Campylobacter fetus Induced Proinflammatory Response in Bovine Endometrial Epithelial Cells

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Abstract

Campylobacter fetus subsp. fetus is the causal agent of sporadic abortion in bovines and infertility that produces economic losses in livestock. In many infectious diseases, the immune response has an important role in limiting the invasion and proliferation of bacterial pathogens. Innate immune sensing of microorganisms is mediated by pattern-recognition receptors (PRRs) that identify pathogen-associated molecular patterns (PAMPs) and induces the secretion of several proinflammatory cytokines, like IL-1β, TNF-α, and IL-8. In this study, the expression of IL-1β, TNF-α, IL-8, and IFN-γ in bovine endometrial epithelial cells infected with C. fetus and Salmonella Typhimurium (a bacterial invasion control) was analyzed. The results showed that expression levels of IL-1β and IL-8 were high at the beginning of the infection and decreased throughout the intracellular period. Unlike in this same assay, the expression levels of IFN-γ increased through time and reached the highest peak at 4 hours post infection. In cells infected with S. Typhimurium, the results showed that IL8 expression levels were highly induced by infection but not IFN-γ. In cells infected with S. Typhimurium or C. fetus subsp. fetus, the results showed that TNF-α expression did not show any change during infection. A cytoskeleton inhibition assay was performed to determine if cytokine expression was modified by C. fetus subsp. fetus intracellular invasion. IL-1β and IL-8 expression were downregulated when an intracellular invasion was avoided. The results obtained in this study suggest that bovine endometrial epithelial cells could recognize C. fetus subsp. fetus resulting in early proinflammatory response.

Key words: bacterial infection, pathogenicity, virulence, pathogen-host interaction (MesH)

Introduction

The innate immune system senses microbial infections and triggers an immediate response to control pathogens’ invasion. Microbial sensing is mediated by pattern-recognition receptors (PRRs), which include Toll-like receptors (TLR), Nucleotide-binding Oligomerization Domain (NOD), Leucine-rich repeat-containing receptors (NLRs), C-Type Lectin-Like Receptors, and Cytoplasmic Nucleic Acid Sensors. These receptors are important in innate and adaptive immune response because they identify Pathogen Associated Molecular Patterns (PAMPs) and determine the type of immune response required (Bryant et al. 2015). The innate immune response includes proinflammatory cytokines secretion, which recruits and activates phagocytic cells to eliminate the pathogenic microorganisms (Iwasaki and Medzhitov 2015).

The female reproductive tract’s mucosal surface forms a physical and immunological barrier that can interact with sexually transmitted pathogens and spermatozoa. Therefore, innate immune mechanisms have an important role in maintaining its integrity (Amjadi et al. 2014). The cells in mucosal epithelia recognize pathogens and stimulate the underlying immune cells like macrophages, inducing an inflammatory reaction via cytokines’ production, resulting in adaptive immunity activation. They also produce antimicrobial peptides that eliminate several bacterial and viral agents (Turner et al. 2014).
Campylobacter fetus subsp. fetus is frequently isolated from the intestinal tract of asymptomatic cattle, goats, and sheep. In animals, C. fetus subsp. fetus exhibits a tropism for placental and reproductive tract tissues and is one of the major causes of sporadic and epidemic septic abortions (Viejo et al. 2001; Iraola et al. 2012). C. fetus subsp. fetus can attach in an irreversible way to bull spermatozoa and affect sperm quality (Cagnoli et al. 2020). The diseases produced by C. fetus subsp. fetus generate considerable economic losses, representing a significant problem in animal production (Mshelia et al. 2010). Heifers infected with C. fetus showed a light inflammatory reaction with few mononuclear and polymorphonuclear cells distributed diffusely beneath the epithelia of vagina and cervix, and moderate endometritis and salpingitis (Cipolla et al. 1994). Such light inflammation reaction can be due to the composition of the external membrane of microorganisms. C. fetus possesses lipooligosaccharides (LOS) instead of lipopolysaccharide (LPS) (Preston and Penner 1987; Moran et al. 2002). Also, it has a protein surface layer known as S-layer, which protects C. fetus against complement and opsonization-phagocytosis response; it also prevents recognition by host innate immune system (Blaser et al. 1987; Fogg et al. 1990; Blaser et al. 1993).

