Theriogenology 86 (2016) 1999–2003

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Interaction of bovine viral diarrhea virus with bovine cumulus–oocyte complex during IVM: Detection in permissive cells

Erika A. González Altamiranda [a,*], Germán G. Kaiser [b], Glenda L. Ríos [b], María R. Leunda [c], Anselmo C. Odeón [c]

[a] Animal Health Group, Department of Animal Production, CONICET, INTA EEA Balcarce, Balcarce, Argentina
[b] Reproduction Biotechnology Group, Department of Animal Production, INTA EEA Balcarce, Balcarce, Argentina
[c] Animal Health Group, Department of Animal Production, INTA EEA Balcarce, Balcarce, Argentina

ARTICLE INFO

Article history:
Received 11 November 2015
Received in revised form 9 June 2016
Accepted 17 June 2016

Keywords:
Bovine viral diarrhea virus
Bovine cumulus–oocyte complex
IVM

ABSTRACT

Structural changes in the zona pellucida (ZP) of bovine oocytes seem to modulate their interaction with various viral agents, facilitating the viral infection in *in vitro* production systems. To evaluate the susceptibility of bovine oocytes to noncytopathogenic bovine viral diarrhea virus (ncp-BVDV), cumulus–oocyte complexes were exposed to 10^7 tissue culture-infective doses ($TCID_{50}$)/mL of an ncp-BVDV strain during IVM (*in vitro* maturation). After that, cumulus cells and the ZP were removed by hyaluronidase and pronase treatment, respectively, and the percentages of oocytes with polar body were analyzed as a sign of nuclear maturation. After passage through cell culture, the virus was isolated from granulosa cells, ZP-free mature oocytes, and ZP-intact mature oocytes. These results were confirmed by reverse transcription–polymerase chain reaction. After consecutive washes, the virus remained associated with ZP-free oocytes, maintaining its replication and infectivity in permissive cells. Based on these findings, it is concluded that the classical viral isolation procedure has a predictive value to detect BVDV associated with ZP-free oocytes and that it was novelty demonstrated that both washing and trypsin treatment of oocytes were ineffective to remove BVDV infection.

1. Introduction

Assisted reproductive techniques, such as *in vitro* embryo production (IVP), have allowed a significant increase in cattle production, thereby improving milk and meat production worldwide [1]. However, there is wide concern that the application of these techniques is associated with an increased risk of transmission of infectious diseases, such as bovine viral diarrhea [2]. Bovine viral diarrhea virus (BVDV) is an important pathogen of cattle, which primarily causes immune suppression and reproductive disorders. Several studies have reported the impact of BVDV on the procedures of IVF when ovaries, follicular fluid, cumulus cells, oocytes, uterine tubal cells, and serum are obtained from infected animals [3–5]. In addition, other studies have shown differences in the susceptibility of bovine embryos produced *in vitro* according to the biotype, genotype, and virulence strain [3,6,7]. The main consequences of the association of noncytopathic BVDV strains with transferred IVF embryos might result in infection of embryo recipients, early embryonic death, abortion, or birth of persistently infected calves [4].

Several studies have shown that all the main organs within the female reproductive tract are permissive to BVDV and that the distribution of the virus in animals with acute infection is similar to that of those with persistent infection [8]. Mammalian oocytes and preimplantation-stage embryos are

* Corresponding author. Tel.: +542266439100; fax: +542266439112.
E-mail address: galtamiranda.erika@inta.gob.ar (E.A. González Altamiranda).

