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Transplacental infection by bovine alphaherpesvirus type 1 induces protein expression of COX-2, iNOS and inflammatory cytokines in fetal lungs and placentas

Mercedes M. Burucúa*[a], María A. Risalde*[b], Felipe A. Cheuquepán[a], Silvina Quintana[c,d], Sandra E. Pérez[e], Germán J. Cantón[a], Dadin P. Moore[a,f], Anselmo C. Odeón[f], Irene Agulló-Ros[b], María Valeria Scioli[a], Claudio Barbeito[g], Eleonora L. Morrell[#a], Maia S. Marin[a]

[a] *Instituto de Innovación para la Producción Agropecuaria y el Desarrollo Sostenible (IPADS Balcarce), INTA-CONICET, Balcarce, Buenos Aires, Argentina.*

[b] *Departamento de Anatomía y Anatomía Patológica Comparadas y Toxicología, Grupo de Investigación GISAZ, UIC Zoonosis y Enfermedades Emergentes ENZOEM, Universidad de Córdoba, Córdoba, Spain.*

[c] *Instituto de Investigaciones de Producción, Sanidad y Ambiente (IIPROSAM), FCEyN, UNMDP-CONICET, Mar del Plata, Buenos Aires, Argentina.*

[d] *Instituto de Biología Molecular Aplicada, Mar del Plata, Buenos Aires, Argentina.*

[e] *Centro de Investigaciones Veterinarias de Tandil (CIVETAN) – CONICET, Facultad de Ciencias Veterinarias, UNCPBA, Tandil, Buenos Aires, Argentina.*

[f] *Facultad de Ciencias Agrarias, UNMdP, Balcarce, Buenos Aires, Argentina.*

[g] *Laboratorio de Histología y Embriología Descriptiva Experimental y Comparada (LHYEDEC), Facultad de Ciencias Veterinarias, UNLP. CONICET. Buenos Aires, Argentina.*

*Mercedes M. Burucúa and María A. Risalde have equally contributed to this work.

[#]Corresponding author: National Institute of Agricultural Technology, Route 226 km. 73.5, Balcarce (7620), Argentina. +542266439120. morrell.eleonora@inta.gob.ar

Abstract

Bovine alphaherpesvirus type 1 (BoAHV-1) is associated with respiratory and reproductive syndromes. Until present the immunologic mechanisms involved in BoAHV-1 abortion are partially known. We studied key elements of the innate immune response in the placentas and fetal lungs from cattle experimentally-inoculated with BoAHV-1. These tissues were analyzed by histopathology. Furthermore, virus identification was performed by qPCR and the expression of the inflammatory cytokines such as tumor necrosis factor-alpha, interleukin 1-alpha and inflammatory mediators like inducible nitric oxide synthase and cyclooxeganse-2 was evaluated by immunohistochemistry. The viral transplacental infection was confirmed by the detection of BoAHV-1 by qPCR in the placenta and fetal organs, which revealed mild inflammatory lesions. Inducible nitric oxide synthase immunolabelling was high in the lungs of infected fetuses and placentas, as well as for tumor necrosis factor-alpha in the pulmonary parenchyma and cyclooxeganse-2 in fetal annexes. However, the expression of interleukin 1-alpha was weak in these organs. To our knowledge, this is the first study that provides strong evidence of an early immune response to BoAHV-1 infection in the conceptus. Advances in the knowledge of the complex immunological interactions at the feto-maternal unit during BoAHV-1 infection are needed to clarify the pathogenesis of abortion.

Keywords:

abortion, alfaherpesvirus, bovine, histopathology, immunohistochemistry, innate immunity, fetal lung, placenta, qPCR

Introduction

Bovine alphaherpesvirus type 1 (BoAHV-1) is associated with different conditions in cattle involving the respiratory and reproductive tract such as, rhinotracheitis, pneumonia, balanoposthitis and vulvovaginitis (Muylkens et al., 2007). Other clinical manifestations of the infection include conjunctivitis, fatal systemic infection in neonatal calves, infertility and abortions (Muylkens et al., 2007). The pathogenesis of BoAHV-1-abortion is partially known. Although previous studies strongly support an hematogenous spread of the virus from the placenta to the fetus via the umbilical vein (Rodger et al., 2007), the immunologic mechanisms that participate in the bovine conceptus during transplacental BoAHV-1 infection remains undefined (O'Toole et al., 2014). The placenta represents an immunological barrier against microbial infection that protects the fetus ensuring its correct development (Entrican, 2002). Initially, this function is performed through the recognition and response to different pathogens via sensors of the innate immunity (Reis et al., 2020). Such innate response to pathogens results in cytokine production, recruitment of immune cells and up-regulation of co-stimulatory molecules that are crucial for the control of infection during pregnancy (Entrican, 2002). Among the effector molecules of the innate immune response, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and the pro-inflammatory cytokines, tumor necrosis factor alpha (TNFα) and interleukin 1-alpha (IL-1α), play a central role enhancing the vascular permeability and modulating the migration of immune cells in cattle (Risalde et al., 2013).

