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Short Communication

The Effects of Cytopathic and Noncytopathic Bovine Viral Diarrhoea Virus with Sperm Cells on *In Vitro* Fertilization of Bovine Oocytes

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Abbreviations: BSA, bovine serum albumin; BVD, bovine viral diarrhoea; COC, cumulus–oocyte complexes; CP, cytopathic; FCS, fetal calf serum; IVF, *in vitro* fertilization; MEM, minimum essential medium; NCP, noncytopathic; PCR, polymerase chain reaction; PI, persistent infection; TALP, Tyrode's solution supplemented with albumin, lactate and pyruvate; $TCID_{50}$, 50% tissue culture infectious dose; TCM, tissue culture medium; ZP, zona pellucida

INTRODUCTION

Bovine viral diarrhoea (BVD) virus is one of the most important causes of bovine reproductive disorders that can severely affect the developing embryo and fetus. This Pestivirus of the family *Flaviviridae* is classified into two biotypes designated as NCP (noncytopathic) and CP (cytopathic) depending on their effect on tissue culture cells (Deregt and Loewen, 1995). The NCP biotype is most commonly isolated in the field. It replicates in cultured cells without inducing cell death, and crosses the placenta to establish a persistent and lifelong infection. In contrast, the CP biotype induces apoptotic cell death in cultured cells (Zhang *et al*., 1996). BVD virus has a tropism for the germ line cells of both sexes and negative effects on reproductive performance and fertility may be observed in bulls and in cows (Kafi *et al*., 1994; Kommisrud *et al*., 1996). Embryos appear to be resistant to infection until they hatch from the zona pellucida around day 10 of gestation. In addition, it is unlikely that embryos are susceptible to a systemic BVD virus infection before implantation at 30 days of gestation (Baker, 1995). It has been shown that the presence of CP and NCP BVD virus during bovine IVF results in lower cleavage rates and embryo development (Vanroose *et al*., 1998).

The objective of this study was to examine the effect of incubation of sperm cells with CP and NCP BVD virus on the process of bovine IVF.

MATERIALS AND METHODS

Materials free of BVD virus and anti-BVD virus antibodies

Materials from animal sources used for this study were bovine serum albumin (BSA), fetal calf serum (FCS) and Hyclone serum. They were tested to be free of BVD virus by PCR

and virus isolation (Van Soom *et al*., 1994). They were also tested negative to anti-BVD virus antibodies by microtitration virus neutralization test.

In vitro *oocyte maturation*

Bovine oocytes were matured using routine techniques (Mahmoudzadeh *et al*., 1995). Briefly, compact cumulus–oocyte complexes (COC) were selected after aspirating antral follicles (2–6 mm) of ovaries collected from a slaughterhouse. They were washed four times in Hepes-buffered TALP medium, followed by a further washing with the maturation medium (Van Soom *et al*., 1994). Maturation was accomplished in groups of 100 COC in 500 μl of maturation medium (no oil overlay) during 24 h of incubation at 38.5°C under 5% CO_2 atmosphere. Maturation medium consisted of TCM 199 (Gibco BRL, Merelbeke, Belgium)) bicarbonate-buffered medium supplemented with 7.5% Hyclone serum (v/v), 0.5 μg/ml follicle-stimulating hormone, 5 μg/ml lutenizing hormone, 0.2 mmol/L sodium pyruvate (Sigma, Bornem, Belgium), 0.4 mmol/L glutamine (Sigma) and 50 μg/ml gentamicin sulphate (Gibco BRL).

Oocytes with a compact multilayered cumulus investment and homogeneous ooplasm were classified as good quality COC. The oocyte maturation process included the completion of two cellular programs of *nuclear maturation*, the resumption of meiosis from the germinal vesicle stage to the formation of the second metaphase plate, and *cytoplasmic maturation*, the molecular and structural changes that provide the mature oocyte the capacity to support fertilization.

To obtain cumulus-free oocytes, matured COC were vortexed in Hepes-buffered TALP medium (Van Soom *et al*., 1994) for 2–4 min at maximum speed to remove adherent cumulus cells before being used in IVF.

Virus

The BVD viruses used in this study were the CP strain Oregon C24V and the NCP isolate No. 22146 (Vanroose *et al*., 1998). They were propagated in MEM +5% FCS.

