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SHORT COMMUNICATION

Upregulation of interferon-alpha gene in bovine embryos produced in vitro in response to experimental infection with noncytophatic bovine-viral-diarrhea virus

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Abstract

In-vitro fertilization is a routine livestock-breeding technique widely used around the world. Several studies have reported the interaction of bovine viral-diarrhea virus (BVDV) with gametes and in-vitro-produced (IVP) bovine embryos. Since, gene expression in BVDV-infected IVP bovine embryos is scarcely addressed. The aim of this work was to evaluate the differential expression of genes involved in immune and inflammatory response. Groups of 20–25 embryos on Day 6 (morula stage) were exposed (infected) or not (control) to an NCP-BVDV strain in SOF medium. After 24 h, embryos that reached expanded blastocyst stage were washed. Total RNA of each embryo group was extracted to determine the transcription levels of 9 specific transcripts related with antiviral and inflammatory response by SYBR Green real time quantitative (RT-qPCR). Culture media and an aliquot of the last embryos wash on Day 7 were analyzed by titration and virus isolation, respectively. A conventional PCR confirmed BVDV presence in IVP embryos. A significantly higher expression of interferon-α was observed in blastocysts exposed to NCP-BVDV compared to the controls ($p < 0.05$). In this study, the upregulation of INFα and TLR7 genes involved in inflammatory and immune response in BVDV-infected IVP bovine embryos is a new finding in this field. This differential expression suggest that embryonic cells could function in a manner like immune cells by recognizing and responding early to interaction with viral pathogens. These results provide new insights into the action of BVDV on the complex molecular pathways controlling bovine early embryonic development.

Keywords Bovine embryos · Gene expression · Bovine-viral-diarrhea virus · Interferon-α

Introduction

Bovine viral diarrhea is a disease with a worldwide distribution and endemic in most cattle populations. BVDV is a single-stranded RNA virus that causes respiratory disease, immunosuppression, abortions, and a reduction in reproductive efficiency that consequently undermines herd health and production [1]. Previous studies have suggested various mechanisms through which BVDV infection can impact reproduction. Among these can be mentioned effects on reproductive organs, gametes, embryo, and fetus. Most of the mechanisms associated with early embryonic losses have however not yet been described clearly [2]. In this sense assisted reproductive techniques, and especially the in vitro production of bovine embryos used for commercial purposes, has also emerged as a useful model for primary research.

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Each of the assisted reproductive techniques available has the potential to increase the spread of infectious diseases of cattle [3]. Systems to produce in-vitro fertilized (IVF) embryos provide unique critical control points for the monitoring and prevention of viral infections [4]. Several authors have demonstrated how easily BVDV can contaminate these systems and the risk of transmission of the infection through IVP embryos [3–5]. These results are particularly associated with IVP embryos owing to the zona pellucida nature. The structure of the ZP is such that it allows at least partial penetration of some viruses and can be carried in cells adhering to this [6]. These differences affect the persistence and the potential outcome of viral association with IVP embryos.

Early embryonic development is a dynamic process controlled by complex molecular pathways that are regulated by the differential expression of many genes [7]. Most of the embryonic losses occur during the first days after fertilization and during the process of implantation (before days 16 to 17) and its cause is multifactorial [8]. Strong evidence suggests that infection with bovine viral diarrhea virus (BVDV) is one of many potential causes of pregnancy failure [2]. Since previous studies have demonstrated that the apoptosis and the interferon (IFN) response become manifest from the earliest stages of pregnancy [9, 10], an evasion of those two key elements of the innate immune system may be crucial, not only for transmission to the fetus, but also for the maintenance of immunotolerance [9]. Many studies on the contribution of the innate immune system in recognizing and subsequently initiating a host response to an invasion of RNA virus has been performed over the last decade [11]. Toll-like receptors (TLRs) have been described to sense RNA virus invasion and they are of substantial importance in the initiation of an antiviral response upon infection (TLR3, TLR7 and TLR8) [10]. However, the studies on these receptors in bovine embryos are limited. Likewise, several studies have reported the regulation of the gene expression related to the stress response in IVP bovine embryos [12, 13]. Nevertheless, studies on gene expression in response to BVDV infection are still scarce. In this study, we examined genes related to the innate immune response (toll-like receptor [TLR]-3 and 7, interferon-α and ß, interleukin-10 and 12), apoptosis [BAX, BCL2], antioxidant protection [SOD2] and inflammatory response and embryonic implantation [LIF]) in *in vitro* produced bovine embryos that were exposed or unexposed to a NCP-BVDV strain.