Nitric oxide (NO) is one of the major molecules with pleiotropic activity at systemic and cellular levels (Krause et al., 2011). NO participates in the regulation of vascular tone, synaptic transmission, and host protection against infection and/or immunosuppression (Yaman and Aydemir, 2021). There are three main isoforms of nitric oxide synthase (NOS) with distinct functions and patterns of expression: constitutive neuronal NOS (nNOS) and endothelial NOS (eNOS), and inducible NOS (iNOS). This

latter is highly expressed in immune cells and plays a crucial role during many inflammatory and infectious processes (Barros et al., 2019).

COX-2 is an inducible enzyme and is involved in the synthesis of prostaglandins from arachidonic acid (Tanabe and Tohnai, 2002). COX-2 has multiple roles in bovine female reproduction: 1) temporary luteo-protective function for the establishment of pregnancy, 2) immunomodulatory mediator at fetal-maternal interface, 3) angiogenic factor, and 4) myometrial relaxant or stimulant (Arosh et al., 2004). In addition, COX-2 has an important function during the inflammatory response as well as during its resolution (Tanabe and Tohnai, 2002).

TNFα and IL-1α are proinflammatory cytokines produced by macrophages and epithelial cells (Kasprzak et al., 2004) being relevant in the induction and regulation of innate immune response (Risalde et al., 2011). During normal pregnancy, TNFα regulates different cellular processes (Haider and Knöfler, 2009). Nevertheless, TNFα also induces adverse effects for pregnancy, and depending on its concentrations, distribution of TNF receptors, and cell types involved, it is implicated in the pathogenesis of several disorders (Haider and Knöfler, 2009). According to this, it has been shown that TNFα is differentially modulated during active BoAHV infections in both the respiratory tract and the nervous system (Burucúa et al., 2019, 2020). Furthermore, previous works demonstrated increases of IL-1α during viral infections, such as those produced by human herpes virus-6B (Wang et al., 2022) and respiratory syncytial virus (Shilovskiy et al., 2021).

In the last years, several studies demonstrated the involvement of the innate immune response against placental infections (Marin et al., 2017; Hecker et al., 2022). However, the mechanisms underlying transplacental BoAHV-1 infection remain undefined. To our knowledge, this is the first study that shows the expression of key elements of the innate immune response in fetuses and placentas from cows experimentally-infected with BoAHV-1, providing strong evidence of an early immune response to the infection.

Material and methods

Animals and experimental design

Initially, twenty-four, five years old Aberdeen Angus cows were involved in the experiment. These animals were in good body condition and belonged to a beef herd located at INTA Balcarce, Argentina. Cows were genitally examined by rectal palpation and bled twice prior to breeding to evaluate their serological status to different reproductive infectious agents. All animals were serologically negative to *Neospora caninum* by an indirect fluorescent antibody test (IFAT) (Venturini et al., 1999), *Leptospira* spp by microscopic agglutination test (MAT) (Campero, 2017), and bovine viral diarrhea virus (BVDV-1) / BoAHV-1 by serum neutralization tests (OIE, 2018). The herd was also free to other abortive diseases including *Campylobacter fetus* and *Tritrichomonas foetus* by isolation from mucus cervicovaginal (Campero, 2017). In addition, the negative diagnosis of the animals to brucellosis (serology) and tuberculosis (intradermal tuberculin test) was performed according to the Argentina animal health legislation for the eradication of these diseases (SENASA).

Cows were estrus synchronized using Estradiol benzoate (Von Franken®) according to the manufacturer's instructions. Subsequently an intravaginal device (Dispocel Monouso, Von Franken®) was placed in each animal during eight days. When the intravaginal device was withdrawn, all cows received Estradiol Cyprionate (Cipiosyn, Zoetis®, Villa Adelina, Argentina) and Prostaglandin F2 alpha (Lutalyse, Zoetis®) according to the manufacturer's instructions. Cows were allocated into a pen with three healthy Angus bulls (*Campylobacter fetus* and *Tritrichomonas foetus*-free) for natural breeding over 7 days. All the animals were fed on a natural grassland and maintained under standard animal husbandry conditions. Transrectal ultrasonography was performed at 60 days after mating and 11 pregnant cows carrying a single fetus were selected for experimental infection.

At 270 days of gestation, cattle were randomly allocated into two groups. Group A cows (n = 7) were challenged with 2 ml of BoAHV-1 Cooper strain (1 x $10^{5.87}$ $TCID_{50}$/mL) by intravenous injection (Rodger et al., 2007), and Group B cows (n = 4) were inoculated intravenously with 2 ml of sterile PBS (control group). Animals were slaughtered at 285 days of gestation [15 days post-infection (dpi)] according to the protocol approved by the Animal Ethics Committee at INTA Balcarce (CICUAE#187/2019). After slaughter, all fetal organs and the placenta of each cow were immediately removed and examined following standard gross pathology procedures (Morrell et al., 2019). Subsequently, samples of these tissues were collected and fixed in 10% buffered formalin during 24 hours for further histopathological and immunohistochemical studies. In addition, placentomes and fetal tissue samples of each fetus were frozen at -80 ˚C until processing by qPCR.