0093-691X/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2016.06.020

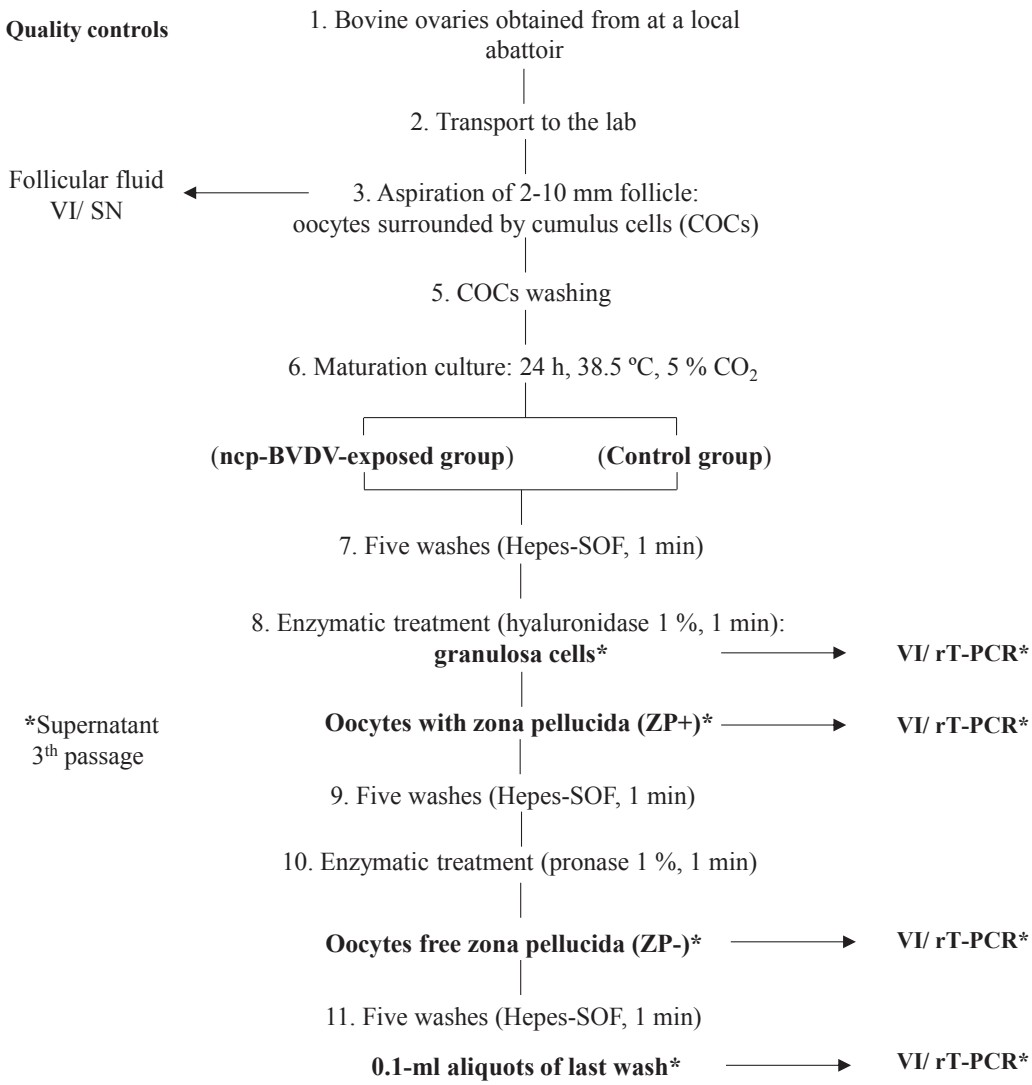

Fig. 1. Experimental design: procedures and experimental infection with ncp-BVDV during IVM of bovine oocytes. Viral titer: ncp-BVDV-98/204 (2.5×10^7 $TCID_{50}$/mL); n = 4 to 5 oocytes for virus isolation. "*," Virus isolation was performed from the third passage from tissue culture plates. COCs, cumulus–oocyte complexes; ncp-BVDV, noncytopathogenic bovine viral diarrhea virus; rt-PCR, reverse transcription–polymerase chain reaction; SN, seroneutralization; VI, virus isolation; ZP, zona pellucida.

surrounded by the zona pellucida (ZP), which plays an essential role in several physiological functions and protects the oocyte/embryo from infectious agents. However, some authors have postulated that the ZP could also act as a carrier of infectious agents [2,9]. The ZP of *in vitro*–produced embryos is structurally different from that of embryos conceived naturally. Evidence indicates that structural changes seem to modulate the interaction of embryos with BVDV, facilitating viral infection in IVP [10]. These differences can also affect both the capacity and persistence of infection in IVP systems [11]. The aim of this study was to evaluate the ability of BVDV to infect bovine oocytes during IVM.

