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Review

Disinfection procedures for controlling microorganisms in the semen and embryos of humans and farm animals

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Abstract

Semen and embryos generated by assisted reproductive techniques (ARTs) may be contaminated with numerous microorganisms. Contamination may arise from systemic or local reproductive tract infections in donors or the inadvertent introduction of microorganisms during ARTs, and may lead to disease transmission. This review describes sanitary procedures which have been investigated to ascertain whether they are effective in rendering semen and embryos free of pathogenic microorganisms, including internationally adopted washing procedures, which can be supplemented by antibiotics and enzymatic treatments. Other methods include treatment with antibodies or ozone, photoinactivation, acidification, and the use of novel antiviral compounds. In conclusion, despite the wide range of antimicrobial procedures available, none can be recommended as a universal disinfection method for rendering semen and embryos free from all potentially pathogenic microorganisms. However, some procedures are unsuitable, as they can compromise the viability of semen or embryos. In humans, washing by the gradient centrifugation method appears to be effective for reducing the microbial population in semen and is harmless to the spermatozoa. A useful procedure for embryos involving multiple washes in sterile medium has much to commend it for the prevention of disease transmission; furthermore, it is recommended by the International Embryo Transfer Society (IETS).

Keywords: Semen; Embryos; Disinfection; IVF; ART

Contents

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1. Introduction

In recent years, a variety of new assisted reproductive technologies (ART), e.g. various forms of IVF and cryopreservation, have been developed and made available in human and veterinary medicine with the aim of infertility treatment or improvement and preservation of livestock genetics.

Although ARTs are used for different purposes in humans and farm animals, a common objective is prevention of disease transmission. The method used for livestock to ensure that germplasm are specific-pathogen-free relies on continuous clinical surveillance of donors for presence of infection before and after semen and embryo production. This strategy has been applied in AI centres to sires used for semen production. In contrast, the more recent approach to ensure that animal embryos do not pose a risk of disease transmission is based on the sanitary status of embryos and the associated washing media. This approach has resulted in extensive embryo-pathogen experimentation, which has clearly demonstrated that livestock embryos could be transferred without transmitting pathogens, regardless of whether the donor is infected, provided that standardized methods of collection and processing embryos are used in accordance with the guidelines of the International Embryo Transfer Society (IETS) and the Office International des Epizooties (OIE) [1,2]. It is noteworthy that these guidelines have become the basis for certifying animal germplasm for international movement.

Although, for medical and ethical reasons, some of the veterinary sanitary measures cannot be applied in humans, guidelines have been established for screening germplasm donors for some infectious (e.g. HIV, hepatitis B and C, chlamydia) and also for the quarantine of cryopreserved semen and embryos before their use in ARTs [3–5]. However, in oocyte/embryo donation programs, the majority of embryos are transferred unfrozen in order to increase pregnancy rates, which precludes their quarantine. Nevertheless, successful application of quarantine to cryopreserved embryos as a mean of preventing transmission of HIV infection has been reported [6].

Viral, bacterial, fungal and parasitic organisms, some pathogenic, have been identified in association with semen and embryos [7–13]. There are only limited data regarding the effect of pathogenic agents on embryonic development and the minimal infectious dose of viral and bacterial agents associated with transferred ova and embryos. Nevertheless, despite an enormous number of artificial insemination AI and ET [14–16] procedures performed over the years, only a few cases of suspected disease transmission in humans and animals have been reported [7,17–24]. This success can be credited, at least in part, to the advances in knowledge gained through research on the interaction of pathogenic microorganisms with semen and embryos, and the consequent recommendations of the IETS and the Office International des Epizooties on sanitary procedures for handling semen and embryos of livestock [1,2].

The rapid evolution of microbial resistance to antibiotics warrants further investigations into methods of their control. It is noteworthy there is no universal disinfectant or procedure to render germplasm free from all microbes.

Some therapeutic compounds or disinfectants are only effective against one type of microorganisms (e.g. antibiotics for bacteria but not viruses, and trypsin treatment against certain viruses, e.g. herpes). Other procedures, such as washing, may be effective against a

range of both bacterial and viral organisms. Some of these methods may not completely inactivate or remove the infectious agent, but may reduce the microbial load associated with germplasm to a level below intrauterine infectivity. This review describes procedures for rendering human and animal semen and embryos free of microbial agents.

In general, any agent or combination of agents added to embryo culture media or semen diluters for the purpose of suppressing microbial growth must have the following characteristics: (1) be effective against microorganisms at the concentration used and under the circumstances applied, (2) be non-toxic to germplasm relative to viability and fertilizing activities, (3) not interact unfavourably with other substances present in the embryo culture, medium or semen extender mixture and, (4) the treatment should be short in duration, and simple for practical application.

2. Origin and risk of microbial contamination of semen

It has been recognized that systemic and local infections of the reproductive tract, as well as the inadvertent introduction of microorganisms during processing, may potentially contribute to the contamination of semen. In general terms, microorganisms can already be present in the semen of an infected male when it is ejaculated or they can gain entry during collection, processing, or storage. Spermatozoa can become infected by a microorganism in the testes or during their transit through the epididymis, ductus deferens, and urethra. Microorganisms may be present in semen when they are associated with blood cells or there is inflammation or trauma of the accessory sex glands (prostate, seminal vesicle or bulbourethral gland) [8]. Furthermore, some microorganisms can contaminate semen due to their high concentration in the urine or the preputial cavity. In addition, some potential contaminants (e.g. mycoplasmas) may be introduced into semen with animal-derived supplements used in diluents and extenders (egg yolk or milk). Environmental microbes may also contribute to semen contamination or a result of poor laboratory hygiene [25,26].

Normal semen consists of seminal fluid, spermatozoa and immature germ cells and non-sperm cells (e.g. epithelial cells, CD4+ T lymphocytes, monocytes, polynuclear leukocytes and macrophages); each component is a potential vehicle for the transport of pathogenic agents. Therefore, identification of the semen component involved in pathogen transmission is important.

Frequently, ejaculated semen is not free from bacterial flora. The saprophytic bacteria of the prepuce in a healthy male comprises numerous species that may become associated with the semen. Some of these bacteria may behave as opportunistic pathogens (e.g. *Pseudomonas aeuruginosa*) and may be a potential risk to the inseminated female. For example, the most common potentially pathogenic microorganisms isolated from bull semen are *P. aeruginosa*, *Streptococcus* spp., *Staphylococcus* spp., *Proteus* spp., and *Bacillus* spp. [27]. That spermatozoa could function to transport surface-bound human bacteria has been reported for *Chlamydia trachomatis*, *Chlamydia psittaci*, *Escherichia coli* (*E. coli*), *Neisseria gonorrhoea*, *Veillonella parvula*, *Peptostreptococcus* spp., *Ureaplasma urealyticum*, *Mycoplasma* spp., and *Candida albicans* [28–31].

A number of viral pathogens have also been identified in association with the semen of infected animals and humans [7,9,10,13]. Some of the viruses can adhere to the surface of spermatozoa, whereas others are associated with the seminal plasma or non-sperm cells present in the semen. Several reports, some of which are controversial, have suggested an ability of some viruses to penetrate the sperm head and integrate their nuclei acid into the sperm genome; these viruses include human immunodeficiency virus-1 (HIV-1) [32], human papillomavirus (HPV) [33], bluetongue virus (BTV) [34], porcine circovirus (PCV) [35], porcine reproductive and respiratory syndrome virus (PRRSV) [36], hepatitis B virus (HBV)[37], bovine herpesvirus-1 (BHV-1) [38], human herpesvirus type 8 [39], herpes simplex 1 and 2 (HSV-1, HSV-2) [40], and murine cytomegalovirus (MCMV) [41]. Therefore, the complete elimination of these viral agents from semen and sperm cells may be difficult or impossible to achieve.

