chloride (Sigma 108mM), 0.11 g potassium chloride (Sigma 3mM), 1.045 g sodium bicarbonate (Sigma 25 mM), 450 mL water, 0.275 g hemicalcium lactate (Sigma 0.5 mM), 22.2 mg pyruvate (Sigma 0.4 mM), 10 mL Eagle's Basal Medium (BME) amino acids (50X, Sigma), 0.073 g l-glutamine (Sigma 1 mM), 5 mL MEM nonessential amino acids (Sigma), 5 mL penicillin/streptomycin (Invitrogen, 10,000U penicillin/10 mg streptomycin/mL), and 0.5 mL phenol red (Sigma 0.005 μg/mL).

Presumptive zygotes were cultured from Day 3.5 to Day 7.5 in IVC drops containing CR2 and 10 % FBS (HyClone). The equine serum, BSA, and FBS used in this research were determined to be free of BHV-1 and anti-BHV-1 antibodies by virus isolation and virus neutralization.

Cell lines

Madin Darby bovine kidney cells (MDBK), purchased from the American Type Culture Collection (Manassas, VA), were propagated and cultured in Minimum Essential Medium (MEM).

Antiviral Compounds

Lactoferrin from bovine milk was purchased from Sigma. It was supplied as a dry powder and dissolved in 1X MEM. The solution was filter sterilized (0.2 μm) before use. Cidofovir was purchased from Gilead Sciences (Foster City, CA).

Virus

Bovine herpesvirus 1 (Colorado strain) was obtained from the American Type Culture Collection (catalog number VR-864, lot number 1222901). It was propagated in MDBK cells. Virus was harvested by freezing and thawing the infected cell cultures and stored in cryovials at -80 °C until needed. The PFU/mL of stock virus was determined by quantifying plaques on MDBK cells.

Virus Isolation

Virus isolation procedures were used to determine the effect of the antiviral agent on the presence of BHV-1. One hundred μL of diluted antiviral agent was added to each well of a 96-well plate (0.32 cm^2) previously seeded with MDBK cells. The plate was incubated for 15 minutes at 38.5 °C prior to adding BHV-1. Bovine herpesvirus 1 was diluted to provide a multiplicity of infection (MOI) of 0.008 to 0.8. The plate was incubated for 5 days at 38.5 °C in an atmosphere of 5 % CO_2 and air. The cells were examined daily for evidence of cytopathic effect (CPE). At Day 2, a portion of all cultures showing CPE were frozen for later viral quantification using plaque assay.

Plaque Assay

Plaque assays were used to quantify the amount of virus remaining after culture with an antiviral agent. Day 2 samples containing the cell lysate were thawed and assayed. The samples were ten-fold serially diluted to 10^{-8}. Two hundred μL of dilutions 10^{-3} to 10^{-8} for each sample were inoculated onto a single well of a 24-well plate (2 cm^2) previously seeded with MDBK cells. The plate was incubated for 1 hour at 38.5 °C in an atmosphere of 5 % CO_2 and air. Three mL of 3 % agarose/2X MEM (50:50) were placed in each well, and the plates were refrigerated for 8 minutes. The plates were then incubated for 5 days at 38.5 °C in an atmosphere of 5 % CO_2 and air. On Day 5, the plaques in each well were counted.

Cytotoxicity Assay

The effects of 4 concentrations of lactoferrin from bovine milk (10, 5, 2.5, 1.25 mg/mL) combined with each of 3 concentrations of cidofovir (62.5, 31.3, 15.6 μg/mL) on MDBK cells were quantified using Cell Counting Kit-8 (Dojindo, Gaithersburg, MD). A water-soluble tetrazolium salt (WST-8) is reduced by dehydrogenases in cells to give a yellow-colored product (formazan). The amount of formazan dye produced by the dehydrogenase activity in the cells is directly proportional to the number of living cells.

A 96-well plate was seeded with MDBK cells for a final cell count per well of approximately 4 X 10^3. The plate was incubated at 38.5 °C for 24 hours. Ten μL of each antiviral concentration was added into the culture media. Each drug was evaluated in triplicate. The plate was incubated for an additional 48 hours at 38.5 °C. Cell Counting Kit-8 solution (10 μL) was added to each well. The absorbance at 450 nm was measured after 2 hours of incubation.

Mini-prep Percoll-gradient Separation of Spermatozoa

One straw of cryopreserved bull semen previously characterized in the IVF system and confirmed to be free of BHV-1 by virus isolation was used for fertilization. Semen was thawed in a water bath at 37 °C for 30 to 60 seconds. Percoll gradients were prepared as follows. One-half mL of 90 % percoll (Sigma) was placed in the bottom of a 1.5 mL microcentrifuge tube. Then, 0.5 mL of 45 % percoll was placed over this layer, and finally the thawed semen was gently placed on top of the two layers of percoll.

Following centrifugation for 7 minutes at 2000 x g, the layers of percoll were removed, being careful not to disturb the sperm pellet at the bottom. The sperm pellet was suspended in 1 mL of TL-Hepes and centrifuged for 1 minute at 2000 x g and resuspended in 60 μL of CR2 and BSA. The supernatant was discarded and the sperm concentration was determined using a hemocytometer. The total number of spermatozoa added to each fertilization drop was approximately 5 x 10^4 in a volume of 2 μL.

In Vitro Fertilization (IVF)

Following arrival of oocytes, cumulus-intact, matured oocytes were washed twice in TL-Hepes and once in CR2 and BSA, a modified synthetic oviductal fluid formulation. Ten to twelve oocytes in about 2 μL were placed in 42-μL fertilization drops overlaid with mineral oil. Two μL of heparin (0.025 mg/mL) and 2 μL of hypotaurine/penicillamine were added to each drop before adding the sperm. Two μL of spermatozoa were added to each drop for 5 x 10^4 spermatozoa/IVF drop and a total sperm concentration of 1 x 10^6 spermatozoa/mL. Fertilization plates were incubated for 6 hours at 38.5 °C in an atmosphere of 5 % CO_2 and air. Following the incubation period, the fertilization drops were examined for hyperactivated sperm as an indication of capacitation.

In Vitro Culture (IVC)

Six hours after fertilization, presumptive zygotes were removed from the fertilization drops and washed twice in TL-Hepes and once in CR2 and BSA to remove excess sperm. Ten to twelve presumptive zygotes were placed in each in vitro culture drop containing 30 µL of CR2 and BSA and overlaid with mineral oil. In vitro culture plates were incubated for 3.5 days at 38.5 °C in an atmosphere of 5 % CO_2 and air.

On Day 3.5, presumptive zygotes were cleaned using a sterile stripping tool. This is a glass pipette with a tapered end. Embryos are repeatedly aspirated into the pipette and expelled resulting in removal of the cumulus cells. The nude embryos that had cleaved to 4-cell or greater were washed 3 times in TL-Hepes and once in CR2 and BSA. Up to 20 to 25 developing embryos were placed in each IVC drop containing 30 µl of CR2 and FBS and overlaid with mineral oil. Also, clusters of cumulus cells were placed in each drop. The stage of embryo development was recorded. In vitro culture plates were incubated for 4 days at 38.5 °C in an atmosphere of 5 % CO_2 and air. On Day 7.5 of culture, embryonic development and quality were assessed as previously described [271].

Experimental Design

After performing cytotoxicity assays, the project was divided into 3 experiments. The first experiment determined the nontoxic concentration of lactoferrin from bovine

milk that was effective against BHV-1 in MDBK cells. The second experiment evaluated the inhibition of BHV-1 in MDBK cells resulting from the combination of cidofovir and lactoferrin from bovine milk. The third experiment assessed the development of bovine embryos cultured in the presence of lactoferrin from bovine milk.

Experiment 1

Four replicates were performed. Two-fold dilutions of lactoferrin from bovine milk (10, 5, 2,5, 1.25, and 0.625 mg/mL) were tested. All antiviral dilutions were made in 1X MEM with 10 % fetal bovine serum. The media over actively dividing monolayers of MDBK cells in single wells of a 96-well cell culture plate (0.32 cm^2) was supplemented with concentrations of each compound to be tested. After 15 minutes, each well was inoculated with 5 x 10^{3-5} PFU/mL of BHV-1 for an MOI of between 0.008 and 0.8, and culture plates were incubated (38.5 ºC) in an atmosphere of 5 % CO_2 and air for 5 days. During the incubation period, cells were examined daily with an inverted cell culture microscope at a magnification of 400X for presence or absence of cytopathic effect (CPE) as an indicator of whether or not the virus was inhibited. In addition, the inhibition of BHV-1 in incubating cells was quantified via plaque assay for virus in media (Day 2). Controls included the following: 1) MDBK cells with test antiviral but no virus (to evaluate toxicity of agent to cells). 2) MDBK cells with BHV-1 (positive control for viral cytopathic effect). 3) MDBK cells but no test antiviral or virus (negative control). The percent viral inhibition for each treatment was determined.

Experiment 2

Four replicates were performed. The 4 following concentrations of lactoferrin from bovine milk (10, 5, 2.5, and 1.25 mg/mL) were combined with each of 3 concentrations of cidofovir (62.5, 31.3 and 15.6 µg/mL). All antiviral dilutions were made with 1X MEM with 10 % fetal bovine serum. The media over actively dividing monolayers of MDBK cells in single wells of a 96-well cell culture plate (0.32 cm^2) were supplemented with each combination to be tested. After 15 minutes, each well was inoculated with 10^{5-6} PFU/mL of BHV-1 for an MOI of between 0.08 and 0.8, and culture plates were incubated (38.5 ºC) in an atmosphere of 5 % CO_2 and air for 5 days. Controls and assessment of viral inhibition was performed as described in experiment 1. The percent viral inhibition for each treatment was determined.

Experiment 3

Six replicates were performed. During each replicate, 300 to 400 oocytes were collected from an abattoir and shipped to our facility. They matured in transit and were distributed as follows: 1) 60 to 103 oocytes were used as an untreated control. 2) the remaining oocytes were used in treatments, with the specified antiviral concentration in IVC media. Thus, the IVC media were supplemented with either 10, 5 or 2.5 mg/mL of lactoferrin from bovine milk (Figure 1).

After embryonic development was assessed on Day 7.5, embryonic quality and viability were evaluated. Embryonic quality was assessed by grading the embryo as described in the Manual of the International Embryo Transfer Society (IETS) [271]. The IETS guidelines grade embryos according to the following codes. Code 1 describes

excellent or good embryos. The blastomeres should be uniform, and at least 85 % of the cellular material should be intact. Code 2 describes fair embryos. There are moderate irregularities in the shape of the embryonic mass, and at least 50 % of the cellular material should be intact. Code 3 is used for poor embryos. There are major irregularities in the shape of the embryonic mass, and at least 25 % of the cellular material is intact. Code 4 is used for dead or degenerating embryos. Embryonic viability also was assessed by counting the nucleated cells of developed blastocysts [231]. The embryos were stained using a rapid whole-mount staining procedure with Hoeschst 33342. A Nikon Eclipse E800 fluorescence microscope was used to visualize the cells. A picture was taken using Spot Advanced, and the cells were counted using Image J 1.32j software (National Institutes of Health).

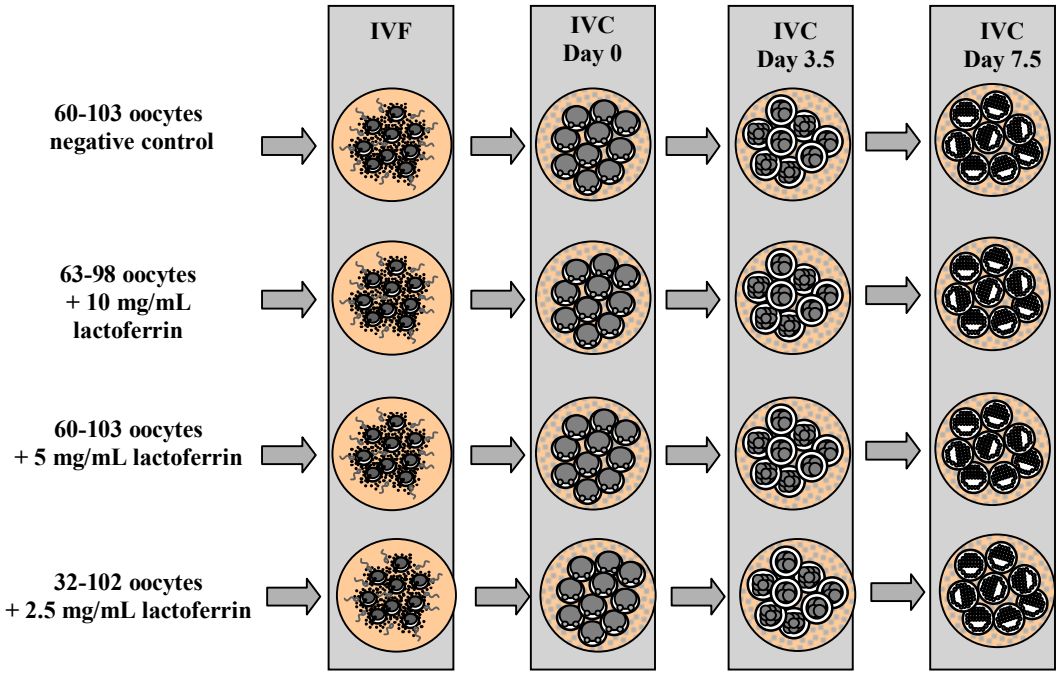


Figure 1. Experimental design for experiment 3. IVF, in vitro fertilization; IVC, in vitro culture of embryos to Day 7.5.

Statistical Analysis

The cytotoxicity assay was analyzed using ANOVA, Tukey-Kramer, and Student's t test (JMP Software, SAS Institute).

For experiment 1 and 2 the percent of virus inhibited for each test antiviral agent at each concentration was determined by comparison to equivalent samples from control cultures in which no compound was added (Percentage of virus inhibited = [Quantity of virus in the control sample - Quantity of virus in the compound sample]/ Quantity of virus in the control sample X 100).

In experiment 3, the percent of embryos that developed to blastocysts in each treatment group was compared to those in the control group using a 2-tailed Fisher's exact test. The number of nucleated cells from the embryos in each treatment group was compared to those in the control group using an ANOVA paired T test with JMP Software, SAS Institute. Also, the grades of the embryos from each treatment group were compared to those in the control group using an ANOVA paired T test with JMP Software, SAS Institute.

RESULTS

Cytotoxicity Assay

None of the combinations of lactoferrin from bovine milk (10, 5, 2.5, and 1.25 mg/mL) and cidofovir (62.5, 31.3 and 15.6 µg/mL) were cytotoxic to MDBK cells.

Experiment 1

In MDBK cells, lactoferrin from bovine milk (10 mg/mL) inhibited 100 to 25,000 plaque forming units (PFU)/mL of virus (99 % viral inhibition) (Figure 2). At a concentration of 5 and 2.5 mg/mL of lactoferrin, 10 to 1,000 PFU/mL of virus (90 to 99 % viral inhibition) were inhibited, and a concentration of 1.25 and 0.625 mg/mL inhibited 1 to 100 PFU/mL of virus (80 to 99 % viral inhibition).

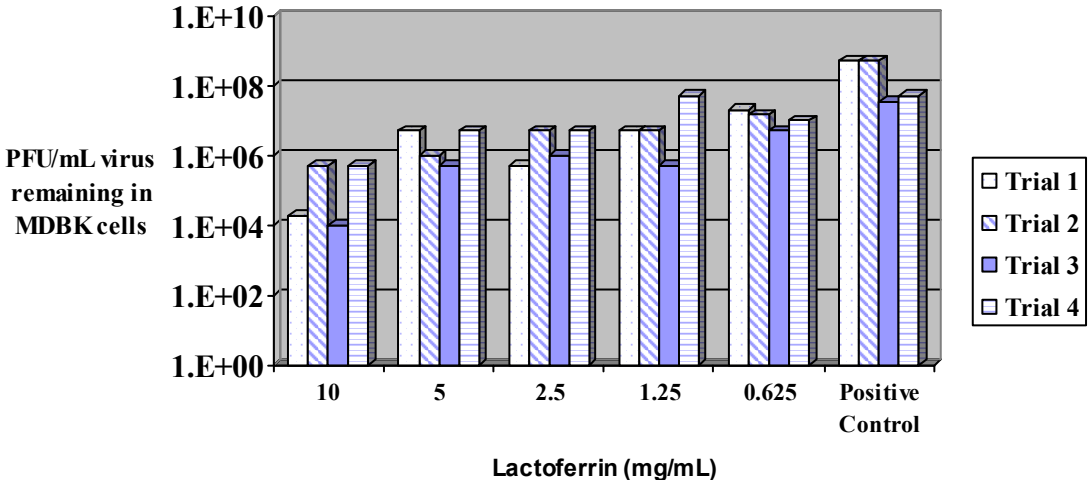


Figure 2. Results of experiment 1 are shown. The amount of virus present following treatment with lactoferrin is given in log scale. PFU, plaque forming unit; MDBK, Madin Darby bovine kidney

Experiment 2

Lactoferrin from bovine milk combined with cidofovir did have a synergistic effect (Figure 3). Lactoferrin from bovine milk (10 and 5 mg/mL) inhibited 500 to 100,000 PFU/mL of virus (99.8 to 99.9 % viral inhibition) when combined with each cidofovir concentration. At a concentration of 2.5 mg/mL lactoferrin, 100 to 10,000 PFU/mL of virus (99 to 99.8 % viral inhibition) were inhibited when combined with each cidofovir concentration, and a concentration of 1.25 mg/mL lactoferrin inhibited 10 to 5000 PFU/mL of virus (90 to 99.9 % viral inhibition) when combined with each cidofovir concentration.

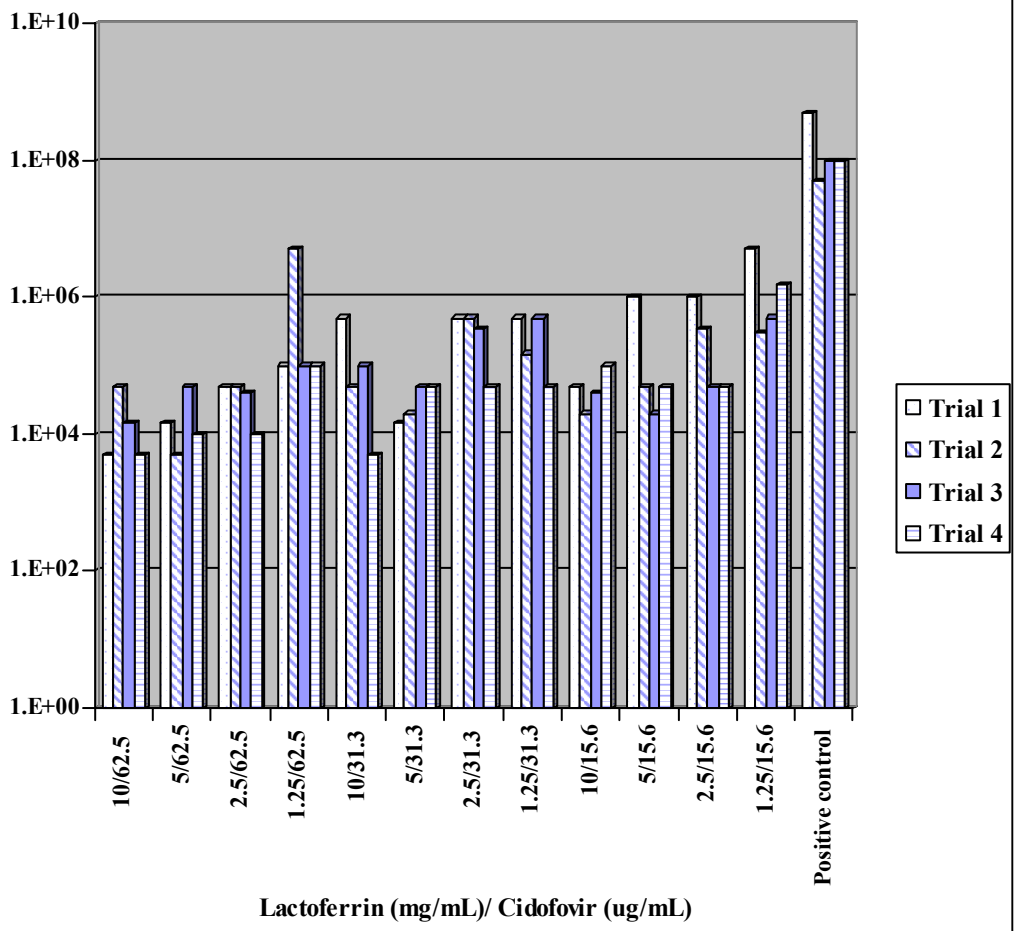


Figure 3. Results of experiment 2 are shown. The amount of virus inhibited is given in a log scale. PFU, plaque forming unit

Experiment 3

Embryos were able to develop in the presence of lactoferrin from bovine milk (Table 1). Lactoferrin did not affect the nucleated cell count or embryo grade of the treated embryos. However, lactoferrin did adversely affect blastocyst development.

