Evaluation of the effectiveness of semen processing techniques to remove bovine viral diarrhea virus from experimentally contaminated semen samples

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A B S T R A C T

The aim of this study was to evaluate the capacity of three semen processing techniques, Percoll gradient centrifugation, Swim-up and a combination of Swim-up and Percoll gradient centrifugation, to reduce the viral load of bovine viral diarrhea virus (BVDV) in experimentally infected semen samples. The evaluation was performed using two approaches: first, searching for the presence of virus in the processed samples (*via* virus titration and RT-PCR) and second, ascertaining the possible interference on *in vitro* embryo production. The sperm count and DNA integrity (Comet assay) of the processed samples were analyzed (Experiment 1). The amount of virus in the processed samples was determined by titration in cell culture (Experiment 2). The samples processed by Swim up/Percoll gradient centrifugation were utilized for *in vitro* embryo production, and the embryos produced were tested for BVDV by RT-PCR (Experiment 3). Sperm concentration, Comet assay and embryo production were analyzed by chi-squared tests ($P < 0.05$). There was a significant difference between sperm separation techniques when the sperm count and Comet assay were analyzed. The sperm count obtained from the Swim up/Percoll gradient centrifugation group was lower than that obtained in either of the two other groups (Swim up and Percoll gradient centrifugation), and the Comet assay showed that the combination of the two semen processing techniques (Swim up/Percoll gradient) produced a 1.1% prevalence of Comet level 2, which was not observed in the other groups. The BVDV titer ($10^{6.68}$ TCID$_{50}$/mL) added to experimentally infected semen samples decreased after Percoll gradient centrifugation to $10^{2.3}$–10^1 TCID$_{50}$/mL; for the Swim up group, the titer range was $10^{3.3}$–$10^{1.87}$ TCID$_{50}$/mL, and in the Swim up/Percoll gradient centrifugation group, BVDV was undetectable. The decreases in titer varied from 99.9% in the Swim up-processed group to 100% in the Swim up/Percoll gradient centrifugation group. *In vitro* embryo production displayed similar blastocyst development rates among all groups, and RT-PCR was negative for the produced embryos. The data showed that the combination of Swim up/Percoll gradient centrifugation promoted the elimination of BVDV from the semen samples without damaging spermatozoa cells and also allowed successful *in vitro* embryo production free of BVDV. Hence, the risk of BVDV contamination is negligible for the embryo recipient.

1. Introduction

Bovine viral diarrhea virus (BVDV) has a broad distribution among cattle populations. It is associated with cells and fluids from infected animals and represents a potential hazard in the application of assisted reproduction techniques (Gard et al., 2007, 2009). BVDV is a single-stranded, enveloped RNA virus from the family *Flaviviridae* (Ridpath and Flores, 2007). It has two distinct genotypes (1 and 2) and two biotypes (cytopathic and noncytopathic) (Ridpath and Flores, 2007). This virus causes both clinical and subclinical disease as well as a spectrum of clinical symptoms that include leucopenia, fever, depression, anorexia, excessive salivation and nasal discharge, ulcers of the nose, mouth and muzzle, diarrhea,

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dehydration, respiratory disease, early embryonic death, teratogenesis and abortion (Ridpath and Flores, 2007). Although acute BVDV infections are the most common, persistent infections can also occur when a fetus is exposed to a noncytopathic biotype prior to developing immune competence (Houe, 1999; Gard et al., 2009). The virus is maintained in persistently infected animals, which are the main source of virus in a herd (Brock et al., 1991; Deregt and Loewen, 1995). Additionally, it is important to note that BVDV causes significant economic losses due to decreases in productivity and to reproductive failure of infected animals (Gregg et al., 2010). These facts corroborated the need to develop safe manipulation protocols for application in assisted reproduction techniques. Reports indicate that bulls infected either acutely or persistently with BVDV are capable of shedding virus in their semen (Kirkland et al., 1997; Givens et al., 2006; Walz et al., 2008) and can transmit the virus to cows *via* insemination (Kirkland et al., 1997; Gard et al., 2007). Persistently infected bulls shed large amounts of virus ($10^{7.6}$ $TCID_{50}$/mL) in their seminal fluid, and the virus may survive both cryopreservation and processing of semen for artificial insemination (Kirkland et al., 1991; McGowan and Kirkland, 1995; Walz et al., 2008). Although the virus was found to be present in the semen of both acutely and persistently infected bulls, it was not determined whether the virus was within the spermatozoa, free within the seminal plasma or within the non-sperm cellular components (Wrathall et al., 2006).

