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Effect of bovine viral diarrhea virus biotypes exposure on bovine gametes in early embryonic development *in vitro*

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Abstract

Bovine viral diarrhea virus (BVDV) is an important viral agent causing reproductive failure in cattle. The objectives of the current study were to investigate the interaction between two BVDV biotypes, cytopathic (CP) and Non-cytopathic (NCP) and bovine gametes during *in vitro* fertilization (IVF) processing, the existence of the virus within embryonic cells and early embryonic development rates. Sperm and ova were exposed separately to CP and NCP BVDV at two concentrations of $10^{4.5}$ and $10^{5.5}$ tissue culture infectious dose 50.00% ($TCID_{50}$) mL^{-1} prior to IVF, respectively. After five days post-IVF, early embryonic development rates of infected groups were assessed. Several embryos of each group, normal and degenerated, were selected for a viral assay using reverse transcription polymerase chain reaction technique. The result showed that the early embryonic development rates were decreased in treatment groups. The rates in the CP groups were lower than the NCP groups. In the CP groups, the proportions were, respectively, 10.00, 6.00 and 11.00, and 6.00% in the infected sperm and oocyte groups ($10^{4.5}$ and $10^{5.5}$ $TCID_{50}$ mL^{-1}) that were higher than 50.00% in the control group. In NCP groups, the rates were, respectively, 25.00, 18.00 and 24.00, and 21.00% in the infected groups compared to 48.00% in the control group. In the CP groups, no BVDV was detected in normal embryos, whereas, all degenerated embryos were completely virus-positive. In the NCP groups, the virus was detected in both normal and degenerated embryos. In conclusion, this study supported detrimental impacts of CP and NCP BVDV on early embryonic development and the role of sperm and the zona pellucida layer as carriers of the virus.

Introduction

Bovine viral diarrhea virus (BVDV) is an important cattle pathogen that induces many destructive effects on health and production of livestock. BVDV is an RNA virus and a member of the genus *Pestivirus* within the family *Flaviviridae*. BVDV isolates can be classified in cytopathic (CP) and non-cytopathic (NCP) biotypes based on their effects on cell cultures.[1] Ova and embryos can become contaminated when exposed to microorganisms in follicular fluids or uterine cavities of naturally or experimentally infected donor.[2] Some small pathogens, mainly viral agents, can potentially penetrate and pass via the zona pellucida (ZP) layer before, during or after fertilization.[3] The BVDV biotypes have been shown to affect fertility during *in vitro* fertilization (IVF) following male and female contaminating processes.[4] Viruses can contaminate and multiply in cells during *in vitro* embryo production (IVEP) procedures. Likewise, contaminations can be introduced using untested semen, infected donors, infected ova and IVF by-products, particularly fetal bovine serum (FBS).[5] Viral contaminations are considered as one of the greatest risks, since they can be present without causing any cytopathic effects (CPEs) on cells. One of the most common viral agent is BVDV.[6] However, it was shown that BVDV could contaminate embryos without affecting their development.[7] According to a survey, more than 260 million doses of bull semen were produced worldwide which 95.00% were deep-frozen, 20 million were moved internationally[8] and more than 300,000 cryopreserved bovine embryos produced *in vivo* and *in vitro* were transferred.[9] Despite that numerous cases of embryo transfer (ET) are conducted worldwide over the years, only two cases of BVDV transmission by cryo-

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preserved embryos have been reported.[10,11] Therefore, there is the possibility that the virus penetrates the oocyte via sperm during fertilization and impresses the early embryonic development. Herein, the purpose of the current study was to investigate the role of male and female gametes distinctly as carriers of CP and NCP BVDV during IVF and the early embryonic development rates of embryos created by infected gametes.

