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CÂMPUS DE JABOTICABAL

TOLL-LIKE RECEPCTORS (TLRs) AND RETINOIC ACID INDUCIBLE GENE – I   (RIG-I) ACTIVATION BY VIRAL ANALOGS IN BOVINE ENDOMETRIAL CELLS

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TÍTULO: TOLL-LIKE RECEPTORS (TLRs) AND RETINOIC ACID INDUCIBLE GENE - I (RIG - I) ACTIVATION BY VIRAL ANALOGS IN BOVINE ENDOMETRIAL CELLS

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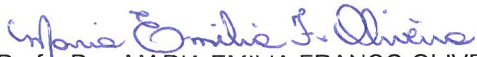
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AUTHOR CURRICULUM DATA

LUISA CUNHA CARNEIRO- was born in Uberlândia, Minas Gerais state, Brazil, on May 14th of 1986. In 2004 she joined the Faculty of Veterinary Medicine at the Federal University of Uberlandia (UFU), concluding her academic experience on December 2008. In 2009, she began a Masters in Animal Reproduction Science, at Federal University of Uberlandia (UFU), where during this time she lived for four months in Riverdale, California, United States of America (USA), participating in projects on dairy cows reproduction and production at Ruann and Maddox Dairy Farm. In 2011 she concluded her Master's degree and on 2012 she began her PhD at Faculty of Agricultural and Veterinary Sciences - FCAV UNESP - Jaboticabal, (Animal Reproduction) where in the first year she moved to the Guelph, Ontario, Canada, to join researches on cows endometritis at University of Guelph during four months. On 2014, she had a one year scholarship to develop part of her PhD project at Swansea, Wales, United Kingdom (UK), at Swansea University focusing on immune uterine defense in cows.

"The two most important
days in your life are the
day you are born and the
day you find out why."
- Mark Twain

To my Dad, Mom
and Brother with love.

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SUMMARY

TOLL-LIKE RECEPCTORS (TLRs) AND RETINOIC ACID INDUCIBLE GENE – I (RIG-I) ACTIVATION BY VIRAL ANALOGS IN BOVINE ENDOMETRIAL CELLS

ABSTRACT- In general, the objective of this study was to determine if bovine endometrial cells replied to virus analogs of pathogen associated molecular pattern (PAMPs) by production of proinflammatory cytokines after Toll-Like Receptor (TLR) activation in the cell endosome and after retinoic acid inducible gene – I (RIG-I) stimulation in the cell cytoplasm. In the first experiment, uterine samples from post pubertal cross-breed beef cows were dissected using a protocol to obtain epithelial and stromal cells. A negative control and four different PAMPs: LPS, ssRNA, Poly I:C (LMW), Poly (I:C) HMW were used. Two treatments (transfected and non-transfected) groups were investigated during 24 hours. In the other experiment, endometrial cells were treated with only Poly (I:C) LMW and a negative Control group. All incubated at 0, 2, 6, 12, 24, 36, 48 and 72 hours. Supernatants were collected to develop Elisa for IL-6 and IL-8. Epithelial cells produced IL-6 in response do Poly I:C (HMW) compared to Control ($P< 0.05$), otherwise, LPS induced IL-6 and IL-8 in stromal ($P< 0.05$). The transfection Reagent differ between cells and treatments ($P> 0.05$). Still, in stromal cells treated by Poly I:C (LMW) the production of IL-6 was higher at 48 and 72 hours ($P< 0.05$), and for IL-8 at 6, 12, 24, 36, 48 and 72 hours when compared to the Control ($P< 0.05$). In the second experiment, uterine samples from others post pubertal mixed-breed beef cows were used. To obtain stromal and epithelial cells, uterine samples were dissected with the same protocol as the first experiment. The PAMP Poly (I:C) LMW and a negative control were used. Proteins for RIG-I and p65 were collected after 12, 24, 48 and 72 hours. In response to Poly (I:C) LMW induction, stromal cells activated RIG-I at 48 hours ($P< 0.05$) were compared to the Control group. On the other hand, epithelial cells were not sufficient stimulated Poly (I:C) LMW to activate RIG-I at any time point evaluated ($P> 0.05$). The protein p65 after stimulated by Poly (I:C) LMW was activated at 12 hours by stromal ($P< 0.05$) and at 24 hours by epithelial cells ($P< 0.05$). In conclusion, bovine endometrial cells were elemental factors in the activation of both TLR and RIG-I pathway in order to start an immune defense against viral infection.

Keywords: Cows, immunity, proinflammatory cytokines, uterus

ATIVAÇÃO DE RECEPTOTRES "TOLL-LIKE" (TLRS) E DE GENES INDUTORES DE ÁCIDO RETINÓICO – I (RIG-I) POR ANÁLOGOS VIRAIS EM CÉLULAS ENDOMETRIAIS BOVINAS

RESUMO- De modo geral, o objetivo deste estudo foi determinar se as células endometriais bovinas responderam a análogos virais de padrões moleculares associados a patógenos (PAMPs) mediante a produção de citocinas pró-inflamatórias após ativadas pelos receptores "Toll-Like" (TLRs) no endossoma celular e no citoplasma celular pelo genes indutores de ácido retinóico tipo I (RIG-I). No primeiro experimento, amostras uterinas de vacas de corte mestiças pós-púberes foram dissectadas para obtenção de células endometriais epiteliais e estromais. Um controle negativo e quatro PAMPs: LPS, ssRNA, Poly I:C (LMW), Poly (I:C) HMW foram utilizados. Dois grupos de tratamentos (transfectados e não transfectados) foram analisados durante 24 horas. Em outro experimento, células endometriais foram tratadas apenas com o PAMP Poly (I:C) LMW e um grupo Controle Negativo. Neste, os grupos foram incubados às 0, 2, 6, 12, 24, 36, 48 e 72 horas. Sobrenadantes foram colhidos para desenvolver o teste de ELISA para IL-6 e IL-8. Células epiteliais produziram IL-6 em resposta ao Poly I:C (HMW) quando comparadas com o Controle (Grupo DOTAP positivo; $P < 0.05$), enquanto que o LPS induziu produção de IL-6 e IL-8 em células estromais ($P < 0.05$). O uso de um reagente de transfecção entre as células e tratamentos demonstrou efeito ($P > 0.05$). Ainda, células estromais tratadas por Poly I:C (LMW) demonstraram uma maior produção de IL-6 às 48 e 72 horas ($P < 0.05$), e para o IL-8 às 6, 12, 24, 36, 48 e72 horas quando comparadas com o grupo Controle ($P < 0.05$). No segundo experimento, outras amostras uterinas de vacas de corte pós-púberes foram utilizadas. A obtenção de células endometriais estromais e epiteliais foram isoladas pelo mesmo protocolo do primeiro experimento. O PAMP Poly (I:C) LMW e um controle negativo foram utilizados. Proteínas para o RIG-I e p65 foram colhidas após 12, 24, 48 e 72 horas de tratamento. Em resposta a Poly (I:C) LMW, células estromais ativaram o RIG-I às 48 hours ($P < 0.05$) quando comparadas com o grupo controle. Enquanto que, as células epiteliais não foram suficientemente estimuladas pelo Poly (I:C) LMW na ativação do RIG-I em nenhum momento testado ($P > 0.05$). A proteína p65 depois de estimulada pela Poly (I:C) LMW foi ativada às 12 horas pelas células estromais ($P < 0.05$) e às 24 horas pelas células epiteliais ($P < 0.05$). Conclui-se que, células endometriais bovinas foram essenciais na ativação das vias exercidas tanto pelos TLR como RIG-I com função de iniciar uma defesa imunológica contra infecções virais.

Palavras-chave: Citocinas pró-inflamatórias, imunidade, útero, vacas

LIST OF ABBREVIATIONS

A	Ampere
AMPs	Activated proteins kinase
AP-1	Activator protein 1
APP	Acute phase proteins
BSA	Bovine Serum Albumin
CARD	Caspase activation and recruitment domain
cm	Centimeter
CO2	Carbon Dioxide
CpG ODN	CpG oligodeoxynucleotides
DExD/H	Box proteins with the domain DEAD or DEAH box helicases
DMSO	Dimetil Sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
g	Gravitational force
GPI	Glycosylphosphatidylinositol
HBSS	Hanks Balanced Solution
IFN I	Type I Interferon
IFNs	Interferons
IFN-α	Interferon *alpha*
IFN-β	Interferon- *beta*
IgG	Immunoglobulin G
IKKα/β	Inhibitor of nuclear factor kappa-B kinase subunit *alpha/beta*
IL-1	Interleukin 1
IL-12	Interleukin 12
IL-12A	Interleukin 12A
IL-17	Interleukin 17

IL-1A	Interleukin 1A
IL-1B	Interleukin 1B
IL-24	Interleukin 24
IL-6	Interleukin 6
IL-8	Interleukin 8
ILs	Interleukins
IRF	Interferons regulated factors
IRF7	Interferon regulatory factor 7
Km	Kilometers
LBP	LPS-binding protein
LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral-signaling protein
MDA5	Melanoma differentiation-associated protein 5
µL	Microliter
µm	Micrometer
mg	Milligram
ml	Milliliter
mm	Millimeter
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test
MyD88	Myeloid differentiation primary response gene 88
ng	Nanogram
NLRs	NOD-like receptors
nm	Nanometer
NF-κB	Nuclear Factor Kappa B
p65	Protein 65
PAMPS	Pathogen-associated molecular patterns
PBS	Phosphate Buffer Saline
PMN	Polymorphonuclear