A study by Bielanski et al. (1992) concluded that none of the simple physical methods used for the separation of sperm from semen prior to *in vitro* fertilization completely removed BVDV from the sperm samples. Five methods were examined: washing, Swim up, glass bead filtration, glass wool filtration and Percoll gradient centrifugation. Viral detection was performed by virus isolation in cell culture followed by virus titration. However, Semprini and Levi-Setti (1992) showed that Percoll gradient centrifugation followed by Swim up effectively removed infected cells with HIV-1 from the semen of HIV-seropositive men. Since then, various forms of sperm washing have been developed (Bielanski, 2007). Of all the methods available for disinfecting contaminated semen samples, such as antibiotics or medium acidification, washing procedures may be used for controlling and eliminating microorganisms (Bielanski, 2007). The aim of the present study was to evaluate the capacity of the semen processing techniques, Percoll gradient centrifugation, Swim up and a combination of these, to reduce or eliminate BVDV from experimentally infected semen samples. Two approaches for evaluation were used: first, searching for the presence of virus in the final pellet of processed samples (*via* virus titration and RT-PCR) and second, evaluation of possible interference of BVDV on *in vitro* embryo production.

2. Materials and methods

2.1. Manufacturer of the chemicals

Unless otherwise indicated in the text, the reagents used were from Sigma Chemical (St. Louis, MO, USA), and the catalog numbers are provided in parentheses.

2.2. Experimental design

2.2.1. Experiment 1

Swim up and Percoll gradient centrifugation are based on a sequence of centrifugation steps that can affect both the sperm cell count and sperm function (Fatehi et al., 2006). The aim of Experiment 1 was to evaluate the quantity and quality of spermatozoa following processing with Swim up and Percoll gradient centrifugation by sperm count and DNA integrity analysis (Comet assay).

The experimental groups for the quantity and quality of spermatozoa and sperm count were as follows: semen samples free of BVDV and semen samples experimentally infected with BVDV. The Comet assay was performed only in semen samples free of BVDV.

Total sperm count was obtained using a Neubauer improved chamber (New Optik, Lancing, UK) (CBRA, 1998). The Comet assay, which ascertains the integrity of DNA, is based on the ability to visualize alterations in nuclear DNA, such as fragmentation, from the appearance of a trail of DNA loops from the nucleus after electrophoresis (Singh et al., 1988).

2.2.2. Experiment 2

The aim of Experiment 2 was to test the potential of semen processing techniques to eliminate or reduce the viral load of experimentally introduced BVDV ($10^{6.68}$ $TCID_{50}$/mL) in semen samples. The experimental groups were as follows: semen samples free of BVDV and semen samples experimentally infected with BVDV, both of which were processed by Percoll gradient centrifugation, Swim up or Swim up followed by Percoll gradient centrifugation.

2.2.3. Experiment 3

The aim of Experiment 3 was to test the viability of the sperm cells obtained to produce bovine embryos *in vitro* after processing *via* Swim up followed by Percoll gradient centrifugation. The experimental groups were as follows: control 1 – *in vitro* embryo production using semen (free of BVDV) processed by Swim up alone; control 2 – *in vitro* embryo production with semen (free of BVDV) processed by Swim up and then by Percoll gradient centrifugation; and the BVDV group–*In vitro* embryo production with semen (experimentally contaminated with BVDV) processed by Swim up followed by Percoll gradient centrifugation. The blastocysts produced were tested for the presence of BVDV by RT-PCR.