Table 1. Embryonic development and viability of control embryos and those treated with lactoferrin from bovine milk (10, 5 or 2.5 mg/mL) from experiment 3.

		Control	10 mg/mL Lactoferrin	5 mg/mL Lactoferrin	2.5 mg/mL Lactoferrin
Day 3.5 development (>4 cell embryos/ oocytes fertilized)	Trial 1	24/72 (33%)	4/71 (6%)[a]	8/72 (11%)[a]	8/78 (10%)[a]
	Trial 2	35/60 (58%)	5/63 (8%)[a]	17/60 (28%)[a]	3/61 (5%)[a]
	Trial 3	30/70 (43%)	7/70 (10%)[a]	8/70 (11%)[a]	4/66 (6%)[a]
	Trial 4	26/66 (39%)	17/95 (18%)[a]	19/60 (32%)	11/32 (34%)
	Trial 5	34/103 (33%)	18/98 (18%)[a]	25/103 (24%)	19/102 (19%)[a]
	Trial 6	42/99 (42%)	18/94 (19%)[a]	24/96 (25%)[a]	20/96 (21%)[a]
	Cumulative	191/470 (41%)	69/491 (14%)[a]	101/461 (22%)[a]	65/435 (15%)[a]
Day 7.5 development (blastocysts/ oocytes fertilized)	Trial 1	17/72 (24%)	0/71 (0%)[a]	3/72 (4%)[a]	3/78 (4%)[a]
	Trial 2	20/60 (33%)	0/63 (0%)[a]	0/60 (0%)[a]	0/61 (0%)[a]
	Trial 3	14/70 (20%)	0/70 (0%)[a]	1/70 (1%)[a]	1/66 (2%)[a]
	Trial 4	12/66 (18%)	1/95 (1%)[a]	0/60 (0%)[a]	4/32 (13%)
	Trial 5	13/103 (13%)	1/98 (1%)[a]	3/103 (3%)[a]	4/102 (4%)[a]
	Trial 6	13/99 (13%)	1/94 (1%)[a]	2/96 (2%)[a]	4/96 (4%)[a]
	Cumulative	89/470 (19%)	3/491 (1%)[a]	9/461 (2%)[a]	16/435 (4%)[a]
Nucleated cell number (mean of Day 7.5 blastocysts)		90 ± 5	65 ± 25	82 ± 15	91 ± 11
Embryo grade (mean of Day 7.5 blastocysts)		1.2 ± 0.1	1 ± 0.3	1.2 ± 0.2	1.5 ± 0.1

[a]Mean differs significantly from control (2-tailed Fisher's exact test; $P < 0.05$).

DISCUSSION

Lactoferrin is an 80 kDa glycoprotein that is folded into two homologous lobes (N-lobe and C-lobe) [12]. These two lobes are connected by a 'hinge region'. Each lobe is divided into two structural domains (N1, N2 and C1, C2) [261]. A lactoferrin molecule can bind two iron atoms [282]. This is dependent on the simultaneous binding of anions such as bicarbonate or carbonate which play a role in firmly holding iron. Therefore, the lactoferrin has an iron-free (apo) and an iron-bound (holo) state. Its antibacterial effect is

due to depriving bacteria of iron required for their survival [215]. It might also bind directly to the cell wall of bacteria and fungi and thus cause membrane damage and leakage of intracellular components [81]. The antiviral activity of lactoferrin occurs in the early phase of infection at the level of viral attachment without affecting normal host-cell metabolism [20,282]. Because of this lactoferrin was added to the cells 15 minutes prior to addition of the virus [303]. The positively charged N-terminus of the lactoferrin binds to glycosaminoglycans [302]. Binding of lactoferrin to the cell surface glycosaminoglycan prevents gC of HSV-1 from attaching to the cell [176].

Lactoferrin has been shown to inhibit the in vitro replication of HSV-1, HSV-2, human cytomegalovirus, canine herpesvirus, feline herpesvirus, human immunodeficiency virus, respiratory syncytial virus, human hepatitis B virus, human hepatitis C virus, hantavirus, simian SA-11 rotavirus, and poliovirus type 1 [20,113,116,120,122,133,175,212,229,280,282,303].

Tanaka et al. demonstrated that bovine lactoferrin is effective against canine herpesvirus (DFD-6 strain) in Madin Darby canine kidney cells (MDCK) [282]. The concentration of lactoferrin in bovine milk is 0.02 to 0.2 mg/mL and in canine milk is 0.05 mg/mL [183,282]. At concentrations ranging from 0.125 to 1 mg/mL lactoferrin, virus was reduced by 10^2 $CCID_{50}$/0.1 mL [282]. This study demonstrated that bovine lactoferrin was not cytotoxic to the cells. The studyalso evaluated human lactoferrin, bovine transferrin, and bovine ovotransferrin with the result that only bovine and human lactoferrin were effective against canine herpesvirus. Andersen et al. also showed that the bovine form of lactoferrin was more effective against both HSV-1 (strain MacIntyre) and HSV-2 (strain G) than the human or goat form of lactoferrin [6]. This illustrated that

the antiviral effect of lactoferrin was not dependent on the species of origin and that it was not a general property of the iron transporter protein family.

In our study bovine lactoferrin was used because it had been shown to be the most effective antiviral among the transferrins. Both apo- and holo-bovine lactoferrin have been evaluated [282]. Both forms inhibited canine herpesvirus indicating that the iron complex formation is not involved with the antiviral effect. This had also been shown with HSV-1 and HSV-2 [302].

Our results are comparable to those of Beaumont et al. who evaluated the effect of lactoferrin from bovine colostrum against feline herpesvirus (strain 727) on Crandell-Reese feline kidney cells (CRFK) [20]. Exposure of CRFK cells with 0.5 to 10 mg/mL lactoferrin for 30 minutes prior to virus adsorption inhibited viral replication by 91 %. Exposure of virus with lactoferrin prior to virus adsorption inhibited viral replication by 90 %. Addition of the lactoferrin after virus adsorption did not cause an inhibition of viral replication relative to the control. As well, they demonstrated that there was no synergistic effect when lactoferrin was added at more than one stage of virus adsorption. When lactoferrin was added pre-adsorption and during adsorption the mean inhibition of viral replication was 90 %. Exposure to lactoferrin during virus adsorption and post adsorption resulted in a mean inhibition of viral replication of 93 %. Addition of lactoferrin during all three stages of virus adsorption resulted in 93 % viral inhibition. Viral replication in these experiments did not vary with concentration of the lactoferrin. Also, lactoferrin was not cytotoxic at any concentration that was tested. Those results contrast slightly with Hasegawa et al. who evaluated the effect of lactoferrin against HSV-1 and CMV [122]. Hasegawa showed that when human embryo lung cells were

incubated with lactoferrin for 5 minutes prior to virus adsorption, virus replication was inhibited by 93 % [20,122]. However, when lactoferrin was added to the virus inoculum prior to virus adsorption, virus replication was inhibited by only 58 % [122]. This suggests that lactoferrin inhibits herpesviruses in different ways [20].

Different combination therapies have used antiviral compounds that have different mechanisms of action so that simultaneous inhibition occurs at different steps of the viral replication cycle [303]. van der Strate et al. demonstrated that bovine lactoferrin works synergistically with cidofovir to inhibit CMV (RC256 strain) on fetal lung fibroblasts [303]. We showed similar results in our study. Because resistance to antiviral agents can arise and long-term treatment can result in undesirable side effects associated with cytotoxicity, combination therapy is a viable option [303]. Lower concentrations of the toxic antiviral agents can be used to achieve the same result as conventional treatment [302]. van der Strate evaluated the combined antiviral effect of bovine lactoferrin with acyclovir, ganciclovir, foscarnet or cidofovir [6,303]. The combination of lactoferrin with acyclovir and foscarnet resulted in antagonism. The combination of lactoferrin and ganciclovir revealed neither antagonism nor synergy. The combination of lactoferrin (8 μg/mL) with cidofovir (4 μg/mL) resulted in marked synergy. The virus was inhibited 42 % more efficiently than theoretically expected on the basis of the individual dose-response curves of each agent. In addition, lactoferrin has been shown to be non-cytotoxic to fetal lung fibroblasts in concentrations up to 2 mg/mL.

Herpes simplex virus and cytomegalovirus are significant causes of human neonatal morbidity and mortality {787, 789 53, 65, 168, 127, 122}. In addition, they can cause genital infections, oral lesions, keratoconjunctivitis, skin infections, and

encephalitis [226,333]. It is possible for an individual to transmit these viruses yet be seronegative and show no clinical signs of disease [47,174,270,285,340]. Further, they can be shed in semen [10,115,159,174,224,340] and possibly be associated with oocytes [44,134,245]. Therefore, contaminated gametes and serum are potential sources for herpesvirus to enter an IVF system [10]. If this were to occur, the virus could become associated with the embryo which could transmit herpesvirus to the mother as well as result in early embryonic death or fetal anomalies [13]. Addition of an antiviral agent into the IVF and IVC media could reduce the level of exposure of the embryo to herpesvirus, thus protecting the conceptus and the recipient.

An antiviral agent that proved effective against BHV-1 in a bovine IVF system as well as allowed development of a viable embryo would serve as a model for elimination of HSV and CMV in a human IVF system. Bovine herpesvirus 1 is an alphaherpesvirus which causes infertility, abortion, genital disease, and respiratory disease [234,315]. In vitro fertilized embryos are at risk for contamination with BHV-1. This can occur via exposed oocytes and spermatozoa or from contaminated products of animal origin that are used in media for washing, fertilization, and culture [24,315,317]. When oocytes of heifers experimentally inoculated with BHV-1 were matured, fertilized and cultured in vitro, embryos, oviductal cells and uterine fluid were positive for BHV-1 [24]. This demonstrated that apparently normal transferable IVF embryos can have BHV-1 associated with them. Such embryos would provide the potential to spread BHV-1 to the recipient. While trypsin treatment is an effective treatment for in vivo-derived embryos [264,274], it is not completely effective for in vitro-derived embryos [30,79]. Therefore, other antiviral agents are needed to render in vitro-derived embryos free of BHV-1. It

was encouraging to note that lactoferrin was effective at reducing BHV-1 in MDBK cells. Also, lactoferrin combined with cidofovir further decreased the amount BHV-1 present in MDBK cells. However, while significant results were seen, total inhibition of viral replication is needed to render embryos completely free of virus. In addition, while the quality of embryos cultured with lactoferrin was comparable to the control embryos, the number of embryos developing to blastocysts was hindered. Thus, while the cytotoxicity assay demonstrated that the combination of cidofovir and lactoferrin was not toxic to MDBK cells and in vivo studies have not shown appreciable side effects [255], the assessment of embryo development is obviously a much more sensitive test of antiviral toxicity. Therefore, additional research is needed to identify an antiviral agent or combination of agents that will be effective against BHV-1 associated with in vitro-derived embryos while not interfering with normal embryonic development.

VI. EFFICACY OF A RECOMBINANT TRYPSIN PRODUCT AGAINST BOVINE HERPESVIRUS 1 ASSOCIATED WITH DAY 7 IN VIVO-DERIVED AND IN VITRO-DERIVED BOVINE EMBRYOS

ABSTRACT

TrypLE™ (Invitrogen, Carlsbad, CA) is a recombinant, fungal, trypsin-like protease that is used as a substitute for porcine-origin trypsin in cell culture procedures. While porcine-origin trypsin will effectively inactivate or remove bovine herpesvirus 1 (BHV-1) associated with in vivo-derived embryos, TrypLE™ was untested for this purpose. This recombinant protease represents an attractive alternative since it is highly stable at room temperature and does not pose the same threat for contaminating microorganisms as animal-origin trypsin. Thus, the objective of this study was to determine if TrypLE™ Express (1X) or TrypLE™ Select (10X) would be effective at inactivating BHV-1 associated with Day 7 in vivo- and in vitro-derived embryos after they had been exposed to the virus.

TrypLE™ Select (10X) when used for 5 and 10 minutes effectively inactivated BHV-1 associated with Day 7, zona pellucida-intact, in vivo-derived embryos. However, further research is needed to determine if embryo viability is affected by the treatment. Treatment of in vitro-derived embryos with TrypLE™ Select (10X) for 7 and 10 minutes

and with TrypLE™ Select (10X) diluted 1:2 for 10 minutes appeared to be effective at removing BHV-1. However, when these treated embryos were cultured on uterine tubal cells for 48 hours, the cells became infected. While the amount of embryo-associated virus required to infect a recipient is not known, ideally the embryo should be completely free of virus. Therefore, TrypLE™ cannot be recommended for treatment of in vitro-derived embryos. Further research needs to be performed to evaluate other recombinant trypsin products.

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INTRODUCTION

According to the Research Subcommittee of the International Embryo Transfer Society Health and Safety Advisory Committee, BHV-1 is a category 1 disease. "Category 1 diseases or pathogenic agents are those for which sufficient evidence has accrued to show that the risk of transmission is negligible provided that the embryos are properly handled between collection and transfer according to the IETS Manual [219,271]." To prevent transmission of BHV-1, proper handling includes trypsin treatment. Porcine-origin trypsin has been used to treat bovine embryos associated with BHV-1. It has been shown to be an effective treatment of zona pellucida-intact in vivo-derived embryos artificially exposed to BHV-1 [264,274]. This trypsin treatment is also known to prevent transmission of BHV-1 after in vivo exposure of in vivo-derived embryos [263]. However, there are differences between in vivo- and in vitro-derived embryos [275], and trypsin treatment is not completely effective at removing BHV-1 from in vitro-derived embryos [30,69,79]. Bielanski et al. showed that BHV-1 present during IVM or IVC would result in groups of 5 IVF embryos that were positive for virus following trypsin treatment [30]. In a study by Edens et al., groups of 5 Day 7 in vitro-derived embryos exposed to BHV-1 and then treated with trypsin were negative on virus isolation. However, if similarly exposed and treated groups of 5 embryos were then further cultured on bovine uterine tubal cells (UTC) cells for 48 hours, the embryos, UTC cells and IVC medium were positive for virus [79]. Thus, further research is needed to provide a treatment that will completely remove BHV-1 associated with in vitro-derived embryos.

TrypLE™ is a non-animal alternative for porcine-origin trypsin [216]. It is a recombinant fungal protease used for cell dissociation protocols. Unlike trypsin, TrypLE™ is stable at room temperature for up to 6 months. In addition, Pryor has shown that TrypLE™ Express is safe for the treatment of in vitro-derived embryos [230]. The purpose of this study was to determine if TrypLE™ would be an effective treatment of BHV-1 associated with either in vivo-derived or in vitro-derived embryos.

MATERIALS AND METHODS

Media

Tyrode lactate solutions (Hepes-TL, Sperm-TL, and IVF-TL) were made [299]or purchased from Millipore (Phillipsburg, NJ). HEPES-TALP (Tyrode's albumin, lactate and pyruvate) contained Hepes-TL, BSA (Fraction V; 3 mg/ml, Sigma, St. Louis, MO), sodium pyruvate (22 μg/ml, Sigma), and gentamicin (7.5 μg/ml, Sigma). Sperm-TALP contained Sperm-TL, BSA (Fraction V; 6 mg/ml), sodium pyruvate (110 μg/ml), and gentamicin (10 μg/ml). IVF-TALP consisted of IVF-TL, BSA (essentially fatty acid free (EFAF); 6 mg/ml, Sigma), sodium pyruvate (22 μg/ml), gentamicin (5 μg/ml), and heparin (20 μg/ml, Sigma).

Potassium simplex optimized medium- bovine embryo modification 2 (KSOM-BE2; Millipore) or Charles Rosenkrans medium (CR1aa) was used for in vitro culture drops in experiment 3 and 4. The KSOM was purchased, and EFAF BSA 3 mg/mL, gentamicin 0.5 μL/mL, and nonessential amino acids (Sigma) 25μL were added. The

CR1aa consists of CR1 stock, EFAF BSA 3 mg/mL, gentamicin 0.5 μL/mL, nonessential amino acids 10 μL/mL, and essential amino acids (Sigma) 20 μL/mL. CR1 stock consists of 6.7 mg/mL sodium chloride (Sigma 108mM), 0.23 mg/mL potassium chloride (Sigma 3mM), 2.2 mg/mL sodium bicarbonate (Sigma 25 mM), 0.04 mg/mL sodium pyruvate (0.4 mM), 0.15 mg/mL glutamine (Sigma 1 mM), and 0.55 mg/mL hemicalcium lactate (Sigma 0.5 mM).

In experiment 2, fertilization drops contained Charles Rosenkrans medium (CR2) (10 mL) and BSA 6 mg/mL (Sigma). This media was also used for in vitro culture drops up to Day 3.5. The CR2 contains 3.143 g sodium chloride (Sigma 108mM), 0.11 g potassium chloride (Sigma 3mM), 1.045 g sodium bicarbonate (Sigma 25 mM), 450 mL water, 0.275 g hemicalcium lactate (Sigma 0.5 mM), 22.2 mg pyruvate (Sigma 0.4 mM), 10 mL Eagle's Basal Medium (BME) amino acids (50X, Sigma), 0.073 g l-glutamine (Sigma 1 mM), 5 mL MEM nonessential amino acids (Sigma), 5 mL penicillin/streptomycin (Invitrogen, 10,000U penicillin/10 mg streptomycin/mL), and 0.5 mL phenol red (Sigma 0.005 μg/mL). Presumptive zygotes were cultured from Day 3.5 to Day 7.5 in IVC drops containing CR2 and 10 % fetal bovine serum (FBS; HyClone, Logan, UT).

Embryos were washed prior to virus exposure in Dulbecco's phosphate buffered saline (Invitrogen, Carlsbad, CA) with 2 % FBS, 100 IU/mL penicillin G (Sigma), and 100 μg/mL streptomycin (Sigma).

Embryos were washed post-virus exposure in Dulbecco's phosphate buffered saline with 0.4 % bovine serum albumin (Sigma), 100 IU/mL penicillin G, and 100 μg/mL streptomycin.

TrypLE™ products were purchased from Invitrogen. They were used at full concentration or diluted with Dulbecco's Phosphate Buffered Saline without calcium and magnesium (D-PBS, Invitrogen).

Trypsin, 2.5 % (10X; porcine-origin) was purchased from Invitrogen. It was diluted in Hanks' balanced salt solution without calcium and magnesium (Invitrogen) for a concentration of 0.25 %.

Embryos were placed in 1X MEM prior to sonication. This media was also used for virus isolation. In addition, virus was propagated in Madin Darby bovine kidney (MDBK) cells which had been cultured in 1X MEM. This consists of Minimum Essential Medium (MEM) with Earle's salts (Invitrogen) supplemented with 10 % equine serum (HyClone), 0.75 mg/mL sodium bicarbonate (Invitrogen), 0.29 mg/mL L-glutamine (Invitrogen), 100 IU/mL penicillin G, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Invitrogen).

Plaque assays required mixing equal volumes of 2X MEM and 3 % agarose. Minimum Essential Medium (2X) includes 80 mL distilled water, 20 mL 10X MEM with Earle's salts (10X, Invitrogen), 10 mL equine serum (HyClone), 2 mL l-glutamine (Invitrogen), 2 mL penicillin/streptomycin/amphotericin B (Invitrogen), and 2 mL sodium bicarbonate (Invitrogen). Agarose (Sigma) consists of 3 grams of type VII low temperature gelling agarose that was dissolved in 97 mL distilled water and autoclaved.

Fetal bovine serum, equine serum, and BSA were determined to be free of BHV-1 antibodies and BHV-1 by virus neutralization and virus isolation, respectively.