Pol III	RNA polymerase III
Poly (I:C) HMW	Polyinosinic-polycytidylic acid High Molecular Weight
Poly (I:C) LMW	Polyinosinic-polycytidylic acid Low Molecular Weight
Poly (U)	Polyuridylic Acid
Poly (I:C)	Polyinosinic-polycytidylic acid
PKR	dsRNA-dependent protein kinase R
PRRs	Pattern recognition receptors
PVDF	Polyvinylidene fluoride
RIG-I	Retinoic acid inducible gene – I
RLHs	RIG-I-like helicases
RLRs	RIG-I-like receptors family
RNA	Ribonucleic acid
SA-HRP	Streptavidin-horseradish peroxidase
ssDNA	Single-stranded DNA
ssRNA	Single-stranded RNA
TBK 1	Serine/threonine-protein kinase 1
TBST	Tris Buffered Saline Tween
TIR	Toll/interleukin-1 receptor
TLR1	Toll-Like Receptor 1
TLR2	Toll-Like Receptor 2
TLR3	Toll-Like Receptor 3
TLR4	Toll- Like Receptor 4
TLR5	Toll-Like Receptor 5
TLR6	Toll-Like Receptor 6
TLR7	Toll-Like Receptor 7
TLR8	Toll-Like Receptor 8
TLR9	Toll-Like Receptor 9
TLR11	Toll-Like Receptor 11
TLRs	Toll-like receptors
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor necrosis factor
TNFα	Tumor necrosis factor alpha

TRAF	Tumor necrosis receptor-associated factor
TRAF3	Tumor necrosis receptor-associated factor 3
TRAM	Translocation associated membrane protein
TRIF	TIR-domain-containing adapter-inducing interferon-β
UK	United Kingdom
USA	United States of America
v	Volts
VISA	Virus-induced signaling adapter

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and 72 hours of treatment and by a Control group (no Poly (I:C)). Proteins levels were compared with the corresponding β-actin levels of each cell performed by a Western Blot test. Cells were treated with Poly (I:C) at a final concentration of 1µL/ml. Statistical significances were calculated using a two-way ANOVA with Tukey test. *P <0.05; **P <0.01.

CHAPTER 1 – GENERAL CONSIDERATIONS

1.Introduction

Reproductive and immune systems might seem to be functionally separated but a complex interlinks exists. This way, reproductive system encounters various challenges including sexually transmitted diseases, systemic infections that affect reproductive organs and chronic inflammatory conditions, which can be overcome with the help of the immune system (KANNAKIA; SHANMUGAM; VERMA, 2011).

The immune system gives protection against a variety of pathogens and is based on a limited repertoire of germline-encoded receptors called pattern recognition receptors (PRRs). This, receptors have the ability to recognize conserved microbial components, known as pathogen-associated molecular patterns (PAMPs) (ALBIGER et al., 2007).

Several classes of PRRs have been implicated in innate immune defense. These include Toll-like receptors (TLRs) (TAKAEDA; AKIRA, 2005), lectin receptors (HUYSAMEN; BROWN, 2009), retinoic acid inducible gene I (RIG-I) (YONEYAMA; FUJITA, 2007), NOD-like receptors (NLRs) (MARTINON; MAYOR; TSCHOPP, 2006) and cytosolic deoxyribonucleic acid (DNA) sensors (TAKAOKA; TANIGUCHI, 2008; HORNUNG et al., 2009).

According to Kawai and Akira (2010), the science community has given an increasing attention to the TLRs. Studies had focused on the post-transcriptional modifications in molecules regulation that are involved in this receptors signaling and characterization of target genes. As well, additional information on the organization of these genes in different species could help to understand the evolution of these types of PRRs (McGUIRE et al., 2006).

Furthermore, another PRR pathway located in the cytosol is able to activate an immune signaling process resulting in the production of type I interferons (IFN I) and others cytokines. This other activation is carry out by RIG-I and melanoma differentiation-associated protein 5 (MDA5) in discriminating different classes of ribonucleic acid (RNA) virus (KATO et al., 2008). It is important to notice that interplay between TLRs and RIGI-like receptors (RLRs) in different cell types during viral infection plays an important role in antiviral responses (KAWAI; AKIRA, 2011).

In uterine inflammation, an effort needs to be made to understand the risk factors for biological mechanisms and how the uterus is able to detect infection, respond to microorganisms and how infection modulates normal function (SHELDON et al., 2008). In this context, the endometrium defense against infections seems to rely heavily on innate immunity, which when activated leads to an inflammatory response by producing cytokines (WIRA et al., 2005; MOR; CARDENAS, 2010).

The recognizing of bacteria and viruses in the endometrium is dependent on innate immunity. Whereas much is known about bacteria and their recognition by TLRs on endometrial cells, little is known about how viruses are detected. Based on that, the main hypothesis of this study was that bovine endometrial (epithelial/stromal) cells sense and respond to viral PAMPs via TLRs, and cytosolic stimulus induced by RIG-I at different times of stimulation.

In general, the objective of this study was to determine if bovine endometrial cells replied to PAMPs virus analogs by producing proinflammatory cytokines after TLR activation in the cell endosome and after RIG-I activation in the cell cytoplasm.

2. Immune uterine defense system

The immune system can be divided in two; namely innate and adaptive. Although the adaptive immune is more specific and robust, activation of antigen specific effective response stays behind compared to the innate immune (KANNAKIA; SHANMUGAM; VERMA, 2011). While sometimes it is considered that innate immunity is "primitive" or "crude" compared to the adaptive, the opposite is true, as in fact, the innate has been refined for a longer period of time than the adaptive, and is more efficient in almost every way (BEUTLER, et al., 2004).

It's well-known that these two systems are sequentially activated during an infection and work cooperatively to eradicate the microbial agent. The innate immunity is the first line of host defense towards microbial invasion, on the other hand, the adaptive is elicited later, about 4–7 days post infection and includes a specific and long-lasting immunity that is based on the rearrangement and the clonal

expansion of a random repertoire of antigen receptors on lymphocytes (ALBIGER et al., 2007).

Innate immunity in the genital tract is highly dependent on the expression of PRRs that will detect PAMPs. These PRRs, such as TLRs, are highly conserved across phyla and are able to detect a range of different PAMPs associated with fungi, viruses and bacteria (BEUTLER, 2004; AKIRA et al., 2006).

A rapid progress was made in understanding innate immune recognition of microbial components and its critical role in host defense against infection (KAWAI; AKIRA, 2010). Although this recognition plays a major role in uterine defense mechanisms, how it occurs is not fully understood (ESPOSITO et al., 2014).

When studying uterine immune defense, some anatomical barriers work against invading pathogens, as the vulva, vagina and cervix. Other physical barriers in the genital tract include the stratified squamous epithelium of the vagina, the columnar epithelium of the endometrium, the basement membrane of ovarian follicles, and the zona pellucida of the oocyte (SHELDON et al., 2014).

According to Yunhea et al. (2013), the uterus is able to block and eliminate invading pathogens via its mechanical protective barrier and immune functions, thereby maintaining its normal physiological activities. Still, damage of these two defense systems (immune and adaptive) is likely to result in uterine inflammation and lead to disease, infertility or even death.

During cows postpartum period, an elevated expression of genes encoding TLRs, like Toll- Like Receptor 4 (TLR4), inflammatory mediators as Nuclear Factor Kappa b (NF-KB), Interleukin 8 (IL-8), Interleukin 1A (IL-1A), Interleukin 6 (IL-6), Interleukin 12A (IL-12A) and effector molecules, such as acute phase proteins (APP) and activated proteins kinase (AMPs), are characteristic of local uterine innate immune response (CHAPWANYA et al., 2009).

According to Beutler et al. (2004), innate immunity system is broad, and, sometimes, it is difficult to decide where this system ends and the rest of the host begins. In part, this is because innate immune mechanisms are dynamic on an evolutionary time scale, still host population is shaped by selective pressure that microbes are imposed, and it survives as best it can.

3. Cellular response against invading pathogens

Some responses against invading pathogens are observed specially at the cellular level. Uterine leukocytes and polymorphonuclear (PMN) cells are responsible for phagocytizing and cleaning contamination (GILBERT et al., 2007). When innate immune cells, such as macrophages and dendritic cells are activated to recognize different pathogens they initiate an entire defense (CARGILL; WOMACK, 2007).

Macrophages are the major population of tissue-resident mononuclear phagocytes and the predominant targets for infection by intracellular pathogens including mycobacteria. They play a dual role in anti-mycobacterial host defense that, currently, is not very clear. Also, it contributes to cell-mediated immunity and bacterial elimination and, is able to provide an essential niche for intracellular survival and escape from host defense mechanisms (VERRECK et al., 2004).

Dendritic cells are closely related to macrophages, but they are more potent antigen-presenting cells (PLAKS et al., 2008). As related by Schulke et al. (2008), this type of cells need to pass by a maturation process, because of that, they require migration to secondary lymphoid organs. During an infection, microbial factors will trigger dendritic cells maturation, with amplification of the response as a result of the subsequent release of endogenous activators (SKOBERNE; BEIGNON; BHARDWAJ, 2004).

To recognize pathogens, dendritic cells have some molecular sensors and antigen-processing machinery (COLLIN; MCGOVERN; HANIFFA, 2013). Also, they can control an additional checkpoint in the efferent phase of the immunity response (BENNETT; CHAKRAVERTY, 2012).

While much of the research on PRRs has focused on specialized cells of the innate immune system such as neutrophils and macrophages, attention is given to other host cells that play important roles in immunity, including the endocrine cells of the endometrium (WIRA et al., 2005). A tractable *ex vivo* model to study infection and innate immunity in the bovine endometrium can be provided by endometrial organ cultures; also they can generate functional inflammatory responses to Gram-negative and Gram-positive bacteria or their PAMPs (BORGES; HEALEY; SHELDON, 2012).