We have previously demonstrated the ability of C. fetus subsp. fetus to invade bovine endometrial cells (Campos-Múzquiz et al. 2019). This phenomenon was dependent on the viability of C. fetus, since dead bacteria could not invade this type of cells (in press). The ability of C. fetus subsp. fetus to invade endometrial cells raises new questions about the pathogen’s mechanisms to infect these surfaces and induce reproductive diseases. The inflammatory response induced by C. fetus in bovine endometrium has not yet been entirely described; meanwhile, for other Campylobacter species, the induction of proinflammatory cytokines in epithelial cells has been described along with the benefits that this represents for tissue invasion (Al-Salloom et al. 2003; Zheng et al. 2008; Eucker et al. 2014). Hence in this study, the cytokine expression patterns induced in bovine endometrial epithelial cells by infection with C. fetus subsp. fetus were evaluated, to establish the role of inflammation in diseases produced by this species in the bovine reproductive tract.

**Experimental**

**Materials and Methods**

**Bacterial strains and growth conditions.** C. fetus subsp. fetus ATCC 27374 (Salama et al. 1995) was grown at 37°C for 48 h under microaerophilic atmosphere (85% N₂, 10% CO₂ and 5% O₂) on Campylobacter selective agar supplemented with 5% sheep blood. *Salmonella enterica* subsp. enterica serovar Typhimurium ATCC 14028 was also grown on hyperosmolar Luria Bertani broth at 37°C for 12 h.

**Endometrial epithelial cell culture.** Epithelial cells from the endometrium were recovered using Skarzynski protocol (Skarzynski et al. 2015) with some modifications. The uterus was removed from three sacrificed cows 15 min after exsanguination at a slaughterhouse. Tissue was washed with Hank's solution supplemented with 1.6 mg/ml of gentamicin and transported to the laboratory in the same buffer solution on ice. The endometrium was cut and washed three times with phosphate-buffered saline solution pH 7.2 (PBS, NaH₂PO₄ 1.9 mM, Na₂HPO₄ 8.1 mM, NaCl 154 mM). Tissue pieces were treated with digestion solution containing 10% fetal bovine serum, 25 mM HEPES, 30 µg/ml gentamicin) at 37°C. Following the incubation, the cell monolayers were washed three times with PBS and incubated with a 40-µm strainer. Cells were placed on cell culture flasks with HEPES (25 mM) and antibiotic/antifungal solutions (penicillin G 10,000 U, streptomycin 5,000 µg, amphotericin B 12.5 µg). For fibroblast depuration, one-minute of trypsinization was performed daily for three consecutive days. Cell type was confirmed by immunofluorescence and RT-PCR (Campos-Múzquiz et al. 2019).

**Invasion assays.** Gentamicin protection assays were performed with C. fetus (multiplicity of infection – MOI = 100:1) and S. Typhimurium (MOI 50:1). An amount of 200,000 endometrial epithelial cells were cultured in a 25 cm² culture flask. Bacteria inoculum was diluted in DMEM, added to cultures, and incubated 2 hrs at 37°C. Following the incubation, the cell monolayers were washed three times with PBS and incubated with DMEM/gentamicin (10% fetal bovine serum) at 37°C for 2 h. A tissue debris-free supernatant was recovered and centrifuged at 4,000 × g for 10 min. The pellet was suspended in 5 ml of DMEM supplemented with 10% fetal bovine serum and filtered with a 40-µm strainer. Cells were placed on cell culture flasks with HEPES (25 mM) and antibiotic/antifungal solutions (penicillin G 10,000 U, streptomycin 5,000 µg, amphotericin B 12.5 µg). For fibroblast depuration, one-minute of trypsinization was performed daily for three consecutive days. Cell type was confirmed by immunofluorescence and RT-PCR (Campos-Múzquiz et al. 2019).