2. Materials and methods

Ovaries from healthy animals with normal reproductive tracts were collected at a local abattoir and transported to the laboratory in sterile saline added with antimicrobial agents at a temperature of 20 °C to 25 °C. Cumulus–oocyte complexes (COCs) were recovered by aspiration of 2- to 10-mm follicles. COCs with homogeneous ooplasm and more than four complete layers of *cumulus* cells corresponding to grades 1 and 2 according to de Loos et al. [12] were selected under a stereomicroscope and washed three times in modified M199 supplemented with 0.5% HEPES (wt/vol). For all experiments, between 40 and 45 COCs per group were matured in 400 μL of M199 plus 0.1 mg/mL L-glutamine and 2.2 mg/mL $NaHCO_3$ supplemented with 0.01 IU/mL rh-FSH (Gonal F-75; Serone, UK) and 10% FBS at 38.5 °C under 5% CO_2 in air with saturated humidity for 22 to 24 hours. About 400 COCs (in four independent trials) were matured in the absence (control group) or presence of a noncytopathogenic bovine viral diarrhea virus (ncp-BVDV) strain (N° 98/204 type 1b, field strain isolated from

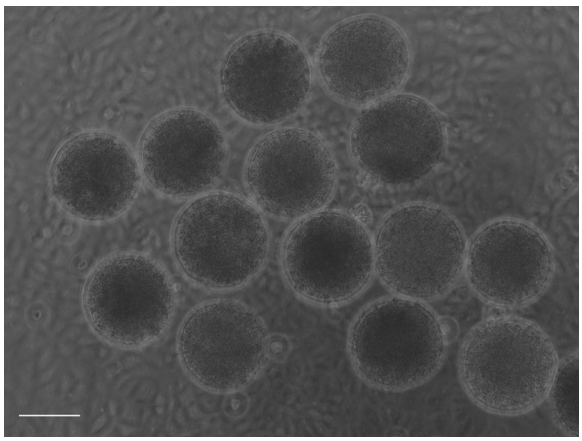

Fig. 2. Oocytes matured *in vitro* and denuded in co-culture with MDBK cells. Before removal of cumulus cells, these oocytes were exposed to an ncp-BVDV strain during IVM. Phase contrast microscopy × 400. Scale bars = 100 μm. A high-resolution version of this slide for use with the virtual microscope is available as eSlide: VM02586. MDBK, Madin–Darby bovine kidney; ncp-BVDV, noncytopathogenic bovine viral diarrhea virus.