Clinical signs and serology of dams

Each animal was monitored for control of temperature, presence of nasal and ocular discharges, inappetence, or any other clinical signs at 0, 3, 7, 10 and 14 dpi. In addition, blood samples of each animal were collected on the same days. Sera were separated and tested for antibodies to BoAHV-1 by serum neutralization. Neutralizing antibodies to BoAHV-1 were evaluated by microtitration on MDBK cells (OIE, 2018). Neutralizing antibody titers were determined as the highest dilution that completely inhibited the cytopathic effect (CPE) after 72 h of inoculation.

DNA extraction and qPCR

Placentomes and fetal tissues (lung, spleen and liver) homogenates from each conceptus were clarified in RNase-/DNase-free 1X PBS by centrifugation for 10 min at 1500 × *g* at 4º C. DNA was extracted from 200 µl clarified tissue samples using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen Inc.), as recommended by the manufacturer. DNA concentrations were measured by absorbance at 260 nm using an Epoch Microplate Spectrophotometer (BioTeK).

qPCR assays to detect BoAHV-1 DNA were performed using primers that amplify a 189 bp fragment from de DNA polymerase gene of BoAHV-1, according to the methodology described by Marin et al. (2016). qPCR reactions were performed in a Rotor Gene Q thermocycler in a final volume of 10 µl using SYBR as an intercalating fluorescent dye, containing 20 µM forward and reverse primers, 1× PCR Master Mix (Master Mix qPCR Sybr/ROX, Productos Bio-Lógicos PB-L) and 1 µl of DNA sample. The cycling conditions consisted of an initial denaturation of 5 min at 94 ºC and 45 cycles of 15 s at 92 ºC and 30 s at 60 ºC. All tests were performed in duplicate. Amplification was followed by a High-Resolution Melting curve analysis. BoAHV-1 identification was performed using Rotor Gene Q software, version 1.7.94. The genotype confidence cut off value of 93% was set up in the software genotyping module.

Histology

Samples of cerebrum, heart, lung, liver, spleen, kidney, striated muscle, tongue, adrenal gland, colon and small intestine from selected fetuses were collected during post mortem examination for microscopic examination. Additionally, samples from randomly selected placentomes and inter-cotyledonary chorion were taken (5 samples of each region). Tissue samples were fixed in 10% buffered formalin, processed by standard methods and included in paraffin blocks. Three µm-thick sections of each tissue block were cut, mounted on glass microscope slides and stained with hematoxylin and eosin (H&E). All samples were observed under an optical microscope (Nikon, eclipse E 200).

Immunohistochemical methods

The evaluation of quantitative changes in iNOS, COX-2, TNFα and IL-1α in fetuses and placentas of cows experimentally-infected with BoAHV-1 was performed by immunohistochemical methods. Three µm-thick sections of the same 10% buffered formalin fixed and paraffin-included tissues (two

placentomes, cerebrum, liver, kidney, spleen, heart and lungs of each fetus) were processed for immunohistochemistry (IHC) using the avidin-biotin-peroxidase complex (ABC) method (Risalde et al., 2013). Each section was placed in silane-coated slides [3-(triethoxysilyl)-propylamine], dewaxed and rehydrated using graded ethanol series. Endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in PBS for 30 min at room temperature (RT). Tissue samples were subjected to different pre-treatments for antigen retrieval depending on the primary antibody used (**Table 1**). After antigen retrieval, the sections were rinsed three times in PBS (pH 7.2) for 10 min and then covered with 20% normal goat serum (Thermo Fisher Scientific) or 3% rabbit serum in PBS for 30 min at RT, for primary rabbit or goat polyclonal antibody (pAb), respectively. After this blocking stage, sections were incubated with the primary antibodies at 4°C overnight. Then, slides were washed in PBS (three times for 5 min each) and subsequently incubated with the secondary antibodies for 30 min at RT. Biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories) diluted 1:200 in PBS containing 10% normal goat serum was used for the primary antibodies done in rabbit. Biotinylated rabbit anti-goat IgG secondary antibody (Vector Laboratories) diluted 1:200 in PBS containing 1% normal rabbit serum was used for the primary antibody done in goat. After three 5 min washes in PBS, samples were incubated with the ABC complex (Vectastain® ABC Elite Kit, Vector Laboratories) for 1 h at RT. All tissue sections were rinsed in TBS and incubated with the chromogen solution (NovaRED® Substrate Kit, Vector Laboratories). Finally, slides were counterstained with Harris' haematoxylin. As positive controls, bovine tissues with previously tested reactivity for the primary antibodies against the cytokines used in this study, were included (Risalde et al., 2013).