This study had the novel advantage of investigating gene expression in BVDV-infected IVP bovine embryos, away from the relatively more numerous previous in vivo and ex vivo typical studies on the integrity, viability, and developmental potential of BVDV-infected bovine embryos.

Materials and methods

Experimental design

This study compared the relative abundance of specific transcripts in embryos in the morula stage (Day 6) infected experimentally with a noncytopathic (NCP)-BVDV or free BVDV (Fig. 1). Briefly, embryos in the morula stage (Day 6) were infected experimentally by coincubation with a noncytopathic (NCP)-BVDV, as described by Vanroose et al. [14]. The embryos were transferred in groups of 20–25 to 400 µL of synthetic oviductal fluid (SOF), covered with mineral oil, and incubated under the same conditions for 24 h. The embryos were randomly allocated into two treatment groups (a BVDV group [infected] and one without infection [control]). Both embryo groups were evaluated on Day 7 (Day 0 = in-vitro fertilization) to determine the proportion of embryos that had reached the blastocyst stage.

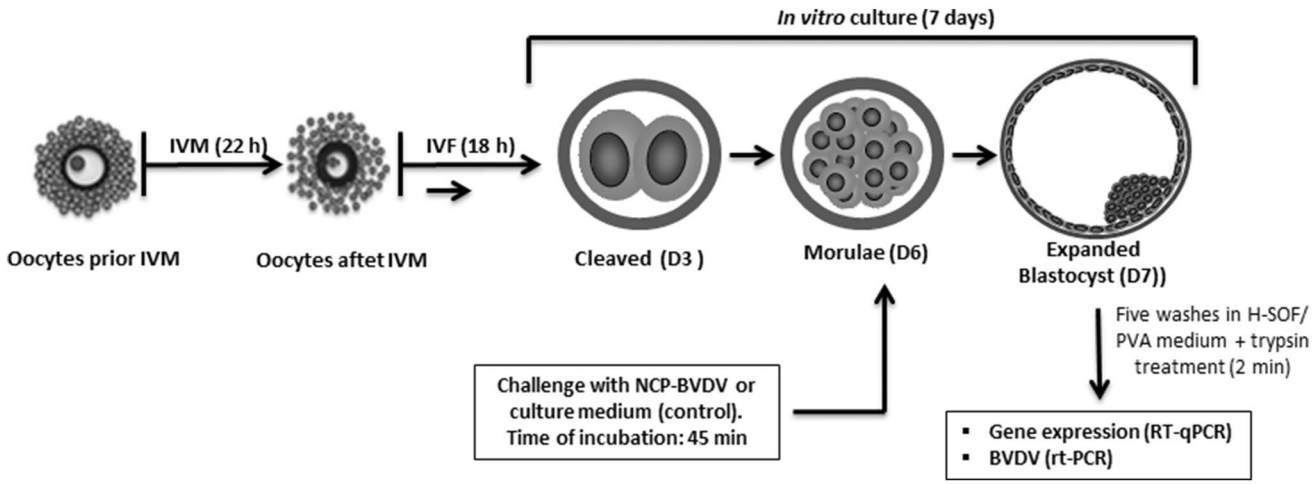

Fig. 1 Experimental design. (Adapted from: Chediek Dall'Acqua et al. 2019 Reprod Dom Anim 2019; 54:666–677)

Only those embryos in expanded blastocyst stage (five per group) were subsequently washed five times in H-SOF/ PVA medium (100 µL/wash) plus one wash with 0.25% (w/v) trypsin (2 min) to remove any residue adhering to the zona pellucida that may interfere with the interpretation of the detection of the virus by the PCR technique. Then, each group was transferred to RNA*later*™, stabilization solution, for storage at − 80 °C. The expression of specific transcripts was quantitated by means of the real-time quantitative polymerase-chain reaction (RT-qPCR) and the presence of BVDV determined by conventional PCR. Four trials were performed during this study in 4 different weeks. The culture media and ovaries used in this study were checked for BVDV free by RT-PCR.