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Intracellular survival evaluation by reverse transcription qPCR. Quantitative reverse transcription PCR using random primers and a commercial kit to synthesize complementary DNA (ProtoScript® First Strand cDNA Synthesis Kit, New England Biolabs) were used to evaluate the intracellular survival ability of C. fetus subsp. fetus. The cDNA was employed to quantify mRNA copies (RC) of C. fetus subsp. fetus and Salmonella Typhimurium (Power SYBR green, Fermentas). The primer sequences used in these assays for C. fetus subsp. fetus were: 5'-GGCAATATCATAGAAACAGTCTCCATCTCAGGAG-3', 5'-GGCTTCTGCTCTCAATCTGC-3', 108 bp; 5'-GGATTTCCACTTCCAGGAG-3', 5'-GGCTGTTCTCCTCAATCTGC-3', 98 bp, as inflammatory genes. The specificity of the PCR product was confirmed by high resolution melting curve. The amplification efficiency (E) of each gene was calculated from each gene using the geometric average of the data. A comparative CT method (2^ΔΔCt) was used to calculate from each gene with treated cells with cytochalasin D to determine if the inhibitors could induce cytokine expression.

Gene expression analysis of proinflammatory cytokines. RNA recuperated from invasion assays was used to synthesize cDNA using commercial kit and Oligo dT primers (ProtoScript® First Strand cDNA Synthesis Kit, New England Biolabs). A quantitative PCR (Maxima SYBR green, Thermo Fisher) was performed using the primers: TAF2 (5'-CATCTCTCTGGAAAACCCAGAAA-3', 5'-GGCTTCTGCTCTCAATCTGC-3', 98 bp), β-actin (5'-AAATCGTGCGTGACATTAAG-3', 5'-GGCTGTTCTCCTCAATCTGC-3', 98 bp), IL-1β (5'-GAAAGAGACAACAAGATTCCCTGTTG-3', 5'-GGTCTACACTTCTCCAGCTGCA-3', 108 bp), TNF-α (5'-CATCTCTCTGAGTGGT-3', 5'-GCAATGCGGCTGATGGT-3', 82 bp), IL-8 (5'-AGTACAGAACCCTTGCAGATG-3', 5'-GGCTGTTCTCCTCCAGTATG-3', 127 bp), IFN-γ (5'-GGTTCTGCTCTCCTCATTG-3', 5'-GGACTTTTATTTTTCCTGAT-3', 144 bp) as inflammatory genes. The specificity of the PCR product was confirmed by high resolution melting curve. The amplification efficiency (E) of each gene was calculated from the standard curves using the equation E = (1 + 10^−1/slope) × 100 (Livak and Schmittgen 2001). For gene normalization, we obtained a geometric average of the three reference genes (Vandesompele et al. 2002). The cytokine expression analysis was realized with treated cells with cytochalasin D to determine if the inhibitors could induce cytokine expression.

Statistical analysis. To analyze differences between gene expression and intracellular microorganisms, an F test was performed to establish the equality of variance of the data. A comparative CT method (2^ΔΔCt) was used to calculate from each gene with using the geometric average of the reference genes (Schmittgen and Livak 2008), and a Student's t-test was used to determine the difference between CT of treatment versus CT of control.

Results

Endometrial epithelial cell culture. The results obtained in primary cell cultures showed that the endometrial epithelial cells presented an epithelial-like appearance in the second week of incubation. In primary cell culture, the expression of Keratin 8 was confirmed by RT-PCR and immunofluorescence. A PCR product of 215 pb corresponding to a segment of the gene encoding for keratin 8 was obtained from RNA recuperated from cell cultures, and more than 90% of cells in the monolayer showed positive results to cytokeratin 18 (data not shown).

Invasion assays. In the intracellular survival assays, the results showed that viable C. fetus subsp. fetus decreased in number over time. At 0 h post-infection (p.i.) the average (~) colony-forming unit (CFU) were 22,408 CFU. At a second time (2 h p.i.), it decreased to ~ 1,316 CFU and 4 h p.i. there were ~ 233 CFU (Fig. 1); these results confirm that there was an intracellular bacterial cells reduction through the time (p = 2.2e−16). In cells infected with Salmonella Typhimurium, the CFU increased at the end of the assay, indicating intracellular replication of bacteria (p = 1.49e−10). At 0 h p.i., the average number of intracellular bacteria was ~ 42,150 CFU, 2 h p.i., CFU decreased to ~ 37,125 CFU, and 4 h p.i. bacteria proliferated, and their number increased to 72,925 CFU (Fig. 1).