2.3. Semen and semen processing techniques

The semen samples used were provided by an Insemination Center from one bull that has been previously tested as giving satisfactory results in terms of *in vitro* embryo production (BVDV negative). Frozen 0.5 mL semen samples had 80 million spermatozoa/mL.

The samples for Swim up processing contained 0.3 mL thawed semen under 1 mL Sperm-TALP prepared with 99.93 mM NaCl (S5886), 3.1 mM KCl (P5405), 2.9 mM NaH_2PO_4 (S5010), 2 mM $CaCl_2$-$2H_2O$ (C7902), 0.4 mM $MgCl_2$-$6H_2O$ (M2393), 0.03 mM phenol red (P5530), 21.69 mM sodium lactate (L1375), 10 mM HEPES (H6147), 25 mM $NaHCO_3$ (S5761), 5 μg/mL gentamicin (G1264), 1 mM sodium pyruvate (P4562) and 0.6 mM bovine serum albumin (GIBCO, Invitrogen, NY, USA). After incubation for 60 min at 38.5 °C at a 45° incline, 0.8 mL of supernatant was collected and centrifuged at 200 × g for 10 min.

Percoll gradient centrifugation (45–90%; Nutricell, Campinas, SP, Brazil) was performed by layering a thawed semen straw (0.5 mL) on top of a 0.5 mL 45% Percoll gradient that was above a 0.5 mL 90% Percoll gradient. The tube was centrifuged at 200 × g for 10 min at room temperature. The supernatant was removed, and the pellet was washed once in Sperm-TALP with centrifugation (200 × g for 10 min).

Previous testing indicated that the combination of both techniques yields the best recovery of spermatozoa when Swim up processing is performed first and the resulting pellet is processed with the Percoll gradient centrifugation method described above.

2.4. Comet assay

The Comet assay is a microgel electrophoresis technique. Cells with greater DNA damage increase the migration of DNA fragments

from the nucleus to the anode (ranging from an intact nucleus – grade 0 to a totally damaged nucleus – grade 4), which then causes a comet-like tail to appear (Singh et al., 1988; Collins et al., 1997). Normal melting point agarose (A9539) and low melting point agarose (A9414) were dissolved in PBS by boiling 3 times. Glass slides were immersed in a 1.5% solution of normal melting point agarose and dried overnight at room temperature. The sample mixture was prepared by mixing 5 μL of spermatozoa suspension in SPERM-TALP with 125 μL of 0.5% low melting point agarose. Next, 130 μL of the sample mixture was pipetted onto the agarose-coated slide and covered with a coverslip (24 mm × 24 mm). The slide was cooled at 4 °C for 5 min to allow the sample mixture to solidify, and then the coverslip was removed. The slide was immersed in lysis buffer (2.5 M NaCl (S5886), 100 mM EDTA (E6758), 10 mM Tris (442833), 1% Triton X-100 (T8532), and 10% DMSO (C6164)) at 4 °C in the dark until use (storage time ranging from 2 h to 14 days). The slides were drained and then placed in a horizontal electrophoresis unit (Bio-Rad, Hercules, CA, USA), which was filled with fresh alkaline electrophoresis buffer containing 300 mM NaOH (38210). Electrophoresis was performed at 25 V and 300 mA for 30 min. The slides were neutralized in 0.4 M Tris–HCl (258148) (pH 7.5), stained with ethidium bromide (20 mg/mL) (46065) and mounted with a coverslip. The slides were analyzed at 200× magnification using an epifluorescence microscope (Carl Zeiss, Göttingen, Germany). Sperm nuclei with mobile fragments of DNA were classified as comets (Singh et al., 1988).