The uterine endometrium is a mucosa comprising of a layer of single columnar epithelial cells overlying a stromal that contains blood vessels and immune cells as well as endometrial stromal cells (DAVIES et al., 2008). Furthermore, the endometrial epithelial and stromal cells appear to have an immunological responsiveness as they express PRRs for the detection of microbes and also to produce a classical inflammatory response to bacteria and their PAMPs (HERATH et al., 2006).

According to Sheldon et al. (2008), in the uterine lumen the first line of defense against microbes are the epithelial cells. Considering that stromal are much more abundant than epithelial cells in the endometrium and closer in proximity to the vasculature and mononuclear cells, this type of cells may have equal importance in the immune response during an inflammatory process (CRONIN et al., 2011).

4. Toll-like receptors in immune responses

4.1. Definition of Toll-like receptors

Toll-like receptors are defined as membrane-bound proteins usually present at the cell surface, but they can also be membrane bound internally such as in endosomes (MEYLAN; TSCHOOP, 2006). In response to virus infections, viral components such as RNA and DNA are recognized by TLRs and (RIG-I)-like helicases (RLHs), and cells are activated to produce type I IFNs and proinflammatory cytokines (HONDA; TAKAOK; TANIGUCHI, 2006).

The founding member of the TLRs was a Toll in *Drosophila melanogaster*, which was first found to instruct dorsal ventral patterning in early embryos, and regulate anti-fungal innate immunity in adult flies. Sequence homology search in mammalian genomes has subsequently identified at least 11 members of TLRs (DU, et al., 2000).

Different cell types can express TLRs; some of these cells include monocytes and myeloid progenitors of dendritic cells (JANEWAY; MEDSHITOV, 2002; KAWAI; AKIRA, 2006; MUZIO et al., 2000). It's well known that these receptors are expressed in different cell types and anatomical tissue locations, although, the mechanisms

regulating TLR gene expression in response to inflammatory mediators remain not fully characterized (MOGENSEN, 2009).

4.2. Toll-like receptors structure

As a conserved innate immune receptors, the Toll-like family belong to a type I transmembrane proteins with an amino terminus (KANNAKIA; SHANMUGAM; VERMA, 2011). In addition to a single transmembrane domain, TLRs are characterized by an extracellular leucine rich repeat (LRR) and a cytoplasmic toll/interleukin-1 receptor (TIR) domain (WERLING; JUNGI, 2003).

The LRR domain in TLRs occurs in proteins ranging from viruses to eukaryotes and appears to provide a structural framework for the formation of protein–protein interactions (CARGILL; WOMACK, 2007). Also, the central part of the LRRs possesses more irregular or longer motifs and varies among different TLRs, implying the functional importance of the central part in ligand recognition (MATSUSHIMA et al., 2007).

The TIR domain usually is involved in downstream signal transduction (KANNAKIA; SHANMUGAM; VERMA, 2011). When this domain is activated, it recruits cytoplasmic adaptor proteins like myeloid differentiation primary response gene 88 (MyD88) and TOLLIP, which in turn associate with various kinases to set off signaling cascades (CARGILL; WOMACK, 2007) (More details were exposed in section 4.5).

Upon ligand recognition, when the protein MyD88 is recruited an association with the cytoplasmic domain of the TLRs via interaction between the TIR domains will be constructed (ALBIGER et al., 2007).

It's important to notice that the over activation of TLRs may break out immune equilibrium and lead to the injury of hosts. Therefore, this over activation must be controlled, in this context, the protein TOLLIP is one of the molecules that can regulate TLR signaling negatively (YUNHEA et al., 2013). Although TLRs are essential for protective immunity against infection, inappropriate TLR responses can contribute to acute and chronic inflammation, as well as to systemic autoimmune diseases (KAWAI; AKIRA, 2010).

4.3. Most important Toll-like receptor ligand: PAMPs

The molecules PAMPs are characterized by being invariant among entire classes of pathogens, essential for their survival and distinguishable from being "self" (JANEWAY, 1989).

These PAMPs are evolutionarily conserved in pathogens and usually critical for infection and pathogenesis Toll-like receptors recognize PAMPs such as peptidoglycan, lipopolysaccharides (LPS), nucleic acids and combinations, in an efficient non-self-reactive manner allowing initiation of a complex signaling cascade to activate various transcription factors and pro-inflammatory cytokines (KANNAKIA; SHANMUGAM; VERMA, 2011).

According to Janeway (1989), this recognition of microbial pathogens is an essential element for the initiation of innate immune responses such as inflammation and is mediated by PRRs that recognize molecular structures that are broadly shared by PAMPs. Different PRRs may recognize the same PAMP; hence TLRs in concert with other PRRs orchestrate both pathogen-specific and cell type-specific host immune responses to fight infections (KAWAI; AKIRA, 2011).

Binding of PAMPs to PRRs activates signal transduction pathways by mitogen-activated protein kinase (MAPK) and NF-κB transcription factors, leading to secretion of prostaglandins, cytokines and chemokines (GHOSH et al., 1998; LI; VERMA, 2002; AKIRA; TAKEDA, 2004).

4.4. Toll-like receptors types

Toll-like receptors are largely divided into two subgroups depending on their cellular localization and respective PAMP ligand. One group is composed of Toll-Like Receptor 1 (TLR1), Toll-Like Receptor 2 (TLR2), TLR4, Toll-Like Receptor 5 (TLR5), Toll-Like Receptor 6 (TLR6) and Toll-Like Receptor 11 (TLR11), which are expressed on cell surfaces and recognize mainly microbial membrane components such as lipids, lipoproteins and proteins. The other group is composed of Toll-Like Receptor 3 (TLR3), Toll-Like Receptor 7 (TLR7), Toll-Like Receptor 8 (TLR8) and Toll-Like Receptor 9 (TLR9), which are expressed in intracellular vesicles such as the

endoplasmic reticulum, endosomes, lysosomes and endolysosomes, where they recognize microbial nucleic acids (KAWAI; AKIRA, 2010).

Signaling of TLR3 and TLR4 has been most extensively characterized, whereas much less is known about how TLR7, TLR8, and TLR9 act in inflammatory responses. Understanding the molecular mechanisms regulating the induction of type I IFNs by these TLRs is likely to reveal novel therapeutic and immune-modulation strategies in order to eliminate acute and chronic viral infections (SCHOENEMEYER et al., 2005).

The TLR3 was originally identified as recognized a synthetic analog of double-stranded RNA (dsRNA) called polyinosinic-polycytidylic acid (Poly(I:C)), it mimics viral infection and induces antiviral immune responses by promoting the production of both type I interferon and inflammatory cytokines, which suggests that TLR3 has an essential role in preventing virus infection (KAWAI; AKIRA, 2010).

The TLR4 is the most extensively studied PRR and it recognizes a variety of ligands (host heat shock proteins, fibrinogen and proteins from virus, pneumolysin, a cytotoxin from *Streptococcus pneumoniae*) but is mostly known to recognize LPS. Still, this is proved by the fact that TLR4 knock-out mice were shown to be unresponsive to LPS (HOSHINO et al., 1999).

The fact is that LPS is recognized by TLR4 but this TLR alone is not sufficient for signaling (MIYAKE, 2006). Some accessory proteins are required for this recognition. Because of this, LPS binds first LPS-binding protein (LBP), which is an acute phase protein that circulates in the bloodstream and binds to glycosylphosphatidylinositol (GPI) linked co-receptor CD14, which is expressed on the cell surface (ALBIGER et al., 2007).

Besides TLR3, the TLR7, 8 and 9 are also specialized in recognition of nucleic acids, with self *versus* nonself discrimination provided by the exclusive localization of the ligands rather than solely based on a unique molecular structure different from that of the host (IWASAKI; MEDZHITOV, 2004). As is shown by Liu (2005), apart from others cells, the TLR7 and TLR9 are highly expressed on plasmacytoid dendritic cells, a cell type known to produce vast amounts of type I IFNs in response to viral infection.

4.5. Toll-like receptor signaling pathways

An improved understanding of the inflammatory pathways at the molecular level which play an important role in normal immune function, metabolism and reproduction, may improve the ability to predict and prevent cows disorders (ESPOSITO et al., 2014).

According to Kawai and Akira (2006), the process of TLR ligand activation induces two signaling pathways, one is MyD88-dependent and the other is MyD88-independent, both ending up with the production of proinflammatory cytokines and type I interferons.

4.5.1. MyD88-dependent signaling

All TLRs, except TLR3, signal through the MyD88 dependent pathway still, only the TLR4 is able to activate MyD88 dependent and independent pathways (ADACHI et al., 1998). Basically, the MyD88 dependent pathway requires a signal transduction intermediates specially by protein kinases that transforms growth factor-activated kinase, to final activate NF-κB in order to induce production of pro-inflammatory cytokines (MEDZHITOV; KAGAN, 2006).

A practical example was showed in the study of Yunhea et al. (2013), when TLR4 recognized LPS and recruited an adaptor protein MyD88 to initiate the MyD88-dependent pathway which had included a series of signal transduction intermediates and activated the NF-κB that started the production of proinflammatory cytokines such as tumor necrosis factor (TNF), Interleukin 1 (IL-1), IL-6 and Interleukin 12 (IL-12).

In case of an over activation of TLR, the protein TOLLIP will be recruited to block the MyD88 dependent signaling pathway by preventing the phosphorylation of associated protein kinases (BULUT et al., 2001; ZHANG; GHOSH, 2002).