Cell counting and statistical analysis

To evaluate the number of immunolabeled cells and to compare the results obtained using different antibodies, fetal tissues of each animal, individually included in a paraffin block, were selected. Cell countings were blinded carried by three experienced observers in 25 randomly-chosen fields of 0.2

mm^2 (M.B, M.A.R and E.M). Cellular identification was based on immunolabeling, morphological features, location and cell size. The results obtained were given as the number of positive cells per 0.2 mm^2.

The results of histopathological and immunohistochemical analysis were expressed as means ± standard errors (SEM). Normality of the data distribution was tested with the Shapiro-Wilk test, using SPSS V18.0 statistical software (IBM Corporation, NY, USA). Statistically significant differences between means were assessed by the Mann–Whitney nonparametric U-test ($p < 0.05$) between BoAHV-1-inoculated and control animals. The statistical analysis was performed with Graph Pad Prism software version 7 (GraphPad Software, Inc.).

Results

Cows have neutralizing antibodies after intravenous BoAHV-1 challenge

Neutralizing antibodies against BoAHV-1 were detected in BoAHV-1-inoculated dams at 14 dpi, demonstrating seroconversion. Clinical signs (including inappetence, nasal and ocular discharges and elevated temperature) were not observed in any of the animals of the control and inoculated groups at 0, 3, 7, 10 and 14 dpi.

BoAHV-1 DNA is detected in fetal tissues and placenta

Results of BoAHV-1 DNA detection by qPCR for selected fetuses are shown in **Table 2**. In the inoculated group (n=7), BoAHV-1 DNA was detected in the spleen of fetus 4 and liver of fetus 6. Furthermore, the placentas corresponding to fetuses 3 and 5 also resulted positive. Fetuses 3-6 were included for further IHC analyses. In addition, in another two fetuses viral DNA was detected in the spleen or lung and in one fetus was undetected. These fetuses were not included in the IHC analysis given the low antibody titers in the dams after challenge and the absence or slight microscopic lesions. In fetuses of the control group (n=4), BoAHV-1 DNA was not detected in any placenta or fetal tissue

analyzed. Two fetuses (fetuses 1 and 2) from this group were randomly selected for histology and IHC analysis.

Microscopic lesions in fetuses from BoAHV-1-infected cows

All selected fetuses of the inoculated group (3, 4, 5 and 6) had microscopic lesions in the liver (**Table 2**), which consisted of mild multifocal, focal or periportal mononuclear hepatitis **(Figure 1A)**. In addition to the hepatic lesions, mild focal mononuclear placentitis was observed in tissue samples collected from cows 3 and 4 **(Figure 1B**). Moreover, fetus 5 showed mild diffuse mononuclear hypercellularity in the alveolar septa **(Figure 1C)**, and mild interstitial mononuclear myocarditis **(Figure 1D)**. In all cases the mononuclear infiltrate was composed mainly by macrophages and occasional lymphocytes. There were no remarkable lesions in fetus 1 (control), whereas fetus 2 (control) occasionally showed mild multifocal mononuclear hypercellularity in the alveolar septa.

BoAHV-1 infection induced the expression of iNOS and COX-2 in placenta and iNOS and TNFα in fetal lungs

BoAHV-1 immunolabelling was negative in fetal and placental tissues from control and inoculated animals. Inflammatory mediators, COX-2 and iNOS, and pro-inflammatory cytokines, TNFα and IL-1α, were not detected by IHC in cerebrum, liver, kidney, spleen or heart. Immunoreactivity of the control and inoculated group for each immune mediator in the placenta **(Figure 2)** and fetal lung **(Figure 3)** were significantly different. In the placenta, COX-2 immunolabeling was occasionally observed in the macrophages of caruncular stroma **(Figure 4A)**, but it was diffusely immunolabeled in trophoblastic cells from the control and inoculated groups **(Figure 4B)**. However, in placentas from inoculated cows, the number of immunolabelled cells were significantly higher (8-fold; $p < 0.05$) when compared with control cows **(Figure 2)**. iNOS immunolabeling was negative in placentas from the control group **(Figure 4C)** and positive in the cytoplasm of macrophages and in occasional fibroblasts

