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Effect of Bovine Viral Diarrhea Virus on the ovarian functionality and *in vitro* reproductive performance of persistently infected heifers

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ABSTRACT

The aim of this study was to study the effect of Bovine Viral Diarrhea Virus on the reproductive female tract by means of analyzing the ovarian follicular population of persistently infected (PI) heifers, and evaluating the performance of oocytes procured form those heifers in *in vitro* fertilization procedures.

Seven BVDV PI Aberdeen Angus and British crossbred heifers ranging from 18 to 36 months of age were spayed and their ovaries used for viral isolation, microscopic examination, and *in vitro* fertilization procedures. Bovine Viral Diarrhea Virus was detected from the follicular fluid and sera of all PI heifers. Microscopic examination of the ovaries from PI heifers showed a significant drop in the number of follicles cortical regions, compared with controls. A comparative analysis of the stages of follicular development showed a significant decrease in the number of primordial and tertiary follicles in the cortical regions of ovaries from PI heifers. Viral antigen was detected by immunohistochemistry, and was widely distributed throughout the ovarian tissues. There were differences in the rate of cleavage and embryo development between oocytes obtained from the ovaries of control animals and PI heifers. Furthermore, two developed embryos obtained from oocytes from one of the PI heifers were positive to BVDV, as well as two media from *in vitro* fertilization (IVF) procedures. The results of this study demonstrate that BVDV PI heifers exhibit alterations in follicular population through of the early interaction between the virus and germ cell line affecting directly the mechanisms involved in the ontogenesis of the ovary.

1. Introduction

The impact of Bovine Viral Diarrhea Virus (BVDV) on the reproductive performance of cattle has important economic consequences (McGowan and Kirkland, 1995). Previous studies reported that many organs of the genital tract are susceptible to BVDV infection, and the presence of virus in such tissues may be one of the factors contributing to repeated infertility in persistently infected (PI) cows (Shin and Acland, 2001; Fray et al., 2000). Non-cytopathogenic (ncp) BVDV was found in all major somatic cells and gametes, including infected semen, serum, oocytes, cumulus cells and follicular fluid, and is on the list of probable contaminants of *in vitro* fertilization (IVF) systems (Perry, 2007). Ovaries have been shown to be one of the sites for BVDV replication. The poor response to

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superovulation treatments in both experimental and natural acutely infected cattle indicates that folliculogenesis did not occur as expected with routine treatments in normal non-infected cattle (Grooms et al., 1996; Kafi et al., 1994). Brock et al. (1991) reported poor *in vivo* embryo production after superovulatory treatment of seven different PI donors where, only nine transferable embryos and 71 total structures were obtained from 45 flushes. These data suggest a link between BVDV acute or persistent infection and changes in normal follicular dynamics, either by compromised hormonal status during folliculogenesis or direct negative effects on ovarian function and oocytes maturation. Considering that follicular development is a critical event in reproductive performance, it is important to understand the dynamic of the interaction between BVDV and follicles during their growth. The affinity of BVDV for the reproductive tract is also reflected by some evidence of ovarian hypoplasia reported in PI cows (McGowan et al., 2003). Fray et al. (1998) demonstrated that BVDV can infect oocytes as they mature within the ovarian follicle. If oocytes are permissive to BVDV, the virus may adversely affect oocyte quality (Fray et al., 2000). Additionally, fertilization of infected oocytes may be responsible for BVDV transmission after embryo transfer (Stringfellow, 1998). Different isolates of BVDV might differ significantly in their potential to replicate in an IVF system, associate with *in vitro*-derived embryos and constitute an infective dose *via* association with washed embryos. Givens et al. (2000) showed that each strain of BVDV had some potential to remain associated with embryos despite washing and then be released into the environment. About several studies have reported differences between Genotype I and II strains and other virus as bovine herpesvirus-1 (BHV-1), in their potential to persist in the IVF systems (Bielanski and Dubuc, 1994; Bielanski et al., 1998). Furthermore, several *in vitro* and *in vivo* studies revealed that after acute infection the presence of BVDV resulted in reduced fertilization and cleavage rates (Kirkland et al., 1990; Booth et al., 1998; McGowan et al., 2003); however this effect was inconclusive in PI cattle, yet.