In Vivo-Derived Embryo Collection

Day 6 embryos were non-surgically recovered from recipients, placed in Emcare holding media (Agtech, Manhattan, KS), and shipped overnight to our laboratory in an incubator. Two hundred twenty-five developed embryos and 125 degenerate embryos were selected for use in this project. Developed embryos were Code 1 and 2 morula or blastocysts. Both developed and degenerate embryos possessed an intact zona pellucida and were free of adherent material [271].

In Vitro-Derived Embryo Production

Mini-prep Percoll-gradient Separation of Spermatozoa

In experiment 2, 1 straw of cryopreserved bull semen previously characterized in the IVF system and confirmed to be free of BHV-1 by virus isolation was used for fertilization. Semen was thawed in a water bath at 37 °C for 60 seconds. Percoll gradients were prepared as follows. One-half mL of 90 % percoll (Sigma) was placed in the bottom of a 1.5 mL microcentrifuge tube. Then, 0.5 mL of 45 % percoll was placed over this layer, and finally the thawed semen was gently placed on top of the two layers of percoll. Following centrifugation for 7 minutes at 2000 x g, the layers of percoll were removed, being careful not to disturb the sperm pellet at the bottom. The sperm pellet was suspended in 1 mL of TL-Hepes (Cambrex, Walkersville, MD) and centrifuged for 1 minute at 2000 x g and resuspended in 60 μL of CR2 and BSA. The supernatant was

discarded and the sperm concentration was determined using a hemocytometer. The total number of spermatozoa added to each fertilization drop was approximately 5 x 10^4 in a volume of 2 μL.

In experiment 3 and 4, three straws of cryopreserved bull semen were used for fertilization. Centrifugation through the Percoll occurred for 10 minutes at 1000 x g. The sperm pellet was suspended in 1.2 mL of Sperm-TALP and centrifuged for 2.5 minutes at 1000 x g and resuspended in 240 μL of KSOM or CR1aa. The supernatant was discarded and the sperm concentration was determined using a hemocytometer. The total number of spermatozoa added to each fertilization drop was approximately 26 x 10^6 spermatozoa/mL.

In Vitro Fertilization (IVF)

In experiment 2, cumulus-oocyte complexes (oocytes surrounded by multiple layers of dense cumulus cells) were aspirated from ovaries at an abattoir, placed in maturation medium in a portable incubator set at 39 ° C, and shipped by courier to Auburn, Alabama. The oocytes were matured while they were in transit (20 to 24 hours). Following arrival of oocytes, cumulus-intact matured oocytes were washed twice in TL-Hepes and once in CR2 and BSA, a modified synthetic oviductal fluid formulation. Ten to twelve oocytes in about 2 μL were placed in 42-μL fertilization drops overlaid with mineral oil. Two μL heparin (0.025 mg/mL) and 2 μL hypotaurine/penicillamine were added to each drop before adding the sperm. Two μL of 1 x 10^6 spermatozoa/mL were

added to each drop for a total sperm concentration of 5 x 10^4 / 50 µL drop. Fertilization plates were incubated for 6 hours at 38.5 °C in an atmosphere of 5 % CO_2 and air. Following the incubation period, the fertilization drops were examined for hyperactivated sperm as an indication of capacitation.

In experiment 3 and 4, following arrival of oocytes, cumulus-intact matured oocytes were washed three times in Hepes-TALP. Twenty-five to thirty oocytes were placed in 600-µL IVF-TALP. Twenty-five µL of 26 x 10^6 spermatozoa/mL were added to each well for a total sperm concentration of 1 x 10^6 / well. Twenty-five µL of a solution containing penicillamine (0.5 mM, Sigma), hypotaurine (0.25 mM, Sigma), and epinephrine (25 µM, Sigma) was added to each IVF well. Fertilization plates were incubated for 18 hours at 38.5 °C in an atmosphere of 5 % CO_2 and air. Following the incubation period, the fertilization drops were examined for hyperactivated sperm as an indication of capacitation.

In Vitro Culture (IVC)

Six hours after fertilization for experiment 2, presumptive zygotes were removed from the fertilization drops and washed twice in TL-Hepes and once in CR2 and BSA to remove excess sperm. Ten to twelve presumptive zygotes were placed in each IVC drop containing 30 µL of CR2 and BSA and overlaid with mineral oil. In vitro culture plates were incubated for 3.5 days at 38.5 °C in an atmosphere of 5 % CO_2 and air. On Day 3.5, presumptive zygotes were cleaned using a sterile stripping tool. This is a glass pipette

with a tapered end. Embryos were repeatedly aspirated into the pipette and expelled resulting in removal of the cumulus cells. The nude embryos that had cleaved to 4-cell or greater were washed 3 times in TL-Hepes and once in CR2 and BSA. Up to 20 to 25 developing embryos were placed in each IVC drop containing 30 µl of CR2 and FBS and overlaid with mineral oil. Also, clusters of cumulus cells were placed in each drop. The stage of embryo development was recorded. In vitro culture plates were incubated for 4 days at 38.5 °C in an atmosphere of 5 % CO_2 and air. On Day 7.5 of culture, embryonic development and quality were assessed as previously described [231].

Eighteen hours after fertilization for experiment 3 and 4, presumptive zygotes were removed from the fertilization media and vortexed in 500 µL Hepes-TALP for 20 sec to remove excess spermatozoa and cumulus cells. The presumptive zygotes were then washed three times in Hepes-TALP and once in IVC media. Twenty-five to thirty presumptive zygotes were placed in each in vitro culture drop containing 50 µL of KSOM-BE2 or CR1aa and overlaid with mineral oil. In vitro culture plates were incubated for 6 days at 38.5 °C in an atmosphere of 5 % CO_2 and air.

On Day 5 after fertilization, 5 µL heat-inactivated FBS (Atlanta Biologicals, Lawrenceville, GA) was added to each drop. On Day 7, embryonic development and quality were assessed as previously described [231].

Pre-exposure Embryo Washing

Developed embryos and non-fertile and degenerate embryos (NFD) were separated and washed according to IETS guidelines [271]. This consisted of 10 washes in Dulbecco's phosphate buffered saline with 2 % fetal bovine serum, 100 IU/mL penicillin G, and 100 μg/mL streptomycin. Groups of 10 or fewer zona pellucida-intact embryos that were free of adherent material were washed. Each wash was a 100-fold dilution. A new sterile micropipet was used each time embryos were moved to a new wash.

Bovine Herpesvirus 1 Exposure

Stock virus of BHV-1 (Colorado strain) was obtained from the American Type Culture Collection (catalog number VR-864, lot number 1222901). It was propagated in Madin Darby bovine kidney (MDBK) cells which had been cultured in minimum essential medium (MEM) with Earle's salts supplemented with 10 % equine serum, 0.75 mg/mL sodium bicarbonate, 0.29 mg/mL L-glutamine, 100 IU/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. Virus was harvested by freezing and thawing the infected cell cultures and stored in cryovials at –80 °C until needed. The plaque forming units per ml of stock virus was determined by quantifying plaques on MDBK. Embryos and NFD were incubated with $10^{5\text{-}8}$ PFU/mL of virus for 1 hour at 38.5 °C in an atmosphere of 5 % CO_2 and air.

Post-exposure Embryo Washing and Treatment

Following exposure to virus, positive control embryos and NFD were washed as described with the pre-exposure embryo washing. Treated embryos were washed according to the IETS guidelines for trypsin treatment [271]. This involved 5 washes in Dulbecco's phosphate buffered saline with 0.4 % bovine serum albumin, 100 IU/mL penicillin G and 100 μg/mL streptomycin. This was followed by 2 washes with 0.25 % trypsin diluted in Hanks' BSS (60 to 90 sec), TrypLE™ Select (10X) undiluted or diluted in D-PBS without calcium and magnesium, or TrypLE™ Express with phenol red for various times of exposure. The embryos were then washed in 5 additional washes identical to the first 5 washes.

Uterine Tubal Epithelial Cells (UTC)

Uterine tubal epithelial cells were collected from cows at a slaughterhouse in Omaha, Nebraska. The UTC were removed from uterine tubes ipsilateral to ovaries with corpora hemorrhagica or early corpora lutea present. The UTC were placed in maturation medium and shipped overnight to our facilities. Upon arrival, the UTC were washed three times in IVC medium, 2.5 μL were placed in IVC2 drops, and the plates were incubated at 38.5 °C in an atmosphere of 5 % CO_2 and air until use. Virus isolation procedures were used to confirm that the UTC were free of BHV-1.

Embryo Culture after Viral Exposure (IVC2)

For experiment 4, after post-exposure washing and treatment, embryos and NFD were placed either individually or in groups of 5 in 50 µl drops of KSOM-BE2 or CR1aa medium containing UTC. Embryos were cultured for 43 to 48 hours at 38.5 °C in an atmosphere of 5 % CO_2 and air.

Post-IVC2 Embryo Washing

Following co-culture for 48 hours, NFD and embryos with their corresponding zona pellucida were washed two times in 35-mm petri dishes containing 1X MEM. Following washing, the embryos were prepared for virus isolation.

Sample Preparation

In vivo-derived embryos and NFD were removed from treatment and placed in 500 µL 1X MEM in groups of 5 or 10 for the first experiment. In vitro-derived embryos were placed as groups of 5 in 500 µL 1X MEM for experiment 2 and 3 and 200 µL 1X MEM for experiment 4. Embryos were sonicated (Microson Ultrasonic Homogenizer Model XL 2000, Misonic Inc., Farmingdale, NY), and the sonicate fluid was frozen at – 80 °C until later testing for BHV-1.

After 48 hours of IVC2, the uterine tubal cells were separated from the IVC2 medium by centrifugation at 14,000 g for 1 minute. One hundred µl of 1X MEM was added to the IVC2 medium for a total volume of 200 µl which was later assayed for BHV-1.

Following separation from the IVC2 medium, the UTCs were washed one time in 200 µl of 1X MEM and centrifuged. The supernatant was discarded and the pellet was resuspended with 200 µl of 1X MEM. The UTC were sonicated, and the sonicate fluid was frozen at -80 °C until later testing for BHV-1.

Virus Isolation

Virus isolation procedures were used to determine the presence of BHV-1. Five hundred µL of sample was inoculated onto a 6-well plate (9.6 cm^2 monolayer) previously seeded with MDBK cells. The plate was incubated for 1 hour at 38.5 °C prior to adding 2 mL of 1X MEM. The plates were incubated for 5 days at 38.5 °C in an atmosphere of 5 % CO_2 and air. The MDBK cells were examined for evidence of cytopathic effect (CPE).

Plaque Assay

Plaque assays were used to quantify the amount of virus the embryos were exposed to in experiments 1 through 3, and they were used to assay all the samples in

experiment 4. In experiments 1 through 3, the virus exposure was serially diluted to 10^{-8}. Five hundred µL of dilutions 10^{-3} to 10^{-8} was inoculated onto a single well of a 6-well plate previously seeded with MDBK cells. In experiment 4, twenty µL of each 200 µL sample (0.1 X) was diluted 10^{-1} to 10^{-8} and inoculated onto a 12-well plate (3.8 cm^2 monolayer). In addition, the remaining 180 µL of sample was placed onto a 12-well plate. The plates were incubated for 1 hour at 38.5 °C in an atmosphere of 5 % CO_2 and air. The 180 µL sample was removed and frozen for later qPCR testing. For the remaining tests, the samples remained on the plates. Three mL (6-well plate) or 1.3 mL (12-well plate) of 3 % agarose/2X MEM (50:50) were placed in each well, and the plates were refrigerated for 8 minutes. The plates were then incubated for 5 days at 38.5 °C in an atmosphere of 5 % CO_2 and air. On Day 5, the number of plaques in each well was counted.

Quantitative Polymerase Chain Reaction

In experiment 4, the 180 µL sample that had been previously frozen was thawed, and the DNA extracted with High Pure PCR Template Preparation Kit (Roche, Indianapolis, IN) to yield 60 µL of sample. Quantitative polymerase chain reaction was performed with primers specific for the BHV-1 ribonucleotide reductase gene. These primers were derived from Schang and amplify both subunits of ribonucleotide reductase [256]. This early gene is responsible for the reduction of ribonucleotides to deoxyribonucleotides. The deoxyribonucleoside triphosphates (dNTP) are used for the

biosynthesis of DNA. This is important for BHV-1 to be able to replicate in neurons where there are low concentrations of dNTP.

Bovine herpesvirus 1 was monitored by the amplification of a 141 bp fragment by using the primers RR-1 (5' TGCCCTACAGGTCGTTGATTA 3') and RR-2 (5' TCCAGCTGCCTCCTCTGTTT 3'). The 5' probe (5' CGTGTGCTTCTCGGCAGTCATCA 3') was labeled with carboxyfluorescein (6-FAM) at the 3' end. The 3' probe (5' CCAAAGGAAAATCGGTCCCAGGA 3') was labeled with Cy5.5 at the 5' end and a phosphate at the 3' end. Primers and probes were synthesized by Operon (Huntsville, AL)

An oligonucleotide mix was prepared containing 5 μM of each primer, 0.5 μM of the 5' probe and 1 μM of the 3' probe. Prior to running each reaction, a master mix was prepared. The master mix contained 20 μL of the oligonucleotide mix, 50 μL QuantiTect Probe PCR Master Mix (Qiagen, Valencia, CA), 2.5 μL RNase-free water, and 2.5 μL uracil-N-glycosalase (Roche; UNG; 1 unit/ μL, Indianapolis, IN). The QuantiTect Probe PCR Master Mix consisted of HotStar Taq DNA Polymerase, QuantiTect Probe PCR Buffer, dNTP mix, and Rox fluorescent dye. The QuantiTect Probe PCR Buffer consisted of TrisCl, KCl, $(NH_4)_2SO_4$, and 8 mM $MgCl_2$. The dNTP mix contained dATP, dCTP, dGTP, and dTTP/dUTP.

For a 100 μL reaction, 75 μL of the master mix was added to each capillary, followed by 25 μL of each sample to be tested. In order to test the entire extracted sample (60 μL), 2 PCR reactions had to be performed. The capillaries were centrifuged and then placed in the Roche LightCycler 2.0. The following program was performed with a 20 °C/second ramp unless otherwise indicated: UNG carry-over prevention at

50 °C for 2 minutes; PCR initial activation step at 95 °C for 15 minutes; 40 cycles of 3 step cycling consisting of denaturation at 95 °C for 0 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 30 seconds; melting curve at 95 °C for 0 seconds, 50 °C for 10 seconds, and then heating to 80 °C for 0 seconds at 0.1 °C/second; and cooling to 38 °C for 30 seconds. The fluorescence channel used was 705/530. The fluorescence data acquisition was quantified during the annealing phase. The results of both PCR reactions were averaged for each sample. The Roche LightCycler Software Version 4.0 was used.

Experimental Design

Experiment 1

This project was divided into 4 experiments (Figure 1). To identify effective treatments of TrypLE™ for in vivo-derived embryos, a total of 225 in vivo-derived embryos and 125 NFD were used in 8 trials for Experiment 1 (Figure 2). During each trial, zona pellucida intact, Day 7, in vivo-derived blastocysts and NFD were washed according to the IETS protocol. Developed embryos were washed separately from NFD embryos. One group of 5 or 10 NFD or developed embryos was not exposed to virus and served as the negative control. The remaining embryos and 10 NFD were exposed to 10^{6-7} PFU/mL BHV-1 for 1 hour. Following exposure, the embryos were assigned to one of the following 4 categories: 1) 1 group of 5 or 10 NFD or developed embryos was washed, 2) 1 group of 10 embryos was treated with porcine origin trypsin for 1.5 minutes,

3) groups of 5 to 10 embryos were treated with TrypLE™ Express for 1.5 or 10 minutes, and 4) groups of 5 to 10 embryos were treated with TrypLE™ Select (10X) for 1.5, 3, 5, or 10 minutes . Following treatment the embryos were sonicated in groups of 5 or 10 in 500 µL 1X MEM and assayed by virus isolation. The virus, to which the embryos and NFD were exposed, was quantified by plaque assay.

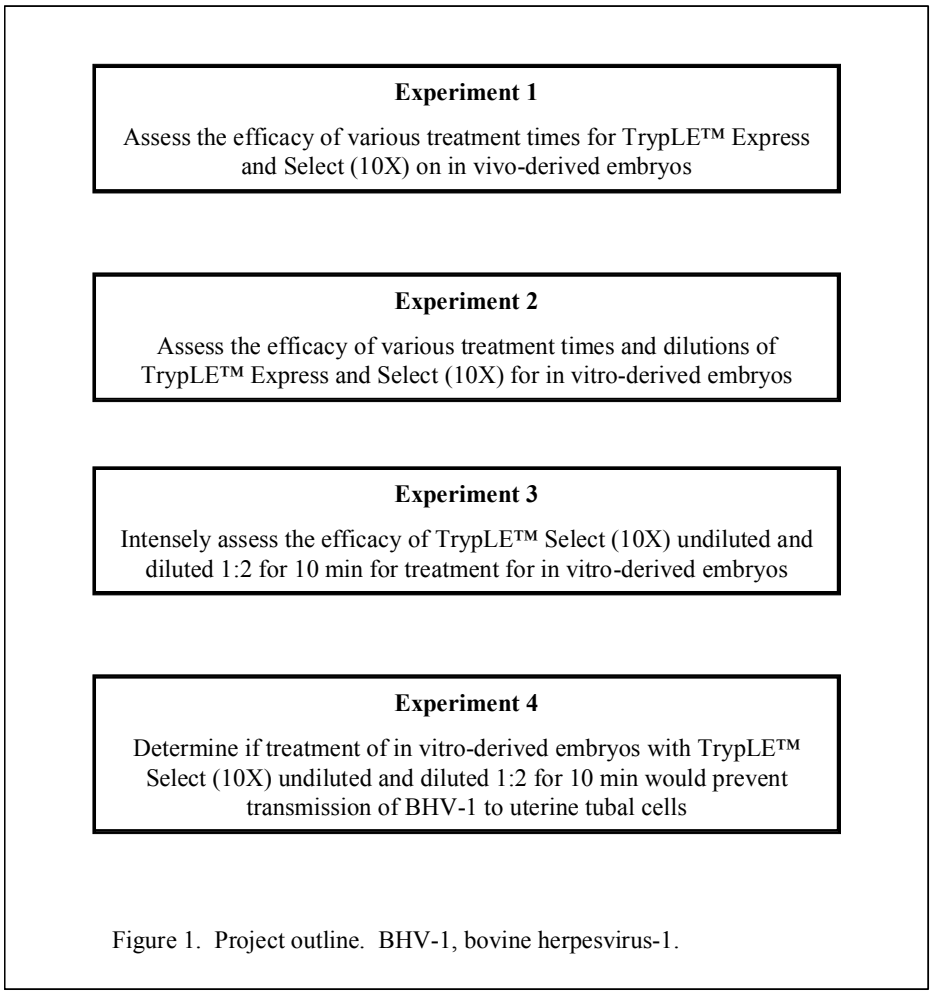

Figure 1. Project outline. BHV-1, bovine herpesvirus-1.

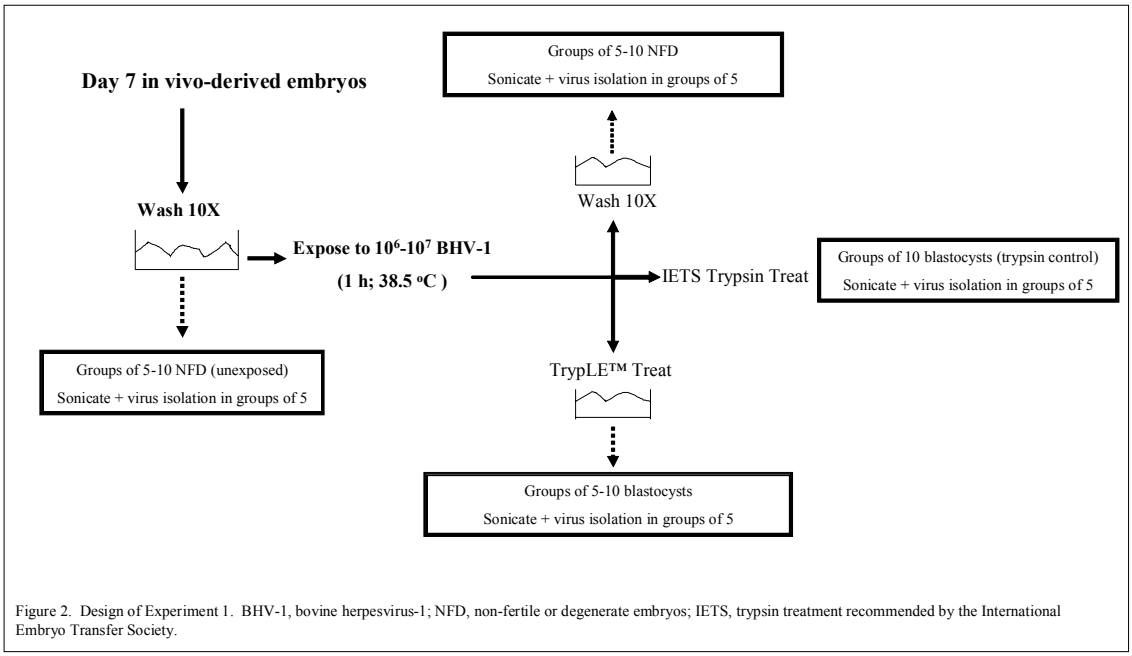

Figure 2. Design of Experiment 1. BHV-1, bovine herpesvirus-1; NFD, non-fertile or degenerate embryos; IETS, trypsin treatment recommended by the International Embryo Transfer Society.