of the chorion from inoculated cows **(Figure 4D)**. TNFα immunolabeling was not observed in the placenta from control cows **(Figure 4E)**; however, few macrophages of the chorion from the inoculated cows were TNFα-positive **(Figure 4F)**. IL-1α was not immunolabeled neither in the placenta of control or BoAHV-1-infected cows. In fetal lung, COX-2 was undetected in both control and BoAHV-1-inoculated cows. iNOS was the main immunolabelled molecule. Immunostaining was highly positive in the cytoplasm of alveolar septal macrophages of the BoAHV-1-inoculated cows in comparison to the control cows **(Figure 5A y B)**. In addition, strong TNFα immunolabeling was observed in this tissue from BoAHV-1-inoculated cows. Occasionally, TNFα-immunolabeled macrophages were observed in the alveolar septum of fetuses from control cows **(Figure 5C)** in comparison to strong positive immunostaining in the fetal lungs from BoAHV-1-inoculated cows **(Figure 5D)**. Furthermore, TNFα positive cells were significantly higher (12-fold; $p < 0.05$) in BoAHV-1-infected group **(Figure 3)**. Within contrast to the placenta, IL-1α occasionally showed a positive immunolabeling in fetal lung. No IL-1α immunlabeling was observed in macrophages of the alveolar septum from fetuses collected from control cows **(Figure 5E)**, but IL-1α reactive septal macrophages were observed in fetal lungs of inoculated cows **(Figure 5F)**.

Discussion

The experimental model of the current study was aimed to recreate the underlying pathologic conditions within the feto-placental environment after BoAHV-1 infection. Antibodies against BoAHV-1 found in the serum of inoculated-cows and BoAHV-1 identification by qPCR in their placental and fetal tissues confirmed viral transplacental infection. Moreover, this study provides novel information regarding the innate immune response in the placenta and fetal lungs after infection of pregnant cows with BoAHV-1, including some aspects not yet fully investigated, such as variations on local TNFα, IL-1α, iNOS and COX-2 expression.

Histopathological analysis revealed mononuclear hepatitis, placentitis, myocarditis and diffuse mononuclear hypercellularity in the alveolar septa of the BoAHV-1-infected fetuses. These lesions were minimal compared to the experiment by Rodger et al. (2007), in which multifocal necrosis in several fetal tissues and placenta was described. These differences could be due to the fact that although in this study the cows were challenged with a similar dosis of the BoAHV-1 Cooper strain by intravenous injection, the moment of gestation in which they received the inoculum was lower (182-220 days) and abortions occurred between 15 and 23 dpi. The reduced time of intrauterine fetal BoAHV-1 exposure, considered adequate for the evaluation of the innate response, may explain the absence of necrosis caused by BoAHV-1 in our study, since cows were slaughtered 15 days post-infection.

iNOS is a potent vasodilator that plays an active role regulating the vascular tone in the placenta during pregnancy (Krause et al., 2011). Additionally, iNOS regulates embryo development, implantation and trophoblast invasion (Krause et al., 2011). Previous studies showed that the peak of iNOS activity occurs at mid and late gestation in the bovine utero-placental unit, supporting that spatiotemporal expression of iNOS may be associated with the regulation of vascular and cellular functions during normal pregnancy (Krause et al., 2011). The placenta from BoAHV-1-inoculated cows revealed strong immunoreactivity to iNOS compared to placentas of control cows at 15 dpi. In this context, it has been demonstrated that placentomes infected with a virulent *N. caninum* strain exhibited high expression of iNOS and pro-inflammatory cytokines which were associated to severe placental damage and the existence of non-viable fetuses (Jiménez-Pelayo et al., 2020). In addition, placentas of cows with retained fetal membranes showed high levels of iNOS that could produce large amounts of NO, inhibiting uterine contraction and increasing the inflammatory reaction (Shixin et al., 2011). However, no clinical reproductive failures were observed in our study, probably due to the short time of intrauterine BoAHV-1 exposure. On the other hand, experimental *in vitro* findings suggest that iNOS

is also an antiviral effector of the immune system and it can inhibit proliferation of several viruses, including Herpes Simplex Virus (HSV) (Zolini et al., 2014), Influenza and Respiratory Syncytial Virus (RSV) (Yaman and Aydemir, 2021). Coinciding with these investigations, NO resulting from iNOS activity could decrease viral replication in the brain of fetuses transplacentally infected with BVDV (Bielefeldt-Ohmann et al., 2008). In agreement with this, the high iNOS immunolabeling in the placentas and fetal lungs of BoAHV-1-infected cows could be related to a mechanism of viral clearance. In the lungs, the role of NO as antiviral effector was demonstrated by Sow et al. (2011). These authors showed that decreased levels of NO in fetal lungs of lambs infected with RSV culminated in an ineffective immune response to viral infection and poor viral clearance. On the other hand, it has been demonstrated that a significant decrease of iNOS expression in the lungs of BVDV/BoAHV-1 co-infected calves has an adverse effect associated to coagulative events, since this mediator can limit the extent and duration of platelet activation (Risalde et al., 2013).

