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The Effects of Cytopathic and Non-cytopathic Biotypes of Bovine Viral Diarrhea Virus on Sperm Vitality and Viability of Holstein Dairy Bulls *in Vitro*

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Abstract

BACKGROUND: Bovine viral diarrhea virus (BVDV) is one of the most important pathogens.

OBJECTIVES: The aim of this study was to investigate the effects of cytopathic (CP) and non-cytopathic (NCP) biotypes of BVDV on vital status, membrane integrity, and motility of sperm cells in Holstein dairy bulls *in vitro*.

METHODS: BVDV-free frozen semen samples were counted after thawing and centrifuged to separate live sperms. A sample containing 10^5 spermatozoa/mL was prepared. CP and NCP BVDV with 3 different doses of 10^5 (high dose), 10^4 (medium dose), and 10^3 (low dose) tissue culture infectious dose (TCID) 50/mL were challenged to sperm cells. After 2 hours of incubation at 38.5°C, eosin-nigrosine staining and hypoosmotic swelling (HOS) test were performed to assess the sperm viability and plasma membrane integrity. Computer assisted semen analysis (CASA) was used to evaluate the sperm motility parameters. The obtained data were analyzed using GLM method in SAS software.

RESULTS: The percentage of live spermatozoa in the control group was 72±3.60%. However, it decreased significantly with the increase of virus concentration in both groups ($P \leq 0.05$). Sperm integrity in the control group showed that the quality of semen was 65± 3.21. But the effect of virus biotypes resulted in a significant decrease in both high (10^5) and low (10^3) concentrations ($P \leq 0.05$). BVDV biotypes are able to reduce different sperm movements as their concentration-increases.

CONCLUSIONS: We concluded that CP and NCP biotypes of BVDV had a significant effect ($P \leq 0.05$) on survival, plasma membrane integrity, and motility of sperm cells *in vitro*.

KEYWORDS: BVDV, *In vitro*, Motility, Sperm, Viability

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Introduction

Bovine viral diarrhevirus (BVDV) is one of the most important cattle pathogens that exerts many destructive effects on the health and production of livestock. BVDV is an RNA virus

and a member of the genus *Pestivirus* within the family *Flaviviridae* (Hopper, 2014). BVDV isolates can be divided into cytopathic (CP) and non-cytopathic (NCP) biotypes, based on their effects on cell culture. Unlike NCP, CP isolates can lead to the formation of vacuoles in cell cytoplasm (cytoplasmic vacuolation) as well as the death of cultured cells (Robert *et al.*, 2004). The NCP biotypes are more prevalent in nature and 60 to 90% of laboratory samples are NCP biotype. Only NCP biotype can cause persistent infection (PI). Epidemiological studies on BVDV in Iran show that it is present in more than 50 to 100% of dairy farms (Garoussi *et al.*, 2019; Rypuła *et al.*, 2020; Foddai *et al.*, 2014). There has been no significant association between the herd size and the prevalence of virus antibody, and animals younger than two years old showed lower serum infection than animals older than two years old (Garoussi *et al.*, 2009). Reproductive diseases following BVDV infection are the most important disorders within the dairy industry. Male and female gonads, as well as all parts of the female reproductive system, can be a suitable medium for BVDV replication (Hopper, 2014). Infected male cows excrete BVDV through semen, but the duration of excretion and its level in persistently infected (PI) animals is much higher than that in cows transiently infected (TI) or acutely infected. Infection of semen with this virus can happen in 4 ways: 1) persistent infection (PI) of bulls during fetal period in uterus, 2) acute infection of bulls after the development of the immune response, 3) chronic testicular infection after prolonged acute infection, and 4) persistent testicular infection from an unknown source (Gard *et al.*, 2007). In laboratory infected bulls, the virus titer in semen varied from 5 to 75 $CCID_{50}$ (cell culture infective dose 50/mL) and the virus was successfully isolated from raw as well as diluted semen (Kirkland *et al.*, 1991; An *et al.*, 2019). Semen from a PI bull contains high levels of virus (10^3 to 10^7 CCID 50/mL) and even the process of freezing and preparing sperm for artificial insemination does not kill the virus, and subsequent insemination of susceptible heifers with semen from these cows will cause widespread infections in industrial and traditional herds and subsequent damage caused by contamination with both BVDV biotypes. *In vitro* studies conducted in Iran have been insufficient in this regard.