It also should be mentioned that there is a potential risk of semen contamination by microorganisms present in liquid nitrogen (LN) when semen is cryopreserved in improperly sealed straws [42–44].

3. Procedures for the disinfection of semen

The standard preparation of semen for AI involves the dilution of ejaculates to achieve an appropriate concentration of sperm in each insemination dose. Depending of the degree of semen dilution, this procedure decreases the concentration of contaminants; to some degree, it may minimize the risk of disease transmission.

The washing procedures described below are primarily applicable to semen preparation for IVF.

3.1. Washing procedures

Over the years, various forms of washing have been developed for both human and animal spermatozoa, and, as with embryos (see below), washing has become the most important procedure for the control and elimination of microorganisms.

The primary purpose of washing is to separate the highly motile sperm fraction from the seminal plasma to optimize the likelihood of successful fertilization. Centrifugation, swim-up, fall down centrifugation, continuous and discontinuous Percoll gradients, albumin gradients, and glass bead and glass wool filtration, are all essential washing procedures that have been used to prepare pure sperm for IVF and other ART. It was soon noticed that these washing procedures not only eliminated abnormal, immotile sperm and debris, but also reduced microbial contaminants associated with sperm. The most common techniques used are swim-up and Percoll gradient centrifugation. Briefly, the first procedure involves the overlaying of a small volume of the semen sample with a larger volume of an appropriate culture medium, followed by the incubation for approximately 1 h. Subsequently, motile spermatozoa, which migrate actively into the overlying medium, are aspirated and then washed by centrifugation. The second method usually involves the placement of an aliquot of semen over two columns of Percoll gradient (e.g. 90 and 45%), followed by brief centrifugation (∼10 min). Motile spermatozoa are collected from the bottom of the tube prior to the washing by centrifugation. However, washing and centrifugation procedures, the concentrations of separating solutions, and the methods used to collect the motile layer of sperm, vary among researchers. Therefore, it is likely that the microorganism elimination rate will vary. Nevertheless, when used with antibiotics, this technique significantly removes or reduces the load of many human viral and bacterial microorganisms [45–49]. To achieve better microbial control during the processing of semen, a double-tube gradient procedure and its modification have been reported [47,50]. This involves the insertion of a simple device into a standard centrifuge tube to prevent contamination of the sperm during the aspiration through Percoll. The most common procedure for human sperm preparation is the two-step gradient centrifugation, followed by swim-up in culture medium. This technique, has been shown to efficiently remove microbes, especially from human semen [46,49,51–54]. Also in humans, a modified swim-up technique, based on a medium containing hyaluronic acid, was even more effective in removing bacteria [55]. Perhaps the highly viscous hyaluronic acid inhibited the movement of microorganisms from semen, but concurrently allowed highly motile spermatozoa to separate. New compounds, such as a silane-coated silica particle solution (‘‘PureSperm’’, Nidacon) which, when recently used for clinical purposes, was also found to be efficient in diminishing bacterial contamination [45]. Since the ‘‘PureSperm’’ lacks an intrinsic bacteriostatic effect and does not contain antibiotics, the use of clean pipettes and tubes between each step of the washing procedure is important. For human AI, a semen washing procedure was devised in Milan and reported in the journal Lancet in 1992, after the birth of the first 10 healthy children resulting from intrauterine insemination of seronegative women with processed HIV-1 positive semen [56]. Furthermore, the data suggested that the application of this methodology for processing sperm for IVF from men infected with HIV-1 and human cytomegalovirus (HCMV) posed no risk of transmission in an oocyte donation program [50,53,57–59]. The risk of transmission of hepatitis C virus (HCV) via semen is still debated, mainly due to the sensitivity of the assay and the presence of PCR inhibitors in the seminal plasma [60]. Nevertheless, Levy et al. [61] reported non-transmission of HCV to women and their children after the transfer of embryos resulting from IVF with previously frozen-thawed semen cleaned by gradient centrifugation and swim-up. Semen from HIV-1 and HCV infected patients was also successfully washed and applied in other IVF techniques, such as subzonal or intracytoplasmic sperm injection (ICSI) resulting in no transmission of disease to the recipients [62]. However, Papaxanthos-Roche et al. [63] noted a higher percentage of HCV RNA-positive oocytes after ICSI versus conventional IVF (85.7% versus 64.7%). Perhaps the washing procedure did not entirely remove viral particles and the microinjection procedure allowed the remaining viruses to be transferred into the oocyte with the spermatozoon; such a possibility previously reported in earlier studies where HIV particles were found in the spermatozoa selected after swim-up and Ficoll centrifugation and such sperm transferred HIV-1 like particles to oocytes [64]. Nevertheless, in clinical practice, it was established that with a combination of gradient density and sperm washing, ICSI could be considered as a safe and effective sanitary procedure that avoids HIV-1 and HCV transmission while concurrently achieving reasonable pregnancy rates [53,65].

From limited studies, it appears that the application of washing procedures to semen associated with human papiloma virus (HPV) will not eliminate the virus from sperm of infected patients. However, when sperm tested by PCR as positive for HPV was used for IVF, healthy children were born [66]. The risk of transmission of pathogens such as *C. trachomatis*, HCMV or HBV during IVF by washed semen remains unknown, but it is of great concern for human infertility clinics.

In animal studies, a similar modified washing procedure using three Percoll density gradient columns, with or without trypsin, was effective in eliminating the PRRS virus from infected boar semen [67]. Moreover, more high-quality embryos were recovered from sows inseminated with trypsin-treated semen compared to untreated control semen.

Attempts to remove BVDV from bull semen prior to IVF by some of the above methods were unsuccessful [68]. Procedures such as washing, swim-up, Percoll gradient, glass wool filtration, and glass beads filtration failed to remove BVDV (titer 10^5–10^6 $TCID_{50}$/mL) from the semen of persistently infected bulls. The final sperm pellets from frozen and fresh ejaculates were positive for BVDV (range, 10^3–10^4 $TCID_{50}$/mL). However, whether the use of such sperm for IVF or AI have the potential to contaminate embryos remains to be determined [69]. Likewise, swim-up and washing procedures did not render the spermatozoa free from bovine leukemia virus [70] and bovine immunodeficiency virus [71].

In contrast, a successful cleaning of equine semen of equine arteritis virus (EAV) by swim-up and density gradient centrifugation was reported by Morrell and Geraghty [72]. However, since EAV was added to the semen, it remains uncertain whether the virus would be eliminated from the semen of naturally infected stallions.

With regard to bacterial infections of human semen, Kaneko et al. [73] found that a discontinuous Percoll gradient (40–80%) was effective in reducing bacterial contamination in the sperm fraction to 0.02% of that present in the raw semen. Bolton et al. [74], using a self-generating 60% Percoll gradient, also found a reduction in the number of bacteria in human separated spermatozoa compared with the semen. The Percoll density gradient centrifugation technique was also used to reduce the bacterial count of boar semen [75]. When bacterial counts of fresh semen were low (i.e. 10^4 mL^{-1}), no bacteria could be detected in the sperm pellet after Percoll gradient centrifugation.