Materials and Methods

All materials were prepared from Sigma-Aldrich (St. Louis, USA) unless otherwise stated and the catalog numbers were provided in parentheses. The NCP and CP BVDV and Razi bovine kidney (RBK) cell line were donated by Razi Vaccine and Serum Research Institute (Karaj, Iran). All animal resource materials used for IVEP including FBS, bovine serum albumin (BSA) and pools of follicular fluids collected from ovaries were evaluated for presence of the virus by reverse transcription polymerase chain reaction (RT-PCR).[12] They were also assessed in terms of anti-BVDV antibodies by micro-titration virus neutralization test.[13] Briefly, they were separately mixed with equal volumes (0.025 mL) of BVDV biotypes at the concentration of 10^2 tissue culture infectious dose 50.00% $TCID_{50}$ mL^{-1} in a 96-well culture plate (Nunclon, Roskilde, Denmark). The mixtures were incubated for 1 hr at 37.00 ˚C and 5.00% CO_2. Then, 0.05 mL of trypsinized RBK cells in Dulbecco's modified Eagle medium (DMEM; Gibco, Gand Island, USA) containing 20.00% fetal Bovine Serum (FBS; Gibco) were added to the plate. For CP biotype, the wells were read after five days incubation when CPEs were observable. For NCP biotype, an RT-PCR assay was performed following five days incubation. Finally, absence of BVDV antibodies were proved by trace of the biotypes.

Virus preparation and titration. The NCP and CP BVDV were propagated in RBK cells previously seeded in a T-25 tissue culture flask (Orange Scientific, Braine-l'Alleud, Belgium) containing DMEM supplemented with 10.00% FBS.[14] Then, the supernatant of the cells was collected following four succeeding passages and stored frozen in aliquots at – 80.00 ˚C. The CP titration was performed using the Reed and Muench method.[15] Briefly, a frozen aliquot of the virus was thawed. Four-fold dilutions of the virus (10^{-4} to 10^{-8}) in quadruplicate were arranged in serum-free DMEM. The dilutions were spilled into a 96-well culture plate containing monolayer RBK cells. The treated cells were examined in terms of CPEs for 72 hr. The NCP titration was performed similar to the CP, however, an RT-PCR[12] was used for viral assay.

Experimental designs. To determine whether CP or NCP BVDV could be penetrated into the oocyte by sperm or ZP during IVF and whether infected gametes could induce adverse effects on the early embryonic development, the sperm and ova were separately exposed to CP and NCP BVDV, for 2 hr prior to IVF in two experimental groups. The virus isolation and RT-PCR techniques were used to enrich and evaluate the presence of BVDV biotypes in embryonic cells. In the current study, embryos were cultured to the morula stage and the titers of CP and NCP BVDV used in each experiment were $10^{4.5}$ and $10^{5.5}$ $TCID_{50}$ mL^{-1}. Totally, 1,843 ova, 921 for CP and 922 for NCP groups, in quadruplicates were used for the experiments. However, 98 oocytes were excluded during IVEP processing. Twenty zygotes in each group were dyed for confirming ZP-sperm binding and fertilization. Briefly, the zygotes were transferred to cold ethanol containing 5.00 µg mL^{-1} of Hoechst 33342 for 15 min, mounted into a small droplet of glycerol on a glass slide and examined under a fluorescent microscope (Eclipse Ti; Nikon Tokyo, Japan).[16]

Experiment 1. First, the sperm was separately infected with $10^{4.5}$ and $10^{5.5}$ $TCID_{50}$ mL^{-1} of CP BVDV for 2 hr at 37.00 ˚C and was centrifuged three times at 300 *g* for 10 min in modified Tyrode's balanced salt solution (mTALP) containing 6.00 mg mL^{-1} fatty acid-free BSA (A6003).[17] Fertilization of the infected sperm and intact ova were implemented and the zygotes were cultured to the morula stage (day five post-IVF). The early embryonic development rates and presence of the virus in embryonic cells were evaluated. Next, the matured ova were distinctly contaminated with $10^{4.5}$ and $10^{5.5}$ $TCID_{50}$ mL^{-1} of CP BVDV for 2 hr at 37.00 ˚C. The infected ova were washed according to the method recommended by IETS[9] including 10 succeeding washes in groups of 10 or less in PBS containing 2.00% FBS accompanied by a trypsin treatment for 60 sec in Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}.[18] Fertilization of the infected ova and non-infected sperm were performed. The morula rates and existence of the virus in embryonic cells were assessed following five days post-IVF.