Given the fact that there are few data regarding the effect of CP and NCP BVDV biotypes on male gametes, this study aimed to investigate the effects of NCP and CP BVDV biotypes on vital status, membrane integrity, and motility of sperm cells from Holstein bulls *in vitro*.

Materials and Methods

BVDV-Free Sperm Samples

Prior to any experiment, all used sperm samples were tested by PCR with 100% specificity and sensitivity to BVDV in order to be informed of the possible presence of the BVDV (Garoussi and Mehrzad, 2011; Saged Hasan, 2012). All sperm samples were BVDV-free.

Sperm Samples

Frozen BVDV-free sperms of bulls were thawed in a water bath at 37°C and introduced to the top of a Percoll gradient (45 and 90%; Pharmacia, Uppsala, Sweden). To separate live sperms from dead ones, the sperms were centrifuged for 30 minutes at 2000 × g. The supernatant was removed after centrifugation and the sperm pellet was re-suspended in TALP + BSA, and centrifuged once more for 10 minutes at 750 × g. The resulting sperm pellet was re-suspended to obtain a final concentration of 10^5 sperm/mL. Frozen sperm samples were obtained from a serial number of the National Breeding Center and Improvement of Animal Production (Karaj-Iran), which were BVDV-free.

Assessment of Sperm Viability

In this study, eosin-nigrosine staining was used to determine whether sperm were alive or dead.

To prepare the eosin-nigrosine dye, 1.45 g of sodium citrate was dissolved in 50 mL of deionized water. Five g of nigrosine and 0.835 g of eosin were poured into a suitable container and then sodium citrate solution was added up to 50 mL. The resulting solution was immersed in boiling water for 20 minutes, then filtered and stored at 5ºC in the refrigerator. After preparing the staining solution, 8 drops were mixed with one drop of semen and waited for 3 minutes and the smear was prepared. Sperms which had red heads were considered dead gametes (Agarwal *et al.*, 2016).

Virus

CP and NCP biotypes with high dose (10^5), medium dose (10^4), and low dose (10^3) (50% tissue culture infectious dose) (TCID 50/mL) were used (Garoussi and Mehrzad, 2011). The virus was cultured in the Minimum Essential Medium (MEM) with fetal calf serum 5% (Vanroose *et al.*, 1998). To observe the cytopathic effects (CPE) of the BVDV, the virus was cultured in Razi bovine kidney (RBK) cells (Razi Vaccine and Serum Research Institute, Karadj-Iran). The virus was then confirmed in both CPE and non-CPE using PCR-specific primers in RBK cells-BVDV co-culture/mixtures. The baseline dose of the virus is usually determined to infect up to 50% of the cells, which is normally done using the virus culture technique in specific cell line. Briefly, the RBK cells were sub-cultured to achieve the desired amount of cells for the RBK cells-BVDV coculture. These RBK cells were then sub-cultured in the 96-well plates until the cells were adhered and completely covered the surface of the wells (usually it takes about 48 hours for RBK cells). The cells were washed to ensure that only adhered cells are used and non-adhered cells are discarded. The BVDV was then prepared with different dilutions (from 10^1 to 10^7 in 8 sterile test tubes), and one test tube was normally used as a control. These virus dilutions were mixed and placed adjacent to the RBK cells for about half an hour to allow the virus to enter the cells. It was then washed and incubated with the new culture medium for 3 days. Finally, the cytopathic changes were checked under microscope and the CPE and non-CPE RBK cells in each well were identified (Andrew *et al.*, 2020). The corres-ponding BVDV doses were calculated using the Reed and Muench method. Indeed, different doses of BVDV had different effects on sperm fertilization and adhesion to the egg (Garousi and Mehrzad, 2011; Garoussi *et al.*, 2019).

Membrane Integrity Evaluation

The hypoosmotic swelling (HOS) test was used to assess the sperm membrane integrity. To prepare the HOS solution, 0.9 g of fructose, 0.49 g of sodium citrate, and 0.04 g of citric acid were mixed together and 20 µL of semen were mixed with 200 µL of HOS solution and incubated for 1 hour at 37ºC. The sperm tails that have an integrated and healthy plasma membrane are twisted, and the spermatozoa which have lost the composition of their plasma membranes have a smooth tail (Baiee *et al.*, 2017).