COX-2 plays an important role in the production of prostaglandins during physiological and pathological conditions; this latter associated to the mechanisms of the inflammatory response (Tanabe and Tohnai, 2002). During normal pregnancy, COX-2 expression is increased in the placenta of several species as gestation progressed, contributing to the mechanisms of labor initiation (Arosh et al., 2004). It had been demonstrated that COX-2-deficient mice show multiple female reproductive failures in ovulation, fertilization, implantation, decidualization and induction of neonatal mortality (Loftin et al., 2002). In pregnant mice infected with Influenza virus, COX-2 expression was reduced in the placenta, but it was activated in the maternal lungs resulting in vasoconstriction and inflammation that lead to respiratory distress with adverse outcomes in fetal health (Littauer et al., 2017). Moreover, in human trophoblast cells and villus explants, the inhibition of COX-2 was a key factor for *T. gondii* proliferation since its inhibition induced a pro-inflammatory response capable of controlling the infection (de Souza et al., 2021). Conversely, COX-2 was highly expressed in human placenta during chronic malaria

infection, but it was not associated with preterm delivery, suggesting that COX-2 was involved in the recovery of placental infection (Sarr et al., 2010). In accordance with this study, the placenta of BoAHV-1-infected cows revealed higher number of COX-2 immunolabelled cells compared to the placentas from control cows. Nevertheless, the implication of COX-2 in the control of the viral infection is difficult to elucidate. The discrepancies on COX-2 expression in the maternal–fetal interface during infection can be related to the different models employed, since the placenta is a unique environment completely different in each species. Moreover, gestational length, hormone production, microorganism related-immune response, time of infection, and several other mechanisms are additional factors that might account for the differences observed (Entrican, 2002; Mor and Cardenas, 2010; Littauer et al., 2017)

Transplacental viral infections depend on interactions between maternal, placental and fetal immune responses; being the type of immune response initiated in the placenta critical for the pregnancy outcome (Entrican, 2002; Mor and Cardenas, 2010). It is well-known that production of high levels of inflammatory cytokines in the placenta, such as TNFα, IFNγ, IL-12 and IL-6, recruit and activate cells that lead to placental damage and abortion. However, certain viral infections in the placenta trigger a mild inflammatory response that does not terminate the pregnancy, but can infect and activate the immune system in the fetus (Mor and Cardenas, 2010). The latter situation was clearly observed in this study, since an increase of TNFα-expression was observed in fetal lungs of BoAHV-1-infected group, while low levels of TNFα were expressed in the placenta. Similarly, the infection of mice with equid alphaherpesvirus 1 demonstrated different expression of inflammatory cytokines in the placenta and uterus, suggesting that immunological mechanisms are complex due to the interplay of different cell types and extracellular components (Zanuzzi et al., 2016). Furthermore, significant TNFα and IL-1-expression showed a disruption of pulmonary homeostasis characterized by the establishment of severe inflammatory infiltrates in the airways of calves co-infected with BVDV and BoAHV-1 (Risalde et al.,

2013) and RSV (Sow et al., 2011). In this work, mild mononuclear hypercellularity in the alveolar septa was observed microscopically in the lungs of BoAHV-1-infected fetuses. This latter scenario would be expected to be severe in the placenta and fetal lungs with longer time of intrauterine BoAHV-1 exposure.

Overall, this study demonstrated that pro-inflammatory immune mediators are highly expressed in BoAHV-1-infected placentas and fetal lungs, which might play an active role in triggering the abortion or controlling the infection. Furthermore, it must be highlighted that this study was conducted using the natural BoAHV-1 host. Thus, our findings may reflect more precisely the changes occurring at the fetal-maternal unit during BoAHV-1 infections. Advances in the knowledge of the innate immunity during pregnancy is a key area for future investigation that will contribute to understand the complex interactions between BoAHV-1, the immunological responses at the maternal-fetal unit and its implications regarding to fetal death or survival.

Declaration of Competing Interest

The author(s) declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure legends

Figure 1. Histopathological findings on BoAHV-1-infected bovine fetuses. Hematoxylin and eosin (HE). **a)** liver, BoAHV-1-infected fetus (#6). Periportal mononuclear hepatitis (asterisk). **b)** placenta, BoAHV-1-infected fetus (#3). Focal diffuse mononuclear placentitis in the base of placentome (asterisk). **c)** lung, BoAHV-1-infected fetus (#5). Mild diffuse mononuclear hypercellularity in the alveolar septa (Inset: higher magnification). **d)** heart, BoAHV-1-infected fetus (#5). Mild interstitial mononuclear myocarditis (asterisk).