In contrast, the potential for infection of IVF embryo cultures with *Candida albicans* and *Enterococcus faecalis* is substantial, since the Percoll gradient centrifugation and washing did not remove these microorganisms from the resulting human spermatozoa [76,77]. Stringfellow et al. [78] described a case of the accidental use of cryopreserved semen contaminated with *Pseudomonas maltophilia* for IVF of bovine oocytes. Despite the processing of the semen in Percoll gradient and the inclusion of antibiotics in the media used for IVF, the microorganism persisted in the IVF system and caused degeneration of the embryos. Other instances where sperm washing and swim-up failed to eliminate human and animal bacterial agents include the mycoplasmas *M. pulmonis* [79], *M. bovis* and *M. bovigenitalium* [80,81].

3.2. Antibiotics

In general, antibiotics are used to prevent and treat bacterial infections and contaminations. There are two main methods for the use of antibiotics for semen decontamination. The first method involves treating the male with antibiotics; the second method, sometimes used in conjunction with the first, is where antibiotics are added to the semen extenders or, in the case of IVF, to the swim-up, washing, and incubation media. This approach has been applied for many years in ART in animals.

Foote and Salisbury [82] and Almquist et al. [83] first proposed that bacterial contaminants in bovine semen could be controlled by adding antibiotics. Early experimentation showed that penicillin, streptomycin and polymyxin were not only effective for controlling bacterial growth in the diluted semen, but had no adverse effect on sperm viability. Furthermore, it was noticed that the addition of antibiotics and sulfanilamide to liquid semen of low-fertility bulls improved their conception rates by 10–15% [84]. These antibiotics, without sulfanilamide which is toxic during freezing, formed the basis for worldwide use to control infectious agents in bull semen. Vibriosis (caused by *Campylobacter fetus venerealis*), disappeared from herds once bulls in AI centres were rendered free of *C. fetus* and antibiotics were added to semen; the increased reproductive performance was worth literally billions of dollars to the AI organizations and the cattle industry they served [85].

Over the years, numerous antibiotics have been screened to determine if they were non-spermicidal in bull semen extenders. It is noteworthy that several antibiotics, e.g. epicillin, flurofamide, aureomycin, and terramycin, and particularly the fungal agents amphotericin B, nystatin, mycostatin, rosaramycin, and clindamycin, are quite spermicidal [12].

Also, the effect of the composition of semen extenders and the method of adding glycerol as a cryoprotectant on the antimicrobial properties of a variety of antibiotics has been investigated. An inhibitory effect of glycerol on the activity of antibiotics in extenders has been suggested [11,12,86–88]. This, and other observations, resulted in the practise of adding antibiotics to raw semen and the non-glycerol portion of the extenders (prior to the addition of glycerol), with the aim of providing more effective control of mycoplasmas, ureaplasmas, *Haemophilus somnus*, and *C. fetus* subsp. *venerealis* [12,89–91]. This treatment usually consists of a mixture of 50 μg tylosin, 250 μg gentamicin, 150 μg lincomycin, and 300 μg of spectinomycin in each millilitre of diluter in a protocol for a two-step method of semen extension. As an alternative, the similar cocktail of antibiotics, but at higher concentrations, can be added to raw semen in one step (one-step method) in a non-fractionated extender that contains 7% glycerol. These protocols are currently recommended by the Certified Semen Services (CSS) for commercial processing of semen for AI use in the North America. In contrast, based on further experiments, French researchers reported that there was not any antimicrobial benefit of adding the combination of gentamicin–tylosin–lincospectin to the raw semen. In addition, such a protocol contributed significantly to a decrease in the percentage of motile spermatozoa. The authors concluded that opportunistic pathogens such as mycoplasmas, ureaplasmas, *C. fetus* and *Haemophilus sommus* may be kept under control with antibiotics added to the diluents used for semen cryopreservation [92].

Recently developed commercial semen extenders (e.g. Biociphos-plus, from IMV) free of egg yolk and milk, eliminated the potential risk of contamination of semen doses with bacteria and mycoplasmas from substances of animal origin [93].

Garcia et al. [94] investigated the survival of various commensal bacteria and enteroccocci, *E. coli*, *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *P. multophilia*, and *P. fluorescens* in human semen cryopreserved in 10% glycerol. Practically all bacteria isolated from the fresh ejaculate were also recovered from the portion frozen in liquid nitrogen. In another study involving human pathogenic microorganisms, only *Neisseria gonorrhea* and *C. trachomatis* were eradicated when semen was frozen in egg yolk-glycerol containing erythromycin (1 mg/mL). It is noteworthy that treatment of the semen contaminated with *U. ureliticum* cryopreserved in the same extender and antibiotic, did not eliminate the microorganism. Also, a broad range of microorganisms was isolated from long term, cryopreserved, commercial bull semen in extenders containing antibiotics, as well as from both nitrogen tank detritus and the liquid nitrogen phase [43].

In humans, antibiotic therapy is often used before IVF when potentially pathogenic bacteria are cultured from the semen. The effect of some antibiotics on the human sperm cell *in vitro* was investigated by King et al. [95]. Various antibiotics were tested: a penicillin derivative, amoxicillin; two forms of quinolones, ofloxacin and ciprofloxacin hydrochloride; a nitrofuran derivative, nitrofurantoin; a tetracycline, doxycycline hyclate, and a cephalosporin, cefuroxime axetil. Within this group of antibiotics, only amoxicillin (7.5 and 750 μg/mL) and ofloxacin (4.6 and 460 μg/mL) had no adverse effect on sperm motility and on the penetration of zona-free hamster oocytes, as compared to the control. The antimicrobial properties of these antibiotics when added to the contaminated semen, were not tested. However, results from other studies showed that antibiotic therapy alone in the male may reduce the incidence of seminal organisms from 92 to 77% and the addition of antibiotics to the wash and swim-up medium reduced the incidence further to 20%. However, the treatment may increase the likelihood of the introduction of antibiotic-resistant bacteria to the culture and may be detrimental to the outcome of IVF [49,96].

The emergence of antibiotic-resistant strains of opportunistic bacteria, such as, *P. aeruginosa*, *H. somnus*, and more recently *Mycoplasma* and *Ureaplasma* spp., has made it difficult to control their spread despite the presence of antibiotics in bull semen extenders [27]. The introduction of antibiotic-resistant strains of *Pseudomonas* spp., *Enterobacter cloacea*, *Staphylococcus sciuri*, *Acinetobacter calcoaceticus*, and *Flavobacterium* spp. and other bacteria into bovine IVF systems by contaminated semen, has also become evident. Particularly relevant has been demonstration that *Stenotrophomonas multophillia* that was detected in cryopreserved semen adversely affected sperm motility and severely suppressed embryonic development [43,78,97].

The common antibiotics used for processing bull semen do not consistently eliminate *P. aeruginosa*. However, experimental insemination of heifers with contaminated semen had no apparent effect on fertilization and early development of embryos [98].

Special attention is required for the control of microbial contaminants in boar semen which is frequently stored in liquid diluents for 3–7 days before AI. Bacteriospermia is a frequent finding in porcine

semen and can affect sperm quality [99]. Adding appropriate antibiotics could prolong the viability and fertility of the sperm. Semen treated with penicillin/streptomycin showed a decrease in sperm motility after Day 3; when treated with gentamicin, sperm motility at Day 7 was reduced to 60% [100]. From other studies, it appeared that aminoglycosides, such as gentamicin, neomycin, kanamycin and dibekacin, at concentrations as low as 6 μg/mL of extender, were more effective than the traditional combination of penicillin and streptomycin. After semen storage in the presence of dibekacin (100 μg/mL) at 15 °C for 7 days, 80% of the samples had no bacterial growth, but normal sperm morphology [101]. Commercial boar semen extenders may contain 200 mg/L gentamicin [102].