fetus) with a final viral titer of 2.5×10^7 tissue culture-infective doses ($TCID_{50}$)/mL (BVDV-exposed group). After maturation, oocytes from both control and BVDV-exposed groups were completely denuded of *cumulus* cells by pipetting in M199-HEPES containing 300 IU/mL hyaluronidase for 1 minute and washed five times in HEPES–SOF (synthetic oviduct fluid) drops. Then, oocytes with polar body were separated into two groups: oocytes with intact ZP (ZP+) and oocytes treated with protease XIV of *Streptomyces griseus* (1 mg/mL) for 1 minute to remove the ZP (ZP−) (Fig. 1). The percentages of oocytes with polar body were analyzed under a stereomicroscope as a sign of nuclear maturation. The maturation rates of both experimental groups were compared using a standard chi-square test. Differences at a probability of $P < 0.05$ were considered significant. Granulosa cells after stripping procedures, oocytes in groups of 5 to 10 (ZP+ and ZP−), and corresponding wash fluids (HEPES–SOF) were tested for viral presence after each of the five consecutive steps of the washing procedure (Fig. 1). Briefly, samples were added in duplicate to 96-well tissue culture plates with confluent Madin–Darby bovine kidney (MDBK). This condition ensures intimate contact between the oocyte and BVDV-permissive cells, allowing its replication (Fig. 2). After three blind passages (each for 48 hours) at 37 °C and 5% CO_2, the presence of BVDV was assessed by a direct fluorescent antibody test using a fluorescein-conjugated porcine polyclonal antiserum (VMRD, Inc., Pullman, WA, USA). An reverse transcription–polymerase chain reaction technique [13] with minor modifications was used to confirm the results of the direct fluorescent antibody test from the supernatant of the third passage of each of the samples. The primers used for the amplifications were HCV 90 5′-CATGCCCATAGTAGGAC-3′ and HCV 368 5′-CCATGTGCCATGTACAG-3′. Cycling conditions were modified for set of primers used: rt reaction, 42 °C (60 minutes) and 94 °C (2 minutes); PCR reaction 94 °C (1 minute), 51 °C (1 minute), and 72 °C (30 seconds); and elongation reaction 72 °C (1 minute). Total RNA was extracted using TRIzol Reagent (Invitrogen Life Technology, Gran Island, NY, USA) according to the manufacturer's instructions. All RNA samples were stored in 30 μL of nuclease-free water at −80 °C until the rt-PCR was performed. Granulosa cells from the BVDV-exposed group were used as positive control because of the susceptibility confirmed to different BVDV strains [8,14]. The amplified products were separated by electrophoresis in a 2% agarose gel and the DNA bands visualized by ethidium bromide staining. The expected amplification product was of 283 bp.

Before the experiments, follicular fluid recovered by aspiration of follicles from each batch of ovaries was routinely tested for the presence of anti-BVDV antibody by virus neutralization using the NADL (type 1 cp-BVDV strain), according to Galik et al. [11]. The experiments were repeated four times. Appropriate noninfected negative controls were used through the steps of IVM.

Table 1
Detection of BVDV by virus isolation and rt-PCR.

Samples	Exposure group		Control group	
	VI	rt-PCR	VI	rt-PCR
Granulosa cells	Pos.	Pos.	Neg.	Neg.
ZP-intact mature oocytes (ZP+)	Pos.[a]	Pos.	Neg.[a]	Neg.
ZP-free mature oocytes (ZP−)	Pos.[a]	Pos.	Neg.[a]	Neg.
Last wash of oocytes (ZP−) (0.1 mL)	Neg.	Neg.	Neg.	Neg.

The total number of oocytes used was control group, n = 315, and BVDV-exposed group, n = 340. All samples were co-incubated in MDBK cells after IVM. The results reported in each column belong to the set of experiments repeated four times.
Abbreviations: Neg., negative; Pos., positive; rt-PCR, reverse transcription–polymerase chain reaction; VI, virus isolation; ZP, zona pellucida.
[a] Each sample contained four to five oocytes.

3. Results and discussion

The present study evaluated the effect of the exposure of COCs to an ncp-BVDV strain on nuclear maturation during IVM. No significant differences were observed in the percentage of oocytes with polar body between the control group and the BVDV-exposed group (71.07 ± 2.7%, n = 154, and 72.45 ± 2.5%, n = 162, respectively). Nevertheless, BVDV was isolated from granulosa cells, mature ZP− oocytes, and mature ZP+ oocytes of the BVDV-exposed group (Table 1). The virus was not isolated from the granulosa cells or oocytes from the control group. The virus was neither isolated from the corresponding wash fluids (HEPES–SOF) of oocyte groups (ZP−). These results were confirmed by reverse transcription–polymerase chain reaction (Table 1; Fig. 3).