The aim of this study was to study the effect of Bovine Viral Diarrhea Virus on the reproductive female tract by means of analyzing the ovarian follicular population of persistently infected (PI) heifers, and evaluating the performance of oocytes procured form those heifers in *in vitro* fertilization procedures.

2. Materials and methods

2.1. Animals

Seven Aberdeen Angus and British crossbred cycling heifers ranging from 18 to 36 months of age from two different beef herds were determined to be infected persistently with BVDV by two repeated isolation of ncp-BVDV from peripheral blood leukocytes (buffy coats) with an interval of 30 days between each attempt of isolation. The heifers were provided from two commercial beef herds free of trichomonosis, campylobacteriosis, and brucellosis located in the Southeast of Buenos Aires province, Argentina. A low fertility performance had been noticed in both herds.

2.2. Ovary collection

Both ovaries were removed from the seven heifers PI by surgical spayed procedure (transvaginal). This procedure was performed by licensed veterinarians using a Hauptner castration device (Ecraseur, Hauptner, Germany). All animals used in this study were handled in strict accordance with good animal practice and the conditions defined by the Animal Ethics Committee at INTA, Argentina. All efforts were made to minimize suffering. Since non-PI heifers were not available for castration from the commercial herd were the PI ovaries were collected, BVDV free control ovaries were obtained from a local slaughterhouse from British crossbred cycling heifers (corpora lutea were observed in at least one ovary) weighing 370–450 kg. BVDV control was performed by viral isolation from follicular fluid.

2.3. Virus isolation and titration of BVDV neutralizing antibodies

Follicular fluid from all tertiary follicles greater than 2 mm in diameter corresponding to both ovaries of each PI and control ovaries was aspirated with a disposable 21 G butterfly attached to a vacuum system and used for virus isolation (Fray et al., 2002). Briefly, 20 μl of follicular fluid sample (dilution 1/120) was inoculated on MDBK (Madin-Darby Bovine Kidney) cells cultured at 37 °C in 5% CO_2. After three blind passages (48 h), the presence of BVDV was detected by a direct fluorescent antibody test (DFAT) using a fluorescein conjugated porcine polyclonal antiserum (American Bioresearch Lab Sevierville, TN, USA).

Viral neutralizing antibody titers were determined in serum and follicular fluid samples from all experimental heifers using a standard microtitration procedure (Frey and Liess, 1971). Briefly, 0.1 ml of each sample was diluted in culture media, in a twofold dilution series, with 1×100^{-1} $TCID_{50}$/ml of the cytopathogenic BVDV reference isolate NADL. Each sample was tested in duplicate and cultured for 72 h at 37 °C. The presence of cytophatic effect (CPE) was detected microscopically. Viral neutralizing antibody titers were calculated by the reciprocal of the maximum serum or follicular fluid dilution that still neutralized the reference cytopathogenic NADL BVDV strain.

2.4. Microscopic studies

Each ovary was cut sagittally and fixed in 10% buffered formalin, embedded in paraffin blocks, sectioned in a serial sequence and the sections were stained with hematoxilin and eosin (H&E). Sections of the middle part from each ovary were analyzed. Follicle counting was performed in the cortical region of each section. The area observed in the microscope under 100× was considered to be a microscopic field. An exhaustive evaluation of the cortical area was done by the observation of an average of 300 fields at

100× per ovary. For follicle counting, each microscopic field that had at least one follicle was considered positive. For comparative analysis, the follicles in each field at 100× were quantified according to their stage of development as follows: (1) primordial follicles: oocytes without zona pellucida and surrounded by flattened granulosa cells; (2) primary follicles: oocytes with zona pellucida and a layer of cubic granulosa cells; (3) secondary follicles: oocytes surrounded by several layers of cubic granulosa cells and (4) tertiary follicles: presence of multiple granulosa cells layers and formation of the follicular antrum containing follicular fluid, adapted from Rodgers and Irving-Rodgers, 2010. The number of observation fields and class of follicle were recorded.