Oocytes collected from abattoir ovaries

Ovaries of cows (*Aberdeen Angus*) were collected at a local abattoir, placed in physiological saline (0.9% [w/v] NaCl) at 37 °C and transported to the laboratory within 2 h after slaughter. The ovaries were washed in fresh Dulbecco's phosphate-buffered saline (pH 7.4) and the cumulus-oocyte complexes (COCs) recovered by aspiration of the follicles (2- to 8-mm diameter) with an 18-guage needle attached to a vacuum pump. COCs with good quality oocytes i.e., more than three layers of compact cumulus cells and homogenous cytoplasm, were selected for use in these experiments [15]. Those COCs were washed three times in TL-HEPES under the stereomicroscope and the follicular fluid of each batch assayed by viral isolation to rule out the presence of the BVDV and other viral pathogens (e.g., bovine herpes virus-1 and − 4).

In vitro maturation and fertilization

COCs were cultured in TCM-199 supplemented (50 µg/mL gentamycin, 0.01 UI/mL rhFSH) and 10% FCS (*Internegocios S.A. FBI code, irradiation certificate*) for 22 h at 38.5 °C in a humidified atmosphere of 5% [v/v] CO_2 in air. The COCs were washed three times in maturation medium and transferred (n = 40–50) into a well of a four-well dish containing 400 µL of in-vitro-maturation medium (IVM). Motile spermatozoa free of BVDV were obtained by centrifugation of thawed semen on a discontinuous Percoll density gradient (30-60-90% [w/v]). Matured COCs were transferred to new four-well plates (400 µL of Tyrode's medium) supplemented with 50 µg/ml heparin. The spermatozoa were counted, and an aliquot of sperm suspension added to each well to obtain a final concentration of 2×10^6 sperm/mL. The plates were incubated for 24 h at 38.5 ºC under 5% [v/v] CO_2 in air at maximum humidity. Loosely associated cumulus cells and spermatozoa were removed by gentle vortexing. The presumptive zygotes were then cultured in groups of 50 in 400 µL volumes of SOF medium covered with mineral oil at 38.5 ºC under an atmosphere of (v/v) 5% CO_2, 5% O_2, 90% N_2 at maximum humidity.

Stock and incubation viral

An NCP-BVDV strain, genotype 1a, (ID 13-558) was propagated in Madin-Darby bovine kidney (MDBK) cell line (provided by the Argentinean Cell Bank http://www.abac.org.ar/), which were cultured in minimum essential medium (MEM). The tissue culture infective dose per mL $TCID_{50}$ of stock virus was determined by virus titration infection in MDBK cells at a multiplicity of infection (MOI) of 1. Aliquots of stock virus (100 µL) with $10^{6.3}$ $TCID_{50}$/mL were frozen at − 80 °C prior to use. The embryos in the morula stage were recorded on Day 6 and were randomly separated in two groups (control and infected), incubated for 45 min at 38.5 °C in 5% CO_2 in air and then were washed five times after the coincubation. After 24 h, expanded blastocysts (Quality Code 1: Excellent or Good) [16] were washed and stored in RNAlater™ stabilization solution.

Total RNA extraction and reverse transcription (RT)

Pools of five expanded blastocysts from each treatment in four biological replicates were lysed in 40 µL of extraction buffer (XB; Arcturus, Carlsbad, CA, USA) by incubation at 42 °C for 30 min, followed by centrifugation (3000× *g*, 2 min) and then stored at − 80 °C until use. The total RNA was extracted from each pool of embryos, and the residual genomic DNA removed by DNAse-I digestion, by the RNase-Free DNase Set™ (Qiagen, Valencia, CA, USA). The total RNA was extracted by means of the PicoPure RNA-Isolation Kit™ (Arcturus, Carlsbad, CA, USA). Reverse transcription was carried out by the RevertAid™ H Minus First Strand Kit™ (Thermo Scientific Inc., Pittsburgh, PA, USA).