Experiment 2. First, the sperm was discretely infested with two concentrations of NCP BVDV ($10^{4.5}$ and $10^{5.5}$ $TCID_{50}$ mL^{-1}) for 2 hr at 37.00 ˚C. The contaminated sperm was centrifuged three times at 300 *g* for 10 min in mTALP medium.[17] The infected sperm and intact ova underwent IVF and the zygotes were cultured to the morula stage. The early embryonic development rates and the embryonic cells in terms of the virus were appraised. Next, the matured ova were separately polluted with two concentrations of NCP BVDV ($10^{4.5}$ and $10^{5.5}$ $TCID_{50}$ mL^{-1}) for 2 hr at 37.00 ˚C. The infected ova were washed based on the IETS procedure.[18] The fertilization of the infected ova and non-infected sperm was accomplished. The rates of early embryonic development and presence of the virus in embryonic cells were evaluated.

***In vitro* embryo production.** Slaughter-derived ovaries were transferred to the laboratory at 30.00 ˚C. They were washed in 0.90% physiological saline. Cumulus-oocyte complexes (COCs) were collected by aspiration of 2.00 to 8.00 mm ovarian follicles using an 18-gauge needle

connected to a 10.00-mL syringe in HEPES-buffered M199 medium with Hank's salts (Gibco) containing 2.00% FBS, 10.00 IU mL^{-1} heparin and 10.00 µL mL^{-1} antibiotic antimycotic solution. The COCs with a uniform granulated cytoplasm surrounding by at least three layers of cumulus cells[19] were washed according to the IETS method within a wash medium (aspiration medium without heparin) proceeded by a trypsin treatment for disinfecting of the virus and sanitary hazards.[18] Next, the COCs were matured in four-well culture plates (Nunclon™) containing bicarbonate-buffered M199 medium with Earle's salts (Gibco) supplemented with 10.00% FBS, 1.00 µg mL^{-1} estradiol, 1.00 IU mL^{-1} human chorionic gonadotrophin (hCG; Intervet, Boxmeer, The Netherlands), 2.00 U mL^{-1} pregnant mare serum gonadotrophin (PMSG; Intervet), and 10.00 ng mL^{-1} epidermal growth factor (EGF) for 24 hr at 38.00 ˚C and 5.00% CO_2. The sperm (0.50 mL) was prepared by the swim-up procedure.[20] The matured ova and motile sperm (approximately 1.00×10^6 mL^{-1}) underwent IVF in Tyrode's albumin lactate pyruvate (TALP) medium containing 6.00 mg mL^{-1} fatty acid-free bovine serum albumin (BSA; Grifols, Barcelona, Spain) 22.00 mg mL^{-1} sodium pyruvate, 20.00 µg mL^{-1} penicillamine, 10.00 M hypotaurine , 1.00 M epinephrine and 25.00 µg mL^{-1} heparin for 18 hr at 38.50 ˚C and 5.00% CO_2. Presumptive zygotes were denuded using a vortex machine and were incubated in modified synthetic oviductal fluid (mSOF) supplemented with 4.00 mg mL^{-1} fatty acid-free BSA, 100 ng mL^{-1} EGF, 5.00 µg mL^{-1} insulin, 5.00 ngmL^{-1} sodium selenite and 5.00 µg mL^{-1} transferrin for 4 days at 38.00 ˚C and 5.00% CO_2.[21] All culture media and chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Viral isolation and RT-PCR. To evaluate the viral contamination of embryos several embryos of each group, normal and degenerated, were selected following five days post-IVF. Briefly, ZP of the embryos was lysed with acid Tyrode's solution (Sigma-Aldrich).[22] The zona-free embryos were frozen and thawed twice for releasing the virus from embryonic cells.