Experiment 2

To identify effective treatments of TrypLE™ for in vitro-derived embryos, a total of 220 in vitro-derived embryos and 200 NFD were used in 10 trials for Experiment 2. Day 7, zona pellucida-intact, in vitro-derived embryos and NFD were washed according to IETS protocol. One hundred NFD were not exposed to virus and served as the negative control. Two hundred-twenty embryos and 100 NFD were exposed to $10^{6\text{-}8}$ PFU/mL BHV-1 for 1 hour. The embryos were treated in groups of 10 according to one of the following treatments: 1) 1 group of 10 NFD was washed, 2) TrypLE™ Express for 10 minutes, 3) TrypLE™ Select (10X) for 1.5, 3, 5, 7, or 10 minutes, 4) TrypLE™ Select (10X) diluted 1:2 for 3, 7, or 10 minutes, 5) TrypLE™ Select (10X) diluted 1:5 for 10 minutes, and 6) TrypLE™ Select (10X) diluted 1:10 for 10 minutes. Following washing and treatment, the samples were sonicated as groups of 5 embryos or NFD in 500 µL

MEM and assayed by virus isolation. The virus, to which the embryos and NFD were exposed, was quantified by plaque assay.

Experiment 3

To further investigate treatments that appeared to be effective for in vitro-derived embryos, a total of 260 in vitro-derived embryos and 180 NFD were used in 9 trials for Experiment 3 (Figure 3). Day 7, zona pellucida-intact, in vitro-derived embryos and NFD were washed according to IETS protocol. Ninety NFD were not exposed to virus and served as the negative control. Two hundred-sixty embryos and 90 NFD were exposed to $10^{5\text{-}8}$ PFU/ mL BHV-1 for 1 hour. The embryos were treated in groups of 10 according to one of the following treatments: 1) washed NFD, 2) porcine-origin trypsin for 1.5 minutes, 3) TrypLE™ Select (10X) for 10 minutes, and 4) TrypLE™ Select (10X) diluted 1:2 for 10 minutes. Following washing and treatment, the samples were sonicated as groups of 5 embryos or NFD in 500 µL MEM and assayed by virus isolation. The virus, to which the embryos and NFD were exposed, was quantified by plaque assay.

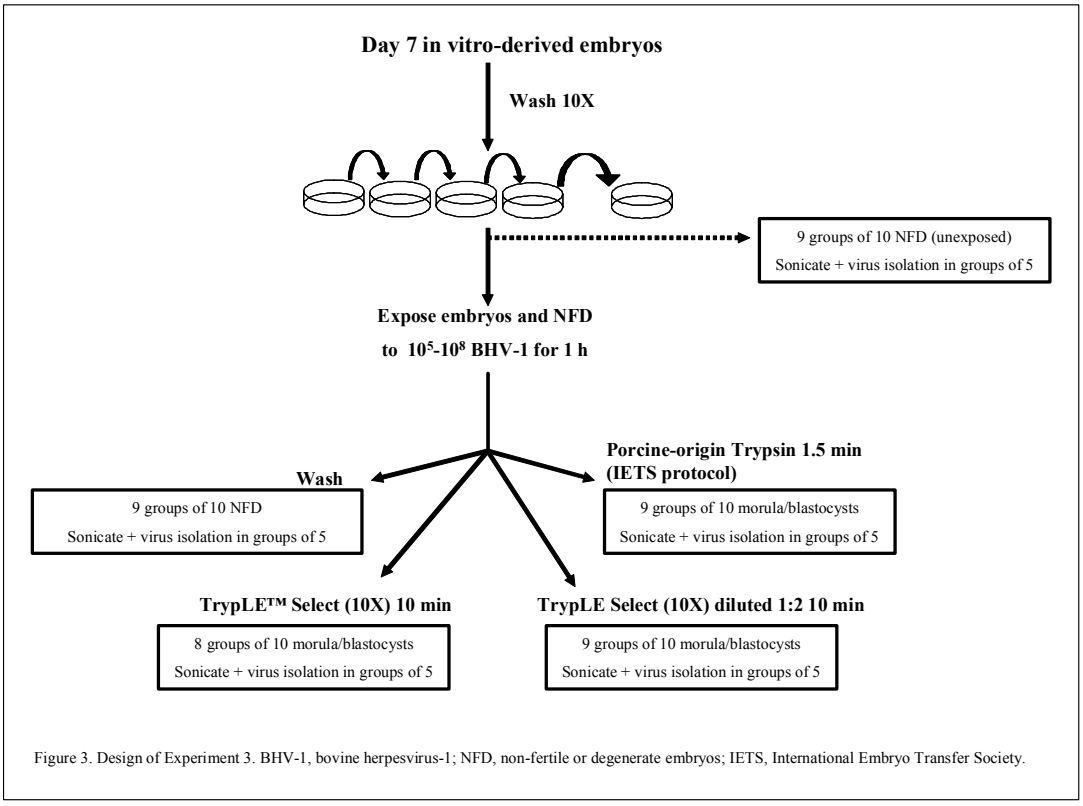

Figure 3. Design of Experiment 3. BHV-1, bovine herpesvirus-1; NFD, non-fertile or degenerate embryos; IETS, International Embryo Transfer Society.

Experiment 4

To assess apparent efficacy of TrypLE™ using more rigorous standards for in vitro-derived embryos, a total of 210 in vitro-derived embryos and 153 NFD were used in 6 trials for Experiment 4 (Figure 4). Day 7, zona pellucida-intact in vitro-derived embryos and NFD were washed according to the IETS protocol. Fifty-five NFD were not exposed to virus and served as the negative control. Two hundred-ten embryos and 98 NFD were exposed to 10^{5-7} PFU/mL BHV-1 for 1 h. Following virus exposure, the embryos were treated in groups of 10 according to the treatments indicated in Experiment 3. Following washing and treatment, 1 group of 5 embryos or NFD from each treatment was sonicated in 200 µL 1X MEM and assayed for BHV-1. The remaining embryos and NFD were placed individually or as groups of 5 in IVC2 drops containing UTC for 43 to

48 h. Following co-culture, the embryos, UTC and IVC2 media were assayed for BHV-1 by plaque assay and qPCR. The results of the qPCR were compared with those of the plaque assay.

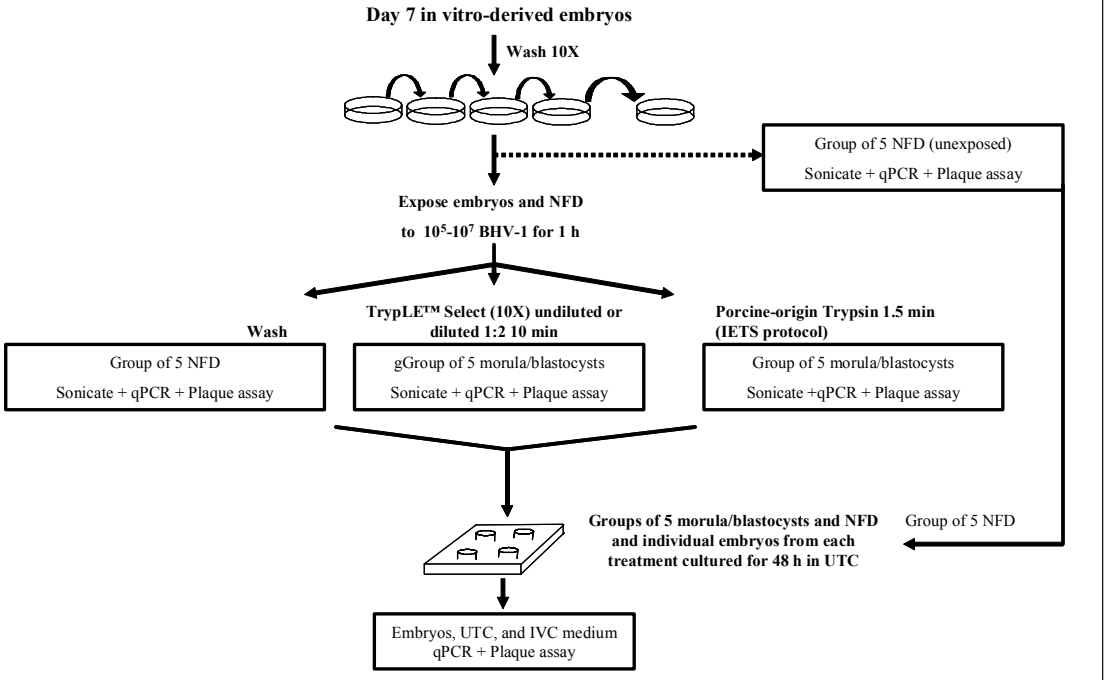

Figure 4. Design of Experiment 4. BHV-1, bovine herpesvirus-1; NFD, non-fertile or degenerate embryos; IETS, International Embryo Transfer Society; UTC, uterine tubal cells.

Statistical Analysis

The treated samples were compared with the washed samples using 2-Tail Fisher's Exact test. The PFU/mL or DNA copies/5 µL, was compared using ANOVA and Tukey-Kramer HSD with JMP IN software version 3.2.1 (SAS Institute).

The results of plaque assay and PCR were compared using Kappa analysis with JMP IN software. The amount of PFU/mL per 1 DNA copy/5 µL was calculated. For

each sample, the amount of PFU/mL was divided by the amount of DNA copies/5 µL. The mean of these results was obtained.

RESULTS

Experiment 1

The groups of unexposed in vivo-derived embryos, porcine-origin trypsin-treated embryos, and in vivo-derived embryos treated with TrypLE™ Select (10X) for 5 and 10 minutes were negative for virus. All washed groups of embryos, and at least a proportion of groups of embryos treated with TrypLE™ Express, and TrypLE™ Select (10X) for 1.5 and 3 min were positive by virus isolation (Table 1).

Table 1. Results of virus isolation from in vivo-derived bovine embryos (5 or 10 embryos per group) that were exposed to BHV-1 and then treated with porcine origin trypsin for 1.5 min, TrypLE™ Express for 1.5 or 10 min, or TrypLE™ Select (10X) for 1.5, 3, 5, or 10 min.

Treatment	Duration of Treatment (min)	BHV-1 positive embryo groups/ total groups assayed
Unexposed		0/13
Washed		13/13
Porcine origin trypsin	1.5	0/6
TrypLE™ Select (10X)	1.5	6/8
	3	2/2
	5	0/2
	10	0/7
TrypLE™ Express (1X)	1.5	11/11
	10	1/2

Experiment 2

The unexposed groups of in vitro-derived embryos, as well as the groups of in vitro-derived embryos treated with TrypLE™ Select (10X) for 7 and 10 min and TrypLE™ Select (10X) diluted 1:2 for 10 min were negative for virus. At least a proportion of the washed groups of embryos and embryos subject to the remaining treatments were positive on virus isolation (Table 2).

Table 2. Isolation of BHV-1 from in vitro-derived embryos (5 embryos per group) following treatment with various dilutions of and treatment times with trypsin (porcine-origin or TrypLE™). These results are compared with those in experiment 1.

Treatment	Duration of treatment (min)	Dilution of product	BHV-1 positive embryo groups/ total groups	
			In vitro-derived embryos (Experiment 2)	In vivo-derived embryos (Experiment 1)
Unexposed			0/20	0/13
Washed			19/20	13/13
Porcine-origin trypsin	1.5			0/6
TrypLE™ Express	1.5			11/11
	10		2/2	1/2
TrypLE™ Select	1.5		2/2	6/8
(10X)	3		2/6	2/2
	5		1/6	0/2
	7		0/6	
	10		0/6	0/7
TrypLE™ Select	3	1:2	2/2	
(10X) diluted	7	1:2	2/2	
	10	1:2	0/7	
	10	1:5	1/2	
	10	1:10	1/2	

Experiment 3

The unexposed groups of in vitro-derived embryos, as well as the groups of in vitro-derived embryos treated with porcine-origin trypsin and TrypLE™ Select (10X) diluted 1:2 for 10 min were all negative for virus. The washed groups of embryos, and 1 group of 10 embryos (assayed as 2 groups of 5 embryos) treated with TrypLE™ Select (10X) for 10 min were positive on virus isolation (Table 3).

Table 3. Isolation of BHV-1 from groups of 5 in vitro-derived embryos following treatment with TrypLE™ or porcine-origin trypsin in Experiment 3.

Treatment	Duration of Treatment (min)	BHV-1 positive embryo groups/ total groups
Unexposed		0/18
Washed		18/18
Porcine-origin trypsin	1.5	0/18
TrypLE™ Select (10X)	10	2/16
TrypLE Select™ (10X) diluted 1:2	10	0/18

Experiment 4

All of the treatment groups assayed immediately and not cultured on UTC were significantly different from the washed groups by plaque assay but not by qPCR (Table 4).

Table 4. Isolation of BHV-1 from groups of in vitro-derived embryos (5 embryos per group) that were assayed immediately and not put into co-culture with uterine tubal cells in Experiment 4. The plaque assay results are given as PFU/mL. The PCR results are given as the average of DNA particles/5 µL.

Treatment	BHV-1 positive samples/total assayed (%) [range]	
	Plaque assay	PCR
Unexposed	0/6 (0 %)[a] [0]	0/5 (0 %)[a] [0][b]
Washed	6/6 (100 %) [5-5 X 10^2]	3/5 (60 %) [0-3.3][b,c]
Porcine origin trypsin	1/6 (17 %)[a] [0 and 5]	5/5 (100 %) [1.5-3.3][c]
TrypLE Select™ (10X)	1/4 (25 %)[a] [0 and 3.5 X 10^1]	2/3 (67 %) [0-2.1][b,c]
TrypLE Select™ (10X) diluted 1:2	0/3 (0 %)[a] [0]	2/3 (67 %) [0 and 2.1][b,c]

[a]The number of positive samples in the treatment is significantly different from the positive samples in the washed treatment in that column. 2-Tail Fisher's Exact test. $P \leq 0.05$.

[b]For the number of PFU/mL or DNA particles/5 µL, differing letters indicate significant differences from one another. The means were compared by Tukey-Kramer HSD.

For inactivating in vitro-derived embryo-associated virus, only treatment of groups of embryos with TrypLE™ Select (10X) significantly reduced detection of BHV-1 by coculture of the groups in UTC (Table 5 and 6). For preventing subsequent infection of co-cultured UTC and contamination of IVC2 media, none of the treatments were significantly effective.

Table 5. In vitro-derived embryos were exposed to BHV-1, treated, and then cultured individually or as groups of 5 on uterine tubal cells for 48 h. Plaque assay results of BHV-1 isolated from embryos, uterine tubal cells, and in vitro culture medium are shown. The plaque assay results are given as PFU/mL.

	BHV-1 positive samples/ total assayed (%) (range)				
	Unexposed	Washed	Porcine origin trypsin	TrypLE™ Select (10X)	TrypLE™ Select (10X) diluted 1:2
Groups of embryos	0/5 (0 %)[a] (0)	8/8 (100 %) (5-1 X 10^4)	6/8 (75 %) (0-1 X 10^3)	1/5 (20 %)[a] (0 and 9 X 10^3)	3/4 (75 %) (0- 5 X 10^5)
Individual embryos		9/28 (32 %) (0-2.5 X 10^2)	6/27 (22 %) (0-2.5 X 10^3)	4/17 (24 %) (0-5 X 10^3)	1/14 (7 %) (0 and 1 X 10^2)
UTC associated with groups of embryos	0/5 (0 %)[a] (0)	7/8 (88 %) (0- 5 X 10^8)	6/8 (75 %) (0-5 X 10^6)	3/5 (60 %) (0-2 X 10^7)	3/4 (75 %) (0-5 X 10^6)
UTC associated with individual embryos		13/28 (46 %) (0-5 X 10^7)	10/27 (37 %) (0-1 X 10^7)	6/17 (35 %) (0-1.5 X 10^7)	2/14 (14 %) (0-3 X 10^4)
IVC media associated with groups of embryos	0/5 (0 %)[a] (0)	7/8 (88 %) (0- 5 X 10^6)	6/8 (75 %) (0-5 X 10^5)	3/5 (60 %) (0- 5 X 10^5)	3/4 (75 %) (0-1.5 X 10^5)
IVC media associated with individual embryos		11/28 (39%) (0-5 X 10^5)	11/27 (41%) (0-5 X 10^4)	6/17 (35 %) (0-5 X 10^5)	1/14 (7 %) (0 and 1.5 X10^3)

[a]The number of positive samples in the treatment is significantly different from the washed treatment in that row. 2-Tail Fisher's Exact test. $P \leq 0.05$.

Table 6. Detection and quantification of BHV-1 by qPCR from in vitro-derived embryos (groups and individuals), uterine tubal cells, and in vitro culture medium following 48 h in co-culture with uterine tubal cells. The qPCR results are given as the average DNA copies/5 µL.

	BHV-1 positive samples/ total assayed (%) (range)				
	Unexposed	Washed	Porcine origin trypsin	TrypLE™ Select (10X)	TrypLE™ Select (10X) diluted 1:2
Groups of embryos	0/4 (0 %)[a] (0)	7/7 (100 %) ($1.5\text{-}3.3 \times 10^3$)	5/7 (71 %) ($0\text{-}1.9 \times 10^4$)	1/5 (20 %)[a] (0 and 2.9×10^3)	3/4 (75 %) ($0\text{-}2.5 \times 10^6$)
Individual embryos		10/25 (40%) ($0\text{-}1.6 \times 10^1$)	9/27 (46 %) ($0\text{-}1.3 \times 10^3$)	5/17 (29 %) ($0\text{-}1.3 \times 10^3$)	2/14 (14 %) ($0\text{-}1.8 \times 10^2$)
UTC associated with groups of embryos	1/4 (25 %)[a] (0 and 0.85)[b,c]	7/7 (100 %) (4×10^1-2.7×10^6)[b]	6/7 (86 %) ($0\text{-}1.3 \times 10^7$)[b]	3/5 (60 %) ($0\text{-}1.2 \times 10^7$)[b,c]	3/4 (75 %) ($0\text{-}6.5 \times 10^7$)[c]
UTC associated with individual embryos		14/25 (56 %) ($0\text{-}3.1 \times 10^6$)	14/27 (52 %) ($0\text{-}1.1 \times 10^6$)	6/17 (35 %) ($0\text{-}1.3 \times 10^7$)	4/14 (29 %) ($0\text{-}5 \times 10^4$)
IVC media associated with groups of embryos	0/4 (0 %)[a] (0)	7/7 (100 %) ($2.1\text{-}3 \times 10^4$)	3/7 (43 %) ($0\text{-}2.1 \times 10^5$)	3/5 (60 %) ($0\text{-}3.2 \times 10^3$)	3/4 (75 %) ($0\text{-}6.8 \times 10^5$)
IVC media associated with individual embryos		10/25 (40%) ($0\text{-}5.5 \times 10^3$)	10/27 (37%) ($0\text{-}1.1 \times 10^4$)	7/17 (41 %) ($0\text{-}4.7 \times 10^4$)	3/14 (21 %) ($0\text{-}2.3 \times 10^2$)

[a]The number of positive samples in the treatment is significantly different from the positive samples in the washed treatment in that row. 2-Tail Fisher's Exact test. $P \leq 0.05$.