2.5. Immunohistochemistry

A dilution 1:500 of monoclonal antibody 15C5 (Kindly provided by Dr. R. Donis, University of Nebraska, Lincoln, USA) was used for specific detection of the viral protein E^{rns} (gp48) in sections of formalin-fixed, paraffin-embedded ovary tissues according to Odeón et al. (1999). Tissues sections were incubated for 1 h at 37° C in a humidified atmosphere. An avidin-biotin-alkaline phosphatase complex (Vectastain ABC kit alkaline phosphatase mouse IgG AK-5002, Vector Burlingame, CA, USA) was employed to reveal locations of BVDV antigen (Odeón et al., 1999). The sensitivity and specificity of the staining procedure was ascertained by the inclusion of adequate positive and negative tissue controls.

2.6. In vitro production of bovine embryos

In vitro maturation – Ovaries collected from PI and control heifers were transported from the collecting places to the laboratory in sterile 0.9% NaCl solution at 20 °C. Cumulus-oocyte complexes (COCs) were recovered by aspiration of 2–10 mm follicles, and diluted with a Tyrode's-lactate solution (TL-HEPES). Only COCs with three layers of compact and unexpanded cumulus cells were selected for IVF procedures. COCs were washed three times and then cultured in TCM-199 supplemented. Maturation was performed in four well plates (Nunc, Roskilde, Denmark) in groups of 50 COCs in 400 μl of maturation media for 22–24 h at 38.5 °C under 5% CO_2 in air with maximum humidity.

In vitro fertilization – Motile spermatozoa were obtained by centrifugation of frozen-thawed semen of commercial BVDV-free bulls on a discontinuous Percoll density gradient (30–60–90%). Matured COCs were transferred to a new four well plate (Nunc, Denmark) containing 400 μl of IVF-SOF (*in vitro* fertilization – synthetic oviduct fluid) supplemented with 50 μg/ml heparin. Spermatozoa were counted and an aliquot of sperm suspension was added to each well to obtain a final concentration of 2×10^6 sperm/ml. Plates were incubated for 24 h under the same conditions described for maturation.

In vitro culture – Loosely associated cumulus cells and spermatozoa were removed by gentle vortexing. Presumptive zygotes were then cultured in groups in SOF-citrate covered with mineral oil, and incubated at 38.5 °C under 5% CO_2, 5% O_2, 90% N_2, with maximum humidity. Seventy-two hours post insemination cleavage was determined. Embryo culture was performed for seven days.

2.7. Extraction of RNA and nested RT-PCR

Total RNA was extracted from IVF procedure media samples (IVF-SOF, IVF washing media, embryo washing media and SOF citrate) using TRIzol Reagent (Invitrogen Life Technology, Grand Island, NY, USA) according to the manufacturer's instructions. Total RNA from single embryos was isolated using the NucleoSpin RNA XS Kit (Macherey-Nagel, GmbH & Co. KG Düren, Germany), according to the manufacturer's instructions. All RNA samples were stored in 30 μl of nuclease-free water at −80 °C until *nested* RT-PCR was performed.

Synthesis and amplification of cDNA by PCR was performed according to Hyndman et al. (1998), with minor modifications. For cDNA Synthesis, 10 μl of RNA, 0.2 μl (0.02 μg/μl) of random hexamers (Biodynamics) and 5.8 μl of dH2O was denatured at 65 °C for 5 min and cooled on ice. The following was added to each reaction tube, 5 μl RT Buffer (0.05 mM Potassium phosphate (pH 7.2), 0.01 mM DTT 0.2% Triton X-100 and 10% glycerol), 1 μl (5 mM) dNTP (Biodynamics), 1 μl MgCl2 (25 mM), 0.5 μl RNA Guard (Promega), 0.5 μl (6 U) Moloney murine leukemia virus reverse transcriptase (Promega) and 1 μl dH_2O to give a final reaction volume of 25 ml, which was then incubated for 90 min at 37 °C. PCR amplification of cDNA was carried out in a total volume of 25 ml containing 4 μl of cDNA, 3 μl MgCl2 (25 mM), 2.5 μl 5× Taq buffer (Promega), 1 μl (5 mM) dNTP, 1 μl of each primer (2.5 mM each) 324:326 (Vilcek et al., 1994), 0.3 μl (1.5 U) Taq Polymerase (Promega) and 12.2 μl dH2O. The reaction mix was subjected to 94 °C 1 min and 30 cycles of 95 °C 1 min, 58 °C 1 min and 72 °C 1 min with elongation step to 72 °C 1 min, using a thermocycler. The nested amplification reagents were identical to the amplification reaction described above. Two microlitres of the first amplification mix was subjected to a second round using 1.5 μl (2 mM) of each internal primers: PESTI 3 (Position in NADL 176-197): PESTI 4 (Position in NADL 345-325) (Hyndman et al., 1998) with the same cycle number. Amplified products were separated by electrophoresis in a 2% agarose gel, and DNA bands were visualized by ethidium bromide staining. The expected amplification product was 171 bp.