2.5. Oocyte collection and in vitro maturation

Bovine ovaries were collected immediately after slaughter at a commercial abattoir and transported to the laboratory (29 ± 2 °C) in modified phosphate-buffered saline (mPBS). The ovaries were washed with fresh mPBS before follicle aspiration. Cumulus-oophorus complexes (COCs) were aspirated from ovarian follicles 2–8 mm in diameter. Oocytes surrounded by multilayers of compact follicular cells and having a uniform cytoplasm (classified as grade I, II and III) were selected for the experiment (Gonçalves et al., 2002). The COCs were washed three times in maturation medium, then matured in 100 μL drops of maturation medium, TCM199 (M2520), containing 0.68 mM L-glutamine and 25 mM HEPES supplemented with 0.2 mM sodium pyruvate (P4562), 26.2 mM NaHCO$_3$ (S5761), 50 μg/mL estradiol-17β, 0.5 μg/mL FSH (Folltropin, Vetrefarm, Ontario, Canada), 0.03 IU/mL hCG (Profasi, MerckSerono, Geneva, Switzerland) and 10% (v/v) fetal calf serum (Nutricell, Campinas, SP, Brazil) under mineral oil (M8410), and then incubated at 38.5 °C in a humidified atmosphere in 5% CO$_2$ in air for 24 h. The COCs were cultured in groups of 15 per drop of in vitro maturation medium.

2.6. In vitro embryo production (in vitro fertilization and culture)

Spermatozoa were selected using Swim up alone or Swim up followed by Percoll gradient centrifugation processing, and the final concentration of sperm was adjusted to 1×10^6 sperm/mL per 100 μL medium drop. Before fertilization, the COCs were washed three times and fertilization was performed in Fert-TALP medium (113.93 mM NaCl (S5886), 3.21 mM KCl (P5405), 2.9 mM NaH$_2$PO$_4$ (S5010), 2 mM CaCl$_2$·2H$_2$O (C7902), 0.78 mM MgCl$_2$·6H$_2$O (M2393), 0.03 mM phenol red (P5530), 10 mM sodium lactate (L1375), 3 mg/mL penicillamine (P5125), 25 mM NaHCO$_3$ (S5761), 5 μg/mL gentamicin (G1264), 0.25 mM sodium pyruvate (P4562), 10 μM hypotaurine (H1384), 1 IU/mL heparin (H3149), 1 μM epinephrine (E4250) and 0.6 mM bovine serum albumin (GIBCO, Invitrogen, NY, USA). The gametes were stored at 38.5 °C in a humidified 5% CO$_2$ atmosphere in air for 18–22 h, after which the presumptive zygotes were mechanically stripped of cumulus

cells, washed three times and cultured in SOF medium (107.7 mM NaCl (S5886), 7.16 mM KCl (P5405), 1.71 mM CaCl$_2$·2H$_2$O (C7902), 0.49 mM MgCl$_2$·6H$_2$O (M2393), 1.19 mM KH$_2$PO$_4$ (P5655), 0.03 mM phenol red (P5530), 2 mM sodium lactate (L1375), 25.07 mM NaHCO$_3$ (S5761), 1.5 mM glucose (G6152), 5 μg/mL gentamicin (G1264), 0.33 mM sodium pyruvate (P4562), 1 mM glutamine-L (G5763), 2% BMEaa (B6766), 1% MEMaa (M7145) and 10% fetal calf serum (Nutricell, Campinas, SP, Brazil). Fifteen presumptive zygotes were cultured per drop at 38.5 °C for 8 days in a humidified atmosphere supplemented with 5% CO$_2$, 5% O$_2$ and 90% N$_2$. For both the negative control and BVDV groups, the blastocysts produced were split into groups of 3–5 for RT-PCR.

2.7. Virus titration

Ninety-six-well flat-bottom plates were seeded with 150 μL MDBK cells (1×10^6 cells/well), 50 μL E-MEM (GIBCO, Invitrogen, NY, USA) and 50 μL of a serial dilution of the sample (10^{-1} to 10^{-8}) and incubated at 37 °C for 5 days under 5% CO$_2$. Following the incubation period, the plate was observed under an optical microscope for evaluation of a cytopathic effect. The viral titer was assessed as previously described (Reed and Muench, 1938).