4.5.2. MyD88-independent signaling

In a MyD88-indepent signaling others proteins need to be recruited apart from MyD88. This independent signaling pathway activated by TLR3 and TLR4 triggers to the induction of a type I IFN response leading to the induction of Interferon *alpha*

(IFN-α) and IFN inducible genes. Whilst TLR3-mediated signaling only requires the adaptor molecule TIR-domain-containing adapter-inducing interferon-β (TRIF), while on the other hand, TLR4-mediated signaling needs in addition to TRIF another adaptor protein known as translocation associated membrane protein (TRAM), both will activate NF-κB to induce expression of proinflammatory cytokines (CUSSON-HERMANCE et al., 2005).

The MyD88-independent pathway is also called interferon regulatory factor 3 (IRF-3) dependent pathways. Because when TRIF is recruited it will also activate a TRIF-related adaptor molecules family members-associated NF-κB activator, known as a tumor necrosis receptor-associated factor (TRAF) that together will activate the enzyme Serine/threonine-protein kinase 1 (TBK1) via tumor necrosis receptor-associated factor 3 (TRAF3). In turn, TBK1 phosphorylates directly two transcription factors, IRF-3 and interferon regulatory factor 7 (IRF-7) allowing them to translocate into the nucleus and induce production of IFN-α and IFN inducible genes (SATO et al., 2003). A schematic figure that shows MyD88 independent and dependent pathway was illustrated in Figure1.
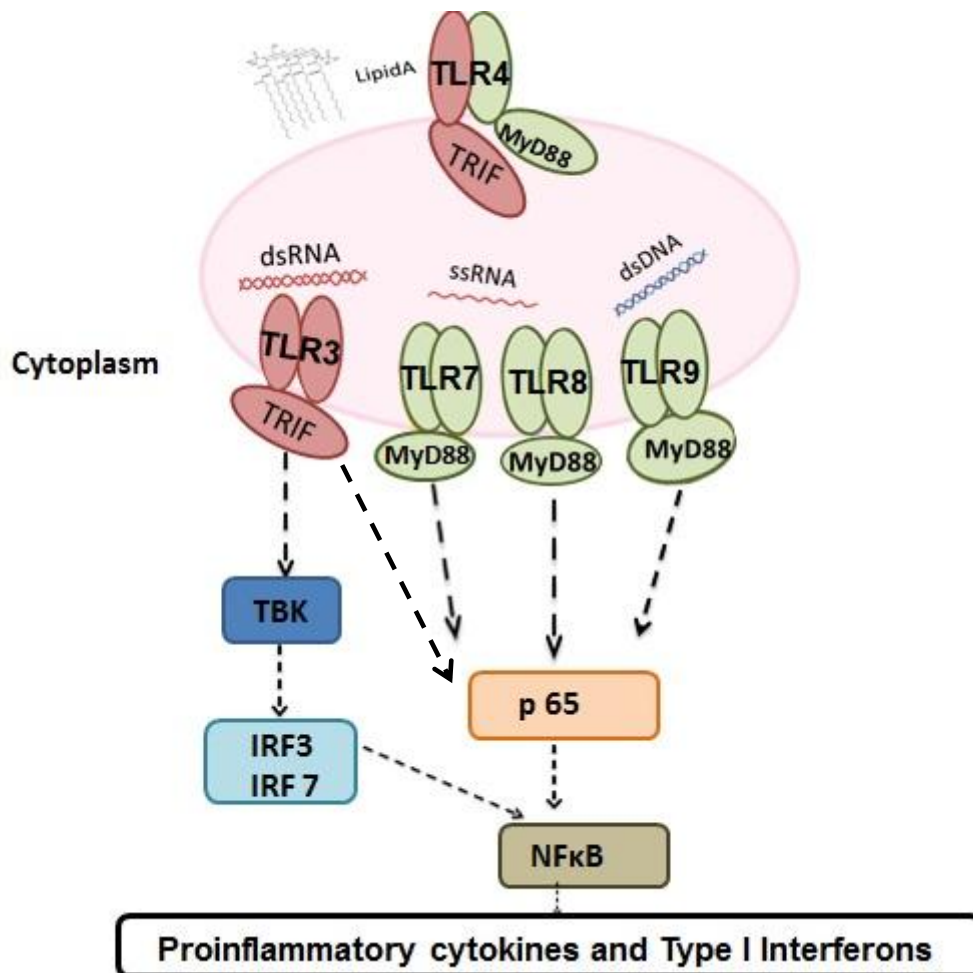
Figure 1. Toll-like receptors 3,4,7,8 and 9 pathways summarized: MyD88 independent pathway (TRIF pathway): when a TLR3 analog (dsRNA) activated this pathway, the adaptor protein TRIF is recruited to phosphorylate TBK and p65 to end up producing proinflamatory cytokines and IFNs mediated by activation of IRF3/7 and NF-κB respectively. MyD88 dependent pathway: when TLR 7, 8 or 9 analogs (ssRNA, dsDNA) activate this pathway, the adaptor protein MyD88 is recruited to phosphorylate p65 resulting in production of proinflammatory cytokines and IFNs mediated by activation NF-κB.TLR4 analog (LIPD A) is able to activate both pathways by recruiting both MyD88/TRIF adaptor proteins to result in the production of proinflammatory cytokines.

4.6. Expression of Toll-like receptors in bovine endometrium

Most of the studies on TLRs are more advanced in mice and humans, although, in the last few years a progress has been made in identifying TLRs in

different species of farm and companion (KANNAKIA; SHANMUGAM; VERMA, 2011).

Different authors have been studied the expression profile of TLRs in selected tissues and cell subsets of cattle and buffalo (MENZIES; INGHAM, 2006; WERLING et al., 2006; VAHANAN et al., 2008); The full-length coding sequence of TLR3 has been well characterized in Water Buffalo (*Bubalis bubalis*) and Nilgai (*Boselaphus tragocamelus*) showing a 98% of homology to that as in cattle (DHARA et al., 2007).

Ten different types (1-10) of TLRs have been identified and physically mapped in cattle having 95% nucleotide sequence identical with human (McGUIRE et al., 2006; MENZIES; INGHAM, 2006).

As related by Herath et al. (2006) and Davies et al. (2008), studies proved that the endometrium of cows express TLRs 1–10, whereas purified populations of endometrial epithelial cells express TLRs 1–7 and 9, and stromal cells express TLRs 1–4, 6, 7, 9 and 10. Apart from that, TLR4 in cows appear to be functional in endometrial epithelial cells as they secreted prostaglandin E2 in response to bacterial PAMPs (KANNAKIA; SHANMUGAM; VERMA, 2011).

5. RIG-I signaling in immune system

5.1. Definition and function of Retinoic Acid Inducible Gene I, RIG-I

In the cytosol, studies have identified NLRs and RIG-I as two additional families of innate immunity receptors that recognize PAMPs (INOHARA et al., 2005; MEYLAN; TSCHOPP, 2006).

The RIG-I with its helicase domain has been demonstrated to be an essential regulator for dsRNA signaling that results in the activation of the transcriptional factors NF-κB as well as IRF-3 (SUMPTER et al., 2005; YONEYAMA et al.; 2004). Moreover, the relationship between RIG-I and TLRs in the recognition of viruses need to be more expolored (KATO el al., 2005).

5.2. RIG-I like receptors (RLRs) family structure

The RLRs consists of three helicases known as: RIG-I, MDA5 and by a protein called laboratory of genetics and physiology 2 (LGP2) (YONEYAMA, et al., 2004; ROTHENFUSSER et al., 2005).

A RNA helicase domain that recognizes viral dsRNA is present in the RLRs family. RIG-I and LGP2 contain a C-terminal regulatory domain that recognizes ssRNA containing 5′-triphosphate, which distinguishes foreign RNAs from self-RNAs that normally contain 5′-modification. Still, RIG-I and MDA5 contain an N-terminal and a caspase activation and recruitment domain (CARD), which interact with the CARD domain of mitochondrial antiviral-signaling protein (MAVS), also known as virus-induced signaling adapter (VISA), which is located out of the mitochondrial membrane (KAWAI et al., 2005; MEYLAN et al., 2005; SETH et al., 2005; XU et al., 2005).

5.3. RLRs signaling

Two CARDs and a protein with the domain DEAD or DEAH box helicases (DExD/H-box) belong to RIG-I and MDA5, the helicase domains of RIG-I and MDA5 recognizes viral RNAs, and their CARDs are responsible for signaling through interaction with a CARD-containing adaptor, MAVS (KAWAI et al., 2005; KUMAR et al., 2006).

Both RIG-I and MDA5 engage the mitochondrial adapter protein MAVS, this protein subsequently triggers downstream signaling and activation of the inhibitor of nuclear factor kappa-B kinase subunit *alpha/beta* (IKKα/β)- NF-κB pathway or the TBK1-IRF3 pathway and transcriptional regulation of proinflammatory cytokines and type I IFN genes, respectively (ABLASSER et al., 2009).

On the other hand, LGP2 does not possess a CARD, but only a DExD/H-box helicase domain, and has been reported to act as a negative regulator factor (ROTHENFUSSER et al; 2005; YONEYAMA et al.; 2005)), specially at the RIG- I mediated pathway (SAITO et al.; 2007; VENKATARAMAN et al., 2007).

5.4. RNA Polymerase III pathway in RIG-I activation

According to Ishii et al. (2006) and Sun et al. (2006), some genetic studies have shown that cytosolic DNA can induce IFN production in mice dendritic cells lacking RIG-I or MAVS, suggesting that the DNA signaling pathway is distinct from the RIG-I pathway.

The RNA polymerase III (Pol III)-RIG-I pathway appears to be functional in both human and mouse cells, but in this last species seems to be redundant with additional DNA sensing mechanisms (ABLASSER et al., 2009).