Relative quantification by real-time polymerase chain reaction (PCR)

Quantification of a panel of 12 specific transcripts (9 genes plus 3 constitutive housekeeping genes) (Table 1) was carried out by reverse transcription via RT-qPCR reaction. Dissociation curves were performed after each PCR run to ensure that a single PCR product had been amplified. A relative qPCR was used for quantification of mRNA expression levels by using the amplification efficiency of each gene as a correction factor. The geometric average of three reference genes were used (*HPRT1, HMBS* and *GAPDH*), being analyzed with the GeNorm Visual Basic Application Program (Version 3.5) [17]. To measure the differences in expression between the two groups of embryos, we used

Table 1 Primers used in real-time quantitative PCR (RT-qPCR)

Gene*	Gene bank	Sequence 5′–3′	Amplification efficiency (%)	Size (bp)
TLR3	WAN	AGGCAGGTGTCCTTGAACTTG GATCTTTCAATAGATTCTGTGTTA CAACGAAA	99.6	98
TLR7	WAN	AACTCTGCCCTGTGATGTCACTCT TGGAGAGATGCCTGCTATGTGGTT	99	150
INF-α	EU276064	GTGAGGAAATACTTCCACAGACTC ACT TGARGAAGAGAAGGCTCTCATGA	97.8	107
IL10	WAN	GGTGATGCCACAGGCTGAG AGCTTCTCCCCCAGTGAGTTC	94.4	68
IL12	WAN	CCAAAGTCACATGCCACAAGG CTGTAGTAGCGGTCCCGGG	97.3	203
BAX	U92569.1	TCCTTCGAGAGCGGCTGC AGGCGGTGAGCACTCCAG	100	123
BCL2	NM001166486.1	CCTGTGGATGACCGAGTA ATACAGCTCCACAAAGGCGT	100	85
LIF	NM173931.1	ACGTCGGCCGTGGTGCTCAG GGTTCTCTTTGGAAAGGTGTTC	90.2	199
SOD2	L22092.1	ACCTCAACGTCGCCGAGG CCAACCGGAGCCTTGGAC	93.5	260
HPRT1	AF176419	TGCTGAGGATTTGGAGAAGG CAACAGGTCGGCAAAGAACT	100	154
HMBS	BC112573.1	CTTTGGAGAGGAATGAAGTGG AATGGTGAAGCCAGGAGGAA	94.4	80
GAPDH	XM583628	TTCAACGGCACAGTCAAGG ACATACTCAGCACCAGCATCA	90.2	119

*TLR, toll-like receptor; INF, interferon; IL, interleukin; *LIF*, leukemia-inhibitory factor; *SOD*, superoxide dismutase; *HPRT*, hypoxanthine-guanine phosphoribosyltransferase; *HMBS*, hydroxymethylbilane synthase; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; WAN, without access number

the pairwise-fixed-reallocation-randomization test in the Relative Expression Software Tool (REST; V2.0.7, Copyright 2008, Corbett Research Pty. Ltd., Munich, Germany). Differences were considered statistically significant when $p < 0.05$. Following RT-qPCR run, the data were obtained by the iCycler iQ Real Time PCR Detection System™ (Biorad iQ Software Version 1.3) and analyzed according to manufacturer's instructions.

Viral detection by RT-PCR

Detection of BVDV nucleic acids by PCR was carried out from the cDNA obtained from both infected and noninfected embryos. A primer pairs specific for the BVDV NS5B region were used [18]. An 84 bp amplicon was generated based on that sequence. The amplified products with the expected size on 1.5% (w/v) agarose gels were visualized with the SYBR® Safe™ DNA stain *Viral isolation and titration*.