[b,c]For the amount of DNA copies/5 µL, differing letters indicate significant differences from one another. The means were compared by Tukey-Kramer HSD.

The qPCR results for all of the samples tested were compared with the plaque assay results (Table 7). The qPCR and plaque assay were in agreement with 85 % of 357 total samples. Twelve samples (3 %) were only detected by plaque assay. Nine of these 12 samples were detecting low concentrations of virus (≤50 PFU/mL). Forty-one samples (11 %) did not grow in culture but were positive using qPCR. Thirty-four of these 41 samples were detecting low concentrations of DNA (≤8.2 DNA copie/5 µL). The qPCR was able to detect the lower and upper limits of the virus cultured (5 to 5 X 10^8 PFU/mL). When 5 PFU/mL was present, qPCR detection occurred in 4 out of 8 samples. As expected, when virus increased to 10 to 65 PFU/mL, qPCR detection occurred in 13 out of 18 samples and when 100 to 650 PFU/mL were present, qPCR detection occurred in 18 out of 19 samples. The plaque assay and qPCR results had a correlation coefficient of 0.7 (Kappa). The conversion was determined to be 1 DNA copy/5 µL equals 1.4 X 10^2 PFU/mL. See Appendix A for further data.

Table 7. Comparison between plaque assay and qPCR results of all samples tested in Experiment 4.

	Detection by both plaque assay and qPCR	No detection by either assay	Detection by plaque assay only	Detection by qPCR only
	119/357 (33 %)	185/357 (52 %)	12/357 (3 %)	41/357 (11 %)
Plaque assay range	5-5 X 10^8 PFU/mL		5-5 X 10^3 PFU/mL	
PCR range	0.49-6.5 X 10^7 DNA copies/5 µL			0.49-4.6 X 10^4 DNA copies/5 µL

DISCUSSION

TrypLE™ is a recombinant fungal protease produced by fermentation [216]. It has cell dissociation ability that is similar to that of porcine trypsin but with lower cell toxicity. Thus, it is recommended as a replacement for dissociating cells that are cultured in monolayers. TrypLE™ cleaves the same 2 amino acid sites as trypsin and has similar activities at comparable pH. Porcine-origin trypsin is unstable at room temperature. However, TrypLE™ is stable for up to 6 months at room temperature and for 1 week at 37 ºC. Because it is not of animal origin there is a reduced risk of contamination with pathogens. It is available as TrypLE™ Express, Select (1X), and Select (10X). TrypLE™ Express is manufactured for mass production and is less expensive than the Select product.

Porcine-origin trypsin has been shown to be an effective treatment of zona pellucida-intact in vivo-derived embryos following in vitro exposure to BHV-1 [274]. In the referenced study, [274] the embryos were treated and assayed in groups of 3 to 5 embryos. Therefore, in the present study, embryos were washed in groups of 10 and assayed in groups of 5 or 10, creating a more stringent evaluation than if single embryos were evaluated. The results with TrypLE™ Select (10X) in this present study with limited numbers of in vivo-derived embryos are similar to those in the referenced study in which porcine-origin trypsin was used. However, further research with TrypLE™ needs to be performed to ensure that treated in vivo-derived embryos would not transfer virus to recipients. It is also not known if the embryos are viable following treatment with

TrypLE™ Select (10X). This additional evaluation was not done in this study due to limited availability of in vivo-derived embryos.

Treatment of in vitro-derived embryos was also evaluated. While TrypLE™ Select (10X) does have some antiviral effect when used for 10 minutes on in vitro-derived embryos, it was not completely effective as shown by the positive virus isolation results of 1 group of 10 embryos. Because this treatment was not completely effective, it would be expected that the diluted treatment would have been less effective. None the less, the groups treated with TrypLE™ Select (10X) diluted 1:2 for 10 min were negative for virus. However, if a larger sample size had been tested, positive groups might have been detected.

Obviously, effectiveness of treatment depends on the type of embryos (in vivo- versus in vitro-derived). Further, it is not known if trypsin inactivates the virus itself or if it disrupts the association of the virus with the embryo. Kim and Carp suggested that trypsin and chymotrypsin have an effect on the envelope and capsid proteins of HSV-1 and CMV [149]; therefore, the effect seen with trypsin might be on the virus itself. Whereas Dunbar et al. showed that trypsin has an effect on the protein patterns of porcine zona pellucida [78]; thus, the effect might be on the disruption of the virus-embryo association. Because trypsin is effective at removing BHV-1 from in vivo-derived embryos [264,274] and semen [28] but not from in vitro-derived embryos [30,69,79] it is possible that the virus interacts differently with in vitro-derived embryos preventing viral dissociation or inactivation. Bureau et al. hypothesize that oviductal proteins coat the zona pellucida of in vivo-derived embryos reducing the binding of virus [51]. Their study showed that enzymatic treatments were effective against several embryo-associated

viruses only after porcine in vitro-derived embryos were incubated with oviductal cells during in vitro maturation [51]. Presumably, because these proteins are not normally associated with in vitro-derived embryos, virus adherence is not reduced.

The perceived effectiveness of the treatment also depends on the sensitivity of the assay. In our previous study, Day 7 in vitro-derived embryos were exposed to BHV-1 for 1 h [79]. Groups of 5 embryos treated with trypsin were negative for virus; however, when groups of 5 embryos were treated with trypsin and then further cultured on UTC cells for 48 h (in vitro model simulating embryo transfer into a recipient) virus could be isolated. Conversely, embryos cultured individually on UTC were negative for virus. In this study, however, trypsin and TrypLE™ treatment of individual in vitro-derived embryos did not render them completely free of virus as shown by their infectivity for UTC in co-culture. A portion of UTC cultures was also infected following culture with the exposed embryos. In the present study, the entire sample was assayed, whereas in the referenced study only a portion of the sample was tested for virus. Therefore, when testing embryos for virus, it is important that the entire sample be assayed for maximum sensitivity. In this study, following viral adsorption for 1 hour on MDBK cells at the initiation of the viral isolation procedure, the samples were collected and frozen for later testing by qPCR. Consequently, the entire sample was subject to both plaque assay and qPCR.

It is not known why groups of in vitro-derived embryos that are treated (trypsin or TrypLE™) and assayed immediately are more likely to be negative for virus compared to those that are cultured on UTC first and then assayed. This demonstrates that the virus is associated with the embryos but only detectable when the embryos are first cultured on

UTC. The qPCR also validated this observation by revealing that viral particles were present even though plaque assay results were negative with some of the embryo groups assayed immediately and not cultured on UTC. However, BHV-1 was detected by plaque assay in association with the washed groups whether they were assayed immediately or first cultured on UTC perhaps due to larger amounts of virus associated with them. We hypothesize that as the ciliated UTC move throughout the drop, the virus is better able to contact the embryos, thus, allowing more virus to adhere to the zona pellucida prior to assaying. This is similar to Blomberg's observations that vibration enhances HSV attachment to cells [33]. The UTC might also be more susceptible than MDBK cells to the virus, allowing greater amplification of virus. Thus, culturing treated embryos on UTC is a very stringent test to determine if infectious virus is indeed associated with embryos. It would be of interest to compare this assay to the actual transfer of treated embryos to recipients and subsequent monitoring of recipients and offspring for infection.

Though using a recombinant trypsin product would be beneficial over an animal-origin product, TrypLE™ Select (10X) does not render a single IVF embryo consistently free of infectious virus. Also, a treatment time of 10 min might not be clinically practical. A product that is effective when used for less than 2 min would be more readily accepted. It is not surprising that the recombinant trypsin would require more time to be effective, due to its purified form. Cleeland and Sugg showed that while it was actually trypsin present in the crystalline and crude form that had antiviral effects against influenza virus, there were other constituents present in crude trypsin that enhanced its action [63]. However, when the incubation time was increased, the two enzymes (crude and

crystalline) were similar. Thus, treatment with recombinant trypsin products may need to be increased from the traditional 60 to 90 sec time period used for animal-origin trypsin. In addition, other recombinant trypsin products need to be evaluated to determine their efficacy against BHV-1 associated with in vitro-derived embryos. Our results are similar to those of Seidel et al. [259] who evaluated TrypZean™. They treated BHV-1 exposed in vivo-derived embryos for 1.5 minutes and assayed for BHV-1. Two out of 18 embryos/ova were positive for virus. In addition to efficacy, further research is also needed to assess the viability of embryos following treatment with recombinant trypsin. While Pryor et al. [230] showed no difference in cell count between in vitro-derived embryos following treatment with TrypLE™ Express and control embryos, similar work would need to be performed to evaluate TrypLE™ Select (10X). Additional research would also need to be performed to evaluate the efficacy of TrypLE™ when embryos are exposed to BHV-1 at different stages of in vitro embryo production.

This research also assessed the efficacy of the real-time qPCR for detection of BHV-1. A total of 357 samples were tested using both plaque assay and qPCR. While it should be noted that qPCR will not discriminate between infectious and noninfectious virus, the samples correlated 85 % of the time with a correlation factor of 0.7. The qPCR also proved able to detect a small amount of virus (5 PFU/mL) in 50 % of those samples and detected with 95 % accuracy samples with 100 to 650 PFU/mL.

In conclusion, we demonstrate the use of a qPCR that can be a useful tool for the detection of small amounts of BHV-1 associated with embryos and present in cells and media. We also show the promising use of recombinant trypsin for the disinfection of in

vivo-derived bovine embryos. However, further research with other recombinant trypsin products is needed for a product that is effective for treatment of in vitro-derived embryos.

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VII. VALIDATION OF A DUPLEX QUANTITATIVE POLYMERASE CHAIN REACTION FOR DETECTION OF BOVINE HERPESVIRUS 1 AND BOVINE VIRAL DIARRHEA VIRUS

ABSTRACT

A variety of procedures associated with embryo production, cloning and transgenics in cattle are currently used not only in research but also for commercial production of calves. Introduction of infectious agents while using these techniques could influence the results of basic research, affect clinical proficiency, or result in unnatural transmission routes of diseases. Two bovine pathogens, bovine herpesvirus 1 (BHV-1) and bovine viral diarrhea virus (BVDV), are widely distributed among cattle populations and have been associated with cells, fluids and tissues that were collected from donor animals for use in reproductive technologies. Thus, an assay that could quickly and simultaneously identify these two viruses in media and materials of animal origin used in embryo production would facilitate quality assurance testing.

In this study we developed a duplex quantitative polymerase chain reaction for detection of both BHV-1 and BVDV type I and II and validated the assay using pooled

samples of bovine follicular fluid. The assay was able to simultaneously detect diluted BHV-1 (1:100) and BVDV I (1:1,000).

INTRODUCTION

Bovine herpesvirus 1 (BHV-1) and bovine viral diarrhea virus (BVDV) cause both acute and persistent or latent infections in cattle [87,190,211,234]. With the exception of a few countries and territories from which these agents have been eradicated, the viruses are distributed worldwide, and each is capable of causing economically significant reproductive and respiratory diseases [128,170,209,341]. Because of their presence in serum [36,37,250] and reproductive tissues [24,31,49,88,102,329], BHV-1 and BVDV have been the focus of risk assessments associated with pathogen transmission via embryos produced by in vitro production or somatic cell nuclear transfer [27,79,99,100,276]. Further, it is well known that neither washing nor trypsin treatment will completely remove these viruses from in vitro-produced embryos [27,30,69,79,100,291]. Thus, efficient quality assurance testing of fluids, gametes and somatic cells used for in vitro embryo production are vital for preventing transfer of embryos that have BHV-1 and BVDV associated with them [272]. Therefore, the primary goal of this study was to develop a real-time quantitative polymerase chain reaction (qPCR) that would simultaneously detect BHV-1 and BVDV and facilitate quality assurance testing.

MATERIALS AND METHODS

Media

Minimum Essential Medium (1X MEM) was used for virus isolation and initial dilution of the follicular fluid. In addition, virus was propagated in Madin Darby bovine kidney (MDBK) cells which had been cultured in 1X MEM. This consists of MEM with Earle's salts (Invitrogen, Carlsbad, CA) supplemented with 10 % equine serum (HyClone, Logan, UT), 0.75 mg/mL sodium bicarbonate (Invitrogen), 0.29 mg/mL L-glutamine (Invitrogen), 100 IU/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Invitrogen).

Plaque assays required mixing equal volumes of 2X MEM and 3 % agarose. Minimum Essential Medium (2X) includes 80 mL distilled water, 20 mL 10X MEM with Earle's salts (10X, Invitrogen), 10 % equine serum, 0.58 mg/mL l-glutamine, 200 IU/mL penicillin G, 200 μg/mL streptomycin, and 0.5 μg/mL amphotericin B (Invitrogen), and 1.5 mg/mL sodium bicarbonate. Agarose (Sigma, St. Louis, MO) consists of 3 grams of type VII low temperature gelling agarose that was dissolved in 97 mL distilled water and autoclaved.

Fetal bovine serum, equine serum, and BSA were determined to be free of BHV-1 and BVDV by virus isolation. They were also free of anti-BHV-1 antibodies by virus neutralization. The fetal bovine serum contained anti-BVDV antibodies detectable at a dilution of 1:80.

Stock Virus

Stock virus of BHV-1 (Colorado strain) was obtained from the American Type Culture Collection (catalog number VR-864, lot number 1222901). Non-cytopathic BVDV I (genotype 1b) was obtained from the serum of the BVDV persistently infected heifer used in this study. The viruses were propagated in MDBK cells. Virus was harvested by freezing and thawing the infected cell cultures and stored in cryovials at –80 °C until needed. The plaque forming units (PFU)/mL (BHV-1) or $CCID_{50}$/mL (BVDV) of stock virus was determined by quantifying plaques or virus titration, respectively.

Virus Isolation and Titration

Virus isolation and titration procedures were used to determine the presence and quantity of BVDV in follicular fluid from a heifer persistently infected with BVDV. Because follicular fluid is toxic to cells in culture[91], the fluid was diluted 1:10 in 1X MEM prior to running the assay. Serial 10-fold dilutions (to 10^{-8}) of samples were performed in a 96-well plate (0.36 cm^2) and tested in triplicate. Fifty µL of MDBK cells was added to each well. The plates were incubated for 3 d at 38.5 ºC in a humidified atmosphere of 5 % CO_2 and air. On Day 3, an immunoperoxidase monolayer assay was performed [3]. Briefly, the samples were dried and fixed with acetone. Cells were incubated with 2 anti-BVDV monoclonal antibodies for 30 min. Antibody D89 (Veterinary Medical Research and Development, Pullman, WA) is specific for gp53/E2, a BVDV major envelope glycoprotein [320,344]. Antibody 20.10.6 (E. Dubovi, Cornell

University) is specific for p80, a conserved nonstructural protein [66]. The cells were washed to remove any unbound antibody, and then incubated with peroxidase-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min. The cells were washed to remove any unbound conjugated antibody. Lastly, aminoethyl carbazole was added (Zymed Laboratories, South San Francisco, CA). A reddish-brown color is visualized under a light microscope when oxidation by horseradish peroxidase has occurred, indicating the presence of BVDV antigen. Reed and Muench calculations were used to quantify the amount of virus present in each sample [236].

Virus isolation of test samples was also performed to detect the presence of BHV-1 or BVDV. Samples were diluted 1:10 in 1X MEM prior to running the assay. A separate plate was set up for detection of each virus. Fifty µL of diluted sample was added to 50 µL of MEM in a 96-well plate. The samples were tested in triplicate. Fifty µL of MDBK cells was then added to each well. The plates were incubated for 3 d (BVDV detection) or 5 d (BHV-1 detection) at 38.5 °C in an atmosphere of 5 % CO_2 and air. Following the 3 d incubation, immunoperoxidase monolayer assay was used to identify BVDV. For BHV-1 detection, the MDBK cells were examined for evidence of cytopathic effect (CPE) at Day 5. Samples showing CPE were frozen in -80 ºC for later confirmation of BHV-1 by BHV-1 qPCR.

Virus Amplification in Monolayers of MDBK Cells

If virus isolation of the samples was negative, the samples were placed on monolayers of MDBK cells to attempt to amplify the virus. Two hundred µL of sample was inoculated into a well of a 12-well plate (3.8 cm^2) previously seeded with MDBK cells. The plate was incubated for 1 hr at 38.5 °C in an atmosphere of 5 % CO_2 and air and then 1 mL 1X MEM was added to each well. The plates were incubated for 5 d. Following incubation the samples were frozen and thawed. Cell lysate samples were then processed as described previously for virus isolation.

Plaque Assay

Plaque assays were used to quantify the amount of BHV-1 present in the follicular fluid from the BHV-1 infected cow. Serial 10-fold dilutions of follicular fluid was performed to 10^{-7}. One hundred µL of each dilution was inoculated onto a single well of a 24-well (2 cm^2) plate previously seeded with MDBK cells. The plates were incubated for 1 h at 38.5 °C in an atmosphere of 5 % CO_2 and air. One mL of 3 % agarose/2X MEM (50:50) was placed in each well, and the plates were refrigerated for 8 min. The plates were then incubated for 5 d at 38.5 °C in an atmosphere of 5 % CO_2 and air. On Day 5, the number of plaques in each well was counted.

Virus Neutralization

Virus neutralization for detection of anti- BHV-1 and -BVDV antibodies was performed on the follicular fluid obtained from the abattoir. A separate plate was set up for detection of antibodies against each virus. The fluid was diluted 1:10 prior to running the assay to prevent cytotoxicity. Two-fold dilutions of the fluid were performed in 50 µL 1X MEM. The test was performed in triplicate. Fifty µL containing 100 to 300 $CCID_{50}$ of BHV-1 (Colorado strain) or BVDV (genotype 1b from the persistently infected heifer) was added to each well. The plates were incubated for 3 d (anti-BVDV antibody detection) or 5 d (anti-BHV-1 antibody detection) at 38.5 °C in an atmosphere of 5 % CO_2 and air. Following the 3 d incubation, immunoperoxidase monolayer assay was applied to the BVDV plates. For anti-BHV-1 antibody detection, the MDBK cells were examined at Day 5 for lack of cytopathic effect (CPE). If virus was not detected, sufficient antibody was present to neutralize the virus. Antibody titer was expressed as the greatest dilution of follicular fluid at which 2 out of 3 wells were free of virus.

Bovine herpesvirus 1 Quantitative Polymerase Chain Reaction

The DNA or RNA in samples was extracted with QIAamp viral RNA kit (Qiagen, Valencia, CA) to yield 60 µL of sample. The extracted sample was assayed using the BHV-1 qPCR, duplex qPCR, and BVDV nested reverse transcribed PCR (RT-nPCR). Bovine herpesvirus 1 qPCR was performed with primers specific for the BHV-1

ribonucleotide reductase gene. These primers were derived from Schang and amplify both subunits of ribonucleotide reductase [256].

Bovine herpesvirus 1 was monitored by the amplification of a 141 bp fragment by using the primers RR-1 (5' TGCCCTACAGGTCGTTGATTA 3') and RR-2 (5' TCCAGCTGCCTCCTCTGTTT 3'). The 5' probe (5' CGTGTGCTTCTCGGCAGTCATCA 3') was labeled with carboxyfluorescein (6-FAM) at the 3' end. The 3' probe (5' CCAAAGGAAAATCGGTCCCAGGA 3') was labeled with Cy5.5™ at the 5' end and a phosphate at the 3' end. Primers and probes were synthesized by Operon (Huntsville, AL)

An oligonucleotide mix was prepared containing 5 μM of each primer, 0.5 μM of the 5' probe and 1 μM of the 3' probe. Prior to running each reaction, a master mix was prepared. The master mix contained 20 μL of the oligonucleotide mix, 50 μL QuantiTect Probe PCR Master Mix (Qiagen), 2.5 μL RNase-free water, and 2.5 μL uracil-N-glycosalase (Roche; UNG; 1 unit/ μL, Indianapolis, IN). The QuantiTect Probe PCR Master Mix consisted of HotStarTaq® DNA Polymerase, QuantiTect Probe PCR Buffer, dNTP mix, and Rox™ fluorescent dye. The QuantiTect Probe PCR Buffer consisted of TrisCl, KCl, $(NH_4)_2SO_4$, and 8 mM $MgCl_2$. The dNTP mix contained dATP, dCTP, dGTP, and dTTP/dUTP.