2.8. RT-PCR

TRIzol® LS Reagent (Invitrogen, NY, USA) was used according to the manufacturer's instructions for total RNA extraction. After RNA extraction, cDNA was synthesized using the SuperScript™ One-Step RT-PCR with Platinum® Taq (Invitrogen, NY, USA) at 65 °C for 5 min, then 55 °C for 60 min and, finally, 70 °C for 15 min. PCR was performed using primers 324 and 326 (Vilcek et al., 1994). PCR products were analyzed by electrophoresis carried out on a 2% agarose gel dyed with Blue Green Loading Dye I (LGC Biotecnologia, SP, Brazil) and visualized under UV light.

2.9. Materials free of BVDV

Bovine serum albumin (BSA) (GIBCO, Invitrogen, NY, USA), fetal calf serum (Nutricell, Campinas, SP, Brazil), frozen commercial semen straws (0.5 mL) and a pool of follicular fluid of each oocyte retrieval collection were free of BVDV as tested by virus isolation and RT-PCR.

2.10. Virus samples

Stocks of the cytopathic strain NADL (National Animal Disease Laboratory, Iowa, USA) were propagated in Madin-Darby bovine kidney (MDBK) cells, which were cultured in E-MEM (GIBCO, Invitrogen, NY, USA). The virus stock was aliquoted and stored at −80 °C until needed. The initial viral titer was $10^{6.68}$ TCID$_{50}$/mL.

2.11. Experimental contamination of semen samples

Semen samples were thawed in a water bath at 37 °C for 30 s, and the viral samples were thawed in a water bath at 37 °C until the ice in the tube was completely melted. The experimental infection was performed by gently mixing semen 1:1 (v/v) with a viral suspension (titer $10^{6.68}$ TCID$_{50}$/mL) in Eagle's Minimal Essential Medium (E-MEM) (GIBCO, Invitrogen, NY, USA) (Garoussi, 2007). For the control group, semen was gently mixed 1:1 (v/v) with E-MEM (GIBCO, Invitrogen, NY, USA). Then, the samples were incubated at 38.5 °C for 1 h under 5% CO$_2$ before initiating the procedures. The final pellet of each separation procedure was tested by virus titration (previously described in detail in Section 2.7).

Table 1
Sperm count after semen processing techniques.

Groups	Semen processing techniques (million spermatozoa/mL)		
	Percoll gradient centrifugation	Swim up	Swim up/Percoll gradient centrifugation
Control	42.38[Aa]	26.13[Ba]	18.88[Ca]
BVDV	21.5[Ab]	13.25[Bb]	14.38[Cb]

Mean of 8 replications; capital superscripts denote significant differences among rows (Percoll gradient centrifugation × Swim up × Swim up/Percoll gradient centrifugation) $P < 0.05$; lowercase superscripts denote significant differences among columns (Control × BVDV) $P < 0.05$.

2.12. Statistical analysis

The sperm concentration was analyzed by chi-squared tests ($P < 0.05$) first between the control and the BVDV group and then as a function of the three processing conditions, Percoll gradient centrifugation, Swim up and Swim up/Percoll gradient centrifugation, for the control and BVDV group (Table 1). Comet assay levels were analyzed by chi-squared tests ($P < 0.05$) as a function of the pre-processing and processing conditions (Table 2). Embryo production was analyzed by chi-squared tests ($P < 0.05$) as a function of the cleavage and blastocyst rates obtained in the control 1, control 2 and BVDV groups (Table 4).