Virus titration was performed on culture media (Day 7) from both experimental groups in 96-well plates previously seeded with MDBK cell line. The virus titrations involved multiple tenfold dilutions by the endpoint method [19]. The plates were incubated for 72 h at 37 °C and the presence of BVDV detected by a direct fluorescent antibody test with a fluorescein-conjugated porcine polyclonal antiserum (American Bioresearch Lab Sevierville, TN, USA). To rule out the possibility of contamination in embryos due to the presence of virus remaining in the culture media, aliquots of the last wash were assayed by virus isolation and after four blind passages (48 h) the presence of BVDV was detected by the same fluorescent antibody probe as for the virus titration.

Results and discussion

The expression of the interferon-alpha (*IFN-α*) gene in embryos infected with the NCP-BVDV strain was higher ($p < 0.001$) than in control embryos. No significant differences were observed in the rest of the panel of genes analyzed (Table 2). To confirm virus infection by BVDV in the IVP embryos, a conventional PCR was carried out. The PCR run with primer pairs specific for the region of the BVDV nonstructural protein NS5B produced an 85 bp product from Day-7 expanded blastocysts exposed to an NCP-BVDV strain. No amplicon was detected in Day-7 expanded blastocysts that were not exposed to the virus (Table 3). BVDV

Table 2 REST* program analysis of gene expression data in blastocysts produced by in-vitro fertilization and exposed to ncp-BVDV strain

Relative gene expression (number of iterations: 6000)		
Gene**	Difference ratios	P(H1)†
TLR3	0.176	0.702
TLR7	10.363	0.053
INF-α	27.225	**0.009 (up)**
IL-10	4.681	0.569
IL-12	1.728	0.423
BAX	3.498	0.337
BCL-2	3.332	0.336
LIF	12.346	0.312
SOD2	13.506	0.137
HPRT1	0.027	Ref gene
HMBS	1.602	Ref gene
GAPDH	22.74	Ref gene

*Relative Expression Software Tool; IVF, in-vitro fertilization; NCP, noncytopathic; BVDV, bovine-viral-diarrhea virus; Ref gene, Reference gene

**TLR, toll-like receptor; INF, interferon; IL, interleukin; *LIF*, leukemia-inhibitory factor; *SOD*, superoxide dismutase; *HPRT*, hypoxanthine-guanine phosphoribosyltransferase; *HMBS*, hydroxymethylbilane synthase; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase

†Probability of the alternate hypothesis that the difference between both groups is caused only by chance. The gene expression indicated in boldface was found to be down-or upregulated in the embryos exposed to NCP-BVDV compared to those not exposed. The reference household genes were selected based on pairwise analysis of their expression stability by the GeNorm program. The difference ratios were obtained by using the randomization and bootstrapping techniques included in the REST program

titration was performed with the culture media from IVF embryos exposed to NCP-BVDV. Table 3 lists the viral titers of each assay at Day 7 of culture. Aliquots from the last medium wash from both groups were evaluated for viral isolation and found all negative.

In the present study, the constitutive expression of IFN-α was high relative to the other studied genes, this result could suggest other roles of IFN-α in early embryonic development. Although the rest of the analyzed genes did not show significant differences, the upregulation of the IFN-α gene in response to infection with BVDV does not have negative effects on early embryonic devolvement despite its antiviral activity. INF-α is a type-1 interferon that is part of the pro-inflammatory cytokines that are induced in response to viral infections and is closely related to the establishment of early pregnancy in ruminants [20]. The expression of the IFN-α gene has been detected previously in mouse preimplantation embryos [21] and more recently in transgenic somatic-cell-nuclear-transfer embryos, which expressed human recombinant IFN-α mRNA [22]. A recent study about IFN induction by BVDV showed alterations in the transcriptome of the PI cattle indicate significant upregulation of innate immune function [23].