To confirm C. fetus subsp. fetus intracellular survival ability, a bacterial mRNA quantification assay was

![Fig. 1. Salmonella Typhimurium and Campylobacter fetus subsp. fetus invasion assays in endometrial cells. A gentamicin protection assay was performed to demonstrate that C. fetus subsp. fetus invades bovine endometrial epithelial cells but does not survive.](image)
performed. For \textit{C. fetus} subsp. \textit{fetus}, \textit{frdA} analysis (the constitutive gene) showed that the number of mRNA copies (genomic copies, GC) decreased significantly during invasion assays \((p = 0.002)\). At 0 h p.i., the number of GC was \(~5.0526\) log, 2 h p.i., it increased to \(~5.7581\) log, and 4 h p.i., it increased to \(~6.9379\) log (Fig. 2).

**Cytokine expression analysis.** A gene expression assay was carried out to evaluate the expression of proinflammatory cytokines in endometrial cells infected with \textit{C. fetus} subsp. \textit{fetus}. Cells invaded by \textit{C. fetus} subsp. \textit{fetus} showed an early IL-1\(\beta\) high expression level (4.65-fold change at 0 h p.i.). Unlike cells invaded by \textit{S. Typhimurium}, the IL-1\(\beta\) highest expression level was reached at 4 h p.i. (3.56-fold change). The cells infected with \textit{C. fetus} subsp. \textit{fetus} had the highest level of IL-1\(\beta\) (1.09 fold change, \(p = 6.645e^{-06}\)). The expression level of IL-1\(\beta\) increased through time (\(p = 7.492e^{-05}\)) in cells infected with \textit{C. fetus} subsp. \textit{fetus}. At 0 h p.i., the fold change was 4.65, and it decreased to 3.78 2 h p.i., and this tendency was continued until 4 h p.i., where the fold change in expression was 1.09-fold. In cells infected with \textit{S. Typhimurium}, the IL-1\(\beta\) expression levels showed an increment in time \((p = 0.0007)\), at 0 h p.i, there was a 4-fold change value and increased to 3.56 at 4 h p.i. (Fig. 3).

The expression levels of IL-8 showed the same pattern in cells infected with \textit{C. fetus} subsp. \textit{fetus} and \textit{S. Typhimurium} throughout the time. In both cases, IL-8 expression levels were high at the beginning of the infection and decreased through time \((p = 0.031, p = 5.127e^{-05},\) respectively). At 0 h p.i., the expression level of IL-8 in cells infected with \textit{C. fetus} subsp. \textit{fetus} there was a 3.41-fold change and 6.14-fold for cells infected with
S. Typhimurium. Simultaneously, the expression levels of IL-8 were highest in cells infected with S. Typhimurium compared to cells infected with C. fetus subsp. fetus (p = 0.015). At 4 h p.i., for the expression level of IL-8 in cells infected with C. fetus subsp. fetus, there was a 2.28-fold change. For cells infected with S. Typhimurium, there was a 2.68-fold change (Fig. 3), so there was no difference in the expression levels between both treatments at this time (p-value = 0.4401) (Fig. 3).

The expression levels of IFN-γ in cells infected with C. fetus subsp. fetus showed a different pattern in comparison with IL-1β and IL-8. In this case, the expression level of IFN-γ increased through time (p = 0.002). At 0 h p.i., the expression level increased 0.89-folds, at 2 h p.i., it increased 2.77-fold, and at 4 h p.i., it reached a 3.62-fold increase (Fig. 3). In cells infected with S. Typhimurium, the expression levels of IFN-γ did not change throughout the time (p = 0.367). At 0 h p.i., the expression level was two times lower, 2 h p.i. it changed by 0.74, and 0.85 4 h p.i. Finally, the expression levels of TNF-α did not change either in cells infected with C. fetus subsp. fetus or S. Typhimurium, and there were no differences when compared to the control (uninfected cells) at any time during the assay.