[23] Finally, the viral enrichment was performed in RBK cells and the supernatant of cells were used for the molecular assay. For RT-PCR, viral RNA was extracted using RNJia Virus Kit (Roje Technologies, Yazd, Iran). Briefly, 560 µL of buffer BFC-carrier RNA was poured into a 1.50 mL microcentrifuge tube and 140 µL of the cell supernatant was added to the tube. The mixture was mixed and incubated at room temperature for 10 min. 560 µL of ethanol (96.00 - 100%) was added to the mixture and mixed for 15 sec. The mixture (630 µL) was pipetted to a Hi-Pure VI column, placed in a 2.00 mL collection tube and centrifuged at 6,000 *g* for 1 min. Flow-through was discarded and the Hi-Pure DR column was placed back into the collection tube. A volume of 500 µL of BWB1 was added to the column and centrifuged at 6,000 *g* for 1 min. Flow-through was discarded and the Hi-Pure DR column was placed back into the collection tube. Five hundred microliter of BWB2 was added to the column and centrifuged at 20,000 *g* for 3 min. The column was placed in a clean 1.50 mL tube, 60.00 µL of ERR was added into the column and centrifuged at 6,000 *g* for 1 min. Eventually, the extracted RNA was stored at – 80.00 ˚C. The cDNA was synthesized using easy cDNA synthesis kit (Kiagene Fanavar, Tehran, Iran). Briefly, 8.00 µL of the extracted RNA, 10.00 µL of buffer-mix (2X) and 2.00 µL of enzyme mix were poured into a 200 µL microcentrifuge tube. RT-PCR was performed at 25.00 ˚C for 10 min 47.00 ˚C for 60 min and 85.00 ˚C for 5 min. The PCR was carried out in a total volume of 20.00 µL consisted of 8.00 µL of cDNA, 10.00 µL of Taq DNA polymerase master mix RED (Ampliqon, Odense, Denmark) and 1.00 µL of each primer (10.00 pmol mL^{-1}) within a 200 µL micro-centrifuge tube. The reaction was overlaid with 10.00 µL of mineral oil (Sigma-Aldrich) and heated in a thermocycler for 35 cycles of 95.00 ˚C for 40 sec, 56.00 ˚C for 30 sec and 72.00 ˚C for 30 sec. Nucleotide sequences of the primers included forward ATGCCCWTAGTA GGACTAGCA and reverse TCAACTCC ATGTGCCATGTAC.[12] PCR products were assayed by electrophoresis on a 1.60% (w/v) ultrapure agarose gel (Invitrogen, Carlsbad, USA), dyed with ethidium bromide stain and visualized under UV light (Uvidoc HD6; Uvitec, Cambridge, UK). The expected amplification product was 288 base-pair.

Statistical analysis. Data were analyzed using logistic regression (GENMOD procedure). All analyses were conducted in SAS Software (version 9.4; SAS Institute, Cary, USA). Differences at $p < 0.05$ were considered statistically significant.

Results

ZP-Sperm binding and fertilization. Regardless of the sperm count bounded to the ZP, ZP-sperm binding and pronuclei existence were confirmed in the most stained zygotes in all experimental and control groups (Fig. 1A).

Experiment 1 (Gametes infected with CP BVDV). The early embryonic development rates were decreased in all CP groups compared to the control group ($p < 0.05$). The proportions of CP-infected sperm and oocyte groups ($10^{4.5}$ and $10^{5.5}$ $TCID_{50}$ mL^{-1}) were shown in Table 1.

Experiment 2 (Gametes infected with NCP BVDV). The early embryonic development rates were reduced in all NCP groups compared to the control group ($p < 0.05$). The proportions of NCP-infected sperm and oocyte groups ($10^{4.5}$ and $10^{5.5}$ $TCID_{50}$ mL^{-1}) were shown in Table 1. In both experiments, the early embryonic development rates were diminished both in lower ($10^{4.5}$ $TCID_{50}$ mL^{-1}) and higher ($10^{5.5}$ $TCID_{50}$ mL^{-1}) viral titers ($p < 0.05$; Fig. 1B-1D). Furthermore, the rates in the CP groups were significantly lower than the NCP groups ($p < 0.05$; Fig. 2).