Computer Assisted Semen Analysis

Computer assisted sperm analysis (CASA) is a useful software for the objective evaluation of sperm motility and hence is now frequently used for evaluating semen quality. CASA software (HFT CASA, Hushmand Fanavar, Tehran, Iran) was used to evaluate the effects of two BVDV biotypes on the motility of infected sperm cells. The motility parameters analyzed by CASA system included: total motility (TMOT, %) and progressive motility (PMOT, %), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head (ALH, µm), mean angular displacement (MAD/D), beat/cross frequency (BCF, Hz), straightness (STR, %), linearity (LIN, %), and wobble (WOB %) as described by Vincent *et al.* (2018).

Experimental Groups

Treatment

Male gametes were exposed to different doses (high, medium, and low) of CP and NCP BVDV in an incubator at 38.5°C for 2 hours at a concentration of 10^5 sperm cells/mL. Samples were evaluated for viability features using eosin-nigrosine staining. Live and dead infected sperm cells were assessed. The HOS test was used to assess sperm integrity. CASA system was used to evaluate different motility parameters.

Control

Semen sample with a concentration of 10^5 sperm/mL without the presence of BVDV biotypes, as well as various treatment groups, were evaluated for viability, membrane integrity, and motility.

All of the above tests for treatment and control groups were repeated 3 times in different time intervals.

Statistical Analysis

The obtained data were analyzed using generalized linear model (GLM) in SAS software.

Results

Table 1 shows the effects of BVDV biotypes on sperm viability and plasma membrane integrity *in vitro*.

It was shown that the number of live sperms in the control group had a range of 72±3.60%, while this value decreased significantly with the increase of virus concentration in both treatment groups ($P≤0.05$). Moreover, the eff-ect of BVDV biotypes on plasma membrane integrity led to a significant reduction in both high (10^5) and low (10^3) CP and NCP BVDV concentrations ($P≤0.05$).

Table 2 represents the effects of BVDV biotypes on sperm motility. The results showed a significant difference ($P≤0.05$) between CP and NCP BVDV biotypes in the control samples and also a significant difference ($P≤0.05$) between CP and NCP BVDV biotypes *in vitro*.

Table 1. The effect of CP and NCP BVDV on viability and membrane integrity of sperm cells *in vitro*

Tests	Groups						
	Control	Treatments					
		CP BVDV($TCID_{50}$/mL)			NCP BVDV($TCID_{50}$/mL)		
		10^3	10^4	10^5	10^3	10^4	10^5
Sperm Viability	72±3.60^a	49±1.52^{be}	35±2.08^c	15±1.15^d	53±3.60^b	39±2.08^{ce}	19±0.57^d
Sperm Plasma Membrane Integrity	65±3.21^a	30.33±2.33^b	29.66±2.72^b	11±1^c	32±1.52^b	29±4.04^b	15±1.52^c

Mean ± SD values in the same rows with different superscripts differ significantly ($P≤0.05$).

Table 2. CP and NCP BVDV biotypes effect on Holstein bull spermatozoa motility parameters *in vitro*.

Parameters	Groups						
	Control	Treatment (BVDV biotypes)					
		CP($TCID_{50}$/mL)			NCP ($TCID_{50}$/mL)		
		10^3	10^4	10^5	10^3	10^4	10^5
TMOT (%)	51.17±1.27^a	15.33±0.52^b	9.38±0.75^c	6.58±0.60^c	42.39±0.93^d	23.64±1.55^e	17.71±0.44^b
PMOT (%)	48.83±1.53^a	15.03±0.72^b	7.81±1.41^c	6.08±0.50^c	40.22±0.95^d	21.32±1.38^e	17.14±0.45^b