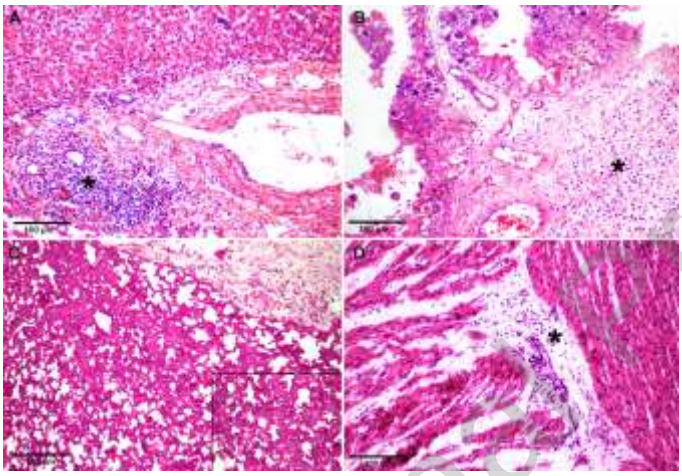

Figure 2. Quantitative evaluation of cells expressing **a)** COX-2, **b)** iNOS and **c)** TNFα in placentas. *Statistically significant differences ($p < 0.05$) between control and infected cows.

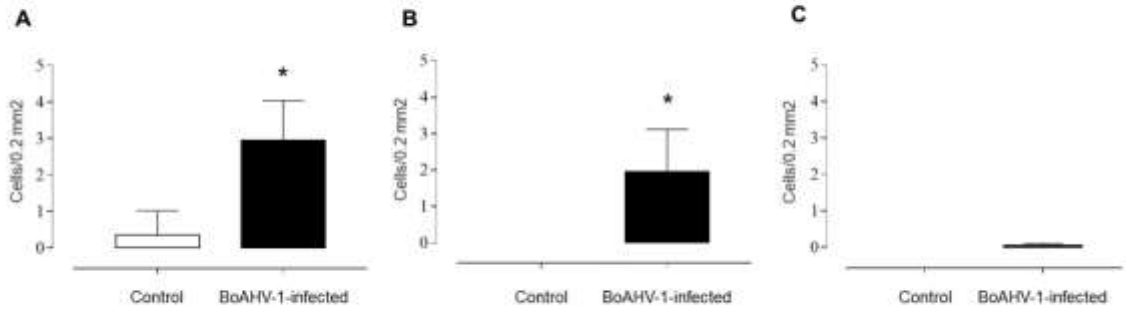

Figure 3. Quantitative evaluation of cells expressing **a)** iNOS, **b)** TNFα and **c)** IL-1α in fetal lungs. *Statistically significant differences ($p < 0.05$) between control and infected cows.

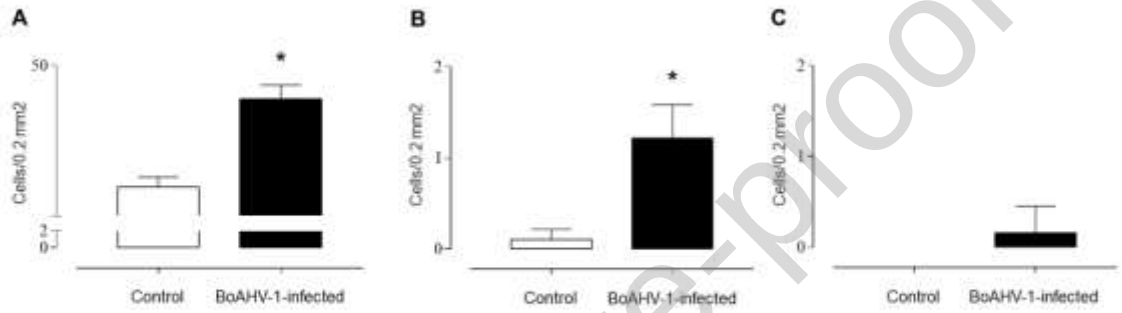

Figure 4. Immunohistochemical labelling in placentas. COX-2-immunolabelling in placentas collected from **a)** control and **b)** BoAHV-1-infected cows. iNOS immunolabelling in placentas collected from **c)** control and **d)** BoAHV-1-infected cows. TNFα-immunolabelling in placentas collected from **e)** control and **f)** BoAHV-1-infected cows. Arrowheads indicate positive cells.