Experimental design

Frozen sperm of a BVD virus-free bull was 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 spermatozoa from dead ones, the sperm were centrifuged for 30 min at 2000*g*. The supernatant was removed after centrifugation and the sperm pellet was resuspended in TALP + BSA and centrifuged once more for 10 min at 750*g*. The resulting sperm pellet was resuspended to obtain a final concentration of 10^7 sperm cells/ml in IVF-TALP with 10 μg/ml heparin and incubated for 1 h at 38°C. Then the sperm cells were centrifuged again, resuspended in IVF-TALP medium without heparin and diluted in IVF-TALP to obtain a concentration of 10^6 and 10^5 sperm cells/ml, respectively.

The study groups were as follows: control group for influence of MEM on fertilization; a group in which 1 ml of MEM was diluted with 1 ml of sperm cells at 10^6 sperm cells/ml; a group in which 1 ml of MEM was diluted with 1 ml of sperm cells at 10^5 sperm cells/ml.

Virus–sperm interaction

Three final concentrations of CP and NCP virus were used as follows:

CP BVD virus: $10^{5.5}$, $10^{4.5}$ and $10^{3.5}$ $TCID_{50}$/ml
NCP BVD virus: $10^{6.3}$, $10^{5.3}$ and $10^{4.3}$ $TCID_{50}$/ml

The final sperm concentrations were adjusted to 10^5 and 10^6 sperm cells/ml. One ml of the appropriate concentration of CP or NCP BVD virus suspensions was mixed with 1 ml of the different concentrations of sperm cells.

All of these samples were incubated for 2 h at 39°C under 5% CO_2 to allow contamination of the sperm cells with the virus. They were centrifuged for 10 min at 1500*g* and the concentrations were adjusted with IVF-TALP + heparin to the initial concentrations of sperm cells. In the meantime, vortexing of COC was performed. Twenty-five μl of the sperm suspension was added to a 25 μl droplet. Ten cumulus-free matured oocytes were added to each droplet under oil and incubated overnight. In total, 1185 oocytes were used; three replications were performed in which 377, 409 and 399 oocytes were used, respectively.

Evaluation of fertilization

After 18–20 h of sperm–oocyte co-incubation, any remaining sperm cells were removed from the oocytes by 1 min of vortexing. Zygotes were then fixed in 2% formaldehyde and 2% glutaraldehyde overnight and stained with Hoechst 33342 to stain the nuclei (Pursell *et al*., 1985). After mounting in 100% glycerol, they were evaluated for the presence of two pronuclei using a Leica DM RBE fluorescence microscope.

Data analysis

The logistic regression test was used for analysis of data.

RESULTS

In total, 589 and 596 oocytes were used during three replications in the present study in which oocytes were incubated with sperm cells infected with CP or NCP BVD virus (Tables I and II).

The study showed that the highest infectious titre of CP BVD virus ($10^{5.5}$ $TCID_{50}$/ml) in combination with the lowest final sperm concentration (10^5 sperm cells/ml) decreased the fertilization rate significantly, compared with the control group (4% vs 19% control) ($p < 0.05$) (Table I). NCP BVD virus had no significant effect on the fertilization rate at the three infectious titres that were examined ($p > 0.05$) (Table II).

TABLE I
The effect of different CP BVD virus concentrations incubated with different sperm cell concentrations on IVF rate of oocytes

		CP BVD virus ($TCID_{50}$/ml)		
Sperm/ml	Control	$10^{3.5}$	$10^{4.5}$	$10^{5.5}$
10^5	19% (14/75)	9% (6/69)	11% (8/75)	4% (3/76)*
10^6	57% (42/74)	71% (53/75)	56% (40/72)	68% (50/73)

*Significant difference with the fertilization rate in the control in the same row ($p < 0.05$)

TABLE II
The effect of different NCP BVD virus concentrations incubated with different sperm cell concentrations on IVF rate of oocytes

		NCP BVD virus ($TCID_{50}$/ml)		
Sperm/ml	Control	$10^{4.3}$	$10^{5.3}$	$10^{6.3}$
10^5	19% (14/75)	26% (18/70)	16% (13/82)	13% (9/68)
10^6	57% (42/74)	60% (46/77)	52% (39/75)	72% (54/75)

No significant differences with the fertilization rate in the control ($p > 0.05$)

In groups with 10^5 sperm cells/ml, the fertilization rate decreased, in comparison to groups with 10^6 sperm cells/ml.