To determine if C. fetus subsp. fetus intracellular invasion was necessary for the induction of IL-1β, IL-8, and IFN-γ, a cytoskeleton inhibition assay was performed, and cytokines expression was evaluated. In cells treated and infected with C. fetus subsp. fetus, the expression level of IL-1β was reduced by 0.34 compared with non-treated cells (p = 0.005). A similar result was observed, with IL-8 expression (0.53-fold change). Nonetheless, the difference was non-significant (p = 0.105). Finally, the expression levels of IFN-γ in cells treated with cytochalasin D and infected with C. fetus subsp. fetus showed an increment (1.90-fold change) in comparison with non-treated cells (p = 0.021) (Fig. 4).

**Discussion**

Pathogen-associated molecular patterns by PRRs upregulate the transcription of proinflammatory cytokines like IL-1β, TNF-α, and IL-8 (Takeuchi and Akira 2010). In this study, endometrial epithelial cells showed a high expression of IL-1β and IL-8 due to the infection with C. fetus subsp. fetus, suggesting that these cells could recognize some molecular patterns in this pathogen through their PRRs. These results agree with other studies that show the induction of proinflammatory cytokines by Campylobacter spp. infection (Wang et al. 2000; Arce et al. 2010b; Man et al. 2010; Yu et al. 2016). In the abovementioned studies, the authors used other types of cells as Caco-2, HEp-2, and HT-29 in their experiments. In this study, primary cell culture of endometrial cells was used, and the same phenomenon was observed during the infection. The early high IL-1β expression in cells infected with C. fetus subsp. fetus (4.65-fold change at 0 h p.i.) suggests an immediate immune recognition that might induce an acute inflammatory response. It could be used by C. fetus subsp. fetus to invade tissues. An invasion study in human trophoblast cells showed that in C. rectus there was a correlation between invasion and cytokine production (Man et al. 2010).

The IL-1β up-expression in bovine endometrial epithelial cells infected with C. fetus subsp. fetus was earlier than in S. Typhimurium (0 h p.i vs 4 h p.i respectively), and it decreased through time. Previously, a similar phenomenon was observed in a 4-week old chicken challenged with S. Typhimurium and Campylobacter jejuni. The peak of IL-1β expression in C. jejuni-infected chicks was at 20 h p.i., and in the Salmonella-infected chicks, it was at 48 h p.i. (Shaughnessy et al. 2009). A possible explanation for this could be that in the C. fetus genome, there are not virulence factors associated with immune suppression as in S. Typhimurium. Therefore, the induction of cytokines could be faster in C. fetus subsp. fetus in comparison with S. Typhimurium, which can modify the immune response (Kienesberger et al. 2014; Hu et al. 2017). Analysis of the present results also showed that the expression of pro-inflammatory cytokines in endometrial cells infected with S. Typhimurium took longer to reach the higher peak of expression, in comparison with cells infected with C. fetus subsp. fetus. Rolhion
et al. (2016) shown that Salmonella Typhi uses SpvD, an effector protein secreted through the type III secretion system, to avoid nuclear translocation of NF-kB transcriptional factor. Therefore, infected cells reduce proinflammatory cytokines secretion.

C. fetus subsp. fetus did not survive in endometrial cells in comparison with S. Typhimurium. In intracellular survival assays, the number of intracellular C. fetus subsp. fetus showed a reduction over time. This result was confirmed by two different methods, quantification of the number of transcripts (mRNA copies of constitutive genes) and CFUs. Considering that each method has different criteria to establish the number of viable microorganisms (gene expression and growth in specific media), we could say that reduction in the CFU number of C. fetus subsp. fetus was not due to the formation of viable non-culturable microorganisms but to the elimination of the pathogen. In the case of S. Typhimurium, the results showed a different pattern, indicating that the bacterium could survive and replicate inside the cells. This phenomenon has been previously reported (Campos-Múzquiz et al. 2019). These could be explained by Salmonella capability adaptation to intracellular niche (Larock et al. 2015) or by an immune stimulation that results in bacteria elimination. Furthermore, when C. fetus subsp. fetus internalization was inhibited, the IL-1β expression was halved. These results showed that C. fetus has been recognized intracellularly and that this recognition was required to induce higher inflammation and cellular mechanisms to eliminate the intracellular bacteria.