Equine semen contains a variety of commensal and potentially pathogenic bacteria originating in the reproductive tract of the stallion and which may be introduced into the mare's uterus at natural service or AI. Both seminal plasma and fresh skim milk provide a suitable medium to facilitate the growth of microbes. To control microorganisms which are of the most concern to breeders (i.e. *E. coli*, *P. aeruginosa* and *Klebsiella pneumoniae*), broad spectrum antibiotics added to seminal extenders was investigated by Squires et al. [103], Jasko et al. [104], and Vainer et al. [105]. Amikacin sulfate (1000 μg/mL) and potassium penicillin (1000 IU/mL) seemed to provide the best antimicrobial action as well as the least adverse effect on sperm motility. Other antibiotics at higher concentrations may affect sperm motility during prolonged storage at lower temperatures (5 °C) [104]. The efficacy of the antibiotics for the control of pathogenic organisms was similar in raw and extended stallion semen [103]. As with bovine semen contaminated with mycoplasmas, equine semen contaminated with *Mycoplasma equigenitalium* was controlled most effectively with lincomycin and gentamicin [106].

Finally, it is worth noting that two fungicides used extensively in embryo culture, amphotericin B and nystatin, were highly spermicidal to bull and rabbit sperm, even in the presence of milk and egg yolk, which are media components used to preserve sperm [107].

It should be remembered that the use of antibiotics may increase the number of antibiotic-resistant bacterial strains. The question then arises as to whether the survival of the microorganisms in antibiotic treated semen should be of concern to animal breeders. As Wierzbowski [27] pointed out in his FAO report, the practice of insemination does not indicate anything about the fertility of bull semen contaminated with potentially pathogenic bacteria. Conversely, the timing of deposition of semen contaminated with ubiquitous bacteria into the uterus during the estrogenic phase of the estrous cycle may diminish the risk of infection. Nevertheless, it is obvious that there is a need for simple, new and more efficient methods of controlling both bacterial and viral contaminants, particularly in semen for IVF where natural defenses in the female genital tract are bypassed. Recently, various procedures for semen disinfection have been explored. These include lowering the pH, treatment with trypsin, and the use of photosensitive dyes.

3.3. Acidification (pH)

Acidification is a method that is particularly effective for eliminating certain viruses from semen. It is well established that, with the exception of adenoviruses and enteroviruses, most viruses are inactivated between pH 5 and 6 [108]. Bluetongue virus (BTV) and rubella virus were inactivated within 1 min below pH 6.0; eastern and western equine encephalomyelitis and Semliki Forest viruses resemble BTV in their sensitivity to low pH, as do a number of other arboviruses [109]. Type A foot-and-mouth disease virus (FMDV) has also been shown to rapidly lose infectivity at pH 5.0 [110]. Therefore, based on the premise that transitory acidification of semen (and embryos, see below) might effectively destroy pH-labile viruses within it, experiments were undertaken to investigate the effect of short-term acidification on the fertilizing capacity of semen, based on the number of embryos recovered from superovulated animals inseminated with acid-treated semen [111]. Ejaculates diluted 1:1 with PBS were acidified to pH 5.0 for 2 or 5 min by adding a small volume of 1N HCL and then gently mixing on a vortex stirrer before being returned to their original pH. The samples were held at the lowered pH at 35 °C. Subsequently, semen pH was returned to that of the original by adding a small volume of 1N NaOH prior to dilution of the sample with milk or with an egg yolk-tris extender, and freezing it. The fertilization rates and the mean percentage of transferable embryos obtained were similar to those reported for superovulated animals bred with frozen-thawed semen in commercial embryo transfer units. The results were supported by acrosome morphology and sperm motility findings, and by an earlier report, in which transitory *in vitro* acidification of semen to pH 5.0 did not result in any morphological sperm defects [112].

In conclusion, transitory acidification of semen is worthy of further investigation as a simple and quick method for rendering semen safe, at least from some

acid-labile pathogens, without affecting its fertilizing capacity.

3.4. Trypsin and other enzymes

The addition of trypsin to semen inactivated BHV-1 without affecting the fertilizing capacity of the spermatozoa [113]. Bull semen contaminated with BHV-1 (10^3–10^4 $TCID_{50}$/mL) was treated with 0.3% trypsin for 5–10 min prior to processing and testing it for the presence of the virus. Virus was not isolated from any trypsin-treated samples, using either a cell culture system or after inoculation into BHV-1-negative calves. Superovulated heifers inseminated with trypsin-treated, frozen-thawed semen yielded transferable embryos [114]. However, a more recent and more detailed study on sperm morphology [115] indicated that trypsin may damage sperm membranes; therefore the use of 0.25% trypsin (rather then 0.3%) was recommended.

The efficacy of multiple semen washings, which included trypsin treatment for removal of infectivity to BHV-1, was confirmed by Guerin et al. [116]. In the same study, it was also concluded that the virus was not present inside the cytoplasm of the sperm, but rather it was associated with the sperm cell membrane.

A novel procedure for removing HIV, HCV, and HBV using 0.25% trypsin incorporated into the density Percoll gradient centrifugation was reported by Loskutoff et al. [47]. The procedure effectively reduced viral copies in the spiked human semen samples to undetectable levels or levels below clinical relevance. The procedure involves use of a propylene tube insert which prevented contamination of the sperm fraction during retrieval after centrifugation.

Other enzymes such as amylase, beta-glucuronidase and catalase were added to bull semen or semen extenders to enhance sperm motility and fertility. However, the antimicrobial properties of those enzymes in contaminated semen were not investigated [88].

3.5. Photosensitive agents and dyes

Hematoporphyrin (HP), hematoporphyrin derivative (HPD), and thiopyronine (TP) are photosensitive agents that have germicidal effects when activated by light [117–119]. Light excitation of HP, HPD or TP results in energy transfer from the excited dye molecule, leading eventually to the formation of singlet oxygen, which has strong oxidative properties. In that regard, HP, HPD, and TP photodynamically inactivated several viruses and bacteria.

The application of HP, HPD, and TP for disinfection of bovine semen was investigated [120]. All three agents, when irradiated with either helium/neon laser light (wavelength 632.8 nm) or substage white light from a 12 V/100 W halogen light, were effective against BHV-1, BVDV, *Mycoplasma bovigenitalium*, *Mycoplasma canadense*, and *Ureaplasma diversum* in culture media. In addition, TP was effective against *Leptospira pomona*. However, when the same microorganisms were added to semen, only BHV-1 was controlled by the photosensitive agents, used at concentrations that did not appear to be harmful to spermatozoa. The fertilizing capacity of semen treated with those agents remains unknown.

3.6. Immunoextenders

The application of immunoextenders containing neutralizing antibodies to viruses was reported by Bartlett [11] and Schultz et al. [121]. Hyperimmune bovine serum, milk and colostrum, and egg yolk were tried. The Ig fraction proved to be most effective for inactivating BHV-1, BVDV, PI3, and BTV. There was no reactivation of the virus and no effect on the quality of the semen or on fertility. The gamma globulin could be added to any of the common extenders.

Similarly Allietta et al. [122] reported that BVDV was eliminated from the semen of a persistently infected bull by supplementing the swim-up medium with purified anti-BVDV IgG from a naturally infected animal. In addition, neutralization of the virus from the semen improved the developmental rate of embryos to a level similar to that of non-infected semen.

Silva et al. [123] reported that incubation of bovine semen spiked with BHV-1 in extender containing 20% hyperimmune egg yolk (1.2 g/mg of IgG) for 60 min at 37 °C reduced the virus concentration by as much as 5×10^5 $TCID_{50}$/mL without affecting sperm viability.