DISCUSSION

The results of this study showed that only exposure of sperm cells at 10^5 sperm cells/ml to CP BVD virus at $10^{5.5}$ $TCID_{50}$/ml affected the fertilization rate significantly, whereas exposure to the other CP BVD virus concentrations ($10^{3.5}$ and $10^{4.5}$ $TCID_{50}$/ml) and to NCP BVD virus did not affect fertilization.

The fertilization rate decreased in lowest final sperm concentration and different CP BVD virus concentrations (Table I). This result was not seen in the group with 10^6 sperm cells/ml with CP BVD virus infections, and it was observed that fertilization rate increased in comparison to the control group (57%) (Table I). This was probably due to the high sperm cell population and an insufficient CP BVD virus concentration for induction of cytopathic effects. However, it can be hypothesized that CP BVD virus when used in high enough concentration on a low sperm cell population may be effective in inducing CP influences on sperm cells.

There were no significant differences in the fertilization rate in treatment groups using sperm cells incubated with NCP BVD virus compared with control group (Table II). It

is also observed that the fertilization rate increased with $10^{4.3}$ and $10^{6.3}$ $TCID_{50}$/ml NCP BVD virus in combination with the higher final sperm concentration (10^6 sperm cells/ml). It is possible that the lower fertilization rate at the lower sperm cell concentration, compared with the control group, was due to interaction of CP BVD virus with sperm cells, which was significant for ($10^{5.5}TCID_{50}$/ml) CP BVD virus. However, this was not seen with NCP BVD virus, even though the concentrations of NCP BVD virus were much greater than those of CP BVD virus used in the treatment group with lower sperm cell concentration. It is possible that the presence of CP BVD virus on sperm cells might affect the motility or cause abnormalities in sperm morphology, resulting in a decreased fertilization rate.

Brock and Stringfellow (1993) have shown that the development of *in vivo*-derived, hatched embryos is reduced in the presence of CP virus, while Vanroose and colleagues, (1998) demonstrated that zona pellucida-free IVP embryos are permissive to both CP and NCP BVD virus, with increasing susceptibility as they get older. These observations are consistent with the view that free virus does not cross the zona pellucida following ovulation and hardening of the zona.

It is known that many pathogens may be bound to spermatozoa by nonspecific binding and can be detected on the surface of sperm cells (Bielanski, 1995). Some enveloped viruses have the ability to interact and fuse with receptors on the surface of bull spermatozoa (Nussbaum *et al*., 1993). This suggests that sperm cells may serve as carriers of viral genes and introduce the virus into oocytes during the fertilization process (Bielanski, 1995). Bovine viral diarrhoea virus was isolated from *in vitro*-produced embryos after use of BVD virus-infected semen (Bielanski, 1995). It has been reported that after the use of persistently intended bull semen, cleavage rates and development to the blastocyst stage were not significantly reduced in IVF (Bielanski *et al*., 1992). In contrast, Guerin and colleagues (1992) demonstrated that fertilization and cleavage were significantly decreased when semen from persistently infected bulls was used for IVF.

The reason for the differences in susceptibility to the two biotypes is not clear. First, it must be mentioned that little is known about the molecular mechanisms responsible for entry of virus into susceptible bovine cells. Glycoproteins, especially gp^{48} and gp^{53}, present in the viral envelope are thought to be involved in attachment of the virus to and(or penetration of the virus into the host cells (Donis and Dubovi, 1987; Boulanger *et al*., 1991). It has been suggested that multiple receptors for BVD virus attachment may be present and that different virus strains do not necessarily have the same receptor. In addition, it is thought that BVD virus utilizes more than one receptor for attachment and that binding of virus to the cell surface may be a multiple event (Schelp *et al*., 1995). In the light of these theories, it is possible that CP and NCP BVD viruses use different receptors for cell entry.

From the results of this study it can be concluded that only the highest infectious titre of CP BVD virus ($10^{5.5}$ $TCID_{50}$/ml) in combination with the lowest final sperm concentration (10^5 sperm cells/ml) decreased fertilization rate significantly, compared with the control group in IVF. There was no significant change in fertilization rate for the other groups of CP BVD virus-contaminated sperm cells. NCP BVD virus had no significant influence on the fertilization rate at the three infectious titres that were used. Nothing is known about mechanisms involved in the interaction of BVD virus with male bovine gametes. Further research is necessary to invesigate how CP BVD virus affects bovine sperm cells during IVF.

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