3. Results

3.1. Experiment 1

After the semen samples were submitted to one of three processing techniques (Swim up, Percoll gradient centrifugation and Swim up followed by Percoll gradient centrifugation), the sperm count (ranging between 13.25 and 42.38 million spermatozoa/mL) and the motility (90% mean motile spermatozoa) were normal. Additionally, the samples exhibited low fragmentation rates, based on the Comet level 2 obtained in only 1.1% of the spermatozoa following processing in the group with the Swim up method followed by Percoll gradient centrifugation.

Data from the evaluation of possible damage caused by the different semen processing techniques tested (Swim up, Percoll gradient centrifugation and Swim up followed by Percoll gradient centrifugation) are presented in Table 1 (sperm count) and Table 2 (Comet assay). The mean motility of the semen samples after processing was 90%.

3.2. Experiment 2

Neither the Swim up method nor the Percoll gradient centrifugation processing alone could completely eliminate BVDV from infected samples because each method resulted in a clear cytopathic effect on the cells. For the Swim up group, the percent reduction in virus titer was between 99.9583% and 99.9985%, and the percent reduction for the Percoll gradient centrifugation group was between 99.9958% and 99.9998%. After using a combination of Swim up and Percoll gradient centrifugation protocols, it was not possible to detect BVDV in the cell culture, indicative of a 100% reduction (Table 3).

3.3. Experiment 3

The *in vitro* embryo production after processing by Swim up followed by Percoll gradient centrifugation displayed normal cleavage and blastocyst rates in all groups (Table 4). RT-PCR was negative in both Control 2 and the infected groups.

4. Discussion

BVDV is known to pose a substantial threat to the application of embryo technologies. In addition, the viral load is likely to be amplified during the culture period of *in vitro* embryo production (Gard et al., 2007). Therefore, it is important either to decrease the viral load in or to eliminate the virus completely from infected semen samples in order to avoid harm to subsequent embryo development. The Swim up/Percoll gradient centrifugation combination is an attractive method to achieve that goal.

The most common procedure for human sperm preparation in human immunodeficiency virus (HIV) discordant couples is a two-step gradient centrifugation, followed by Swim up in culture medium. Percoll gradient centrifugation and Swim up were employed in motile spermatozoa from an HIV-positive patient, and the final spermatozoa suspension was tested for the presence of HIV-RNA. The test showed that Swim up processing after Percoll gradient centrifugation reduced the HIV-1 RNA and HIV-1 proviral DNA loads to undetectable levels (Hanabusa et al., 2000).

In Experiment 1, the sperm count obtained from all the BVDV infected groups were lower than the ones obtained for the control groups which probably could be related to normal variations in the semen samples. However despite the sperm count obtained from the Swim up/Percoll gradient centrifugation group was lower than

Table 2
Comparison of Comet assay results from the spermatozoa nuclei evaluations following semen processing techniques.

Comet level	Percoll gradient centrifugation		Swim up		Swim up/Percoll gradient centrifugation		Pre-processing	
	%	N	%	N	%	N	%	N
0	95.90[a]	209/218	97.90[b]	175/189	94.90[c]	377/397	95.20[d]	80/84
1	4.10[a]	9/218	2.10[b]	4/189	4[c]	16/397	4.80[d]	4/84
2	0[a]	0	0[a]	0	1.10[b]	4/397	0[a]	0

Five replications; different superscripts show significant differences among lines ($P < 0.05$).

Table 3
Results of BVDV titration following sperm processing techniques.

Replicate	Post processing Viral titer BVDV (TCID$_{50}$/mL)		Initial titer $10^{6.68}$ TCID$_{50}$/mL
	Percoll gradient centrifugation	Swim up	Swim up/Percoll gradient centrifugation
1	10^1	$10^{2.97}$	ND
2	$10^{1.97}$	$10^{2.73}$	ND
3	$10^{0.87}$	$10^{1.87}$	ND
4	$10^{1.73}$	$10^{2.3}$	ND
5	$10^{2.3}$	$10^{3.3}$	ND

ND – no cytopathic effect detected.

Table 4
Results of *in vitro* embryo production using semen processed by Swim up followed by Percoll gradient centrifugation.