Parameters	Groups						
	Control	Treatment (BVDV biotypes)					
		CP(TCID50/mL)			NCP (TCID50/mL)		
		10^3	10^4	10^5	10^3	10^4	10^5
VCL (µm/S)	20.76±0.48[a]	11.05±0.38[bf]	7.90±0.97[c]	8.73±0.41[b]	17.35±0.63[d]	14.16±0.58[ef]	11.92±0.72[f]
VSL (µm/S)	9.20±0.30[a]	4.14±0.31[b]	2.26±0.33[b]	2.00±0.13[c]	7.62±0.50[a]	4.90±0.42[b]	3.98±0.55[b]
VAP (µm/S)	11.37±0.64[a]	5.59±0.34[b]	3.44±0.22[c]	3.43±0.23[c]	9.47±0.27[d]	6.77±0.46[b]	5.95±0.24[b]
MAD (D)	45.02±0.52[a]	22.33±0.67[b]	17.07±0.23[c]	16.88±0.50[c]	37.76±0.58[d]	32.70±0.37[e]	22.84±0.87[b]
ALH (µm)	0.98±0.01[a]	0.66±0.00[b]	0.50±0.05[c]	0.54±0.02[b]	0.84±0.02[d]	0.76±0.02[bd]	0.71±0.02[bd]
BCF (Hz)	0.95±0.02[a]	0.34±0.01[b]	0.22±0.00[c]	0.18±0.00[dc]	0.84±0.01[e]	0.55±0.02[f]	0.29±0.01[bc]
LIN (%)	39.98±1.80[a]	28.62±0.51[b]	29.67±0.72[b]	21.84±0.68[c]	39.65±0.86[a]	30.06±0.48[b]	29.33±0.36[b]
WOB (%)	54.27±0.66	43.55±1.10[bc]	44.04±1.03[bc]	4.98±0.72[b]	54.06±0.59[a]	46.06±0.35[c]	49.52±0.40[d]
STR (%)	70.39±0.70[a]	54.74±1.06[b]	54.21±0.55[b]	48.22±0.60[c]	66.79±1.03[a]	57.43±0.65[b]	49.65±1.23[c]

Mean ± SD values in the same rows with different superscripts differ significantly ($P \leq 0.05$).
TMOT: Total motility, PMOT: Progressive motility, VCL: Curvilinear velocity, VSL: Straight line velocity, VAP: Average path velocity, MAD (D): Mean angular displacement, ALH: Amplitude of lateral head displacement, BCF: Beat/cross frequency, LIN: Linearity, WOB: Wobble. STR: Straightness.

Discussion

The present study examined the effects of CP and NCP BVDV biotypes on the viability and integrity of cattle sperm cells membranes, as well as the motility parameters *in vitro*. This study revealed that different concentrations of BVDV (both CP and NCP biotypes) can affect vital features including survival or death of gametes, sperm plasma membrane integrity, and the motility of sperm cells. They lead to a decrease in the abovementioned three factors (Tables 1 and 2). In beef cattle, suppression of the host immune system and respiratory disease are the leading causes of BVDV damage, and reproduction losses have been reported to be the most prominent damage to the dairy industry (Hopper, 2014). Male and female gonads, as well as all parts of the female reproductive tract can be a suitable medium for BVDV to grow and replicate. Widespread genital infection followed by a decreased reproductive function of the animal after infection with the BVDV can be clearly seen in PI animals (Hopper, 2014; Griffin *et al.*, 2019). BVDV infection can have devastating effects on cattle pregnancy, especially infections before the third trimester that are more dangerous. The virus can easily cross the placental barrier, and both biotypes can infect the fetus (Duffell and Harkness, 1985). Infection of the male fetus between the days 45-125 of the fetal period, acute infection after the development of the immune response, chronic testicular infection after prolonged acute infection, and persistent testicular infection from an unknown source are the four ways of infecting male animals with BVDV (Gard *et al.*, 2007). Not much is known about the molecular mechanism of virus entry into the sperm cells. However, the effective role of glycoproteins 48 and 53 (gp^{48} and gp^{53}) in the virus membrane for binding to host cells has been reported in some studies (Garoussi and Mehrzad, 2011). The CD^{46} molecules were identified as receptors for BVDV (Maurer *et al.*, 2004). The present study showed a significant effect of both biotypes of virus on survival, membrane integrity, and the motility parameters of bovine sperm cells *in vitro*. Induction of programmed cell death (apoptosis) can be one of the reasons for the decrease of sperm viability after exposure to CP and NCP BVDV biotypes (Givens,