Immunoextenders offer a promising method of inactivating of some viral agents from bovine semen. Further studies are warranted to confirm, on a larger scale, whether this method can be used without the risk of disease transmission by AI.

3.7. Ozone treatment

Ozone, the triatomic allotrope of oxygen, possesses bactericidal and virucidal properties. Gradil et al. [124] used ozone-saturated milk extender in an attempt to disinfect bull semen. Exposure of *P. aeruginosa*, *E. coli*, and *C. fetus* subsp. *venerealis* for 2 h at 5 °C caused a considerable reduction in the number of live

microorganisms, with only a minor effect on sperm motility.

In summary, it is difficult to conclude whether these experimental semen disinfection procedures are more effective for bacteria than for viruses. It can be only speculated that the efficiency of such procedures is related to the structure of the microbial agent, its size and the binding properties to the spermatozoa. Thus, in practice, the results of these techniques should not be extrapolated from one microorganism to another without further research.

4. Origin and risk of microbial contamination of embryos

Prior to ovulation, oocytes may become infected by contact with an infectious agent present in either the ovarian granulosa cells or the follicular fluid, probably during viremia at the acute stage of a disease [125–128]. At this stage, viruses can be present in the blood and other body fluids and spread to various tissues and organs. For example, in cattle microorganisms have been found in follicular fluid a few days after natural and experimental exposure to BVDV and BHV-1 [127,128]. Therefore, collection of oocytes for IVF at this stage of the disease may result in contaminated embryos. This hazard also can be substantial when ovaries are harvested from asymptomatic persistently or latently infected donors (e.g. BVDV, BHV-1). Following ovulation, oocytes may become infected by a spermatozoan during fertilization, or by contact with a pathogen that has been excreted into the oviduct or uterus [64,129]. Other sources of contamination include agents introduced with culture supplements of biological origin, e.g. serum, trypsin, supporting co-culture cells, or cell lines for nuclear transfers [25,26,130]. For example, when transmission of BVDV by ET was documented, contaminated fetal bovine serum (FBS) or bovine serum albumin (BSA) used for embryo handling were thought to have been responsible [18,20,21].

Environmental microbes associated with an operator, abattoir, or the laboratory may pose risks during the production of embryos *in vitro* when pooled materials are used. In this regard, inadvertent inclusion of the follicular fluid from an infected animal into the pool may cross-contaminate all clean oocytes, leading to batches of contaminated embryos [131–135]. Also transvaginally collected human oocytes are potential sources of microbial contamination for the IVF-ET culture system. Cottel et al. [136] reported that various microorganisms (*Mycoplasma hominis*, *U. urealyticum*, *Staphylococcus epidermis*, *Lactobacilli* sp., *Difteroids*) were isolated from approximately one-third of the needle flushes after oocyte recovery and from more than one-third of the follicular fluids aspirated from the first follicle punctures on each ovary. In another study, 39 of 44 samples of follicular contaminated with blood were positive for HCV RNA [137].

As with semen, oocytes, and embryos can become contaminated by direct exposure to liquid nitrogen during cryopreservation (e.g. vitrification) or when stored in unsealed or improperly sealed containers [42,43]. Therefore, the use of high-security ionomeric resin straws (CBS, Cryo Bio System L'Aigle, France) or ''double bagging'' for safe cryostorage are recommended [138,139].

Oocytes and embryos are surrounded by the zona pellucida (ZP), a spongiform, glycoprotein shell, which protects them from physical injury and infection. The ZP also plays a role in fertilization and in early embryonic development. The specific structural and chemical nature of the ZP is a major factor with regard to its interaction with pathogenic microorganisms and its role in disease transmission [140].

It is well known that the intact ZP of uterine stage and IVF embryos is an effective barrier against penetration by most pathogens, even though some may adhere firmly to the surface [1,141]. New IVF-related ART procedures (e.g. ICSI, embryo sexing, embryo cloning, and gene transfer) involve breaching the ZP or removing the entire ZP from embryos to permit manipulation of the embryonic cells. This is often followed by an extended period of *in vitro* culture. These procedures increase the risk of exposure of embryonic cells to pathogens and their contamination. Therefore, international trade in *in vivo*-derived and *in vitro*-produced embryos, as well as micromanipulated embryos of livestock is regulated; guidance on this matter is given by the IETS [1] and by OIE in Appendices 3.3.1. and 3.3.2 of the Terrestrial Animal Health Code [2]. These involve embryo washing procedures as a principal sanitary tool, along with other related techniques which may be useful in rendering embryos free of pathogens.

5. Methods of rendering oocytes and embryos free of pathogens

5.1. Washing procedures

Sanitary washing procedures for *in vivo*-derived embryos in the presence of antibiotics (e.g. 100 IU/mL penicillin and 100 mg/mL streptomycin) and trypsin were originally developed by Singh [142] and later

adopted and endorsed by the IETS and OIE as a standard sanitary requirement for health certification of livestock embryos. A detailed description of these procedures is given in the Manual of IETS [1].

It was demonstrated that by a simple transfer of embryos from one dish well to another well with clean medium (dilution factor at least 1:100), the viral load of some pathogenic agents decreased to undetectable levels by the 10th wash. In addition, to facilitate the dilution of microorganisms, it has been recommended that the micropipette tip be replaced after each wash, and to limit the number of embryos in each washed group to $\leq$10. This procedure was shown to be effective in rendering bovine, porcine and ovine *in vivo* derived ZP-intact embryos free of a number of viral, bacterial and prion agents of epizootical and economical importance (e.g. foot-and-mouth disease virus (FMDV), bovine leukemia virus (BLV), and bovine spongiform encelophalopathy (BSE) [1,143]. However, there are some agents that reportedly adhere firmly to the ZP of ZP-intact embryos in such a way that they may not be entirely removed using the described methodology. These include BHV-1, BHV-4, vesicular stomatitis virus, *U. diversum*, and *Mycoplasma bovis*, *Mycobacterium paratuberculosis*, and *H. somnus* with bovine embryos; bluetongue virus, *Brucella abortus* and *Brucella ovis* with sheep embryos; African swine fever virus (ASFV), hog cholera virus, pseudorabies virus, swine vesicular disease virus (SVDV), parvovirus, vesicular stomatitis virus (VSV), and *Leptospira bratislava* with pig embryos [1]. It is worth noting, that although some of these pathogens stick to ZP of embryos after experimental exposure *in vitro*, it would appear that embryos are rarely contaminated naturally *in utero*, even during acute maternal infection [144,145].

With the advent of IVF, it was demonstrated that many of the viral and bacterial agents adhered more easily to the ZP of *in vitro*-produced as compared to *in vivo*-derived embryos and therefore rendering them free of microbes by a simple washing technique was more difficult [146–149]. Perhaps the best example of this phenomenon is the tendency of non-cytopathic strains of BVDV to adhere to the ZP of bovine embryos [140,150,151]. Effective washing of cumulus oocyte complexes (COCs) and embryos at the very early stages after IVF can be especially difficult, since the multiple layers of cells that are attached to the ZP at this early post-ovulatory stage may harbour microorganisms which cannot be completely washed out or penetrated by the disinfectant substances [152]. In some cases, although the primary washing procedure of oocytes and early zygotes may not be fully effective in removing the microorganisms, the latter will not survive in the *in vitro* system following the IVC period (e.g. *C. fetus*) [152]. Where washing did not remove an agent from the ZP, enzymatic treatment in conjunction with washing, as described below, is recommended [1]. To the author's knowledge, there is a lack of any comparative studies, similar to those on animal embryos, on the efficacy of rendering human embryos free from bacteria and viruses by washing or other forms of disinfection.