2.8. Statistical analysis

Data from follicle counting was analyzed using the PROC GLM with the Statistical Analysis System software package (SAS, 2001). A P value < 0.05 was taken to denote statistical significance. Chi square test was performed to analyze data of *in vitro* embryo production. A P value < 0.05 was taken to denote statistical significance.

3. Results

3.1. Virus isolation, titration and detection of neutralizing antibodies

Viral isolation from all samples of peripheral blood leukocytes was positive for the seven heifers screened in the two samplings in an interval of 30 days. The virus titration and neutralizing antibody results are shown in Table 1. Virus titers in ovarian follicular fluid collected from all PI heifers were higher than BVDV titers in serum collected from these animals at the same time. Two PI heifers (#5 and #7) had low levels of serum neutralizing antibodies to the reference NADL strain in their follicular fluid. The viral genome of BVDV was identified when these samples were subjected to nested rt-PCR confirming the presence of virus (data no shown).

Table 1
Virus isolation, titration and detection of neutralizing antibodies from cells and fluids of persistently infected heifers with BVDV.

PI heifer	Leukocyte Viral Isolation	Viral titer[a]		Neutralizing antibodies	
		Serum	FF	Serum	FF
1	Pos	$10^{3.50}$	$10^{9.00}$	Neg	ND
2	Pos	$10^{3.10}$	$10^{8.97}$	Neg	ND
3	Pos	$10^{3.87}$	$10^{8.50}$	Neg	ND
4	Pos	$10^{5.30}$	$10^{7.50}$	Neg	ND
5	Pos	Neg	Neg	1:8	1:40
6	Pos	$10^{3.73}$	$10^{5.13}$	Neg	Neg
7	Pos	Neg	Neg	1:16	1:40

PI: persistenly infected; FF: follicular fluid; Pos: positive; Neg: negative; ND: not done.
[a] Viral titers ($Log_{10}TCID_{50}$/ml) of BVDV and neutralizing antibodies levels (inverse of dilution) in serum and follicular fluid of PI heifers.

3.2. Histological analysis and follicle counting

Microscopic examination of the sections of ovaries from PI heifers showed a significant drop in the numbers of cortical follicles, increasing the number of fields without follicles in cortical regions (Fig. 1A) in comparison with control non-PI sections of ovary (Fig. 1B). The SAS GLM procedure indicated statistically significant differences between the two conditions; median values from the total number of fields without follicles were 0.28 and 0.40, respectively. The results showed a significant decrease in the number of primordial and tertiary follicles in the cortical regions of ovaries from PI heifers in comparison with controls. No differences were found for primary and secondary follicles between PI and control ovaries (Table 2).

Several mononuclear (lymphocytic) foci were found sparsely disseminated throughout the ovaries analyzed of PI heifers (Fig. 2). Ovaries from control cattle, at comparable stage of the ovarian cycle, were normal and no mononuclear cells were observed (Fig. 1B).

3.3. Immunohistochemistry

Viral antigen was widely distributed in ovarian tissues from all PI heifers. The virus signal was present in the ovarian stroma, the theca cells of developing follicles, and *cumulus oophorus* cells surrounding some oocytes (Fig. 3).