Studies from Chiu, McMillan, Chen (2009) demonstrated that RIG-I binds to RNA but not to DNA; so the question of how DNA might activate the RIG-I pathway need to be raised. This RNA species contains 5′-triphosphate and forms a dsRNA. The conversion of DNA to RNA can be recapitulated *in vitro* using cytosolic extracts. Due to that, Pol-III seems to be the enzyme responsible for transcribing the DNA template into an RNA ligand that activates RIG-I. These results suggest that Pol-III is a cytosolic DNA sensor that triggers type-I interferon production through the RIG-I pathway

6. Viral infection against immune response

Viruses are obligatory intracellular pathogens; therefore, their replication (and the pathogenic consequences of infection) depends critically on the ability to transmit their genomes from infected to non-infected host organisms and from infected to uninfected cells (MARSH; HELENIUS, 2006). They are also restricted in using metabolic and biosynthetic pathways of the cells that they infect. These pathways vary between cell types, lineage, and stage of differentiation and with the state of cell activation (DONOFRIO et al., 2007).

The innate immune system has evolved several distinct viral recognition systems that integrate complex networks of signaling pathways, which can lead to activate some pathway-specific transcription factors and the induction of immune response genes (SCHOENEMEYER et al., 2005).

In order to infect, first, viruses must bind to the cell surface. This structures that they bind are composed of two general types (RNA, DNA) depending on the

functional consequences of the interaction, that usually are highly specific, and the presence of receptors determines in a large degree which cell types and species can be infected (MARSH; HELENIUS, 2006).

Studies of Seth, Sun and Chen (2006), certified that two basic events are required to trigger an effective anti-viral response: first, a detection of the invading virus by immune system receptors; and second an initiation of protein signaling cascades that regulate the synthesis of IFNs. In viral infections, the induction of IFN I have shown to be primarily due to the recognition of dsRNA that is a sign of replicating viruses (SUMPTER et al., 2005; YONEYAMA et al.; 2004).

Based on Hiscott et al. (2006) comments, the probing of the immune response for millions of years was caused by viruses, also, this investigation of this response is yielding important clues about which pathways must be compromised in order for virus infection perpetuate. Utilization of this knowledge will be a cornerstone in the understanding of molecular aspects of viral pathogenesis and the improvement of strategies for the development of vaccines and antiviral agents.

7. Proinflamatory cytokines (focus on IL-6 and IL-8)

In general, cytokines are considered to have different functions, they are primarily involved in host responses to disease or infection, and their participation in homeostatic mechanisms has been less explored (DINARELLO, 2000). Moreover, cells have mechanisms for regulating both signaling pathways to avoid injurious effects due to overproduction of inflammatory cytokines during microbial infection (KUMAR; KAWAI; AKIRA, 2011).

The production of proinflammatory cytokines and IFN I (an important group of cytokine family) are released in response of the recognition of viruses by PRRs. In particular, type I IFNs, comprised of a multiple interferon-*alpha* (IFN-α) and interferon *beta* (IFN-β), and they are extremely important in eliminating viruses by inducing death of infected cells, conferring resistance to viral infection on surrounding them, and activating acquired immune responses (KATO et al., 2008).

Binding to specific receptors on the neutrophil surface, cytokines can influence different cell functions, for example the ability to localize at the site of inflammation, phagocytic activity, production of oxygen metabolites, and the release of lysosomal enzymes (SEMMANI; KABBUR, JAIN; 1993).

Another important proinflammatory cytokine group is composed by the Interleukins (ILs). They are secreted proteins that bind to their specific receptors and play a role in the communication among leukocytes. Also, investigations of the mechanisms of immune and inflammatory cell functions have identified a growing list of ILs and interactions among different cell types that contribute to their effector and suppressive functions (AKDIS et al., 2011).

According to Fischer et al. (2010), in cows, it is important to study and understand the defense made by some proinflammatory cytokines specially IL-6, IL-8, and TNF, as they accelerate PMN infiltration into the bovine endometrium following infection.

A balance between the effects of proinflammatory and anti-inflammatory cytokines is thought to determine the outcome of disease, whether in the short or long term. In fact, some studies suggested that the susceptibility to disease is genetically determined by the balance or expression of either proinflammatory or anti-inflammatory cytokines (DINARELLO, 2000).

As one of the most important members of the cytokines, IL-6 has a helix bundle structure consisting of four long α-helices. Also, it is a multifunctional, pleiotropic cytokine involved in regulation of immune responses, acute-phase responses, hematopoiesis, and inflammation (AKDIS et al., 2011).

A more detailed study conducted by Hurst et al. (2001), revealed that in innate immunity, IL-6 directs leukocyte trafficking and activation and induces production of acute-phase proteins by hepatocytes cells.

Other researches on cytokines showed that, high concentrations of IL-6 are present in the bovine uterus before parturition and decreased to baseline values by eight days after parturition (ISHIKAWA et al., 2004). Also, high levels of this cytokine were associated with bovine endometritis, while low levels with retention of the placenta (SINGH et al., 2008).

Chemokine is a group of cytokines, and it's well known that chemokine IL-8 is one of the most studied cytokines during immune inflammatory response. Still, the major effector functions of IL-8 are activation and recruitment of neutrophils to the site of infection or injury (MATSUSHIMA et al., 1988).

The chemokine IL-8, is produced by a variety of cells, such as monocytes and macrophages, neutrophils, lymphocytes, and endothelial and epithelial cells after stimulation with IL-1A, interleukin 1B (IL-1B), interleukin 17 (IL-17), tumor necrosis factor *alpha* (TNF-α), or TLRs (COELHO et al., 2005). The presence of recombinant IL-8 has been shown to increase the influx of PMN leucocytes into the bovine uterus (ZERBE et al., 2003).

In a more specific study developed by Yoshimura et al. (1987), IL-8 was identified as a neutrophil-specific chemotactic factor and later classified as a member of the chemokine family. Recent studies have shown that IL-8 promoter contains binding sites for the transcription factor NF-κB and activator protein 1(AP-1) (AKDIS et al., 2011). Others studies developed by Turner et al. (2012), found that endometrial epithelial and stromal cells mounted cellular responses to bacterial lipopeptides typical of innate immunity with secreting both IL-6 and IL-8.

8. What to expect in the next studies

As a result of the researches from Kannakia, Shanmugam and Verma (2011), they assumed that one particular area to focus on the PRRs studies should be the analysis of potential TLR agonists for development of new vaccine/adjuvant to prevent reproductive infection. Moreover, polymorphisms in TLR genes and their association with diseases of economic importance in cattle such as mastitis have been established. In the future these facts could be used as molecular markers in order to select animals in the development of immunogenetically superior stocks.

Even though data is available on the area of TLR signaling in response to bacterial ligands, more about the integrated responses that occur when intact pathogens are infecting the host should be given. Studies on different mechanisms

by which primary pathogens can modulate or suppress innate immune responses by interfering at different levels with TLR signaling, and how opportunistic pathogens may take advantage of, for example, host responses to viral infections to gain access to deep tissue from local sites should be the focus (ALBIGER et al., 2007).

It is clear to observe that the importance of PRRs signaling for both immune homeostasis and for defense mechanisms against pathogens has emerged in the past decade (KONDO; KAWAI; AKIRA, 2012). An advancement has been made not only in the understanding of the structure of TLRs but also in revealing the complexity of TLR-mediated signaling and in the identification of PAMPs derived from microbial pathogens such as mycobacteria, bacteria, viruses, fungi and parasites (KUMAR; KAWAI; AKIRA, 2011).

The virus infection models tested to date support roles for TLRs, rather than RLRs, in instructing the adaptive immune system. However, investigations are required, since these two PRR systems provide different contributions depending on the viruses involved and also may depend on the route of infection (TAKEUCHI; AKIRA, 2008).

Related to bovine immune function, future work should focus on determining which pathogen, bacteria or virus can cause endometritis, and understanding how the host response to infection is regulated in the endometrium. New knowledge of uterine diseases will provide a platform for new therapeutics and vaccines (CARNEIRO; CRONIN; SHELDON, 2015).

Moreover, studies need to be developed in all domestic animals, so it will be possible to understand and verify the real response of immune system mediate by a TLR-RIG-I system that will work adverse pathogens infections that affect important economic rates in cattle.

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CHAPTER 2 – BOVINE ENDOMETRIAL CELLS ACTIVATE IMUNE RESPONSES AFTER A VIRAL dsRNA ANALOG INDUCTION

BOVINE ENDOMETRIAL CELLS ACTIVATE IMUNE RESPONSES AFTER A VIRAL dsRNA ANALOG INDUCTION

ABSTRACT- The aims of this study were: 1) to determine which pathogen associated molecular patterns (PAMPs) were able to activate immune responses mediated by TLR3, TLR7 in bovine endometrial cells; 2) to check if the use of a Liposomal Transfection Reagent (DOTAP) increased PAMPs action during a viral replication and 3) to validate which moment this cells produced proinflammatory cytokines after treated with a dsRNA analog by TLR3 induction. Uterine samples from post pubertal cross-breed beef cows were dissected using a protocol to obtain epithelial and stromal cells. A negative Control and four different PAMPs: LPS, ssRNA, Poly I:C (LMW), Poly (I:C) HMW were used. Two treatments groups were investigated during 24 hours, one with PAMPs diluted in Optimen Media, the other with PAMPs transfected with DOTAP Liposomal Transfection Reagent also diluted in Optimen Media. In another experiment, endometrial cells were treated with only Poly (I:C) LMW, and a negative control group (no PAMP) was incubated at 0, 2, 6, 12, 24, 36, 48 and 72 hours. Supernatants were collected to analyze Elisa for IL-6 and IL-8. Endometrial epithelial cells produced IL-6 in response do Poly I:C (HMW) compared to Control (DOTAP positive; P< 0.05). Stromal cells produced IL-6 (DOTAP negative group) and IL-8 (DOTAP positive and negative groups) in response to LPS (P< 0.05). The use of DOTAP Liposomal Transfection Reagent differ between cells treated with Poly (I:C) LMW (P> 0.05). Still, in stromal cells treated by Poly I:C (LMW) the production of IL-6 was higher at 48 and 72 hours (P< 0.05), and for IL-8 at 6, 12, 24, 36, 48 and 72 hours when compared to the Control group (P< 0.05). In response to TLR3 production bovine endometrial cells sensed do dsRNA analog, by releasing IL-6 after treated by Poly I:C (HMW) (epithelial cells). Still the use of a Liposomal Transfection Reagent did affect the production of cytokines. Furthermore, in response do TLR3 activation, bovine stromal cells treated by Poly I:C (LMW) produced IL-6 at two different time-point, and IL-8 at all time-points tested when compared to the Control.