5.2. Enzymatic treatments

Application of enzymes to disinfect embryos must be carried out in strictly controlled conditions to prevent dissolution of the ZP and damage to the embryonic cell membranes which could, in turn affect embryo survival. Currently, a trypsin-like protease is the only enzyme that is used routinely for rendering embryos free from viruses when a mechanical washing procedure is not fully effective. It was noteworthy that a trypsin-like protease, secreted from hatching mouse embryos, was considered as a hatching enzyme [153].

When embryos are being processed for export, trypsin treatment should be included in the multiple washing protocol, as recommended by the IETS [1] and OIE [2]. Briefly, the protocol involves five washes in culture medium supplemented with 0.4% BSA, followed by two washes in 0.25% trypsin in Hanks balanced salt solution for 90 s at 25 °C and pH 7.6, and five further washes in medium containing 2% FBS or 0.4% bovine serum albumin (BSA) to inactivate trypsin activity [1,142]. The value of using Ca^{2+} and Mg^{2+}-free trypsin (as described in the original reports) medium is not clear. The use of trypsin which is free of these ions likely resulted from a common practice of its application for cell dispersal in a variety of monolayer cultures; whether this facilitates detachment of viral particles from the ZP remains unknown. However, it has been recognized that the presence of Ca^{2+} protects the enzyme from rapid autolysis and inactivation [154]. The activity of the trypsin preparation that is used should be standardized and the preparation should be free of contaminants, e.g. mycoplasma. In order to exclude the possibility of introducing such contaminants via natural porcine trypsin, a recombinant trypsin-like fungal preparation (TrypLE Select, Invitrogen) has recently been investigated [155]. After exposing *in vitro*-produced bovine embryos to BHV-1, a short incubation period (1.5 min) with a 1× concentration of this recombinant trypsin during embryo washing was not effective for removal of the virus, but a longer interval

(10 min) with a 10× concentration of enzyme did remove the virus from the ZP of *in vivo*-derived embryos. A similar result was obtained when *in vitro*-produced porcine embryos exposed to BHV-1 were treated with TrypLE Select [156]. It seems that the recombinant trypsin-like preparation was less potent than natural trypsin and it may require more prolonged incubation with the embryos, which is not practical for routine use. In contrast, it has been recently reported that treatment, which involved use of ''pure'' trypsin recombinant (derived from maize) in medium supplemented with 1 mM EDTA, was as effective as porcine trypsin in removing BHV-1 from bovine embryos within 1 min of incubation [157]. However, more detailed research is needed to determine the optimal conditions for inactivation of BHV-1 by this enzyme preparation.

It was noteworthy that trypsin was not essential for effective removal of FMDV, pseudorabies virus, vesicular stomatitis virus, and hog cholera virus, provided that the embryos were properly washed ten times [1]. It should be noted that trypsin is not a universal disinfecting agent, either for *in vivo*- or *in vitro*-fertilized embryos. In general, most Gram-positive and Gram-negative bacteria are resistant to the action of this enzyme [158]. Trypsin treatment was not effective for removal of African swine fever virus (ASFV), swine vesicular disease virus (SVDV) and bovine mycoplasmas from *in vivo*-derived embryos [144,159,160]. It can be also assumed that trypsin would not be useful as a disinfectant for removing the equine encephalomyelitis virus and swine influenza virus from embryos, since it failed to inactivate these viruses in infected cell cultures [158].

In addition, due to the differences in the properties of the ZP of IVF embryos, treatment was not fully efficient for non-cytopathic strains of BVDV [146,147], BHV-1 [127,148], FMDV [161], Sendai virus [162], BTV [163], epizootic hemorrhagic disease virus-2 (EHDV-2) [164], mycoplasmas [160], and *E. coli* [165].

It is noteworthy that trypsin, at the concentration (0.25%) used for the treatment of ZP-intact embryos, had no subsequent adverse effect on embryonic development and pregnancy rates of transferred Day 7 fresh or frozen bovine embryos [166–169]. Also Kissing et al. [170] suggested that exposure of human oocyte to trypsin may have enhance fertilization in patients with a history of fertilization failure, probably by thinning of the ZP and facilitating sperm penetration.

The effect of other potentially disinfecting proteolytic enzymes, e.g. lipase, phospholipase C, and chymotrypsin, on the viability of preimplantation embryos have been investigated. The last two enzymes were harmful when tested on pre-implantation mouse embryos [171]. The value of antimicrobial properties of these enzymes for decontamination of germplasm was not studied.

Substituting 0.1% hyaluronidase (exposure time 5 min) in combination with enzymes RNase and DNase (exposure time 30 min) for trypsin in the washing procedure was reported to be effective for the removal or inactivation of porcine encephalomyocarditis virus, porcine circovirus type 2 (PCV-2), porcine parvovirus, porcine reproductive and respiratory syndrome (PRRSV), and BVDV from *in vitro*-produced swine embryos which were co-cultured in the presence of bovine oviductal cells (BOC) [172]. In contrast, a similar treatment was less effective, or ineffective, against PCV-2 when the embryos had not previously been exposed to oviductal secretions that, in the former study, presumably were produced by the BOC [172,173].

A non-specific protease (''Pronase'', derived from *Streptomyces griseus*) was used to aid removal of epizootic hemorrhagic disease virus serotype 2 (EHDV-2) from *in vitro*-produced bovine zygotes. In this experiment, the COCs were exposed to the virus (10^6 $TCID_{50}$/mL) during maturation, prior to fertilization with uninfected semen and the protease treatment. This protease (0.1%, 4 units/mL, type XIV, manufactured by Sigma Chemical Co., St. Louis, MO, USA) was substituted for the trypsin in the IETS washing procedure [1]. There was a significant reduction in the amount of EHDV-2 associated with embryos when they were exposed to this protease for 45 s; these results were better than those obtained after the standard trypsin treatment, in accordance with the IETS procedure [174].

5.3. Antibiotics

Antibiotics are routinely added to media used for the long-term culture of cells and tissues, to avoid contamination from bacteria and fungi. As a general rule, the quantities of antibiotics in the media are kept at standard concentrations which are thought to have no detectable toxic effects on the cultured cells. However, antibiotics are biologically active substances that always have the potential to affect cell function. With regard to the collection, *in vitro*-production and storage of embryos, therefore, it seems logical to consider that culture media composition should reflect as much as possible the conditions present in the lumen of the oviduct or uterus. Various media have been formulated with the aim of improving embryo viability on successive

days of culture. Penicillin (100 IU/mL) and streptomycin (100 μg/mL) as well as gentamicin (50 μg/mL) are the most common antibiotics used to control microorganisms in culture media, without any apparent effect on the development of embryos of domestic animals. They are effective against a variety of Gram-positive and Gram-negative bacteria. Amphotericin B (0.25 μg/mL) or mycostatin (50 U/mL) are often added to control fungal contamination. However, Magli et al. [175] reported that antibiotic supplementation of the media used for *in vitro* production of human embryos with standard amounts of penicillin (100 IU/mL) and streptomycin (50 μg/mL) had an adverse effect on embryonic growth. A similar effect was observed with reduced concentrations of these antibiotics. Subsequent work by Zhou et al. [176] confirmed that penicillin and streptomycin together may affect development of pronuclear hamster embryos *in vitro*. In contrast these antibiotics and gentamicin (10 μg/mL) had no adverse effect when used separately. That hamster gametes are extremely sensitive to culture conditions suggests that a combination of these two popular antibiotics may interfere with the timing of cleavage events by delaying or blocking embryo development. This study also suggests that if antibiotics are to be used, then gentamicin is the safest [176].