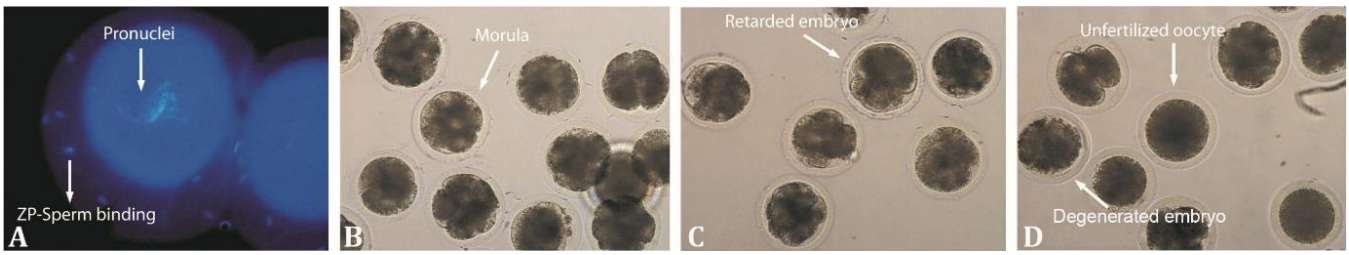

Fig. 1. A) A zygote stained with Hoechst 33,342 under a fluorescent microscope (200×). **B)** Control group. **C)** NCP-infected group **D)** CP-infected group. B-D indicate embryos after five days post-IVF (200×).

Table 1. Early embryonic development rate (%) of cytopathic (CP) and non-cytopathic (NCP) BVDV after 5 days post-IVF. The concentration of the sperm used for IVF was approximately 1.00×10^6 mL^{-1}.

BVDV Biotypes	Control	Treatment			
		Infected sperm ($TCID_{50}$ mL^{-1})		Infected oocyte ($TCID_{50}$ mL^{-1})	
		$10^{4.5}$	$10^{5.5}$	$10^{4.5}$	$10^{5.5}$
CP	49.72 (88/177)[a]	9.83 (17/173)[b]	6.22 (11/177)[b]	10.86 (19/175)[b]	6.25 (11/176)[b]
NCP	47.95 (82/171)[a]	25.15 (43/171)[b]	17.88 (32/179)[b]	24.29 (43/177)[b]	20.71 (35/169)[b]

[ab] Different superscripts in the same row indicate statistically significant differences ($p < 0.05$).

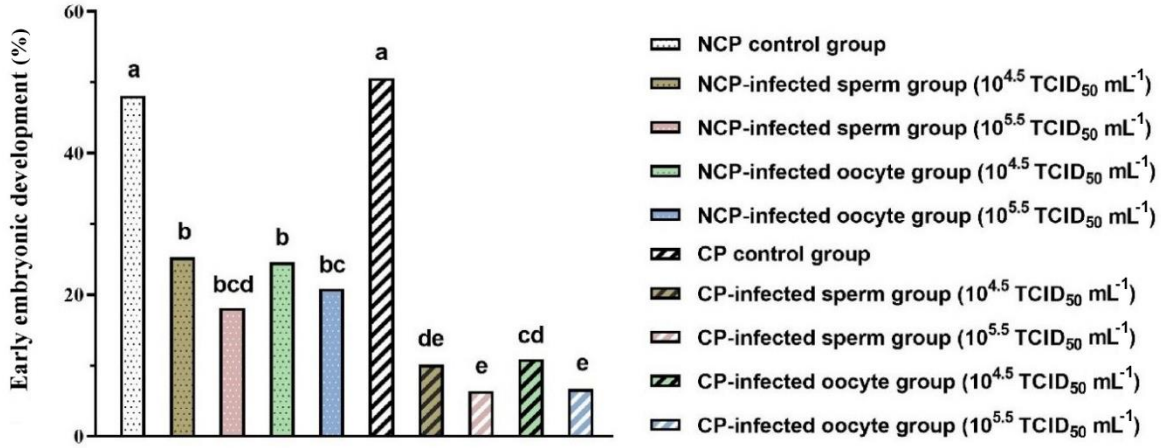

Fig. 2. Early embryonic development rates of cytopathic (CP) and non-cytopathic (NCP) BVDV groups after five days post-IVF.
[a-e] Different superscripts in the same row indicate statistically significant differences ($p < 0.05$).