Keywords: Cows**,** immunity, cytokines

Introduction

       The capacity to detect various pathogens and tailor response to specific agents is critical for the induction of innate immunity and to establish an adaptive response. After infection, innate immune system is able to detect invading pathogens by a variety of PAMPs that are recognized by TLRs (RUDD et al., 2005).

       Toll-like receptors family is characterized by an amino-terminal leucine-rich repeat domains and carboxy-terminal Toll/interleukin (IL-1) receptor signaling domains (ARMANT; FENTON, 2002). Still, they have been identified in mammalian host immune-competent cells, such as dendritic and macrophages, which are ones that most come into direct contact with pathogens from the environment via mucosal epithelia (HORNUNG et al., 2002). On the other hand, less information regarding responses by endometrial epithelial tissue and cell lines on the impact of immune responses to viral infection on the endometrium is available (JORGENSON et al., 2005).

       Whilst the focus of many studies have been mainly on bacterial components, TLR3, TLR4, and TLR7, have been shown to mediate the response to some viral associated PAMPs: the dsRNA analog Poly I:C; the F protein of Respiratory Syncytial Virus and the antiviral therapeutic compounds, known as imidazoquinolines (ALEXOPOULOU et al., 2001; HEMMI et al., 2002; KOPP; MEDZHITOV, 1999; KURT- JONES et al., 2000; TAKEUCHI; AKIRA, 2001; TAKEUCHI et al., 1999).

       Poly (I:C) is a synthetic dsRNA copolymer of inosinic and cytidilic acids, which induces cell activation in part via TLR3, beyond the fact that several different TLR ligands were able to stimulate epithelial cells, this specific PAMP was considered to be the most effective one (ALEXOPOULOU et al. 2001).

       One of the best characterized TLRs is the TLR4, and it can recognize LPS from Gram-negative bacteria, while TLR2 recognizes lipoteichoic acid and peptidoglycan from Gram-positive bacteria (SCHRODER et al., 2003; TAKEUCHI et al., 2000). Although the knowledge of LPS/TLR4 signaling pathway mainly focuses on human and mouse, some studies had investigated the interaction of LPS in bovine endometrial cells (HERATH et al., 2009; SHELDON; ROBERTS, 2010).

It is visible that TLRs activate a variety of inflammatory and immune response in mammals. How the host integrates the information that is signaled through TLRs and any co-receptors will ultimately control the progression of the response against pathogens. Understanding all this process will undoubtedly lead to the development of novel therapeutics and immune adjuvants (ARMANT; FENTON, 2002).

In this research the hypotheses were that bovine endometrial epithelial and stromal cells sense and respond to viral PAMPs analogs via TLR3 and TLR7. Still, the use of a Liposomal Reagent Transfection would enhance cells response by production of proinflammatory cytokines.

The aims of this study were: 1) to determine which PAMP (dsRNA/ssRNA) were able to activate immune response mediated by TLR3 and TLR7 in bovine endometrial cells; 2) to check if the use of a Liposomal Reagent Transfection increased PAMPs action during a viral replication; 3) to validate which moment bovine endometrial cells produced proinflammatory cytokines after treated with a dsRNA analog by TLR3 induction.

Material and Methods

Pre-treating the cells

Bovine samples

The experiment was developed at Swansea University, Wales, UK, at Institute of Life Science. Uterine samples from post pubertal cross-breed beef cows over ten months of age were collected from a local slaughterhouse. At the slaughterhouse, only uterus with no gross evidence of genital disease or macroscopic content that seemed to be infection were selected to be part of the experiment.

Uterine samples were kept on ice (2-8°C) for one hour before processing in the laboratory. When the samples arrived at the University they were washed with ethanol 70% (Appendix 1).

Bovine endometrial cell isolation protocol

To obtain stromal and epithelial cells the steps (1,2,3, and 4) were based on a cell isolation protocol previously described (FORTIER, M.A.; GUILBAULT, L.A.; GRASSO, F., 1988; CHENG, Z. et al., 2003) with modifications adjusted by Herath et al. (2006).

1) The ipsilateral horn of the uterus with active luteal or follicular structure was opened longitudinally with sterile scissors; the endometrium was exposed and washed with Endo Wash (Appendix 2).

2) Endometrium was dissected from the uterine horn thinner as possible in order to avoid myometrium contamination. Strips of the dissected endometrium were removed and placed directly in a pot containing 25 milliliters (ml) of Endo Strip Wash (Appendix 3) and then transferred into a second 60 ml pot with 25 ml of HBSS [(Hanks Balanced Solution (HBSS), Sigma -Aldrich Ltd, Dorset, UK]. The strips were cut into 3-5 mm^3 pieces and kept in a 50 ml falcon tube containing HBSS, then placed into a water bath for 10 minutes at 37°C.

3) In cell culture hood, supernatant from falcon tubes were removed and 25 ml of Digest Media (Appendix 4) were added. Following one-hour incubation in a shaking water bath at 37°C, the cell suspension was filtered through a 40 micrometers (μm) cell strainer (Fisher Scientific, Loughborough, UK) into a falcon tube containing 5 ml of Stop Solution (Appendix 5). The suspension was centrifuged at 700 x g for 7 minutes at 25°C (VWR®, CT6E, Japan) and carefully supernatant was removed and 5 ml of warm sterile water was added into the cell pellet and tubes were vortexed.

4) Immediately, the cell water mixture was top up with Stop Solution (Appendix 5) until a total volume of 40 ml and another centrifuging (700 x g for 7 minutes at 25°C) (VWR®, CT6E, Japan) was performed. Supernatant decant was removed and the cells pellet were re-suspended in a 5 ml of fresh warm Complete Media (Appendix 6).

The cells were cultured in 75 cm^2 flasks (Greiner Bio-One, Gloucester, UK) for 18 hours to allow selective attachment of stromal cells (FORTIER, M.A.; GUILBAULT, L.A.; GRASSO, F., 1988), while the remaining was transferred to a new

flask to obtain epithelial cell (KIM, J.J.; FORTIER, M.A, 1995). The cells were then incubated in a humidified atmosphere of air with 5% of carbon dioxide (CO_2), and the medium changed every 48 hours.

Primary cultured cells were examined under a microscope (Axiovert 40 C, Carl Zeiss Microscopy, USA) every day to ensure sub-confluency. As both epithelial and stromal cells were present within the same culture, differentiation splitting of epithelial and stromal cells was required 72 hours post-dissection.

The epithelial or stromal cells were detached from the flasks using a protocol detachment solution with Accutase (Millipore, Watford, UK), as follows: in each flask, 5 ml of PBS were added to wash the cells, then PBS was discarded and 2 ml of Accutase were added to detached first stromal cells. After 3 minutes the cells were re-suspended in 13 ml of Complete Media (Appendix 6). For epithelial cells, 4 ml of Accutase were added after washed with PBS, and around 7 minutes were waited to re-suspend in 8 ml of Complete Media (Appendidx 6).

After stromal and epithelial cells were put in separated flasks, for each flask 2 ml of Accutase (Millipore, Watford, UK) were added to adherent cells to suspend them. Once the cells were re-suspended, a Complete Media (Appendix 6) was added to neutralize Accutase action. The cells were then centrifuged at 400 x g for 7 minutes (VWR®, CT6E, Japan); supernatant was rejected and the cells were re-suspended in 3 ml of Complete Media (Appendix 6). A hemocytometer FastRead102 (Immune Systems LTD, UK) was used for counting the sample in 7 microliters (µL) of cell suspension.

Stromal and epithelial cells were plated at 1×10^4 cells/ml in a 24-well plates (TPP, Trasadingen, Switzerland), where 1 ml of the re-suspended cells was added in each well respectively. Before any cellular treatment, under a microscope (Axiovert 40 C, Carl Zeiss Microscopy, USA) the plates were incubated and their adhesion and normality were examined until future treatments.

Treating the cells

Experiment 1: Transfection of endometrial cells with a panel of PAMPs

In this first experiment a total of 16 different uterus (n) were used (n=16). Six uterus (n=6) were for stromal cells, and others ten uterus (n=10) for epithelial cells. After cell culture, four different PAMPs were tested: LPS, ssRNA (Polyuridylic Acid Poly (U)), Polyinosinic-polycytidylic acid Low Molecular Weight (Poly (I:C) LMW), Polyinosinic-polycytidylic acid High Molecular Weight (Poly (I:C) HMW). For positive control, LPS from *Escherichia coli* 011:B4 (Invivogen, Toulouse, France) was used, and for negative Control only Complete Media was choose (Appendix 6).

To bind TLR7, Poly (U) (Invivogen, Nottingham, UK) was selected as agonist from ssRNA, while to bind TLR3 Poly (I:C) LMW and Poly (I:C) HMW (Invivogen, Nottingham, UK) were chosen as dsRNA agonists.

