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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