Within the ovary, BVDV has been located in interstitial, luteal, granulosa, and thecal cells and in follicular fluid [14,15]. Although this work evaluated the susceptibility of COCs to an ncp-BVDV strain during IVM, the experimental design allowed us to simulate the outcome of an acute infection. Although the way in which BVDV compromises ovarian function is still uncertain, we may suggest some possible mechanisms: inadequate gonadotropic support, low levels of plasma estradiol associated with anestrus and ovulation failure, and a deficient ovarian leukocyte

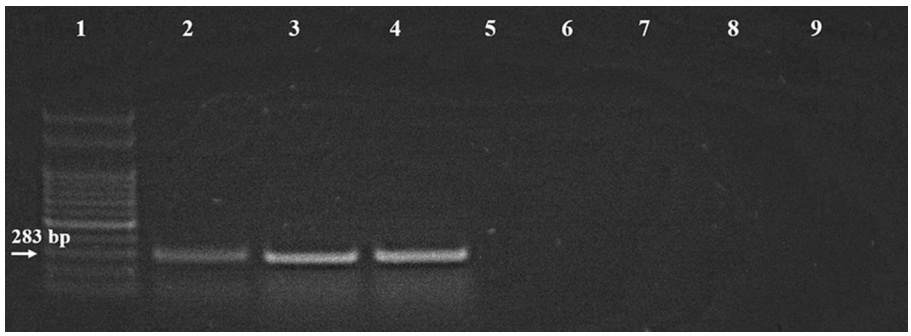

Fig. 3. Electrophoresis in 1.5% agarose gel. Lane 1: molecular weight markers; lanes 2 to 5 (exposed group): 2, granulosa cells; 3, ZP-intact mature oocytes (ZP+); 4, ZP-free mature oocytes (ZP−); 5, last wash of oocytes (ZP−); lanes 6 to 9 (control group): 6, granulosa cells; 7, ZP-intact mature oocytes (ZP+); 8, ZP-free mature oocytes (ZP−); 9, negative control (supernatant of MDBK cells unexposed to BVDV).

population because of the leukopenia that accompanies acute infections [16].

In this work, the virus was isolated from both oocytes with and without ZP after being exposed to an ncp-BVDV strain during IVM. It is relevant to highlight that the virus was isolated from ZP− oocytes. This result shows that the virus retained its infectivity because it was able to replicate in permissive MDBK cells (Fig. 3 in association with Fig. 2). These results are consistent with previous reports and provide new evidence on the susceptibility of mature oocytes to BVDV and its association with them [17–19].

The physical and chemical properties of the bovine ZP of *in vitro*–produced embryos determine the association of embryos with different viral pathogens [20]. Suzuki et al. [21] found that the ZP of immature oocytes is characterized by a network of numerous wide meshes and deep holes in their fine structure and that after maturation, it becomes thinner and the meshes and holes appear to be less deep. These authors also reported that most of these cell processes retract or disintegrate and that the matrix of the ZP closes the channels left by the cytoplasmic processes. Vanroose et al. [22] suggested that the pores on the outer surface of mature oocytes possibly mark the entries of these cell processes. In agreement with these results, other authors have proposed that viral penetration occurs along the channels left when the follicle cell processes are withdrawn [23]. In bovine oocytes, these pores have a mean diameter of 182 nm (nanometers), and the viral particles of BVDV are 45 to 55 nm. Therefore, the ZP surface is considered permeable enough to allow the entry of the virus [22]. Previous studies have shown that BVDV uses more than one receptor to bind to the host cell [24]; however, no studies have reported the presence of some of them in the membrane of germ cells involved in the interaction with BVDV. Some authors have proposed different routes by which BVDV may gain access to the oocytes during an acute infection. The main route proposed is through cumulus cells, which are permissive to BVDV infection [8]. The plasma membranes of cumulus cells and the oocyte are in intimate contact through cell processes that pass through the ZP. These processes may act as a channel through which BVDV can bypass the ZP and infect the oocyte [14]. This is consistent with that observed in our study, where the COCs were susceptible to an ncp-BVDV strain during IVM. It is also relevant to emphasize that the virus was not isolated from corresponding wash fluids (HEPES–SOF) of infected (ZP−) oocytes (Table 1), showing that the virus remains associated with the oocyte, ruling out a possible contamination. Current data show that only two viruses are able to penetrate the ZP of mammalian oocytes: the porcine parvovirus, which is a very small virus of only 20 nm in diameter and the Meningoencephalitis virus (27–28 nm) in mice. These viruses are able to pass through the ZP of early porcine embryos at the four- to eight-cell stage and the ZP of two-cell mouse embryos and morulae, respectively [25,26].