3.4. In vitro fertilization

Out of 43 oocytes retrieved from all ovaries of seven PI heifers, 36 were considered of good quality (with three

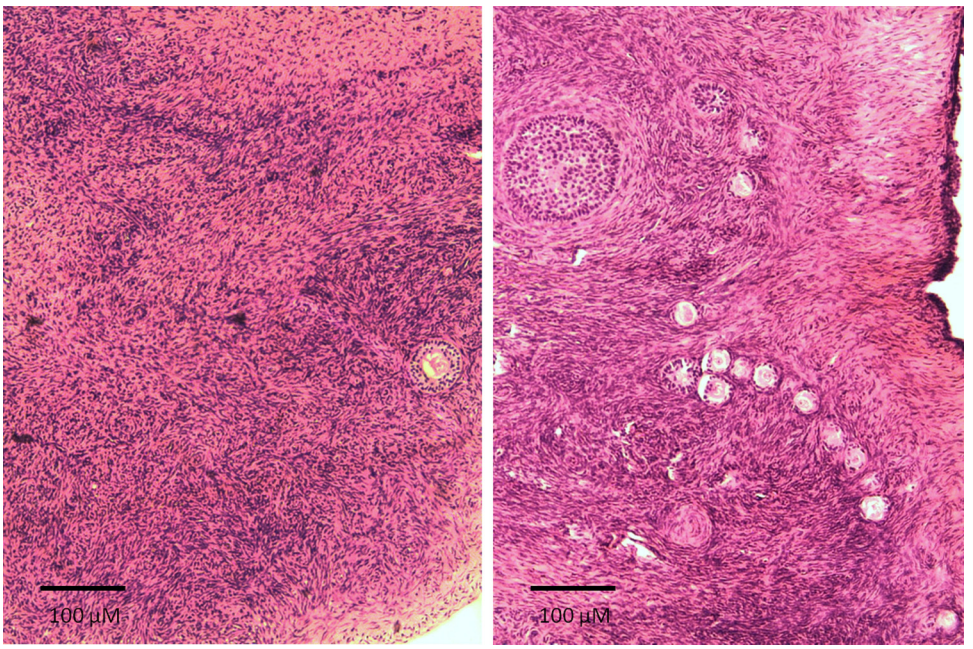

Fig. 1. Microphotograph of the cortical area of an ovary from a PI heifer (left). Note the severe reduction in follicles in the cortical region. Right: the same area from a normal heifer. Note the regular distribution of primary and secondary follicles. H&E 100×.

Table 2
Comparison of follicle stage of development between CN and PI ovaries.

Fields (N)	Follicle stage of development[c]				
	Primordial	Primary	Secondary	Tertiary	Total
CN (992)	1.31[a]	0.32[a]	0.08[a]	0.11[a]	1.82[a]
PI (1037)	0.58[b]	0.26[a]	0.05[a]	0.05[b]	0.94[b]

CN: control; PI: persistently infected.
[c] Total fields observed/number of follicles. Media values with different letters show significant differences between groups ($P < 0.05$).

layers of compact and unexpanded cumulus cells) and were used for *in vitro* fertilization procedures. These oocytes were from three of seven PI heifers a summary of results from the *in vitro* fertilization procedures is shown in Table 3. After *in vitro* fertilization, presumptive zygotes were evaluated until day seven of culture. Even though there were no statistical differences, *in vitro* embryo production rate from the PI oocytes was lower than the control ones (Table 3).

After *nested* RT-PCR of media used for *in vitro* fertilization procedures BVDV viral genome was detected in the Embryo Washing Medium of oocytes from PI heifer #7, and in the Culture Medium of oocytes from PI heifer #5. In addition, BVDV was detected in two developed embryos obtained from oocytes of PI heifer #7. However, BVDV was not detected in three embryos procured from heifer PI #5, as well as in the media and embryos from control procedure of FIV.

4. Discussion

Ovarian dysfunction has been suggested previously in both acute and persistent infections with BVDV (Grooms et al., 1996; Fray et al., 1998). Although the source of infection is different in both cases those findings suggest a link between infection with BVDV and changes in normal follicular dynamics. Several *in vitro* and *in vivo* studies showed that disturbances in follicular or oocyte development can result in significantly higher incidence of fertilization failure and early embryonic death (Grooms et al., 1998; Givens et al., 2000; McGowan et al., 2003). In this study, the decrease in both total follicular population and in the number of not only primordial but also tertiary follicles indicates that follicular development was altered. These findings were reflected in the poor *in vitro* performance of the oocytes harvested from these ovaries.