Viral contamination of embryos. The result of viral isolation and RT-PCR in the embryos, normal and degenerated, clarified that in the CP groups no virus was detected in normal embryos resulting from the fertilization of infected and non-infected gametes, whereas, all degenerated embryos were virus-positive (Fig. 3A). In the NCP groups, the virus was detected in both normal and degenerated embryos (Fig. 3B). Besides, the embryos resulting from both infected male and female gametes were virus-positive.

Discussion

The microscopic evidences in our experiments showed that although ZP-sperm binding and fertilization had accrued and a significant decline was observed in the early embryonic development rates of CP and NCP-infected groups (Table 1). It indicated an interaction between BVDV biotypes and ZP. Thus, the biotypes could penetrate

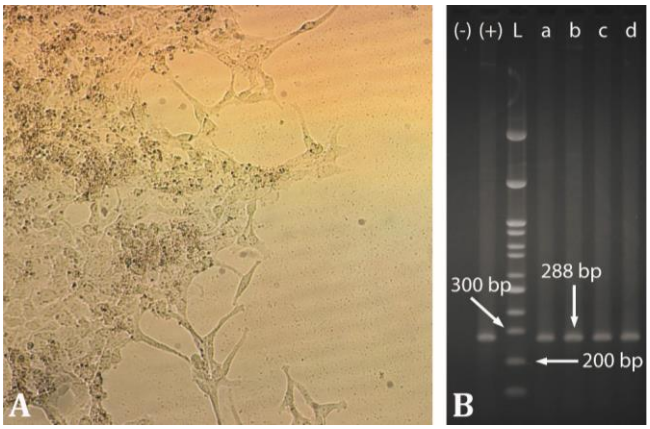

Fig. 3. A) The CP-induced CPEs in RBK cells resulted from viral isolation (150×). **B)** Molecular assay of NCP-degenerated embryos. Lanes (-) and (+): PCR-negative and positive control, respectively. Lane L: ladder 100 bp. Lanes a-d: Degenerated embryos resulted from male and female gametes infected with NCP BVDV. The expected amplification product was 288 bp.

the ZP during IVF and resulted in embryonic death. Additionally, adverse effects of the biotypes on early embryonic development rates were associated with both infected sperm and infected ZP. In fact, in both circumstances, the ZP could not act as a protective barrier against the virus during IVF. According to other reports, viruses such as BHV-1 and BVDV could adhere to heparin receptors of sperm and disturb the sperm capacitation and ZP-sperm binding.[24,25] Thus, it is a common practice to use discontinuous gradient centrifugations and swim-up procedures to remove or reduce the load of various viruses in frozen-thawed semen prior to fertilization. Moreover, it has been shown that this procedure could decrease the risk of disease transmission to recipients and contaminations of IVEP systems in the human and animals.[26,27] Mammalian ova and preimplantation-stage embryos are surrounded by ZP which is a glycoprotein acellular matrix. The ZP plays important roles in many physiological functions including fertilization, blocking polyspermy, transporting embryos through oviducts and preserving blastomers.[28] Furthermore, the ZP acts as a protective shell against infections of embryonic cells and the spread of livestock diseases through ET practices.[3] Some authors proposed that manipulations such as eliminating cumulus cells and washing embryos could lead to virus inactivation.[29] In contrast, some others believed that the ZP acts a viral carrier[30,31], and can lead to contaminating embryos.[32] These conflicting results are likely related to viral strains. The genotypic variances of strains might cause high affinity to their receptors.[33,34] These features might affect the intensity of responses to the virus. Several reports demonstrated that BVDV was embedded into outer layer holes of ZP and fissures were shaped with sperm enzymatic digestion during IVF. However, these paths were blocked swiftly and it was unlikely that non-motile BVDV could penetrate the ZP.[35,36] Therefore, the role of sperm as a viral carrier is more prominent.[37] Although the ZP was intact in the current study, BVDV biotypes could impress IVEP processing. This was confirmed by virus detection inside the degenerated embryos using RT-PCR (Fig. 3). Nevertheless, Vanroose *et al*. distinguished that no viral replication without signs of embryo degeneration was detected in the ZP-intact embryos, though the virus could infect ZP-free embryos without affecting the embryo development *in-vitro*.[7] Totally, the effects of CP has been much more severe than NCP. This effect was due to cytopathic effects of CP which could induce cell apoptosis in early developmental stages of the embryos. Consequently, it was shown that CP and NCP BVDV could penetrate the ZP during IVF. Although the developmental rates of infected embryos were declined, several morphologically normal embryos, infected and non-infected were formed, particularly in NCP BVDV.