In the case of BVDV, the results obtained with *in vitro* infections are more controversial. Vanroose et al. [19] did not detect virus titer in *in vitro*–matured ZP− oocytes after exposure to the ncp-BVDV strain. Likewise, Tsuboi and Bielanski [27] determined that immature oocytes were unable to replicate the ncp-BVDV *in vitro* challenge after the 24-hour maturation period. However, these authors were unable to conclude whether the virus detected after the 24-hour maturation period was infectious and whether it could be transmitted to embryos after oocyte fertilization.

Although there are previous works on *in vitro* experimental infections of bovine oocytes, these were performed in oocytes with intact ZP (ZP+) and under different IVM conditions [17,18,28]. In the present study, the virus was isolated from ZP-free oocytes (ZP−) previously matured *in vitro* in the presence of an ncp-BVDV strain. This finding allows concluding that bovine oocytes are susceptible to the ncp-BVDV strain (genotype 1b) during IVM and that the virus retains its infectivity. Although the mechanism of interaction between BVDV and the ZP remains to be determined, we were able to define that the virus remains associated with the oocyte membrane (ooplasm). Further research is needed, however, to determine whether the virus is able to establish a productive infection in embryos produced from these oocytes.

Acknowledgments

We would like to thank Dra. Andrea Verna for the technical help and Dra. Maria Victoria Eusevi for language advice. This work was partially supported by FONCyT, grant

"PICT 2006-2410," and INTA Specific Projects AESA-203971 and AESA-203981.

Competing interests

The authors declare no conflicts of interest.