The name, TLR binder, stock and worked concentrations of the PAMPs chosen for this experiment were described in Table 1.

Table 1. Toll-like receptor binder, stock and empirically concentrations of LPS, ssRNA, Poly I:C (LMW) and Poly I:C (HMW) used in bovine endometrial stromal and epithelial cells.

PAMP	Name	TLR Binder	Stock Concentration (µl/ml)	Calculated worked concentration (µl/ml)
LPS	LPS	TLR4	1000	0.1
ssRNA	Poly (U)	TLR7	1000	1
dsRNA	Poly (I:C) - LMW	TLR3	20,000	1
dsRNA	Poly (I:C) - HMW	TLR3	1000	1

Final concentrations of the PAMPs were based on manufacturer recommendations and on previous tests (unpublished data) developed at the same Laboratory from this experiment.

In this study, two treatments groups were investigated. The first group was composed only with the PAMPs. The cells were treated with each PAMP tested diluted in 15 µl of Optimen Media (Appendix 9), then this PAMP/Media dilution was

added in a tube containing 30 µl of Optimen Media (Appendix 9) and to make the final concentration to1 ml, 955 µl of Complete Media were added (Appendix 6).

The second group had the same treatment as described in the first group, however each PAMP/Media dilution after added in a tube containing 30 µl of Optimen Media (Appendix 9) received more 10 µl of DOTAP Liposomal Transfection Reagent (10 - 20 DOTAP cells/ml culture Media) (Roche, Brasileia, Switzerland). To make the final concentration to 1 ml, 945 µl of Complete Media were added (Appendix 6).

The use of DOTAP Liposomal Transfection Reagent (Roche, Brasileia, Switzerland) was choose to verify if there was any kind of difference on PAMPs action when cells were transfected. DOTAP was selected for this experiment as a liposomal transfection reagent for the delivery of negatively charged biomolecules specially DNA, RNA, oligonucleotides, and protein into eukaryotic cells.

In both groups cells were incubated in a humidified atmosphere of air with 5% CO_2 for 24 hours prior to supernatant collection.

Experiment 2: Time evaluation for endometrial cells treated with dsRNA analog

For this experiment, a total of 12 uterus were used, six (n=6) to evaluate stromal cells and six (n=6) to evaluate epithelial cells. The cells were cultured in a Complete Media (Appendix 6) at $1x10^4$ cells/ml/well in 24-well-plates (TPP, Trasadingen, Switzerland), and treated only with one PAMP: Poly (I:C) LMW, (Invivogen, Nottingham, UK), used as a dsRNA analog in order to activate the TLR3 pathway.

In the PAMP group, the cells were treated with Poly (I:C) LMW (1µl/ml) diluted in 15 µl of Optimen Media (Appendix 9), then this PAMP/Media dilution was added to a tube containing 30 µl of Optimen Media (Appendix 9). After that, the solution received more 10 µl of DOTAP Liposomal Transfection Reagent (10 - 20 DOTAP cells/ml culture Media) (Roche, Brasileia, Switzerland). To make the final concentration to 1 ml, 945 µl of Complete Media were added (Appendix 6). A negative Control group (no PAMP, only Optmem Media, Appendix 9) was also used.

Different time-points were chosen to evaluate the best moment which Poly (I:C) LMW acted on endometrial cells. The cells were incubated in the same plate in

a humidified atmosphere of air with 5% of CO_2 with treatments at 0, 2, 6, 12, 24, 36, 48 and 72 hours before collecting supernatants for future analyses.

Post-treating the cells

Enzyme-Linked Immunosorbent Assay (ELISA)

After evaluating by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (MTT) (data not shown) Elisa test was developed. Concentrations of IL-6 and IL-8 in epithelial and stromal supernatants were measured according to the manufacturer's instructions. Bovine IL-6 Screening Set (ESS0029; ThermoFisher Scientific) and Human CXCL8/IL-8 DuoSet (DY208; R&D Systems Europe Ltd., Abingdon, UK)] were used. The human CXCL8/IL-8 DuoSet has been previously validated for the measurement of bovine IL-8 (RINALDI et al. 2008).

Basically, after an overnight incubation using the coating antibody, the 96 well-plate was blocked with 150 µl of blocking buffer, followed by adding 50 µl of each sample and specific standards to put later the detection antibody: Streptavidin-horseradish peroxidase (SA-HRP); with the exception of IL-8 which had a tertiary biotinylated Goat Anti Rabbit detection antibody (Dako, P0448). After 30 minutes, 50 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added to finally put 50 µl of stop solution (Elisa IL-6 and IL-8 detailed assay are described in Appendix 7 and 8 respectively).

A plate washer was used to wash the plates thrice between treatments and the absorbance was read with a plate reader (Omega Polarstar®, BMG, Ortenberg, Germany). The raw data collected was analyzed using Mars software (BMG Labtech, Ortenber, Germany) and concentration calculated.

Statistical analysis

In experiment 1 a 5 x 2 factorial was developed (five PAMPs in the presence and absence of DOTAP). The effect of the variables [IL-6] and [IL-8] in both epithelial

and stromal cells factors were analyzed using a two-way ANOVA and means were compared by Tukey test analysis [AgroEstat (BARBOSA; MALDONADO, 2015)].

In experiment 2, the effect of the variables [IL-6] and [IL-8] in both epithelial and stromal cells at different time-points was analyzed by the General Linear Model Procedure (ANOVA, SAS) and means were compared by Tukey test analysis. Differences were considered significant when $P < 0.05$.

Results

Experiment 1
Transfection of endometrial cells with a panel of PAMPs

In this experiment, ELISA test was performed in order to further investigate the production of proinflammatory cytokines and chemokines as IL-6 and IL-8, in response to virus analogs such as dsRNA and ssRNA analogs after 24 hours of treatment.

Cows endometrial epithelial cells were stimulated with a panel of PAMPs, and it was observed that when the cells were treated with Poly I:C (HMW) with DOTAP Liposomal Reagent Transfection the production of IL-6 was higher when compared to Control group also treated with DOTAP Liposomal Reagent Transfection ($P < 0.05$) (Figure 1A). However, when epithelial cells were treated with others PAMPs as Poly I:C (LMW), ssRNA, and LPS no difference was observed neither in DOTAP positive nor Negative groups when compared to Control groups or to those cells treated with Poly I:C (HMW) from both groups ($P > 0.05$) (Figure 1A).
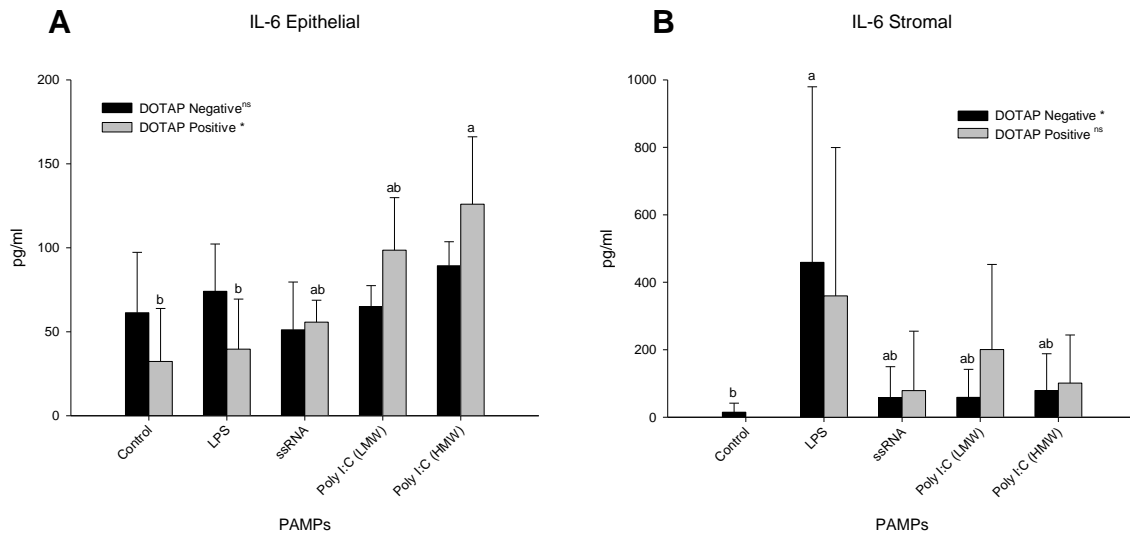
Figure 1 (A,B). ELISA IL-6 (pg/ml) results from bovine endometrial epithelial and stromal cells transfected (DOTAP positive) or not (DOTAP negative) treated with different PAMPs and a Control. The P values were calculated by a two way ANOVA and means were compared by Tukey test. Means followed by the same letter on each DOTAP Positive and Negative were not different at 5%; [ns] Non-significant; * Signifcantly diferent at 5%* (n=3 for epithelial; n=5 for stromal).

Stromal endometrial bovine cells were subjected to ELISA IL-6 after treated with the same PAMPs. It was observed that when DOTAP was not included, the cells treated with LPS produced more IL-6 when compared to Control DOTAP negative group ($P < 0.05$; Figure 1B). In the other PAMPs tested (Poly I:C (HMW), Poly I:C (LMW) and ssRNA), the groups from DOTAP positive and negative were not different in IL-6 production when compared to respective Control groups ($P > 0.05$; Figure 1B).