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Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

1. Hopper RM, King EH. Evaluation of breeding soundness: Basic examination of the semen.In: Hopper RM (Ed). Bovine reproduction. New Jersey, USA: John Wiley & Sons, Inc. 2014; 68-78.
2. Yavru S, Kale M, Gulay M, et al. Effects of bovine viral diarrhoea virus on the fertility of cows. Acta Vet Hung 2013; 61(2): 281-289.
3. Van Soom A, Wrathall AE, Herrler A, et al. Is the zona pellucida an efficient barrier to viral infection? Reprod Fertil Dev 2010; 22(1): 21-31.
4. 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(6): 1067-1075.
5. Gard JA, Givens MD, Stringfellow DA. Bovine viral diarrhea virus (BVDV): epidemiologic concerns relative to semen and embryos. Theriogenology 2007; 68(3): 434-442.
6. Gard JA. Research challenges involving embryo pathogen interactions. Anim Reprod 2015; 12(3): 444-449.
7. Vanroose G, Nauwynck H, Van Soom A, et al. Replication of cytopathic and noncytopathic 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(3): 857-866.
8. Thibier M, Wagner HG. World statistics for artificial insemination in cattle. Livest Prod Sci 2002; 74(2): 203-212.
9. Stroud B. IETS 2011 statistics and data retrieval committee report. The year 2010 worldwide statistics of embryo transfer in domestic farm animals. Available at:https://www.iets.org/Portals/0/Documents/Public/Committees/DRC/December2011.pdf. Accessed 02 Feb, 2023.
10. Lindberg A, Ortman K, Alenius S. Seroconversion to bovine viral diarrhea virus (BVDV) in dairy heifers after embryo transfer. In proceedings: 14th International