For a 100 μL reaction, 75 μL of the master mix was added to each capillary, followed by 25 μL of each sample to be tested. The capillaries were centrifuged and then placed in the Roche LightCycler 2.0. The following program was performed with a 20 °C/sec ramp unless otherwise indicated: UNG carry-over prevention at 50 °C for 2 min; PCR initial activation step at 95 °C for 15 min; 50 cycles of 3 step cycling consisting of

denaturation at 95 °C for 0 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec at 2 °C/sec; melting curve at 95 °C for 0 sec, 50 °C for 10 sec, and then heating to 80 °C at 0.1 °C/sec; and cooling to 38 °C for 30 sec. The fluorescence channel used was 705/530 nm. The fluorescence data acquisition was quantified during the annealing phase. The Roche LightCycler Software Version 4.0 was used.

Duplex Quantitative Polymerase Chain Reaction

This 2-step qPCR was performed with primers specific for the BHV-1 ribonucleotide reductase gene [256] and primers specific for the BVDV I and II 5' non-translated region [279].

Bovine herpesvirus 1 DNA was amplified and detected using the primers and probes previously described for BHV-1 qPCR. Bovine viral diarrhea virus I was monitored by the amplification of a 137 bp fragment, and BVDV II was detected with a 134 bp fragment. The primers BVDu3 (5' CATGCCCAAAGCACATCTTA 3') and BVDl1 (5' TGCCATGTACAGCAGAGATTT 3') were used [279]. The 5' probe (5' AYGRAYACAGCCTGATAGGGTGY 3') was labeled with carboxyfluorescein (6-FAM) at the 3' end. The 2nd, 6th, and 23rd bp contained a Y (C+T) and the 4th bp contained an R (A+G) so that it could detect both BVDV I and II. Two 3' probes were used, one for detection of BVDV I and one for detection of BVDV II. The 3' probe for detection of BVDV I (5' CAGAGGCCCACTGTATTGCTACTAAA 3') was labeled with Bodipy® 630 at the 5' end and a phosphate at the 3' end. The 3' probe for detection of BVDV II

(5’ CAGAGACCTGCTATTCCGCTAGTAAA 3’) was labeled with Bodipy® TR-X at the 5' end and a phosphate at the 3' end. Primers and probes for detection of BVDV were synthesized by Qiagen.

The Roche LightCycler 2.0 is able to detect multiple reporter dyes in the same capillary. However, each reporter dye can be detected by more than one detection channel. This was corrected by creating a color compensation file that was applied to each analysis.

Reverse transcription (RT) was performed separately from the qPCR. A master mix for reverse transcription was prepared containing 2 µL RNase-free water, 2 µL buffer RT (10X, Qiagen), 2 µL dNTP (Qiagen), 1 µL RNasin® RNase inhibitor (40 U/ µL, Promega, Madison, WI), 1 µL Omniscript RT (Qiagen), and 2 µL BVDV primer mix (10 µM BVDVu3 and l1). Ten µL of master mix was added to each tube followed by 10 µL of sample. The tubes were vortexed for 5 sec, briefly centrifuged, and incubated in a heating block set at 37 °C for 1 h. Following reverse transcription, the qPCR was performed.

An oligonucleotide mix was prepared for each virus. The mix for BHV-1 contained 10 µM of each primer and 2 µM of each probe. The mix for BVDV I and II contained 4 µM of each primer and 2 µM of each probe. Prior to running each reaction, a master mix was prepared. The qPCR master mix contained 2 µL of each oligonucleotide mix, 10 µL QuantiTect Multiplex PCR NoRox Master Mix (Qiagen), 0.8 µL RNase-free water, and 0.2 µL uracil-N-glycosalase (Roche; UNG; 1 unit/µL, Indianapolis, IN). The QuantiTect Multiplex PCR NoRox Master Mix consisted of HotStarTaq® DNA

Polymerase, QuantiTect Multiplex PCR Buffer, dNTP mix, and 11 mM $MgCl_2$. The dNTP mix contained dATP, dCTP, dGTP, and dTTP/dUTP.

Reactions were performed in 20 µL capillaries. Fifteen µL of the qPCR master mix was added to each capillary, followed by 0.5 µL of reverse transcribed sample and 4.5 µL of extracted sample that had not undergone RT. Samples were tested in duplicate. The capillaries were centrifuged and then placed in the Roche LightCycler 2.0. The following program was performed with a 20 °C/sec ramp unless otherwise indicated: UNG carry-over prevention at 50 °C for 2 min; PCR initial activation step at 95 °C for 15 min; 40 cycles of 3 step cycling consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec at 2 °C/sec; melting curve at 95 °C for 0 sec, 50 °C for 10 sec, and then heating to 80 °C at 0.1 °C/sec; and cooling to 40 °C for 1 min. The fluorescence channels used were 705 nm for detection of BHV-1, 640 nm for detection of BVDV I, and 610 nm for detection of BVDV II. The fluorescence data acquisition was quantified during the annealing phase. The Roche LightCycler Software Version 4.0 was used.

Bovine viral diarrhea virus Standard Reverse Transcription Nested Polymerase Chain Reaction (RT-nPCR)

The single closed-tube RT-nPCR was performed as described previously [99]. Bovine viral diarrhea virus was monitored by the amplification of a 290 and 213 bp fragment. The outer primers, BVD 100 (5' GCTAGCCATGCCCTTAG 3') and HCV 368

(5' CCATGTGCCATGTACAG 3') are specific for the 5' non-translated region of the viral genome. The inner primers, BVD 180 (5' CCTGAGTACAGGGDAGTCGTCA 3') and HCV 368 amplify a sequence within the first amplicon. The 14th base, D, is degenerate (G+A+T) to enable detection of different virus strains. Primers were synthesized by Qiagen.

Prior to setting up the RT-nPCR, a trehalose mix was prepared containing the inner primers and placed in the lid of a 200 µL microcentrifuge tube. The mix consisted of 5 µL trehalose (22 % w/v; Sigma), 0.4 µL BVD 180 (50 µM), 0.4 µL HCV 368 (50 µM), 1 µL dNTP (10 mM, Promega), and 0.25 µL GoTaq® Flexi DNA polymerase (Promega). The tubes were then air dried for 2 hours prior to use or storage. Prior to running each reaction, a master mix was prepared with reagents purchased from Promega. The master mix contained 20.5 µL RNase-free water, 10 µL 5X Green GoTaq® Flexi buffer, 8 µL $MgCl_2$, 2 µL dNTP (400 µM), 1 µL BVD 100 (0.1 µM), 1 µL HCV 368 (0.1 µM), 1 µL Triton X-100, 0.25 µL DL-dithiothreitol (DTT, 0.5 mM), 0.25 µL RNasin® RNase inhibitor (0.2 U/µL), 0.5 µL GoTaq® Flexi DNA polymerase (0.05 U/µL), and 0.5 µL Moloney murine leukemia virus (M-MLV) reverse transcriptase (2 U/µL).

For each reaction, 45 µL of the master mix was added to a tube containing the inner primers in the lid, overlayed with 50 µL mineral oil, and followed by 5 µL of each sample added to the bottom of the tube. The tubes were then placed in the RoboCycler Gradient 96 (Stratagene, Cedar Creek, TX). The following program was performed: 37 °C for 45 min; 95 °C for 5 min; 20 cycles of 3 step cycling consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min; and final

extension at 72 °C for 10 min. The tubes were then inverted several times to mix the product with the inner primer reagents in the lid of the tube. The tubes were centrifuged at 14,000 x G for 15 sec. A second program was performed in the PCR machine that consisted of the following parameters: 30 cycles of 3 step cycling consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 45 sec; and final extension at 72 °C for 10 min. Ten μL of each PCR product were separated by 1.5 % agarose gel electrophoresis. The agarose gels contained ethidium bromide (0.5 μg/mL) to allow visualization of RT-nPCR products using an ultraviolet transilluminator.

Experimental Design

Ovaries were removed from a BHV-1 acutely infected heifer and a BVDV persistently infected heifer. The follicular fluid was aspirated from the ovaries, assayed and quantified for BHV-1 (plaque assay, duplex qPCR, and BHV-1 qPCR) and BVDV (virus titration and isolation, duplex qPCR, and RT-nPCR) (Figure 1). Ten ovaries from dairy cows were obtained from an abattoir (Laceyville, PA). The follicular fluid was aspirated, pooled, and shipped on ice packs overnight to our facility. The fluid was assayed for BVDV and BHV-1 (duplex qPCR, RT-nPCR, and BHV-1 qPCR) and anti-BVDV and -BHV-1 antibodies (virus neutralization). When performing virus isolation, quantification or neutralization, the follicular fluid was first diluted 1:10 in 1X MEM to prevent cell cytotoxicity.

The BVDV and BHV-1 infected follicular fluid was diluted with the pooled follicular fluid (1:5, 1:10, 1:50, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, $1:10^6$, $1:10^7$). Each dilution was assayed by duplex qPCR, RT-nPCR, and BHV-1 qPCR. In addition, virus isolation was performed on each dilution. Immunoperoxidase monolayer assay was used to confirm the presence of BVDV, and BHV-1 qPCR was used to confirm the presence of BHV-1. Samples that were negative for virus after virus isolation were passaged on monolayers of MDBK cells once and retested by virus isolation. Four trials were performed.

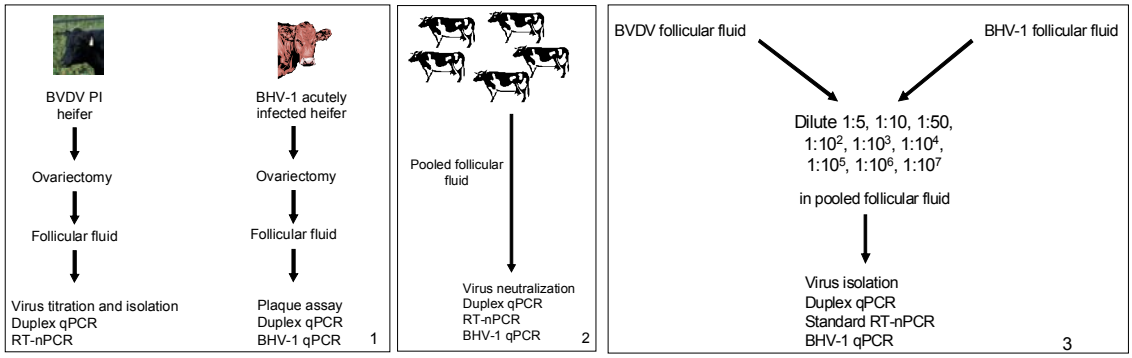

Figure 1. Experimental Design. 1. Follicular fluid was collected from the ovaries of a BHV-1 acutely infected heifer and a BVDV persistently infected heifer. The samples were assayed as indicated. 2. Follicular fluid was collected and pooled from 10 ovaries of dairy cows and assayed as indicated. 3. The pooled follicular fluid was diluted with the BHV-1 and BVDV infected follicular fluid and assayed as indicated. BVDV, bovine viral diarrhea virus; PI, persistently infected; BHV-1, bovine herpesvirus 1; qPCR, quantitative polymerase chain reaction; RT-nPCR, reverse transcription nested polymerase chain reaction.

RESULTS

Prior to performing this study, the duplex qPCR was evaluated using different techniques. A one-step program was examined which combined the RT and qPCR step in 1 tube. The detection with this method was not as sensitive as that with the two-step

program as performed in this study. As well, varying ratios of extracted and RT sample were tested. It was found that samples consisting of 0.5 µL of reverse transcribed sample and 4.5 µL extracted sample optimized the detection of both the RNA and DNA virus. The duplex qPCR was also evaluated against different strains of BHV-1 and BVDV I and II. The viruses were detected in the appropriate detection channels.

The follicular fluid collected from the BHV-1 acutely infected heifer contained 4 X 10^5 PFU/mL of BHV-1 (Table 1). The BHV-1 qPCR detected 1.1 X 10^4 DNA copies/5 µL, and the duplex qPCR detected 1.7 X 10^4 DNA copies/5 µL. No BVDV was detected in this sample with the duplex qPCR.

The follicular fluid collected from the BVDV PI heifer contained 3.5 X 10^6 $CCID_{50}$/mL of BVDV. The RT-nPCR detected both the 213 and 290 bp fragments indicative of BVDV. The duplex qPCR detected 1.9 X 10^6 BVDV I RNA copies/5 µL. No BHV-1 or BVDV II was detected in this sample with the duplex qPCR.

The follicular fluid collected from the pooled ovaries did not contain BHV-1 or BVDV as indicated by the BHV-1 qPCR, duplex qPCR, and RT-nPCR. It did contain anti-BVDV I antibodies detectable at a dilution of 1:80 and anti-BHV-1 antibodies detectable at a dilution of 1:40.

Table 1. The amount of virus detected in the follicular fluid obtained from the BHV-1 and BVDV infected heifers are shown. qPCR, quantitative polymerase chain reaction; BHV-1, bovine herpesvirus 1; BVDV, bovine viral diarrhea virus; PFU, plaque forming units; $CCID_{50}$, cell culture infectious dose; PI, persistently infected.

Follicular fluid source	Duplex qPCR		BHV-1 qPCR	Virus titration	
	BVDV I	BHV-1		BVDV	BHV-1
BHV-1 infected heifer		1.7×10^4 DNA copies/ 5 µL	1.1×10^4 DNA copies/ 5 µL		4×10^5 PFU/mL
BVDV PI heifer	1.9×10^6 RNA copies/ 5 µL			3.5×10^6 $CCID_{50}$/mL	

Preliminary research demonstrated accurate detection of 2 strains of BHV-1 (Colorado and LA), 5 strains of BVDV I (81,180,526,66, KY147), and 2 strains of BVDV II (AU501 and 134F) by the duplex qPCR. The strains of BVDV tested were isolated from PI animals. This duplex qPCR also routinely detected external standards containing greater than 10^2 nucleic acid copies/5 µL and detected 10^2 and 10^1 nucleic acid copies/5 µL in a proportion of samples (Table 2).

Table 2. The external standards containing 10^2 and 10^1 nucleic acid copies/5 µL are shown for each virus detected by the duplex qPCR. BHV-1, bovine herpesvirus 1; BVDV I, bovine viral diarrhea virus type I; BVDV II, bovine viral diarrhea virus type II; qPCR, quantitative polymerase chain reaction.

	BHV-1	BVDV I	BVDV II
	External standard detected/Duplex qPCRs performed		
External standard containing 10^2 nucleic acid copies/5 µL	12/12	6/12	12/12
External standard containing 10^1 nucleic acid copies/5 µL	12/12	3/12	7/12

The detection results of the various assays for the diluted follicular fluid are shown in Table 3. The RT-nPCR was able to consistently detect BVDV at a dilution of 1:1,000. The detection of BVDV by the duplex qPCR and RT-nPCR had corresponding results. The BHV-1 qPCR consistently detected BHV-1 at a dilution of 1:100. The duplex qPCR had corresponding results with the BHV-1 qPCR for detection of BHV-1. Virus was not detected by virus isolation in any of the samples.

Table 3. The detection ability of each assay is shown. BHV-1, bovine herpesvirus 1; BVDV I, bovine viral diarrhea virus type I; RT-nPCR, reverse transcription-nested polymerase chain reaction; qPCR, quantitative polymerase chain reaction.

Dilution of virus in follicular fluid	Samples positive for virus/total samples tested					
	Virus isolation		RT-nPCR	BHV-1 qPCR	Duplex qPCR	
	BHV-1	BVDV I	BVDV	BHV-1	BHV-1	BVDV I
1:5	0/4	0/4	4/4	4/4	4/4	4/4
1:10	0/4	0/4	4/4	4/4	4/4	4/4
1:50	0/4	0/4	4/4	4/4	4/4	4/4
$1:10^2$	0/4	0/4	4/4	4/4	4/4	4/4
$1:10^3$	0/4	0/4	4/4	2/4	2/4	4/4
$1:10^4$	0/4	0/4	1/4	0/4	0/4	1/4
$1:10^5$	0/4	0/4	0/4	0/4	0/4	0/4
$1:10^6$	0/4	0/4	0/4	0/4	0/4	0/4
$1:10^7$	0/4	0/4	0/4	0/4	0/4	0/4

The lowest limit of detection by the duplex qPCR for BHV-1 was 2.64 DNA copies/5 µL at the 1:1,000 dilution of follicular fluid. The lowest limit of detection by the BVDV I was 49.8 DNA copies/5 µL at the 1:10,000 dilution of follicular fluid. The

BHV-1 qPCR was able to detect down to 10.2 DNA copies/5 µL at the 1:1,000 dilution of follicular fluid.

DISCUSSION

Bovine herpesvirus 1 was isolated from cumulus-oocyte complexes, follicular fluid, granulosa cells and uterine tubal cells that were collected from acutely infected cattle [24]. Further, the virus was isolated from IVF embryos produced from materials collected from acutely infected cattle or when BHV-1 was artificially introduced at various stages of in vitro embryo production [24,30]. Bovine viral diarrhea virus was also isolated from cumulus-oocyte complexes, follicular fluid, granulosa cells and uterine tubal cells from cattle that were either acutely or persistently infected with BVDV [25,31,38,295], and BVDV antigen was detected in a proportion of oocytes in the ovaries of persistently infected cattle [49]. Both BHV-1 and BVDV have been associated with serum collected from infected cattle or fetuses [36,37,153,250]. Thus, the potential exists for introduction of these viruses during in vitro embryo production, and the production of embryos associated with each of these viruses has been demonstrated [29,31,275].

Since washing and trypsin treatment are not effective for completely removing these viruses from in vitro-produced embryos [27,30,79,291] quality assurance testing of materials of animal origin used in embryo production and perhaps of other samples (e.g. follicular fluid, embryo culture media, or nonfertile or degenerated ova) might be required as a means of ensuring that these viruses will not be transmitted during transfer

of in vitro-produced embryos [286]. Such a requirement is most likely to be invoked when embryos from endemic countries are to be exported to countries where the diseases have been eradicated [128,170,341].

We undertook this study to develop and validate a duplex qPCR for detection of both BHV-1 and BVDV to facilitate any such testing of materials of animal origin that might be required to confirm that neither of these viruses were associated with in vitro-produced embryos. In this study, the assay was validated using pooled follicular fluid. It has been shown that follicular fluid can be contaminated with BHV-1 and BVDV [24,29,31,91,295]. When oocytes are taken from ovaries obtained from an abattoir for use in in vitro embryo production, the follicular fluid aspirated from these ovaries could be tested to determine the presence of these viruses. Avery et al. showed that 12 % of their in vitro embryo production experiments were contaminated with BVDV when oocytes and oviductal epithelial cells were used from an abattoir [9]. The oocytes from each of their experiments were pooled from approximately 24 animals. Therefore, it is possible that a single infected animal could have contaminated the entire batch for that experiment. Thus, cumulus oocyte complexes associated with contaminated follicular fluid could be confirmed to be free of the viruses.

Previously, the RT-nPCR used in this study was shown to be able to detect virus in semen from a PI bull diluted 1:1000 with virus-free semen [103]. Using a RT-PCR, Renshaw et al. were able to detect the presence of BVDV in milk samples diluted 1:600 with a PI animal [238], and Radwan et al. was able to detect BVDV diluted 1:640 [235]. Also, Weinstock et al. used a RT-PCR to detect the presence of BVDV in pools of serum containing up to 100 samples [330]. In the present study, the duplex qPCR was shown to

be as sensitive for detection of BVDV in follicular fluid as the RT-nPCR [99,101], while having the added advantage of quantitating the virus, detecting BHV-1, and differentiating BVDV type I and II. These 2 PCRs (RT-nPCR and duplex qPCR) were able to detect follicular fluid from a known PI animal diluted 1:1000 in pooled follicular fluid. Because the BVDV was from a PI heifer, it contained a higher concentration of virus than might be detected in a BVDV acutely infected animal [127]. Therefore, if the samples were contaminated with BVDV from an acutely infected animal the detection might be lower than 1:1000.

Additionally, BHV-1 was detected by both the duplex qPCR and BHV-1 qPCR to the 1:100 dilution. These results are similar to Wang et al. who demonstrated a qPCR that detected 1:100 dilution of BHV-1 positive semen with virus-free semen [325] and up to a 1:160 dilution of BHV-1 positive semen with MEM [326]. As well, they showed that with virus-free semen spiked with BHV-1, detection was possible to the $1:10^6$ dilution [325]. Grom et al. also demonstrated that virus-free semen spiked with BHV-1 could be detected at 1:256 dilution by a standard PCR [108].