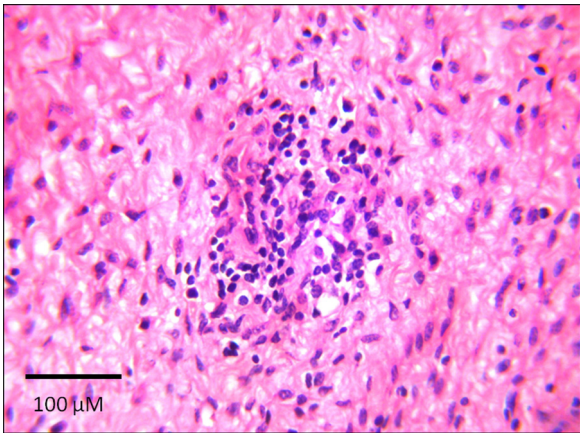

Fig. 2. Microphotograph of a perivascular lymphocytic focus in the ovary cortex of a PI heifer. H&E 400×.

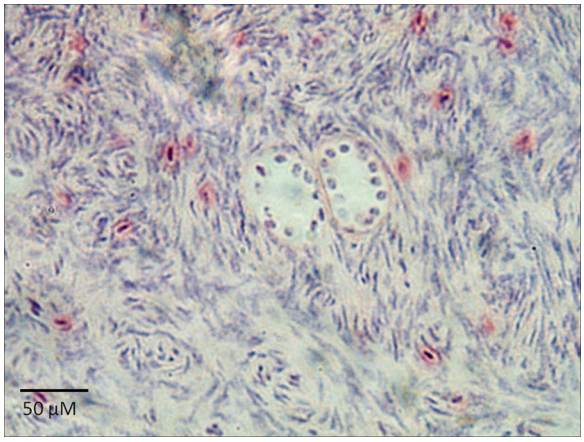

Fig. 3. Microphotograph of BVDV antigen detected by IHQ in an ovary section from a PI heifer. H&E 100×.

Even though various authors demonstrated that BVDV infection of susceptible breeding cattle can cause ovarian dysfunction (Kafi et al., 1997; Grooms et al., 1998), the mechanisms involved are not yet fully understood. Currently, most studies about the causes of poor fertility in ncp-BVDV infected cattle are focused on the interaction between the virus and ovarian-endometrial function. In agreement with previous results (Grooms et al., 1996; Marley et al., 2009), the significant decreased number of follicles observed in the cortical regions of ovaries from PI heifers compared to the same regions from uninfected animals suggests that the virus has a deleterious effect on the follicular population starting as early as the first stage of follicular development. After migration of the primordial germ cells through gonadal ridges, they arrive at the gonads and are embedded in the proliferating tissue, and continue to multiply by mitotic division (Lin et al., 2002). If these cells are permissible to the virus, the mitotic division rate could be reduced and therefore affect the number of primordial germ cells. The decreased number of primordial follicles observed reflects significant morphological changes in the ovaries from PI heifers with BVDV, suggesting a reduction in normal ovarian activities. It was assumed that the significant difference in primordial follicular population is neither affected by the body condition, nor by the heat cycle stage. The PI and control

Table 3
Comparison of *in vitro* fertilization procedures with oocytes from control and persistently infected (PI) ovaries. Chi square $P < 0.05$.

Group	Ovaries (n)	Recovered oocytes	Cultured oocytes	Cleaved oocytes (%)	Embryos (%)
Control	6	38	31	24 (77.4)	14 (45.2)
PI	6	36	25	13 (60)	5 (20)

heifers studied were from the same range age and despite their condition of persistently infected the heifers had good body condition.

On the other hand, as reported by other authors (Grooms et al., 1996), we also found a significant drop in the number of tertiary follicles, but the decrease in the number of primordial follicles is a novel data which shows that follicle formation is being altered in PI heifers.