congress on animal reproduction. Stockholm, Sweden 2000; 250.
11. Nettleton PF, Gunn G. BVD virus genotype 2 in British cattle. Vet Rec 2002; 151(20): 616.
12. Vilcek S, Herring AJ, Herring JA, et al. Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. Arch Virol 1994; 136(3-4): 309-323.
13. Deregt D, Smithson S, Kozub GC. A short incubation serum neutralization test for bovine viral diarrhea virus. Can J Vet Res 1992; 56(2): 161-164.
14. Fay PC, Cook CG, Wijesiriwardana N, et al. Madin-Darby bovine kidney (MDBK) cells are a suitable cell line for the propagation and study of the bovine poxvirus lumpy skin disease virus. J Virol Methods 2020; 285: 113943. doi: 10.1016/j.jviromet.2020.113943.
15. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. Am J Epidemiol 1938; 27(3): 493-497.
16. Al-Essawe E, Wallgren M, Wulf M, et al. Addition of seminal plasma reduces binding of stallion spermatozoa to bovine oocytes. In proceedings: 33rd meeting of the association of embryo transfer in Europe. Bath, UK 2017; 910.
17. 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(3): 365-370.
18. Ponsart C, Pozzi N. Sanitary requirements for bovine gametes and embryos in international trade. Anim Reprod 2013; 10(3): 283-296.
19. Madison V, Avery B, Greve T. Selection of immature bovine oocytes for developmental potential *in vitro*. Anim Reprod Sci 1992; 27(1): 1-11.
20. Magdanz V, Boryshpolets S, Ridzewski C, et al. The motility-based swim-up technique separates bull sperm based on differences in metabolic rates and tail length. PLoS One 2019; 14(10): e0223576. doi: 10.1371/journal.pone.0223576.
21. Wei Y, Idrees M, Sidrat T, et al. BOEC–Exo addition promotes *in vitro* maturation of bovine oocyte and enhances the developmental competence of early embryos. Animals (Basel) 2022; 12(4): 424. doi: 10.3390/ani12040424.
22. Saini S, Ansari S, Sharma V, et al. Folate receptor-1 is vital for developmental competence of goat embryos. Reprod Domest Anim 2022; 57(5): 541-549.
23. Afshar A, Dulac GC, Dubuc C, et al. Comparative evaluation of the fluorescent antibody test and microtiter immunoperoxidase assay for detection of bovine viral diarrhea virus from bull semen. Can J Vet Res 1991; 55(1): 91-93.
24. Okazaki K, Honda E, Kono Y. Heparin-binding domain of bovid herpesvirus 1 glycoprotein gIII. Arch Virol 1994; 134(3-4): 413-419.
25. Zanrosso D. The hidden risk: transmission of pathogens micro by mean of embryo transfer in cattle [Italian]. Bachelor Thesis. University of Parma, Parma, Italy: 2019.
26. Maertens A, Bourlet T, Plotton N, et al. Validation of safety procedures for the cryopreservation of semen contaminated with hepatitis C virus in assisted reproductive technology. Hum Reprod 2004; 19(7): 1554-1557.
27. Morfeld KA, White B, Mills G, et al. 186 A novel method for eliminating porcine reproductive and respiratory syndrome virus from boar semen and its effects on embryo development. Reprod Fertil Dev 2004; 17: 243-243.
28. Boccaccio A, Frassanito MC, Lamberti L, et al. Nanoscale characterization of the biomechanical hardening of bovine zona pellucida. J R Soc Interface 2012; 9(76): 2871-2882.
29. Bielanski A, Dubuc C. *In vitro* fertilization of ova from cows experimentally infected with a non-cytopathic strain of bovine viral diarrhea virus. Anim Reprod Sci 1995; 38(3): 215-221.
30. Blondin P. Health and safety of IVF embryos: challenges for the international ET industry. Anim Reprod 2014; 11(3): 270-277.
31. Queiroz-Castro VLD, da Costa EP, Alves SVP, et al. Bovine herpesvirus 1 can cross the intact zona pellucida of bovine oocytes after artificial infection. PLoS One 2019; 14: e0218963. doi: 10.1371/journal.pone.0218963.
32. Wrathall AE, Simmons HA, Van Soom A. Evaluation of risks of viral transmission to recipients of bovine embryos arising from fertilisation with virus-infected semen. Theriogenology 2006; 65(2): 247-274.
33. Brodersen BW. Bovine viral diarrhea virus infections: manifestations of infection and recent advances in understanding pathogenesis and control. Vet Pathol 2014; 51(2): 453-464.
34. Ambrose RK, Gravel JL, Commins MA, et al. In vivo characterisation of five strains of bovine viral diarrhoea virus 1 (Subgenotype 1c). Pathogens 2018; 7(1): 12. doi: 10.3390/pathogens7010012.
35. 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(4): 421-430.
36. Vanroose G, Nauwynck H, Van Soom A, 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(2): 463-469.
37. Stringfellow DA, Givens MD. Infectious agents in bovine embryo production: hazards and solutions. Theriogenology 2000; 53(1): 85-94.