Although 5 µL of reverse transcribed sample can be added to the PCR mix, the sensitivity for detection of BHV-1 was increased when only 0.5 µL reverse transcribed sample plus 4.5 µL extracted sample was used. Nonetheless, only using 0.5 µL reverse transcribed sample did not diminish the ability of the duplex qPCR to detect BVDV. The detection ability was the same as the RT-nPCR. While it is possible to perform individual qPCRs for detection of BHV-1 or BVDV, the duplex qPCR results in more rapid results and is less expensive due to fewer reactions needed for performing 1 PCR rather than 2.

Obviously, specific antibodies in the pooled follicular fluid might be expected to neutralize some virus and impair our ability to isolate virus. Thus, our results are similar to Galik et al. who showed that BVDV could be detected by RT-nPCR in samples of pooled follicular fluid while virus isolation of the samples was negative [91]. In the present study, we also showed that virus was not isolated from any of the diluted samples even following viral amplification in monolayers of MDBK cells. Therefore, while the PCR does detect viral nucleic acid, it is not an indication of the infectivity of the virus. However, it would show that in a pool of oocytes obtained from the contaminated follicular fluid, some of the oocytes had been exposed to virus. The use of these oocytes and the likely contaminated cells around them for in vitro embryo production might result in embryos associated with virus [29,31] or result in contamination of the in vitro embryo production system [295] leading to contamination of all developing embryos [275,348].

In summary, we showed that a duplex qPCR can be used to simultaneously detect and quantify BHV-1 and BVDV in follicular fluid. Detection of BVDV was accurate to the 1:1000 dilution and detection of BHV-1 was accurate to the 1:100 dilution. While not determined in this study, the use of the duplex qPCR should be examined to include detection of BHV-1 and BVDV in serum, semen, reproductive tract cells, and sonicated embryos, as well as somatic cells used for cloning. This can be a useful tool for screening products of animal origin for these viruses that are known frequent contaminants prior to their use in embryo production or prior to embryo transfer.

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VIII. GENERAL SUMMARY AND CONCLUSIONS

Bovine herpesvirus 1 can cause reproductive failure in cattle [233]. The virus can be associated with an embryo without infecting embryonic cells due to the effective barrier provided by the ZP [277,316]. However, virus associated with the ZP might infect other cells, when the embryo is transferred or co-cultured. It is important that transferable embryos be free of infectious BHV-1, especially if they are to be shipped to BHV-1 free countries.

Porcine-origin trypsin has been shown to be effective against BHV-1 associated with in vivo-derived embryos [264,274]. In these studies, Day 5 to 7 embryos, in groups of 3 to 8, were exposed to virus and then trypsin treated. All of the groups were negative for BHV-1. Additionally, in a preliminary study (data not shown), we showed that groups of in vivo-derived embryos treated and then cultured on UTC did not infect the UTC. Trypsin treated embryos have also been transferred to recipients for many years with no evidence of recipients becoming infected. On the other hand, there are mixed reports of effectiveness of trypsin on in vitro-derived embryos. Bielanski et al. showed that when embryos were exposed to BHV-1 during IVM, both individual and groups of 5 embryos were positive for virus after trypsin treatment [30]. However, when embryos were exposed during IVC, groups of 5 embryos were positive for virus but individual embryos were negative after trypsin treatment. We also showed in a previous study that

when groups of 5 in vitro-derived embryos were exposed to virus at Day 7 and then trypsin treated, they were negative for virus [79]. These results are similar to the study done on in vivo-derived embryos [274]. However, we took this a step further and put the treated embryos in co-culture with UTC for 48 hrs before assaying. We showed that 80 % of the treated groups of embryos had enough virus associated with them to infect the UTC. The treated individual embryos were negative, but less sensitive methodology was used in the assays. In the most recent study, in which the entire sample of sonicate fluid used in the co-culture was assayed, a portion of both trypsin treated individual and groups of embryos and UTC were positive. While it is not known if the treated individual in vitro-derived embryos would have sufficient infectious BHV-1 associated with them to transmit virus to the recipients, the optimal goal is for them to be completely free of virus.

In light of this need, additional methods were evaluated in an attempt to produce virus-free, in vitro-derived embryos. We evaluated phosphonoformic acid, lactoferrin and TrypLE™. Both phosphonoformic acid and lactoferrin decreased BHV-1 in cell culture but were detrimental to the viability of the embryos. TrypLE™ Select (10X) significantly inhibited the virus associated with Day 7 in vitro-derived embryos exposed to virus in vitro. When embryos were sonicated and assayed directly for virus, only 1 group (10 embryos/group) out of 22 groups was positive. However, when the treated embryos were subsequently cultured on UTC, both individual and groups of embryos as well as the UTC were positive for virus. Thus, results are similar to the studies using traditional trypsin treatment on IVF embryos. The research with trypsin treated in vitro-derived embryos reveals that if the embryos are assayed (virus isolation) immediately

after treatment they are generally negative for virus. However, if the embryos are further cultured on UTC, they and the UTC are likely to become positive. Based on sensitivity of UTC co-culture, it is suggested that treated embryos not be tested for virus immediately after treatment. Instead, they should be further cultured in recipients or on UTC to determine if virus is still associated with the embryo and will infect the cells or recipient.

There are other recombinant trypsin products that are available and potentially effective against embryo-associated virus. Seidel et al. has shown similar results using TrypZean™ to our results with TrypLE™ [259]. When they treated BHV-1 exposed in vivo-derived embryos and then assayed for virus, 2/18 were positive. Their results though differed from Stringfellow et al. [274] in that they had 3/17 positive following trypsin treatment. However, this difference might be due to their use of bovine-origin trypsin as opposed to porcine-origin trypsin. One advantage of TrypZean™ over TrypLE™ is that it could be used for 1 minute. However, it is not known if the virus would be transmitted following co-culture on UTC or embryo transfer into recipients. It is also not known if these embryos would be viable following treatment. And further, it is not known if this treatment would be effective for treatment of in vitro-derived embryos.

During this research we used a high concentration of virus (10^{5-8} PFU/mL) for in vitro exposure. Bielanski et al. showed that $10^{2.5-9.6}$ $TCID_{50}$/mL virus can be associated with follicular fluid, oocytes, UTC, granulosa cells, and uterine fluid [24,29]. For this study we wanted to create a worst case scenario. While, this high concentration of virus associated with the embryos did infect the UTC, it is not known if this amount of virus

would infect the recipient via a transferred embryo. This evaluation is also performed without the aid of the recipient's nonspecific immune system helping to prevent infection. Thus, the intrauterine infectious dose remains to be determined.

In addition to the above treatments, we also examined recombinant bovine interferon (IFN-τ) (PBL Biomedical Laboratories, Piscataway, NJ) and novel antiviral drugs (Dr. D. Boykin, University of Georgia) to determine if they would be effective against BHV-1 (Appendix B). These were preliminary results and were not reported. Bovine IFN-τ did not decrease BHV-1 on cell culture. While IFN-τ and IFN-α are similar [59], work by Peek et al. showed that recombinant human IFN α-2a and α-B/D did not have antiviral activity against BHV-1 [228]. Babiuk et al. also suggested that recombinant bovine IFN- α1 does not have a direct antiviral effect on BHV-1 [11]. As well, while a few of the novel antiviral agents (DB1031 and DB1032) decreased BHV-1, the effect was not significant. Thus, an effective and safe treatment for in vitro-derived embryos remains to be found.

Failure of these treatments to produce virus-free IVF embryos and the unacceptably long time (10 minutes) required for exposure to TrypLE™ Select (10X) to achieve significant reduction in embryo-associated virus, raises the question of whether a better approach to producing virus-free embryos might be development and use of a rapid assay for detecting the presence of viral contamination.

During this research, we developed a real-time qPCR to detect BHV-1. Results with the qPCR were comparable to those with plaque assays (kappa correlation coefficient 0.7). This will provide a good tool for detecting BHV-1 in samples that might be toxic to cell culture (i.e. semen, milk, follicular fluid) and to prevent amplification of

live virus in the laboratory. Also, a duplex real-time qPCR was developed to detect both BHV-1 and BVDV types I and II. Efficacy of this assay was tested on follicular fluid that was contaminated with both BHV-1 and BVDV I. Bovine viral diarrhea virus and BHV-1 are the 2 most common viral reproductive pathogens in cattle. These viruses have been shown to be associated with cumulus oocyte complexes, spermatozoa, follicular fluid, granulosa cells, and fetal bovine serum. This duplex qPCR has the potential to be of great benefit for detection of BHV-1 and BVDV in eradication programs and for detection in materials of animal origin (bovine serum, cell cultures, stocks of virus, cumulus oocytes, granulosa cells, and bovine oviductal epithelial cells). These materials of animal origin can be assayed prior to their use in cloning or in vitro embryo production. It can also be used as a validation following in vitro embryo production, through the testing of cells and NFD, to ensure that the viruses were not present in the system; thus, allowing production of embryos and cell lines that are free of BHV-1 and BVDV.

In conclusion, while an effective and safe anti-BHV-1 treatment was not found for treatment of bovine in vitro-derived embryos in this study, there are other products that are available for testing that might be of benefit. In addition, we showed that UTC co-culture of treated embryos provides increased sensitivity for virus detection. We suggest that when evaluating embryo-associated virus, the embryos should be further cultured on UTC or in recipients to determine if virus is still associated with the embryo and will infect the cells or recipient. We also demonstrated the use of both a qPCR for detection of BHV-1 and a duplex qPCR for detection of both BHV-1 and BVDV I and II. We perceive these assays as being very beneficial for the prevention and detection of

contamination with BHV-1 and BVDV in cloning and in vitro embryo production systems.

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APPENDICES

APPENDIX A

ADDITIONAL DATASHEETS FOR CHAPTER VI.

Distribution pattern of plaque assay results between embryos, UTC, and IVC media.

Treatment	Embryos	Uterine tubal cells	IVC2 medium	Number of groups or individual embryos in each pattern
Negative Group	-	-	-	5
Positive Group	+	+	+	7
	+	-	-	1
Positive Individual	-	-	-	14
	+	+	+	8
	-	+	+	3
	-	+	-	2
	+	-	-	1
Trypsin Group	+	+	+	6
	-	-	-	2
Trypsin Individual	-	-	-	16
	+	+	+	6
	-	+	+	4
	-	-	+	1
TrypLE™ Group	-	-	-	2
	-	+	+	2
	+	+	+	1
TrypLE™ Individual	-	-	-	11
	+	+	+	5
	-	+	+	1
TrypLE™ 1:2 Group	+	+	+	3
	-	-	-	1
TrypLE™ 1:2 Individual	-	-	-	12
	+	+	+	1
	-	+	-	1

Distribution pattern of PCR results between embryos, UTC, and IVC media.

Treatment	Embryos	Uterine tubal cells	IVC2 medium	Number of groups or individuals in each pattern
Negative Group	-	-	-	3
	-	+	-	1
Positive Group	+	+	+	7
Positive Individual	-	-	-	8
	+	+	+	5
	-	+	+	3
	-	+	-	3
	+	+	-	3
	-	-	+	1
	+	-	+	1
	+	-	-	1
Trypsin Group	+	+	+	3
	+	+	-	2
	-	-	-	1
	-	+	-	1
Trypsin Individual	-	-	-	10
	+	+	+	6
	-	+	-	4
	-	-	+	2
	-	+	+	2
	+	+	-	2
	+	-	-	1
TrypLE™ Group	-	-	-	2
	-	+	+	2
	+	+	+	1
TrypLE™ Individual	-	-	-	8
	+	+	+	3
	-	+	+	3
	+	-	-	2
	-	-	+	1
TrypLE™ 1:2 Group	+	+	+	3
	-	-	-	1
TrypLE™ 1:2 Individual	-	-	-	9
	-	+	+	2
	+	+	+	1
	-	+	-	1
	+	-	-	1

Distribution pattern of individual embryo development after 48 hours in UTC and the plaque assay results between embryos, UTC, and IVC media. Negative and positive treatments were not evaluated because they should have been NFD.

Treatment	Embryo development after 48 h in culture with UTC	Embryos	Uterine tubal cells	IVC2 medium	Number of groups or individual embryos in each pattern
Trypsin Individual	Hatched	-	-	-	4
		-	+	+	1
		-	-	+	1
	Blastocyst	-	-	-	9
		+	+	+	4
		-	+	+	2
	NFD	+	+	+	2
		-	-	-	2
		-	+	+	1
TrypLE™ Individual	Hatched	-	-	-	2
	Blastocyst	-	-	-	6
		+	+	+	2
		-	+	-	1
	NFD	+	+	+	3
		-	-	-	3
TrypLE™ 1:2 Individual	Hatched	-	-	-	1
		+	+	+	1
	Blastocyst	-	-	-	5
		-	+	-	1
	NFD	-	-	-	5

Distribution pattern of individual embryo development after 48 hours in UTC and PCR results between embryos, UTC, and IVC media. Negative and positive treatments were not evaluated because they should have been NFD.

Treatment	Embryo development after 48 h in culture with UTC	Embryos	Uterine tubal cells	IVC2 medium	Number of groups or individual embryos in each pattern
Trypsin Individual	Hatched	-	-	-	4
		-	+	+	1
		-	+	-	1
	Blastocyst	-	-	-	5
		+	+	+	4
		-	+	-	2
		-	-	+	2
		+	+	-	1
		+	-	-	1
	NFD	+	+	+	2
		+	+	-	1
		-	+	-	1
		-	+	+	1
TrypLE™ Individual	Hatched	-	-	-	1
		+	-	-	1
	Blastocyst	-	-	-	5
		+	+	+	2
		-	+	+	1
		-	-	+	1
	NFD	-	-	-	2
		-	+	+	2
		+	+	+	1
		+	-	-	1
TrypLE™ 1:2 Individual	Hatched	+	+	+	1
		-	+	-	1
	Blastocyst	-	-	-	4
		-	+	+	1
		+	-	-	1
	NFD	-	-	-	5

APPENDIX B

UNPUBLISHED PRELIMINARY WORK

Interferon-tau was evaluated for efficacy against BHV-1.

Interferon-tau ((PBL Biomedical Laboratories, Piscataway, NJ)) was evaluated for efficacy against BHV-1. In 96-well plates, an IFN-τ concentration of 0.2 μg/mL was placed in the first row and diluted 1:10 down the columns for 8 dilutions to 0.2×10^{-7} μg/mL. Fifty μL of MDBK cells were added, and the plate was incubated for 24 hours. Ten μL of BHV-1 (50, 10, and 5 PFU) was added to each well in 15 replicates performed over 3 trials. The IFN-τ did not inhibit 50 or 10 PFU of virus. It is not known if the IFN-τ inhibited 5 PFU of virus or if the virus did not grow (Table 1). The control titrations of virus did not grow consistently. Only 1 out of 9 wells was positive for virus in the control titrations for 5 PFU of virus. However, this was a 10-fold dilution of what was used in the IFN-τ assay.

Table 1. Wells of virus (5 PFU) that were inhibited by IFN-τ.

IFN-τ concentration (μg/mL)	Negative wells/ total wells
0.2	7/15
0.2×10^{-1}	7/15
0.2×10^{-2}	5/15
0.2×10^{-3}	10/15
0.2×10^{-4}	7/15
0.2×10^{-5}	7/15
0.2×10^{-6}	10/15
0.2×10^{-7}	7/15

Novel antiviral agents (Dr. D. Boykin, University of Georgia) were screened for efficacy against BHV-1.

Experiment 1

One replicate was performed to screen 79 antiviral compounds for cytotoxicity and their ability to inhibit replication of BHV-1. A high (25 μM) and a low (5 μM) concentration of each antiviral agent was placed into individual wells (2 cm^2) of a 24-well cell culture plate previously seeded with MDBK cells. After 15 minutes, each well was inoculated with 2 x 10^5 to 1 x 10^6 plaque-forming units (PFU) BHV-1/mL for a multiplicity of infection (MOI) of 0.01 to 0.1. Control wells also included on the plates were as follows: 1) MDBK cells with test antiviral (25 μM) but no virus (to evaluate toxicity of agent to cells). 2) MDBK cells with BHV-1 (positive control for viral cytopathic effect). 3) MDBK cells but no test antiviral or virus (negative control). 4) MDBK cells with BHV-1 and phosphonoacetic acid (200 μg/mL; Sigma) which is known to inhibit BHV-1 in cell culture [126]. All plates were incubated at 38.5 ^oC for 5 days in an atmosphere of 5 % CO_2 and air. At 24-hour intervals, cell monolayers were examined with an inverted cell culture microscope for typical viral cytopathic effect (CPE) or cytotoxicity. At 48 hours, a sample (300 μL) of medium over monolayers exposed to BHV-1 was collected, and virus was quantified via plaque assay [79]. The percent of virus inhibited for each test antiviral agent at both the high and low concentrations was determined by comparison to equivalent samples from control cultures in which no compound was added [percentage of control = (Quantity of virus in the compound sample)/(Quantity of virus in the control sample lacking the compound)].

Two agents, DB1031 and DB1032, effectively inhibited BHV-1 (99.8 %) at a concentration of 5 μM, yet neither was toxic to cells at a concentration of 25 μM (Table 2).

Table 2. Results from the more effective novel antiviral agents against BHV-1 on MDBK cells.

Antiviral Agent	Concentration (μM)	Logs of virus inhibited		
		Trial 1	Trial 2	Trial 3
DB630	25	4	1	
	5	Complete inhibition	0	
DB187	25	2	0	
	5	2	0	
DB302	25	2		
DB185	25	2	0	
	5	2	1	
BBE	25	2		
DB1030	25	2		
	5	2		
DB1031	25	1	1	
	5	2	2	
DB1032	25	3	2	3
	5	2	1	1
DB1034	25	4	0	
DB1035	25	2		
48-701	12.5	2		
DB458	25	Complete inhibition		
DB494	25	2		

Experiment 2

Four replicates were performed. The purpose of this experiment was to identify the minimal inhibitory concentrations and maximal nontoxic concentrations of antiviral compounds that were shown in screening assays to be effective against BHV-1 and non cytotoxic at the high concentration (25 μM). This was necessary to determine the

therapeutic index (margin of safety between cytotoxic dose and effective antiviral dose). Thus, two-fold dilutions of DB1031 and DB1032 (100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, and 0.19 µmol) were tested. In addition, acyclovir (800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, and 0.195 µmol) was tested. All antiviral dilutions were made with 1X MEM with 10% equine serum. Except for the testing of multiple concentrations of antiviral, trials to determine the minimal inhibitory concentration is very similar to those in experiment 1. As before, the media over actively dividing monolayers of MDBK cells in single wells of a cell culture plate (0.32 cm^2) was supplemented with concentrations of each compound to be tested. Subsequently, BHV-1 (Colorado strain) was added to the cells in a single well at a multiplicity of infection of 0.05 to 0.5, and culture plates were incubated at 38.5 ^oC in an atmosphere of 5 % CO_2 and air for 5 days. During the incubation period, cells were examined daily for presence or absence of CPE. In addition, the inhibition of BHV-1 in incubating cells was quantified via plaque assay for virus in media (Day 2). Controls were as in experiment 1. The percent of virus inhibited for each test antiviral agent at both the high and low concentrations was determined as in experiment 1. The IC_{99} was calculated by using the JMP software (SAS Institute).

The IC_{99} was shown to be 7.546 µmol for DB1031 and 10.571 µmol for DB1032. DB 1031 caused cytotoxicity to the MDBK cells at 100, 50, and 25 µmol. DB 1032 was cytotoxic to the MDBK cells at 100 µmol (Table 3). Acyclovir was not inhibitory to the virus.

Experiment 3

Four replicates were performed to evaluate selected antivirals at selected concentrations for efficacy against BHV-1 in primary cell cultures (cumulus cells). Cumulus oophorus cells are somatic cells that are commonly used in co-cultures with bovine and human IVF embryos. Inhibition of viral replication and non toxicity in cumulus cells would provide additional evidence that selected antiviral compounds could be used effectively in IVF.

The purpose of this phase was to identify the minimal inhibitory concentrations and maximal nontoxic concentrations of antiviral compounds that were shown in screening assays to be effective against BHV-1 and non-cytotoxic at the high dose (100 µM). This is necessary to determine the therapeutic index (margin of safety between cytotoxic dose and effective antiviral dose).