2013). In addition, the increasing level of oxidative stress and subsequent damage to the cell membrane lead to a decrease in the movement and eventually cell death (Givens, 2013). Spermatozoa are highly vulnerable to oxidative stress due to their lack of enough cytoplasmic space to accumulate defensive enzymes and antioxidants, and in particular the sperm membrane is highly sensitive to oxidative attacks due to its abundant unsaturated fatty acids (Aitken, 2017). On the other hand, the ability of sperm cells depend on the production of high levels of reactive oxygen species (ROS), the production of which can cause oxidative stress in sperm and the loss of its optimal function (Aitken, 2020). However, the cell can be diverted to apoptosis, which is accompanied by the production of mitochon-drial ROS, loss of mitochondrial membrane potential, caspase activity, expression of phosphatidylserine, and DNA oxidative damage (Aitken, 2020). Therefore, any factor that increases the production of these substances in sperm cells can affect its fertility (Drevet and Aitken, 2019). This adverse effect can be due to the disruption of vital parts of the sperm, such as the plasma membrane, mitochondria, or acrosome. The significant effect of CP biotype versus NCP on total and progressive sperm motility as well as other motility parameters can be investigated in the ability of CP biotype to cause cell damage in the cell culture medium (Hopper, 2014). Significant differences between high (10^5) and low (10^3) doses in terms of survival, membrane integrity, and all sperm motility parameters indicate the effect of intensity of virus infection on male fertility. Contamination of experimental oocytes and sperm cells with CP and NCP BVDV biotypes led to a significant reduction in fertilization rate *in vitro*; this could be due to the destructive effects of the virus on the sperm plasma membrane and disruption of the binding process of spermatozoa to zona pellucida (ZP) and a decrease in sperm total and progressive motilities or problems with capacitation and acrosomal reaction (Vanroose *et al.*, 2000, Garoussi and Mehrzad, 2011; Garoussi *et al.*, 2019). Fetuses obtained by *in vivo* methods could have been infected if they had been collected from a PI or TI donor, or if a virus-infected serum had been used in the embryo transfer, culture, or freezing solution. However, the threat of virus infection appears to be much greater in laboratory-produced embryos than in *in vivo* produced fetuses since the virus can be transmitted through serum or oocytes from slaughterhouse-derived ovaries. In addition, the virus can bind to the ZP since washing and treatment processes with trypsin to clear the virus from IVF embryos are less effective than in vivo embryos (Garoussi and Mehrzad, 2011). Use of male PI cows in IVF reduced sperm fertilization and cell division (Alietta *et al.*, 1995; Duncan *et al.*, 2016). The quality of semen obtained from PI animals can be normal or abnormal in terms of concentration, motility, and morphology. The use of semen from PI bull can reduce the conception rate due to several reasons such as low quality of semen, bull's sickness, and a negative effect on the female reproductive system (Kirkland, 1994; Grooms, 2004). Experimental contamination of oocyte and sperm with CP and NCP BVDV biotypes leads to a significant reduction in fertilization through *in-vitro* fertilization (IVF) and this can be due to the adverse effects of the virus on the sperm membrane and the disruption of sperm attachment to the ZP by reducing total and progressive sperm motilities or making problems for sperm capacitation and acrosome reaction (Garoussi and Mehrzad, 2011). Despite the fact that virus has been detected in genital fluids of TI and PI bulls, the virus presence in spermatozoa or seminal plasma is still controversial (Wrathal *et al.*, 2006). In acutely infected bulls, the production of neutralizing antibodies leads to the clearance of the virus from most organs though the chronic infection of testicular tissue has also been reported despite the antibody production (Givens and Marley, 2013). This was first tested on a seropositive bull that was negative for the virus in the blood but the virus was continuously excreted in the semen, and an acute infection was reported before the blood-testis barrier was completed (Voges *et al.*, 1998; Gad *et al.*, 2018). Localized and generalized testicular infections have been reported in laboratory infected and intranasal inoculated cows, as well as those vaccinated with BVD vaccine through subcutaneous injection before puberty

(Givens *et al.*, 2007; Givens *et al.*, 2003). However, the development of blastocyst from BVDV-infected eggs fertilized with PI male sperm significantly decreased compared to the control group (Bielanski and Loewen, 1994; Bromfield *et al.*, 2017). In addition, the cumulus oophorus complex (COC) obtained from slaughterhouses and cows with unknown history can increase the risk of infection in the fetus (Givens and Waldrop, 2004). It has been shown that the virus can affect not only the female but also the male gametes. However, the results of this study showed that BVDV can effect sperm viability and activity (Tables 1 and 2).