Experimental groups	Sperm concentration (10^6/mL)	Cleavage rate % (n)	Blastocyst rate % (n)
Control 1	46.67	73.04 (214/293)	25.31 (41/162)
Control 2	21.83	73 (219/300)	18.51 (52/281)
BVDV	26.33	71.35 (127/178)	30.07 (43/143)

Mean of 5 replications; Control 1 – swim up; Control 2 – swim up/Percoll; no significant difference among groups.

that obtained in either of the other two groups (Swim up or Percoll gradient centrifugation) the resulting count was high enough to perform an *in vitro* fertilization procedure (Tables 1 and 4). A similar experiment performed with human semen revealed that the experimental sperm parameters do not significantly differ from those that the World Health Organization considers to be normal (Garrido et al., 2005). The Comet assay showed that the combination of the two semen processing techniques (Swim up/Percoll gradient) produced a 1.1% occurrence of Comet level 2, which is considered to be a low damage rate (Table 2). It has been shown that a 24% bovine blastocyst production rate is possible using a semen sample with 25% of its sperm cells damaged by X-ray irradiation (Fatehi et al., 2006).

Experiment 2 showed that the Swim up/Percoll gradient centrifugation combination reduced the BVDV viral load in the semen sample so that it was undetectable in cell culture (Table 3). In the other two single-process experimental groups (Swim up and Percoll gradient centrifugation), the viral load was detectable in cell culture. These results were similar to those reported by Bielanski et al. (1992). In Experiment 2, the viral titers following Percoll gradient centrifugation ranged from $10^{2.3}$ to 10^1 TCID$_{50}$/mL; in the Swim up group, the viral titer ranged from $10^{3.3}$ to $10^{1.87}$ TCID$_{50}$/mL (Table 3). The titers obtained in Experiment 2 were lower than those reported by Bielanski et al. (1992), which ranged between $10^{3.4}$ and $10^{3.8}$ TCID$_{50}$/mL. However, it is important to note that the study performed by Bielanski et al. (1992) used persistently infected bull semen, which likely possessed a larger initial BVDV concentration. A recent study showed that with an initial infectious virus titer of $10^{3.82}$–$10^{3.25}$ TCID$_{50}$/50 μL, a viral load reduction of more than 99% was achieved in porcine circovirus type 2 (PCV2) spiked semen samples with a double processing method consisting of single layer centrifugation followed by Swim up (Blomqvista et al., 2011).

The effect of BVDV infection at the time of *in vitro* fertilization on *in vitro*-produced embryos has been the focus of several studies (Guerin et al., 1992; Wrathall et al., 2006; Gard et al., 2009). The presence of BVDV was shown to be detrimental in cell culture experiments and causes a reduction in maturation, fertilization and/or development rates (Gard et al., 2009). Guerin et al. (1992) found that fertilization and embryo cleavage rates were significantly reduced when semen from a persistently infected bull was used to inseminate oocytes and that development to the blastocyst stage was only 2.1% compared with 19.6% when semen from an uninfected control bull was used. Therefore, considering that the presence of BVDV can depress *in vitro* fertilization parameters, the cleavage and blastocyst rates observed in Experiment 3 are important data that bolster the result that the combination of Swim up and Percoll gradient centrifugation was effective in reducing and/or eliminating BVDV from semen samples. Similar results were obtained in all groups evaluated (Table 4). This finding was also corroborated by negative results from RT-PCR.

5. Conclusions

The results obtained in this study demonstrated that the combination of Swim up followed by Percoll gradient centrifugation completely eliminated BVDV in artificially contaminated semen samples and allowed the subsequent production of blastocysts. The protocol described here could be easily incorporated into the daily routine of a commercial *in vitro* embryo production system to avoid introduction of BVDV into *in vitro* fertilization procedures, thereby enhancing sanitary control and preventing unnecessary embryo loss.

Acknowledgements

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