The following dilutions of the selected chemicals were tested: DB1031 (50, 25, 12.5, 6.25, and 3.125 µmol) and DB1032 (100, 50, 25, 12.5, and 6.25 µmol). The antiviral agents were diluted in 1X MEM with 10 % equine serum. Subsequently, BHV-1 (Colorado strain) was added to the cells in a single well at a multiplicity of infection of 0.005 to 0.5, and culture plates were incubated at 38.5 ºC in an atmosphere of 5 % CO_2 and air for 5 days. During the incubation period, cells were examined daily for presence or absence of CPE. In addition, the inhibition of BHV-1 in incubating cells was quantified via plaque assay for virus in media (Day 2). Controls included the following: 1) Monolayer plus BHV-1 but no antiviral. 2) Monolayer of cumulus cells and no chemical or virus. The percent viral inhibition for each treatment was determined.

DB1031 effectively inhibited greater than 4 or 5 logs of virus at 12.5 µmol . DB1032 inhibited more than 4 logs of virus at 50 µmol (Table 3). DB1031 was cytotoxic to the cumulus cells at 50 µmol. DB1032 caused cytotoxicity at 100 µmol.

Table 3. Comparison of results of experiment 2 and 3. T, concentration of antiviral agent cytotoxic to the cells; MDBK, Madin Darby bovine kidney cells.

Antiviral Agent	**Antiviral Concentration**	**Logs of Virus Inhibited**							
		MDBK Cells Experiment 2				**Cumulus Cells Experiment 3**			
		Trial 1	Trial 2	Trial 3	Trial 4	Trial 1	Trial 2	Trial 3	Trial 4
DB1031	100 µM	>4 T	>4 T	>4 T	>4 T				
	50 µM	3 T	>4 T	3 T	>4 T	>5 T	>5 T	>5 T	>4 T
	25 µM	2 T	>4	3 T	4 T	>5	>5	5	>4 T
	12.5 µM	2	>4	1	>4	>5	>5	3	>4
	6.25 µM	0	3	1	3	4	>5	2	0
	3.125 µM	1	2	1	2	0	2	1	0
	1.563 µM	1	0	0	1				
	0.781 µM	0	0	0	0				
	0.391 µM	0	0	0	0				
	0.195 µM	0	0	0	0				
DB1032	100 µM	3 T	>4 T	3 T	>4 T	5 T	>5 T	>5 T	>4
	50 µM	2	>4	2	>4	5	>5	>5	>4
	25 µM	2	4	2	3	3	5	2	4
	12.5 µM	1	3	1	3	2	4	1	1
	6.25 µM	1	3	1	1	1	3	1	1
	3.125 µM	0	2	0	0				
	1.563 µM	0	0	1	1				
	0.781 µM	0	0	0	0				
	0.391 µM	0	0	0	1				
	0.195 µM	0	0	0	0				

Experiment 4

One trial was performed. Five replicates of each virus concentration were performed during this experiment. The purpose of this experiment was to determine if the selected antiviral agents would completely inhibit BHV-1 in MDBK cells. The following dilutions of the selected chemicals were tested: DB1031 (50, 25, 12.5, 6.25, and 3.125 μmol) and DB1032 (100, 50, 25, 12.5, and 6.25 μmol). The antiviral agents were diluted in 1X MEM with 10 % equine serum. Subsequently, BHV-1 (Colorado strain) was added to the cells in a single well. The following dilutions of virus were used 6×10^5 PFU/mL, 6×10^4 PFU/mL, 6×10^3 PFU/mL, and 6×10^2 PFU/mL. This allowed for a multiplicity of infection of 0.2, 0.02, 0.001, and 0.001 respectively. Culture plates were incubated (38.5 ^{o}C) in an atmosphere of 5 % CO_2 and air for 5 days. During the incubation period, cells were examined for CPE. Controls included the following: 1) Monolayer plus BHV-1 but no antiviral. 2) Monolayer of MDBK cells and no chemical or virus. 3) Phosphonoacetate (200 μg/mL) plus BHV-1. The fifty percent end point in virus titration was determined using the Reed-Muench method [236].

DB1031 and DB 1032 were unable to completely inhibit the virus at the four viral concentrations (Table 4).

Table 4. Effect of DB1031 and DB1032 (5 concentrations) against BHV-1 (10^5, 10^4, 10^3, 10^2 PFU/mL) on MDBK cells.

Antiviral Agent	Concentration	BHV-1 Infected Wells of MDBK Cells			
		BHV-1 Concentration			
		1 X 10^5 PFU/mL	**1 X 10^4 PFU/mL**	**3 X 10^3 PFU/mL**	**6 X 10^2 PFU/mL**
DB 1031	50 µM Toxic	5/5	5/5	5/5	5/5
	25 µM Toxic	5/5	5/5	5/5	0/5
	12.5 µM	5/5	5/5	5/5	2/5
	6.25 µM	5/5	5/5	5/5	0/5
	3.125 µM	5/5	5/5	5/5	3/5
DB 1032	100 µM Toxic	5/5	5/5	4/5	2/5
	50 µM	5/5	5/5	5/5	2/5
	25 µM	5/5	5/5	5/5	3/5
	12.5 µM	5/5	5/5	5/5	3/5
	6.25 µM	5/5	5/5	5/5	4/5

Cidofovir and Lactoferrin from bovine milk and bovine colostrum were screened for efficacy against BHV-1.

Experiment 1

Three trials of experiment 1 were performed. Each trial was performed in triplicate. The purpose of this phase was to determine if the selected antiviral agents would completely inhibit BHV-1 in MDBK cells. The following dilutions of the selected antiviral agents were tested: Lactoferrin from bovine milk (10000, 5000, 2500, 1250, 625, and 313 µg/mL) and Cidofovir (2000, 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 2 and 1 µg/mL). The dilutions were made with 1X MEM with 10 % fetal bovine serum. Subsequently, BHV-1 (Colorado strain) was added to the cells in a single well. The

following dilutions of virus were used 6 x 10^5 PFU/mL, 6 x 10^4 PFU/mL, 6 x 10^3 PFU/mL, and 6 x 10^2 PFU/mL. This allowed for a multiplicity of infection of 0.1, 0.01, 0.001, and 0.0001 respectively. Culture plates were incubated (38.5 ^oC) in an atmosphere of 5 % CO_2 and air for 5 days. During the incubation period, cells were examined for CPE. Controls were the same as the above experiment 4. The fifty percent end point in virus titration was determined using the Reed-Muench method [236].

Cidofovir (125, 62.5, and 31.3 µg/mL) was able to completely inhibit the virus concentration of 6 x 10^2 PFU/mL (Table 5). The 125 µg/mL concentration of Cidofovir was also able to completely inhibit 6 x 10^3 PFU/mL. In addition, Cidofovir (125 µg/mL) inhibited 89 % of the virus concentration 6 x 10^4 PFU/mL. Cidofovir did show cytotoxicity at the following concentrations: 2000, 1000, 500, and 250 µg/mL.

Lactoferrin from bovine milk (10000, 5000, 2500, and 1250 µg/mL) was able to completely inhibit the virus concentration of 6 x 10^2 PFU/mL (Table 5). Also, the concentrations of 10000, 5000 and 2500 µg/mL completely inhibited 6 x 10^3 PFU/mL and 10000 µg/mL completely inhibited 6 x 10^4 PFU/mL. However, lactoferrin at concentrations 10000 to 1250 µg/mL showed some toxic changes to the cells.

Table 5. The efficacy of lactoferrin from bovine milk (6 concentrations) against BHV-1 (10^5, 10^4, 10^3, 10^2 PFU/mL) was evaluated on MDBK cells. The plates were set up with 1X MEM with equine serum but the samples were diluted with 1X MEM with fetal bovine serum.

		BHV-1 Infected MDBK cells			
		BHV-1 concentration			
Antiviral Agent	**Concentration**	**6 X 10^5 PFU/mL**	**6 X 10^4 PFU/mL**	**6 X 10^3 PFU/mL**	**6 X 10^2 PFU/mL**
Lactoferrin	10000 µg/mL Toxic	3/4	0/4	0/4	0/4
	5000 µg/mL Toxic	4/4	1/4	0/4	0/4
	2500 µg/mL Toxic	4/4	2/4	0/4	0/4
	1250 µg/mL Toxic	4/4	3/4	1/4	0/4
	625 µg/mL	4/4	4/4	1/4	0/4
	313 µg/mL	4/4	4/4	4/4	2/4

Experiment 2

Four replicates were performed. The purpose of this experiment was to identify the minimal inhibitory concentrations and maximal nontoxic concentrations of antiviral compounds. This was necessary to determine the therapeutic index (margin of safety between cytotoxic dose and effective antiviral dose). Thus, two-fold dilutions of Cidofovir (125, 62.5, 31.3, 15.6, 7.8, 3.9 µg/mL) were tested. All antiviral dilutions were made with 1X MEM with 10 % fetal bovine serum. As before, the media over actively dividing monolayers of MDBK cells in single wells of a cell culture plate (0.32 cm^2) was supplemented with concentrations of each compound to be tested. Subsequently, BHV-1 (Colorado strain) was added to the cells in a single well at a multiplicity of infection of

0.01 to 0.8, and culture plates were incubated (38.5 ^{o}C) in an atmosphere of 5 % CO_2 and air for 5 days. During the incubation period, cells were examined for CPE. In addition, the inhibition of BHV-1 in incubating cells was quantified via plaque assay for virus in media (Day 2). Controls were the same as experiment 1 for novel antiviral agents. The percent viral inhibition for each treatment was determined.

Cidofovir (125 µg/mL) inhibited 3 to 4 logs of virus (Table 6). At a concentration of 62.5 µg/mL, 1 to 3 logs of virus were inhibited, and at 31.3 µg/mL 0 to 2 logs of virus were inhibited. Further dilution of cidofovir (15.6, 7.8, and 3.9 µg/mL) only inhibited 0 to 1 log of virus.

Table 6. Cidofovir (6 concentrations) was evaluated for efficacy against BHV-1 ($10^5$and 10^2 PFU/ml) on MDBK cells.

Cidofovir concentration	**Logs of Virus Inhibited**							
	Trial 1		**Trial 2**		**Trial 3**		**Trial 4**	
	10^5 PFU/ml	10^2 PFU/ml	10^5 PFU/ml	10^2 PFU/ml	10^5 PFU/ml	10^2 PFU/ml	10^5 PFU/ml	10^2 PFU/ml
125 µg/ml	3	Complete inhibition	4	Complete inhibition	4	Positive control did not have any plaques	4	Inaccurate data
62.5 µg/ml	3	Complete inhibition	1	Complete inhibition	2		2	Complete inhibition
31.3 µg/ml	2	Complete inhibition	1	Complete inhibition	1		0	3
15.6 µg/ml	1	0	1	Complete inhibition	1		1	Complete inhibition
7.8 µg/ml	1	1	1	2	0		1	0
3.9 µg/ml	1	1	1	Complete inhibition	0		0	Complete inhibition

Experiment 3

Three trials of experiment 3 were performed. Each trial was performed in triplicate. The purpose of this experiment was to determine if lactoferrin from bovine colostrum would completely inhibit BHV-1 in MDBK cells. The results of this experiment were compared with those from experiment 1 using lactoferrin from bovine milk. The following dilutions were tested 10000, 5000, 2500, 1250, 625, and 313 μg/mL. The dilutions were made with 1X MEM with 10 % fetal bovine serum. Subsequently, BHV-1 (Colorado strain) was added to the cells in a single well. The following dilutions of virus were used 3×10^5 PFU/mL, 3×10^4 PFU/mL, 3×10^3 PFU/mL, and 3×10^2 PFU/mL. This allowed for a multiplicity of infection of 0.1, 0.01, 0.001, and 0.0001 respectively. Culture plates were incubated (38.5 ^{o}C) in an atmosphere of 5 % CO_2 and air for 5 days. Cells were examined for CPE. Controls were the same as experiment 4 of novel antiviral agents. The fifty percent end point in virus titration was determined using the Reed-Muench method [236].

Lactoferrin from bovine colostrum (10000, 5000, and 1250 μg/mL) was able to completely inhibit the virus concentration of 3×10^2 PFU/mL (Table 7). Also, the concentrations of 10000, and 5000 μg/mL completely inhibited 3×10^3 PFU/mL. The lactoferrin from bovine colostrum did not appear to by cytotoxic to the cells.

Table 7. Lactoferrin from bovine colostrum was evaluated for efficacy against BHV-1.

		BHV-1 Infected Wells of MDBK cells			
		BHV-1 concentration			
Antiviral Agent	**Concentration**	**3 X 10^5 PFU/mL**	**3 X 10^4 PFU/mL**	**3 X 10^3 PFU/mL**	**3 X 10^2 PFU/mL**
Lactoferrin from bovine colostrum	10000 µg/mL	9/9	2/9	0/9	0/9
	5000 µg/mL	8/9	5/9	0/9	0/9
	2500 µg/mL	9/9	6/9	1/9	1/9
	1250 µg/mL	9/9	8/9	1/9	0/9
	625 µg/mL	9/9	9/9	6/9	2/9
	313 µg/mL	9/9	9/9	9/9	4/9

Experiment 4

Four replicates were performed. The purpose of this experiment was to identify the minimal inhibitory concentrations and maximal nontoxic concentrations of lactoferrin from bovine colostrum. The results of this experiment were compared with those of experiment 2 using lactoferrin from bovine milk. Two-fold dilutions of lactoferrin from bovine colostrum (10000, 5000, 2500, 1250, 625 µg/mL) were tested. All antiviral dilutions were made with 1X MEM with 10 % fetal bovine serum. These trials to determine the minimal inhibitory concentration were very similar to those in experiment 1. As before, the media over actively dividing monolayers of MDBK cells in single wells of a cell culture plate (0.32 cm^2) was supplemented with each concentration to be tested. Subsequently, BHV-1 (Colorado strain) was added to the cells in a single well at a multiplicity of infection of 0.1 to 1.0, and culture plates were incubated (38.5 ^oC) in an atmosphere of 5 % CO_2 and air for 5 days. Cells were examined for CPE. In addition, the inhibition of BHV-1 in incubating cells was quantified via plaque assay for virus in

media (Day 2). Controls were the same as experiment 2. The percent viral inhibition for each treatment was determined.

Lactoferrin from bovine colostrum (10000 µg/mL) inhibited 1 to 3 logs of virus (6 x 10^5 PFU/mL) (Table 8). At a concentration of 5000 µg/mL, 0 to 4 logs of virus were inhibited, and 2500 µg/mL inhibited 0 to 2 logs of virus. A concentration of 1250 µg/mL inhibited 0 to 1 log of virus and 625 µg/mL did not inhibit any virus.

Table 8. Efficacy of lactoferrin from bovine colostrum (5 concentrations) was evaluated against BHV-1 (10^5 PFU/mL) on MDBK cells.

Antiviral Agent	**Concentration**	**Logs of Virus Inhibited**			
		Trial 1	**Trial 2**	**Trial 3**	**Trial 4**
Lactoferrin from bovine colostrum	10000 µg/mL	1	3	2	1
	5000 µg/mL	0	1	4	0
	2500 µg/mL	0	2	1	0
	1250 µg/mL	0	0	1	0
	625 µg/mL	0	0	0	0

Experiment 5

Three trials of experiment 5 were performed. The purpose of this experiment was to determine if lactoferrin from bovine milk and cidofovir combined would completely inhibit BHV-1 in MDBK cells. An 8 X 8 2-fold dilution test was set up. The following dilutions of the selected antiviral agents were tested: Lactoferrin from bovine milk (10000, 5000, 2500, 1250, 625, 313, 156, and 78 µg/mL) and Cidofovir (250, 125, 62.5,

31.3, 15.6, 7.8, 3.9, 2 μg/mL). The dilutions were made with 1X MEM with 10 % fetal bovine serum. Subsequently, BHV-1 (Colorado strain) was added to the cells. This allowed for a multiplicity of infection of 1 to 11. Culture plates were incubated (38.5 ^{o}C) in an atmosphere of 5 % CO_2 and air for 5 days. Cells were examined daily for CPE. Controls were the same as in experiment 1. The fifty percent end point in virus titration was determined using the Reed-Muench method [236].

The highest concentration of cidofovir (250 μg/mL) combined with all 8 concentrations of lactoferrin from bovine milk was cytotoxic to the cells. Cidofovir at 125 μg/mL combined with all 8 concentrations of lactoferrin from bovine milk was slightly toxic to the cells. All of the other concentrations were not toxic to the cells.

Virus was completely inhibited at the following combined concentrations: Cidofovir at 125 and 62.5 μg/mL combined with lactoferrin (10000, 5000, 2500 and 1250 μg/mL) and cidofovir at 31.3 μg/mL combined with lactoferrin (10000 and 5000 μg/mL) (Table 9).

Table 9. 8 X 8 2-fold dilutions of lactoferrin from bovine milk and cidofovir against BHV-1 (10^6 PFU/mLl) on MDBK cells. BHV-1 infected wells are shown per number of wells tested.

	BHV-1 infected wells/ wells tested							
	Cidofovir 250 µg/mL	125 µg/mL	62.5 µg/mL	31.3 µg/mL	15.6 µg/mL	7.8 µg/mL	3.9 µg/mL	2 µg/mL
Lactoferrin 10 mg/mL	0/3 Toxic	0/3 Slightly toxic	0/3	0/3	1/3	3/3	3/3	3/3
5 mg/mL	0/3 Toxic	0/3 Slightly toxic	0/3	0/3	2/3	3/3	3/3	3/3
2.5 mg/mL	0/3 Toxic	0/3 Slightly toxic	0/3	1/3	3/3	3/3	3/3	3/3
1.25 mg/mL	0/3 Toxic	0/3 Slightly toxic	0/3	2/3	3/3	3/3	3/3	3/3
625 µg/mL	3/3 Toxic	1/3 Slightly toxic	2/3	3/3	3/3	3/3	3/3	3/3
312.5 µg/mL	3/3 Toxic	3/3 Slightly toxic	3/3	3/3	3/3	3/3	3/3	3/3
156 µg/mL	3/3 Toxic	3/3 Slightly toxic	3/3	3/3	3/3	3/3	3/3	3/3
78 µg/mL	3/3 Toxic	3/3 Slightly toxic	3/3	3/3	3/3	3/3	3/3	3/3

Experiment 6

The effects of various antiviral agents on MDBK cells were quantified using Cell Counting Kit-8 (Dojindo, Gaithersburg, MD). A water-soluble tetrazolium salt (WST-8) is reduced by dehydrogenases in cells to give a yellow-colored product (formazan). The amount of formazan dye produced by the dehydrogenase activity in the cells is directly proportional to the number of living cells.

A 96-well plate was seeded with MDBK cells for a final cell count per well of approximately 4 X 10^3. The plate was incubated at 38.5 ° C for 24 hours. Ten µL of each antiviral concentration was added into the culture media. Each drug was evaluated in triplicate. The plate was incubated for an additional 48 hours at 38.5 °C. Cell Counting Kit-8 solution (10 µL) was added to each well. The absorbance at 450 nm was measured after 2 hours of incubation. The cytotoxicity assay was analyzed using Student's t test (JMP Software, SAS Institute).

Phosphonoacetic acid (200 and 100 µg/mL) diluted in 1X MEM with 10 % FBS was not cytotoxic to MDBK cell growth. However, phosphonoacetic acid (200 and 100 µg/mL) diluted in 1X MEM with 10 % equine serum was cytotoxic to MDBK cell growth.

Cidofovir at 2 mg/mL significantly hindered MDBK cell viability. But the other concentrations of cidofovir (62.5, 31.3 and 15.6 µg/mL) did not affect cell viability.

Lactoferrin from bovine colostrum (10, 5, 2.5, 1.25 and 0.625 mg/mL) was not cytotoxic to MDBK cell growth ($P<0.05$). All of the concentrations tested enhanced cell growth. Lactoferrin from bovine milk (10, 5, 2.5, 1.25 and 0.625 mg/mL) was also not cytotoxic to MDBK cells.

Cidofovir was evaluated for safety in a bovine embryo production system.

Cidofovir (62.5, 31.3, and 15.6 µg/mL) was placed into the IVC1 and IVC2 media. Blastocyst development was significantly different from the control ($P<0.01$)