Conclusion

To conclude, CP and NCP BVDV can affect sperm vitality, viability, and dynamic biological features *in vitro*. The present study confirmed the negative effects of CP and NCP BVDV on sperm cells. This effect could mainly result from the reduction of sperm .activity in comp-arison with the control group Nevertheless, the exact mechanisms of BVDV attachment to the male gamete need more fundamental inves-tigations.

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Conflict of Interest

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

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مجله طب دامی ایران، ۱۳۹۹، دوره ۱۵، شماره ۲، ۱۹۷-۲۰۷

بررسی اثرات بایوتایپ‌های CP و NCP ویروس BVD روی وضعیت حیاتی و ماندگاری اسپرم گاوهای نر نژاد هلشتاین در شرایط برون‌تنی

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زمینه مطالعه: ویروس اسهال ویروسی گاو یکی از مهمترین پاتوژن‌ها است.

هدف: هدف از این مطالعه، بررسی اثرات بایوتایپ‌های CP و NCP ویروس BVD روی وضعیت حیاتی، یکپارچگی غشاء و حرکات مختلف اسپرم گاو در شرایط برون‌تنی است.

روش کار: اسپرم منجمد عاری از ویروس BVD پس از یخ‌گشایی و سانتریفوژ برای جداسازی اسپرم‌های زنده شمارش شدند. نمونه‌ای حاوی ۱۰۵ سلول اسپرماتوزا در میلی‌لیتر آماده شد. با سه دز مختلف ۱۰۵ (دز بالا)، ۱۰۴ (دز متوسط) و ۱۰۳ (دز پایین) TCID50/mL بایوتایپ‌های CP و NCP ویروسBVD ، مجاور گشت. پس از ۲ ساعت هم‌جواری اسپرم و ویروس در انکوباتور با دمای ۳۸/۵°C، آزمون‌های ائوزین نیگروزین و هایپواسموتیک برای ارزیابی ماندگاری و یک‌نواختی غشای اسپرماتوزوا انجام گرفت. به‌منظور ارزیابی دینامیک حرکات اسپرماتوزوا از نرم‌افزار CASA استفاده شد. اطلاعات به‌دست‌آمده با استفاده از دستورالعمل GLM و نرم‌افزار SAS تجزیه و تحلیل شد.

نتایج: میزان اسپرم‌های زنده در گروه شاهد دارای دامنه‌ای به میزان ۳/۶۰±۷۲ درصد بود. این میزان با افزایش غلظت ویروس در هر دو بایوتایپ کاهش چشمگیری یافت ($P\geq ۰/۰۵$). یکپارچگی اسپرم‌ها در گروه شاهد نشان داد که دامنهٔ آن در ۳/۲۱±۶۵ درصد اسپرم‌ها یکنواخت بود. اما تاثیر بایوتایپ‌های ویروس منجر به کاهش چشمگیر در دو غلظت بالا (۱۰۵) و پایین (۱۰۳) شد ($P\geq ۰/۰۵$). بایوتایپ های ویروس BVD قادر به کاهش حرکات مختلف اسپرم شدند به گونه‌ای که هر چقدر غلظت بایوتایپ های ویروس افزایش پیدا کرد، میزان حرکات مختلف اسپرم‌ها کمتر شد.

نتیجه‌گیری نهایی: نهایی: نتیجه آن‌که در شرایط برون‌تنی، بایوتایپ‌هایCP و NCP ویروس BVD تاثیر معنادار (P≥۰/۰۵) روی زنده‌مانی، یکپارچگی غشای پلاسمایی و دینامیک حرکتی اسپرم دارند.

واژه‌های کلیدی: BVDV، برون‌تنی، حرکت، اسپرم، ماندگاری

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