Riddell et al. [177] investigated the toxicity of a combination of penicillin, streptomycin, and amphotericin B on murine and bovine embryos. Penicillin and streptomycin alone, even at 10 times the recommended concentration, for 72 h at 37 °C had no effect on the development of Day 7 bovine embryos. However, these antibiotics, together with amphotericin B or amphotericin B alone at higher concentrations, were toxic.

The effect of Ceftiofur sodium (Naxcel) on bovine oocyte maturation, fertilization and development *in vitro* was investigated in detail by Holyoak et al. [178]. This antibiotic had a broad spectrum of activity against Gram-positive and Gram-negative bacteria, including beta-lactamase-producing strains. Although there was no detrimental effect on oocyte and fertilization at concentrations of 10 and 50 μg/mL, post-fertization development of embryos was adversely affected *in vitro*.

It appears that effective antimycoplasmic treatment of embryos requires a long exposure to high concentrations of antibiotics. The antimicrobial effects of kanamycin, gentamicin, tetracycline, tylosin, lincomycin, spectinomycin, and penicillin and streptomycin at concentrations similar or higher than that used for semen treatment was investigated [160,179]. Of these antibiotics, only the treatment for 4 h with tylosin (200 μg/mL) or kanamycin (1000 μg/mL) was effective in disinfecting bovine embryos after the latter had been exposed *in vitro* to *M. bovis*. These antibiotics had no obvious effect on embryonic development at the time of hatching [177]. However, exposing embryos for only 10 min and to lower concentrations of these antibiotics was not effective for the removal of *M. bovis* [158].

The efficacy of antibiotic treatment, in conjunction with the standard IETS washing procedure [1] for removal of the bacteria *Streptococcus agalactiae*, *Actinomyces pyogenes*, and *E. coli* from *in vitro*-produced bovine embryos, was studied by Otoi et al. [180]. Groups of embryos were exposed to bacterial suspensions without antibiotics for 18 h, washed 10 times in medium containing gentamicin (50 μg/mL), and then tested for the presence of bacteria, but the procedure was ineffective. On some occasions antibiotic-resistant strains of *Pseudomonas* spp., *Streptococcus* spp., *Staphylococcus* spp., and fungi may occur in *in vitro*-production systems and their presence may lead to reduced fertilization rates followed by degeneration and death of the embryos. Although the source of such contamination is often difficult to establish, some opportunistic pathogens, such as *P. aeruginosa* are common contaminants of bovine semen and these may invade the IVF system via this route. Another possibility is that contaminated oviductal cells might be used for co-culture. *Leptospira borgpetersenii* serovar *hardjobovis* has also been found in association with washed oocyte and embryos generated *in vivo* and *in vitro*, but in these cases supplementation of the media with penicillin and streptomycin will remove the infection and ensure that the embryos are not contaminated [181,182].

5.4. Immunological methods

It is well recognized that natural exposure to pathogenic microorganisms, or to vaccination, usually results in the production of neutralizing antibodies not only in the serum but also in the ovarian follicular fluid and oviductal and uterine secretions. Therefore, these antibodies may provide some protection for recently ovulated ova and developing embryos [183,184].

Singh et al. [185] investigated the possibility of using exogenous antibodies to inactivate BHV-1 from *in vivo*-fertilized embryos exposed *in vitro* to 10^6 and 10^8 $TCID_{50}$ virus. They noted the same neutralizing effect on the virus as had been obtained using trypsin, but it required a 1-h incubation with the antibodies to render the embryos free of the virus. In another study, incubation for 1 h of COC or IVF embryos in the presence of a mixture of monoclonal BHV-1 antibodies and guinea pig complement (GPC) or the GPC alone eliminated the virus [186].

Tsuboi et al. [187] reported that the use of FBS with a high titer of neutralizing antibodies in the *in vitro*-production system prevented the transmission of BVDV from COCs derived from a persistently infected cow to COCs that had been derived from healthy cows. In contrast, Stringfellow et al. [188] noted that BVDV could persist in the system, despite the presence of antiviral antibody in *in vitro* maturation and *in vitro* culture media. Indeed, Whitmore and Archbald [184] and Galik et al. [135] reported that the presence of anti-BVDV antibody in the pooled follicular fluid collected from vaccinated and virus-exposed cows may neutralize the virus and thereby interfere with its isolation, but not necessarily kill it. Thus, in some cases, failure to transmit disease via embryo transfer may result from the presence of serendipitous neutralizing antibodies in either follicular fluid or the FBS used for embryo culture [189].

It can be concluded that use of immunological methods, such as addition of antibodies to media, could result in binding but not killing of a pathogenic microorganism and thus lead to a false sense of biosecurity if the treated embryos were subsequently transferred.

5.5. Photosensitive dyes and chemical compounds

Other chemical substances or procedures shown to have a germicidal effect are hematoporphyrin (HP) and hematoporphyrin derivative (HPD); furocoumarins: 8-methoxypsoralen (8-MOP) and 4,5′,8-trimethypsoralen (TMP) (trioxalen) and the heterocyclic dye, thiopyronine (TP) [117–119]. The effect of these substances on embryonic development has been investigated. It was found that BHV-1 and BVDV were photoinactivated without any obvious harmful effect on embryonic development when infected embryos were exposed for 5 or 10 min to HP and HPD followed by helium neon laser light (250 J/cm^2) or white light [190].

In similar experiments, Dinkins et al. [164] used HP (15 μmol) or hypericin (1 and 10 μmol) and a 1 mW helium laser light (633nm, red) to inactivate EHDV-2 from *in vitro*-produced embryos. Both agents reduced the percentage of contaminated embryos that were contaminated after 3 min of light exposure, but did not eliminate the virus completely. The treatment had no effect on the subsequent development of the zygotes.

5.6. Antiviral agents

A novel antiviral agent selected from the group of compounds with aromatic cationic molecules, 2-(4-[2-imidazolinyl]phenyl)-5-(4-methoxyphenyl)furan (DB606) was investigated by Givens et al. [191] for its ability to inhibit or remove BVDV replication in tissue culture and in an *in vitro* embryo production system. Zygotes that resulted from IVF were cultured for 7 days in medium supplemented with 0.4 FM DB606 in the presence of infected uterine-tubal-cells (UTC). The agent effectively inhibited replication of BVDV in the embryo culture system. Furthermore, blastocyst development, pregnancies per transferred embryo, and development of calves did not differ significantly from controls. It was concluded that BD606 can be safely used as an additional safeguard agent to reduce exposure of IVF embryos to BVDV in the culture system [192].

Another antiviral compound, ‘‘Foscarnet’’ (phosphonoformic acid), was evaluated for its ability to inhibit or remove bovine herpesvirus 1 (BHV-1) in cumulus cells commonly used for co-culture with bovine *in vitro*-produced embryos. This compound inhibited viral replication by preventing cleavage of pyrophosphate from deoxynucleotide triphosphate. At 200 and 400 μg/mL, phosphonoformic acid inhibited 4 logs of BHV-1. Subsequently, phosphonoformic acid (200 and 400 μg/mL) added to both the *in vitro* fertilization and *in vitro* culture media and resulted in a decrease in the proportion of developed blastocysts, and in the number of cells per blastocyst, in the treated embryos. Therefore, although phosphonoformic acid can effectively inhibit replication of BHV-1 in co-culture cells, it also inhibited development of *in vitro*-produced bovine embryos [193].