References

[1] Foote RH. Artificial insemination to cloning: tracing 50 years of research. Ithica, NY: The Internet-First University Press; 1998. Archive 001, 30 Apr 05 Books and Articles.
[2] Gard JA, Givens MD, Stringfellow DA. Bovine viral diarrhea virus (BVDV): epidemiologic concerns relative to semen and embryos. Theriogenology 2007;68:434–42.
[3] Givens MD, Galik PK, Riddell KP, Brock KV, Stringfellow DA. Replication and persistence of different strains of bovine viral diarrhea virus in an in vitro embryo production system. Theriogenology 2000;54:1093–107.
[4] Gard JA, Givens MD, Marley MS, Galik PK, Riddell KP, Stringfellow DA, et al. Bovine viral diarrhea virus (BVDV) associated with single *in vivo*-derived and *in vitro*-produced preimplantation bovine embryos following artificial exposure. Theriogenology 2009; 71:1238–44.
[5] González Altamiranda EA, Kaiser GG, Mucci NC, Verna AE, Campero CM, Odeón AC. Effect of Bovine Viral Diarrhea Virus on the ovarian functionality and *in vitro* reproductive performance of persistently infected heifers. Vet Microbiol 2013;165:326–32.
[6] Garoussi MT, Mehrzad J. Effect of bovine viral diarrhoea virus biotypes on adherence of sperm to oocytes during in-vitro fertilization in cattle. Theriogenology 2011;75:1067–75.
[7] Garoussi MT. The effects of cytopathic and noncytopathic bovine viral diarrhoea virus with sperm cells on in vitro fertilization of bovine oocytes. Vet Res Commun 2007;31:365–70.
[8] Booth PJ, Stevens DA, Collins ME, Brownlie J. Detection of bovine viral diarrhoea virus antigen and RNA in oviduct and granulosa cells of persistently infected cattle. J Reprod Fertil 1995;105:17–24.
[9] Stringfellow D. Recommendations for the sanitary handling of *in-vivo*-derived embryos. In: Stringfellow DA, Seidal SM, editors. Manual of the International Embryo Transfer Society. Third edition. Savoy, IL: IETS; 1998. p. 79–91.
[10] Stringfellow DA, Givens MD. Epidemiologic concerns relative to *in vivo* and *in vitro* production of livestock embryos. Anim Reprod Sci 2000;60–61:629–42.
[11] Galik PK, Givens MD, Stringfellow DA, Crichton EG, Bishop MD, Eilertsen KJ. Bovine viral diarrhea virus (BVDV) and anti-BVDV antibodies in pooled samples of follicular fluid. Theriogenology 2002; 57:1219–27.
[12] de Loos FA, Bevers MM, Dieleman SJ, Kruip TA. Morphology of preovulatory bovine follicles as related to oocytes maturation. Theriogenology 1991;35:527–35.
[13] Ridpath JF, Bolin SR. Differentiation of types 1a, 1b and 2 bovine viral diarrhoea virus (BVDV) by PCR. Mol Cell Probes 1998;12: 101–6.
[14] Fray MD, Prentice H, Clarke MC, Charleston B. Immunohistochemical evidence for the localization of bovine viral diarrhea virus, a single-stranded RNA virus, in ovarian oocytes in the cow. Vet Pathol 1998;35:253–9.
[15] Grooms DL, Ward LA, Brock KV. Morphologic changes and immunohistochemical detection of viral antigen in ovaries from cattle persistently infected with bovine viral diarrhea virus. Am J Vet Res 1996;57:830–3.
[16] Fray MD, Paton DJ, Alenius S. The effects of bovine viral diarrhea virus on cattle reproduction in relation to disease control. Anim Reprod Sci 2000;60–61:615–27.
[17] Bielanski A, Lutze-Wallace CL, Nadin-Davis S. Adherence of bovine viral diarrhea virus to bovine oocytes and embryos with a hardened zona pellucida cultured in vitro. Can J Vet Res 2003;67:48–51.
[18] Lalonde A, Bielanski A. Efficacy of the International Embryo Transfer Society (IETS) washing procedure for rendering oocytes matured *in vitro* free of bovine viral diarrhea virus (BVDV). Theriogenology 2011;76:261–6.
[19] Vanroose G, Nauwynck H, Van Soom A, Vanopdenbosch E, de Kruif A. Replication of cytopathic and noncytopahatic Bovine Viral Diarrhea virus in zona-free and zona-intact *in vitro*-produced bovine embryos and the effect on embryo quality. Biol Reprod 1998; 58:857–66.
[20] Stringfellow DA, Givens MD, Waldrop JG. Biosecurity issues associated with current and emerging embryo technologies. Reprod Fertil Dev 2004;16:1–10.
[21] Suzuki H, Yang X, Foote RH. Surface alterations of the bovine oocyte and its investments during and after maturation and fertilization *in vitro*. Mol Reprod Dev 1994;38:421–30.
[22] Vanroose G, Nauwynck H, Van Soom A, Ysebaert M-T, Charlier G, Van Oostveldt P, et al. Structural aspects of the zona pellucida of *in vitro* produced bovine embryos: a scanning electron and confocal laser scanning microscopic study. Biol Reprod 2000;62:463–9.
[23] Gwatkin RB. Effect of viruses on early mammalian development. III. Further studies concerning the interaction of Mengo encephalitis virus with mouse ova. Fertil Steril 1966;17:411–20.
[24] Iqbal M, McCauley JW. Identification of the glycosaminoglycan-binding site on the glycoprotein E(rns) of bovine viral diarrhoea virus by site-directed mutagenesis. J Gen Virol 2002;83:2153–9.
[25] Bane DP, James JE, Gradil CM, Molitor TW. *In vitro* exposure of preimplantation porcine embryos to porcine parvovirus. Theriogenology 1990;33:553–61.
[26] Gwatkin RB. Passage of mengovirus through the zona pellucida of the mouse morula. J Reprod Fertil 1967;13:577–8.
[27] Tsuboi T, Bielanski A. Resistance of immature bovine oocytes to non-cytopathogenic bovine viral diarrhoea virus *in vitro*. Vet Rec 2005;156:546–8.
[28] Bielanski A, Algire J, Lalonde A, Nadin-Davis S. Transmission of bovine viral diarrhea virus (BVDV) via *in vitro*-fertilized embryos to recipients, but not to their offspring. Theriogenology 2009;71: 499–508.