Although, there is little evidence about lesions in ovarian tissue from PI cattle (Cutlip et al., 1980), our microscopic findings of scarce focal lymphocytic inflammatory reactions in the ovarian stroma of PI heifers might be associated with the abnormal development of the follicular population (Grooms et al., 1998). Although, some authors have not observed an association between the presence of viral antigen and tissue lesions in the ovaries of PI heifers, as has been reported in acute BVDV infections (Liebler-Tenorio et al., 2004). Even though was not a goal of the present work to perform a quantitative analysis of BVDV positive follicles, a wide distribution BVDV antigen was observed throughout the ovaries of PI heifers. This observation was consistent with the erratic distribution of BVDV in other tissues as reported previously (Shin and Acland, 2001; Baszler et al., 1995; Sopp et al., 1994). A broad BVDV signal of NS3 protein and E2 glycoprotein was also demonstrated within ovarian stroma and follicular cells from the ovaries of PI heifers (Fray et al., 1998, 2000). These results demonstrate that an intact zona pellucida (ZP) does not guarantee that the enclosed oocyte will be free from BVDV, suggesting that cumulus oophorus processes that contact oocytes through the ZP may offer a possible route of infection (Fray et al., 1998).

High BVDV titers detected in the follicular fluid of PI heifers in this study are consistent with reports which showed titers of $1 \times 10^{7.0}$ $TCID_{50}$/ml in ovarian tissue collected from PI heifers (Fray et al., 2000). Interestingly, virus titers in sera were lower than in the corresponding follicular fluids; however, the serum titers were comparable to those reported previously from PI cattle elsewhere (Brock et al., 1998). Differences observed between titers of follicular fluid and serum could be attributed to the fact that serum concentrations of BVDV gradually declined in all PI animals over at least a 2-years period (Brock et al., 1998). In addition, follicular fluid viral titers could remain more stable due to the virus affinity for the reproductive tract tissues. The absence of virus in serum and follicular fluid from PI heifer's #5 and #7 might be related to the presence of neutralizing antibodies in both serum and follicular fluid. These findings have important implications for the use of serum for virus isolation to identify persistently infected animals. A similar observation was reported by Brock et al. (1998).

Regarding to the efficiency of oocyte recovery after ovarian aspiration, its total number from PI heifers was comparable with the control group, but lower in quality. The number of recovered oocytes was similar to the average obtained in the routine procedures used in our laboratory when processing ovaries from slaughterhouse (4.5–5 good quality oocytes per ovary). This fact was in somehow reflected on its cleavage rate and embryo production obtained (although not statistically different). Cumulus oocytes complexes (COC's) obtained from acutely infected or PI cattle constitute a biohazard for BVDV infection by the IVF procedure, since the virus is protected inside the oocytes and cumulus cells against neutralizing antibodies present in the extracellular environment, mainly the in follicular fluid (Galik et al., 2002). The use of oocytes from BVDV PI heifers in an IVF procedure, showed a decrease in both the cleavage and embryo production rates regard to from free – virus oocytes. Therefore the developmental potential of ncp-BVDV infected oocytes can be established in the present study. *In vitro* fertilization techniques may be responsible for BVDV transmission after embryo transfer (Stringfellow, 1998). In the IVF procedure carried out in our work, the detection of viral genome BVDV in two developed embryos highlighted the potential for germ line transmission of BVDV. In addition, the screening of media used in the IVF procedure showed the presence of BVDV in several of them. BVDV was detected in two washing media from different IVF procedures, which indicates infection of oocytes/embryos of PI heifers to the IVF's media and the amplification of the contamination toward *in vitro* systems. These results are consistent with recent work in which BVDV was detected in the media used for IVF employing oocytes from PI heifers (Marley et al., 2009).

Detection of the viral genome in embryos collected from PI heifers has also been demonstrated, (Tsuboi and Imada, 1998). Non-cytophatic BVDV genome was detected from 2/17 (11.8%) of *in vivo* embryos derived from PI cows. This finding clearly indicates the high risk of spreading the virus by means of micromanipulation techniques. Viral particles, either from granulose cells or the oocyte itself can be the source of infection in IVF protocols. The risk of viral transmission in an *in vitro* production system is high, especially when considering that cumulus-oocyte complexes are often pooled from several follicles and ovaries of unidentified cows obtained from an abattoir without BVDV screening.

In conclusion, the present study demonstrated that BVDV PI heifers exhibit alterations in their follicular populations. We inferred that the lower number of primordial follicle will indicate an early interaction between the virus and germ cell line affecting directly the mechanisms involved in the ontogenesis of the ovary. Our findings could help to explain the low rates of cleavage and embryo development, highlighting the effect of Pestivirus infections in an *in vitro* embryo production system.

Conflict of interest statement

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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