The results obtained in this study show a decrease in IL-1β expression through time. Stephenson et al. (2014) showed that C. jejuni flagellum binds to Siglec-10 of dendritic cells and increases IL-10 expression. This molecule has been described in human uterine cells (Sammar et al. 2016) but not in bovine endometrial cells. More studies are necessary to evaluate if the reduction of IL-1β was mediated by this molecule or only by infection control in the endometrial cells.

The pattern expression of IL-8 was like that of IL-1β, suggesting that C. fetus subsp. fetus infection promotes early neutrophil recruitment (Foley et al. 2012). In epithelial cell lines IPEC-J2 and IPI-21, a high IL-8 expression was shown in response to S. Typhimurium LPS (Arce et al. 2010a), suggesting a prompt recognition by cells. The decrease of IL-8 also is due to an IL-10 increase described for IL-1β (Méndez-Samperio et al. 2002) or IFN-γ upregulation since it represses genes involved in leukocyte recruitment (Hoeksema et al. 2015). Moreover, when C. fetus subsp. fetus internalization was inhibited, the expression of IL-8 was not altered, indicating that the external sensing and not the bacteria's internalization induce polymorphonuclear recruitment (Takeuchi and Akira 2010).

Unlike our observation on IL-1β and IL-8 expression, IFN-γ showed an inverse pattern that increased over time in C. fetus subsp. fetus infected cells. These results are related to the studies observed in INT-407 cells infected with C. jejuni in which IFN-γ peak was recorded at 12 hours post stimulation (Al-Amri et al. 2008). IFN-γ alters epithelial barrier function during inflammation by disrupting tight cell junctions and increasing permeability in polarized epithelial cells, which leads to cytosolic translocation of occludins and claudins (Bruewer et al. 2005). The increment of IFN-γ expression could be used by C. fetus subsp. fetus to invade submucosae.

In the presence of IFN-γ, C. jejuni was able to translocate across the monolayer more efficiently than in the absence of IFN-γ. This IFN-γ expression suggests also that epithelial cells from the endometrium might be associated with macrophage and adaptive immunity activation in campylobacteriosis (Hoeksema et al. 2015).

On the other hand, IFN-γ expression showed a slight increase when cells were treated with cytochalasin D. This could be due to a synergistic effect of C. fetus subsp. fetus sensing and Campylobacter toxins, for example, cytolethal distending toxin, which alters DNA (Rees et al. 2008). It has been reported before that cytochalasin D activates the p53 transcriptional factor, which controls DNA damage stress signaling (Rubtsova et al. 1998); however, more research is necessary to describe this phenomenon. The expression of IFN-γ in the cells infected with S. enterica subsp. enterica serovar Pullorum was non-significant. These results are not surprising since Salmonella possesses an immune response modulator LpaI that decreases IFN-γ expression in macrophage cell line HD11 (Yin et al. 2018). Also, the principal sources of IFN-γ during Salmonella spp. infection are the neutrophils and NK cells (Pham and McSorley 2015).

In the cells infected with S. Typhimurium or C. fetus subsp. fetus, TNF-α expression did not change. A similar phenomenon was reported by Cronin et al. (2012) in bovine endometrial epithelial cells challenged with lipopolysaccharide. The same result was observed in bovine endometrial epithelial cells challenged with the danger-associated molecular patterns (DAMPs) (Healy et al. 2014). The TNF-α expression in these cells was possibly due to different conditions since the endometrium’s primary function is blastocyst implantation (Kaneko et al. 2013).

In conclusion, the present study examined the proinflammatory response to C. fetus subsp. fetus in bovine endometrial epithelial cells. These bovine endometrial epithelial cells were able to recognize C. fetus subsp. fetus resulting in early proinflammatory response. Additionally, the internalization of the bacteria was necessary to induce IL-1β expression but not IL-8, suggesting the
importance of intracellular C. fetus subsp. fetus recognition. Their inability to survive inside of epithelial cells, the early induction of cytokines, and the upregulation of IFN-γ imply that the principal pathogenesis mechanism of C. fetus subsp. fetus in the uterine cavity is only to pass through the epithelium. This movement induces an inflammatory response that alters the tight junctions, as demonstrated in human HT-29/B6 and Caco-2 cells (Baker et al. 2010, Bücker et al. 2017).

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### Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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