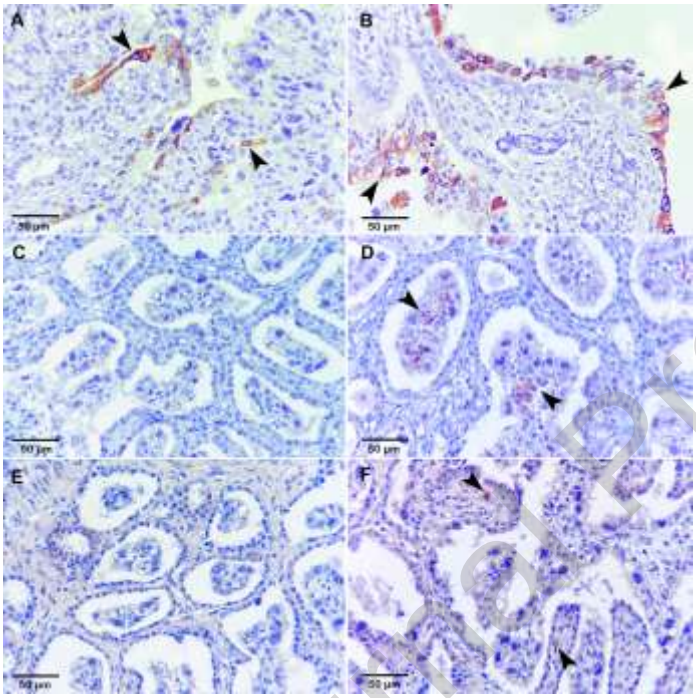

Figure 5. Immunohistochemical labelling in fetal lungs. iNOS-immunolabelling in lungs of **a)** control and **b)** BoAHV-1-infected fetuses. TNFα-immunolabelling in lungs of **c)** control and **d)** BoAHV-1-infected fetuses. IL-1α-immunolabelling in lungs of **e)** control and **f)** BoAHV-1-infected fetuses. Arrowheads indicate positive cells. Insets represent higher magnifications of the fields framed in black.

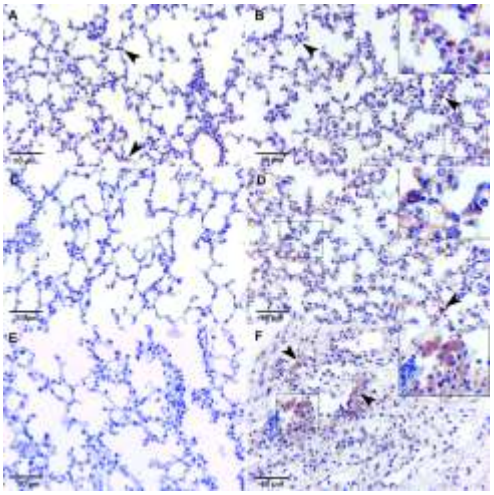

Table 1 Details of the antibodies used in the immunohistochemical study

Primary antibodies						***Secondary antibodies***		
Specificity	***Species of origin***	***Target***	***Dilution***	***Antigen retrieval treatment***	***Source*** (Cat. No.)	***Specificity***	***Species of origin***	***Source*** *(Cat. No.)*
Anti-BoAHV-1	Goat (pAb)	BoAHV-1	1:1000	TC-microwave[a]	VMRD (210-70-IBR)	Anti-goat	Rabbit (pAb)	Dako (P0449)
Anti-bovine TNFα	Rabbit (pAb)	TNFα	1:25	TC-37°C[b]	Bio-Rad AbD (AHP852Z)			
Anti-human IL-1α	Rabbit (pAb)	IL-1α	1:100	Tween-20[c]	Endogen (P420A)	Anti-rabbit	Goat (pAb)	Vector Labs. (BA-1000)
Anti-murine iNOS/NOS Type II	Rabbit (pAb)	iNOS	1:100	TC-autoclave[d]	BD Transduction Lab. (610332)			

Anti-murine COX-2	Rabbit (pAb)	COX-2	1:75	TC-autoclave[d]	Cayman Chemical (160106)	

.pAb, polyclonal antibody; BoAHV-1, bovine alphaherpesvirus type 1; TC: 0.01 M tri-sodium citrate dehydrate.

[a]Incubation with 0.1 M citric acid (pH 6) and microwave for 6 min at sub-boiling temperature.
[b]Incubation with 0.1 M citric acid (pH 3.2) at 37° C in oven for 30 min.
[c]Incubation with 0.1 M citric acid (pH 6) and autoclaved for 10 min at 121° C, 1 atm.
[d]Incubation with 0.1% Tween 20 (Merck) in 0.01 M PBS, pH 7.2, for 10 min at room temperature.

Table 2. Results of BoAHV-1 qPCR and microscopic lesions of placentas and fetal tissues from control and inoculated group.

Animal	***Treatment***	***BoAHV-1 qPCR***	***Microscopical lesions***
1	Control	-	No lesions
2	Control	-	Occasional mild multifocal mononuclear hypercellularity in the alveolar septa of
3	BoAHV-1-infected	+ (placenta)	Multifocal mononuclear infiltrate in liver and focal mononuclear infiltrate in
4	BoAHV-1-infected	+ (fetal spleen)	Focal mononuclear infiltrate in liver and placenta
5	BoAHV-1-infected	+ (placenta)	Mononuclear infiltrate in hepatic periportal spaces, alveolar septa in lung
6	BoAHV-1-infected	+ (fetal liver)	Mononuclear infiltrate in hepatic periportal spaces

References: + (positive) - (negative)

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Highlights

- BoAHV-1 transplacental infection causes an early immune response and mild inflammatory lesions at the feto-maternal unit.
- BoAHV-1 induces protein expression of iNOS in fetal lung and placenta.
- BoAHV-1 induces protein expression of TNFα in fetal lungs and COX-2 in the placenta.
- The expression of pro-inflammatory immune mediators at the feto-maternal unit during BoAHV-1 might play an active role in triggering the abortion or controlling the infection.