5.7. Interferon

Most interferons are glycoproteins; in addition to their ability to alter the function of target cells, they express antiviral actions [194]. In ruminants, interferon-τ is secreted in large quantities from the trophectoderm for a few days prior to implantation and is a critical component of pregnancy recognition. *In vivo*-derived and *in vitro*-produced bovine embryos as well as cloned and demi-embryos expressed varying amounts of interferon, which could contribute to differences in pregnancy rates after transfer to recipients [195].

The induction of an antiviral state in preimplantation bovine embryos treated with interferon was investigated by Bowen [196]. Hatched blastocysts were cultured for 24 h in the presence or absence of human leukocyte interferon (5000 units/mL) and then challenged with either vesicular stomatitis virus or bluetongue virus. Interferon treatment failed to reduce virus replication in the blastocysts and had no effect on the virus-induced

cytopathic effect. In contrast, significant antiviral effects were induced by interferon when fetal bovine cells were treated. The lack of biologic activity of interferon in bovine embryos was similar to that observed previously with undifferentiated murine embryonal carcinoma cells [197].

It was also noted that the interferon-τ produced no observable cytotoxicity in Madin Darby bovine kidney (MBDK) cells and significantly decreased the concentration of BVDV but not BHV-1 in these cell cultures. Based on this observation, Galik et al. [198] postulated that the interferon produced by developing embryos might limit or prevent transmission of BVDV to recipients if this virus was inadvertently associated with embryos which were transferred.

5.8. Lactoferrin

Lactoferrin, a natural glycoprotein excreted in milk, saliva, and tears, has antiviral properties against herpesviruses. Lactoferrin, at concentrations from 2.5 to 10 mg/mL, did not affect embryonic development. Lactoferrin from milk, at a concentration of 10 mg/mL, inhibited 2–5 logs of BHV-1 (Colorado strain) in MBDK cell cultures [199].

5.9. Acidification (pH)

With the aim of inactivating foot-and-mouth disease virus (FMDV) from *in vitro*-produced bovine embryos by low pH, the effect of acidic organic buffer 2-(*N*-morphalino)-ethanesulfonic acid (MES) on embryonic development was investigated. In a previous study, MES destroyed FMDV at a rate of 90%/min at pH 6 and at 90%/s at pH 5 [200]. When groups of non-infected oocytes were exposed to MES at pH 5.5 for 30–60 s, there was no difference between control and treated groups in the number which cleaved and developed to the blastocyst stage [201]. However, it still should be investigated whether such treatment is effective in removing FMDV from bovine embryos.

6. Conclusion

Several antimicrobial procedures are currently available for disinfecting semen and embryos and others are still under development. Unfortunately, none of them fulfill the requirement for a universal disinfectant. Various washing procedures have proven to be generally applicable for rendering the semen and embryos of human and animals free from a range of pathogenic microorganisms; these procedures do not compromise survival and embryonic development. Pathogenic microorganisms that can be removed, inactivated or have their load reduced in the semen of humans and animals by washing with diluents containing antibiotics are listed in Table 1. Similarly, a list of microorganisms that can be removed or inactivated from *in vivo*-derived, ZP-intact embryos of domesticated animals by washing, including trypsin washes in some cases, according to the IETS Manual recommendations [1], is shown in Table 2.

Table 1
Some pathogenic microorganisms which can be inactivated or have their load reduced in human and animal semen by washing with diluents containing antibiotics or trypsin

Microbial agent/disease	Washing method			References
	Gradient centrifugation	Swim-up	Trypsin	
Humans				
Human immunodeficiency virus (HIV-1)	+	+	+	[46,47,50,51–54]
Hepatitis C virus (HCV)	+	+	+	[47,50,61,62,65]
Mycoplasma hominis		+		[48,49]
Ureaplasma urealiticum	+	+		[48,49]
Staphylococcus epidermis		+		[45,48,49]
Gardnerella vaginalis		+		[48,49]
E. coli	+	+		[45,49]
Streptococcus sanguis	+	+		[45]
Animals				
Porcine reproductive and respiratory syndrome virus (PRRS)	+		+	[67]
Equine viral arteritis virus	+	+		[72]
Various aerobic and anaerobic bacteria		+		[75]

Table 2
Pathogenic microorganisms which can be removed or inactivated from *in vivo*-derived, ZP-intact embryos of farm animals by washing according to the IETS recommendation [1]

Pathogen	Disinfection procedure			References
	10 washes	Antibiotics	Trypsin	
Cattle				
Akabane virus (AV)	+			[202]
Bovine leukemia virus (BLV)	+			[214]
Bluetongue virus (BT)	+			[202,203]
Bovine viral diarrhea virus (BVDV, cytopathic strain)	+			[202]
Bovine immunodeficiency virus (BIV)	+			[70]
Foot-and-mouth disease virus (FMDV)	+			[204]
Infectious bovine rhinotracheitis virus (IBRV)	+		+	[205,220]
Bovine herpesvirus-4 (BHV-4)	+		+	[205]
Bovine spongiform encephalopathy (BSE)	+	+		[143]
Brucella abortus	+			[206]
Mycobacterium bovis	+			[207]
Haemophilus sommus	+	+		[208]
Leptospira borgpetersenii	+	+		[209]
Sheep				
Scrapie	+			[219]
BVDV	+			[210]
Campylobacter fetus (*C. fetus*)	+			[211]
Sheep pulmonary adenomatosis (SPA)	+			[218]
Goats				
Caprine arthritis-encephalitis virus (CAEV)	+			[217]
Pigs				
Hog holera virus (HCV)	+	+		[212]
Pseudorabies virus (PrV)	+	+		(in 1)
Vesicular stomatitis virus (VSV)	+	+		[213]
PRRS	+		+	[215]
PCV-1	+			[216]

Note: Embryos were collected from infected donors or were exposed *in vitro* to the pathogenic agent prior to washing and disinfection treatments. Efficiency of washing-disinfecting procedures were based on non-transmission of the agent by ET to recipients and offspring or on testing of embryos *in vitro*.

It is not realistic to expect that processed semen will be completely free of microorganisms. Ubiquitous bacteria, which are not necessarily considered to be primary pathogens, can be controlled to some degree by proper sanitation/hygiene and standard antibiotics (e.g. penicillin and streptomycin). Further measures, such as other more specialized antibiotics, may be use to control potentially pathogenic organisms such as mycoplasmas; this can be accomplished by adding gentamicin, tylosin and possibly lincomycin–spectinomycin in various combinations and concentrations, using appropriate exposure durations and temperatures.

Unfortunately, washing and antibiotics are not effective for a number of important microorganisms; to ensure that these are not present in the semen or embryos of animals, it may be necessary to select donor sires and dams that are guaranteed free from infection (often a very difficult and expensive option). Alternatively, it may be possible to use one or more of the additional disinfection procedures described in this review. In animals, therefore, it is more convenient to adopt other disinfection procedures. Since antibiotics are not effective against viral microorganisms, these additional procedures might, for example, include trypsin treatment for removal or inactivation of herpesviruses from bull semen and from bovine and porcine embryos. Other disinfecting procedures, which are being developed and show promise for dealing with viruses, include semen acidification, the use of photosensitive dyes, and novel antiviral agents. In humans, screening potential donors for infectious agents remains the basic tool for controlling disease transmission in ART. In addition, washing semen by gradient centrifugation and swim-up appears to be effective for reducing the microbial population and is harmless to the spermatozoa.

It is clear from this review that there is a considerable need for more research on procedures for the disinfection of semen/spermatozoa and embryos to ensure that they can be used without fear of disease transmission to recipients and their offspring.

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