The artificial exposure of embryos in morula stage to a high affinity strain of NCP-BVDV was chosen because a previous study had concluded that ZP-free IVP embryos were permissive to infection by both BVDV biotypes, with the susceptibility of the embryos increasing in accordance with their developmental stage [14]. Besides, most isolates of BVDV are NCP and unlike this biotype only the CP-BVDV strains induces cytopathic effects detectable with light microscopy. The presence of the NS5B region from BVDV genome in embryos exposed to an NCP-BVDV strain confirms the association of virus with ZP-intact in-vitro-derived bovine embryos, even after washing and trypsin treatment to avoid any virus remaining from the culture medium. Previous studies have indicated similar results, the virus remains associated with the ZP of IVP bovine embryos even after the conventional washing and trypsin treatment [24, 25]. More recently, our group and other authors have demonstrated that NCP-BVDV strains remain associated with both oocytes and embryos after experimental infections [26, 27], but no evidence has been found for changes in the cellular processes

Table 3 Summary of the results of the polymerase-chain-reaction (PCR) and classical-virology techniques

Trial*	Conventional PCR†		Virus titration¶		Virus isolation‡	
	Control	BVDV[1]	Control	BVDV[2]	Control	BVDV
1	Neg	++	0	1.97	Neg	Neg
2	Neg	+	0	2.1	Neg	Neg
3	Neg	+	0	1.87	Neg	Neg
4	Neg	++	0	1.87	Neg	Neg

*Five expanded blastocysts per pool were evaluated in each trial

†Conventional PCR from washed IVF embryos exposed to BVDV

¶BVDV titration from culture media (Day 7)

‡BVDV isolation from the last medium wash from the *in-vitro*–fertilized embryos of both groups

[1]Intensity of the amplification 85-bp band for bovine-viral-diarrhea virus, BVDV

[2]Final titer, 50% tissue-culture infective dose/ml; Neg, negative

involved in early embryonic development in response to infection by BVDV.

Furthermore, embryos produced in vitro have a greater chance of becoming contaminated by BVDV because of the culture period and the stickier and more porous nature of the ZP surrounding those embryos [6]. The infection with NCP-BVBD was demonstrated through amplification of the genetic loci of the virus by conventional PCR. Likewise, the difference between the titers in the culture medium of the initial morula (Day 6) and final blastocyst (Day 7) stages after inoculation suggested that the virus may have infected the blastocyst cells at this latter stage, thus leading to a decreased virus concentration in the medium. This phenomenon had also been observed in the culture medium of porcine embryos produced in vitro. The copies of porcine circovirus type 2 in the medium of those IVF embryos manifested a significant upregulation at the morula stage and then decreased dramatically at the blastocyst stage [28].

The absence of virus in the last washing of the infected-group embryos in each trial provides new evidence regarding the association of ncp-BVDV strains with IVP embryos and the putative effect of the virus on the pattern of gene expression. Although detection of the virus in the ZP or in an embryo by PCR does not necessarily mean that the virus is still infectious or that it is present in an infectious dose, the embryos produced for this analysis were not under any stress caused by the in-vitro-culture system, other parameters should be compared that were related to BVDV exposure. This aspect represents a critical advantage of the present study in that the differences observed in the INF-α gene expression between both groups of embryos (infected and control) could be monitored and interpreted with certainty. Aside from these results, however, little is known about the mechanisms involved in the activation of the innate immune response by viral infection in IVP embryos. There is extensive evidence that NCP-BVDV can evade the innate immune response in infected hosts by suppressing the induction of type I IFN response [10]. In contrast, new studies have emerged on the induction of type 1 IFN response to in vitro and in vivo infections by NCP-BVDV [29]. The results of this study demonstrated that BVDV infection can modulate the pathway of type-I interferons during early embryonic development in in vitro production systems, suggesting that embryonic cells similarly to immune cells, can recognize and respond at an early stage to the virus challenge.

A slight increase in the expression of TLR7 was also observed in embryos exposed to NCP-BVDV ($P = 0.053$) and this might suggest a role of TLR in early pregnancy. In addition, TLRs as members of the innate immune system are thought to be implicated in fertility, as those receptors are expressed in granulosa and/or cumulus cells [30]. Further studies are needed to understand the multiple mechanisms involved in cell survival during the viral infection of bovine embryos since, within that scenario, the cellular response to virus represents a key point in the association between early reproductive losses and BVDV infections.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

Informed consent The authors declare their consent to publish the data presented in this research.

Research involving human and/or animal participants The authors declare that the present research did not involve Human Participants and/or Animals.

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