The chemokine IL-8 was also measured in this experiment as it is consider an important proinflammatory immune factor. Endometrial epithelial cells did not show any difference in the presence or absence with DOTAP in response to the induction of Poly I:C (HMW), Poly I:C (LMW), LPS, ssRNA when compared to Control group ($P > 0.05$; Figure 2A). Although, when DOTAP was used as a transfected reagent, in epithelial cells treated with Poly (I:C) LMW, higher concentration of IL-8 was observed when compared to those treated with Poly (I:C) from DOTAP negative group.
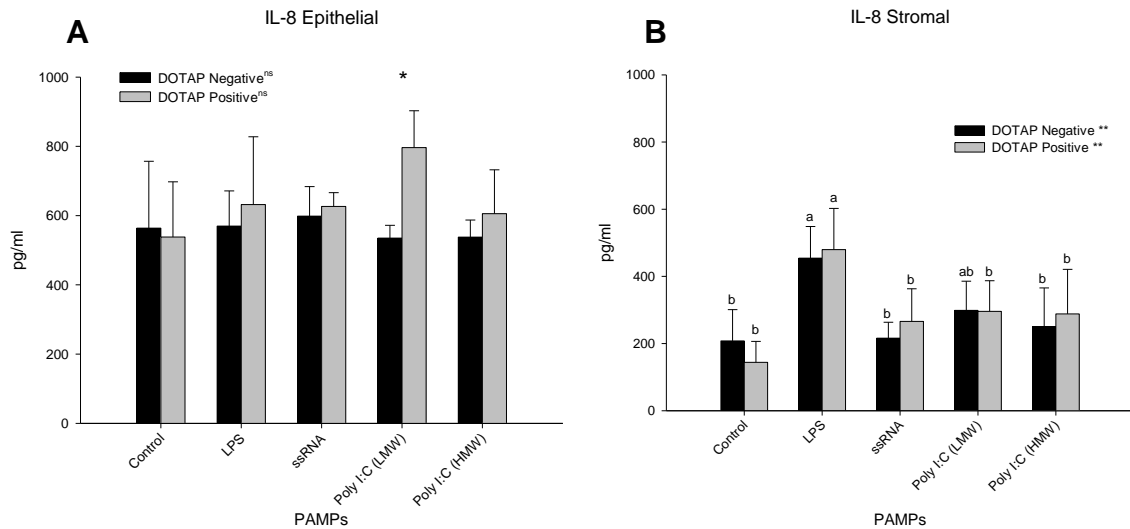
Figure 2 (A,B). ELISA IL-8 (pg/ml) results from bovine endometrial epithelial and stromal cells transfected (DOTAP positive) or not (DOTAP negative) treated with different PAMPs and a Control. The P values were calculated by a two way ANOVA and means were compared by Tukey test. Means followed by the same letter on each DOTAP Positive and Negative were not differen at 5%. [ns] Non-significant; *Significantly different when $P<0.05$; ** Significantly different when $P< 0.01$; (n=3 for epithelial; n=5 for stromal).

On the other hand, in stromal cells when LPS group was compared with Control group, higher concentration of IL-8 was observed ($P <0.01$; Figure 2B) in both treatments (DOTAP positive and DOTAP negative). The others PAMPs as Poly I:C (HMW), Poly I:C (LMW) and ssRNA were not different when compared to Control group ($P> 0.05$; Figure 2B), but when these PAMPs were compared to LPS, the production of IL-8 was higher when DOTAP was included than ssRNA, Poly (I:C) HMW, Poly (I:C) LMW from DOTAP positive group.

Experiment 2

Time evaluation for endometrial cells treated with dsRNA analog

After first experiment was performed, a time course was made to determine the rapid response and to verify if supernatants were analyzed at the appropriate time-point.

As it was shown, supernatants from experiment 1 of this study were collected after 24 hours of treatment with a panel of PAMPs. Differently, in this time-point

experiment, the authors had to check if the production of proinflammatory cytokines exists before and after 24 hours of Poly (I:C) treatment.

In those endometrial epithelial cells treated by Poly I:C (LMW) exposure, no difference at any time point was observed in production of IL-6 when the treated group was compared to the negative Control group ($P > 0.05$; Figure 3).
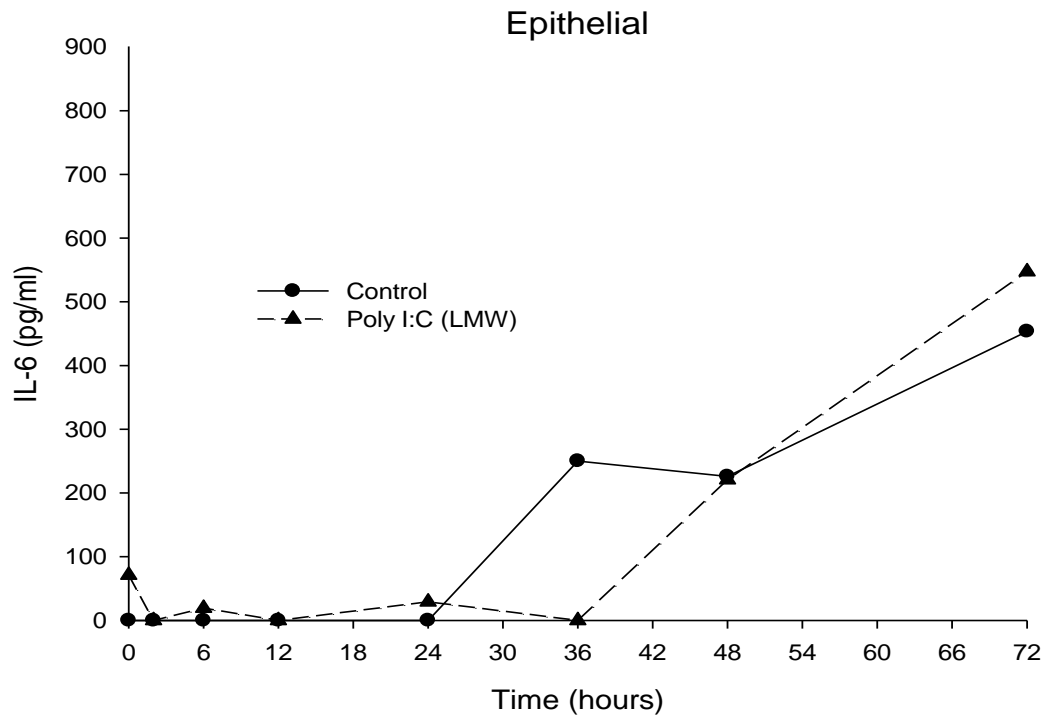
Figure 3. Cows endometrial epithelial cells cytokine production treated with the TLR3 ligand Poly I:C (LMW). Culture medium was collected following 0, 2, 6, 12, 24, 36, 48 and 72 hours of Poly I:C (LMW) stimulation and analyzed for IL-6 by ELISA. Cells were treated with Poly I:C (LMW) at a final concentration of 1µL/ml. The P values were calculated by a two way ANOVA and means were compared by Tukey test. (n=3 cows)

For stromal cells the production of IL-6 was higher at 48 hours ($P < 0.05$) and 72 hours ($P < 0.001$) after Poly I:C (LMW) treatment when compare to Control group (Figure 4).
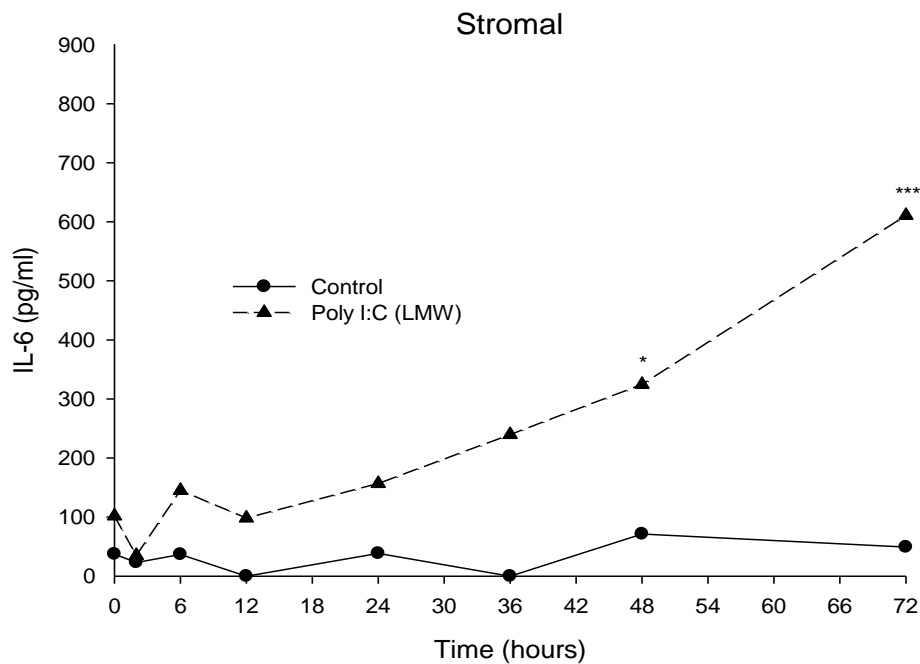
Figure 4. Cows endometrial stromal cells cytokine production treated with the TLR3 ligand Poly I:C (LMW). Culture medium was collected following 0, 2, 6, 12, 24, 36, 48 and 72 hours of Poly I:C (LMW) stimulation and analyzed for IL-6 by ELISA. Cells were treated with Poly I:C (LMW) at a final concentration of 1µL/ml. The P values were calculated by a two way ANOVA and means were compared by Tukey test. Significantly different from control: *$P < 0.05$; ***$P < 0.001$. (n=4 cows)

For the cytokine IL-8 in endometrial epithelial cells, no difference was observed at those different times treated with Poly I:C (LMW) when compared to Control group ($P > 0.05$; Figure 5).
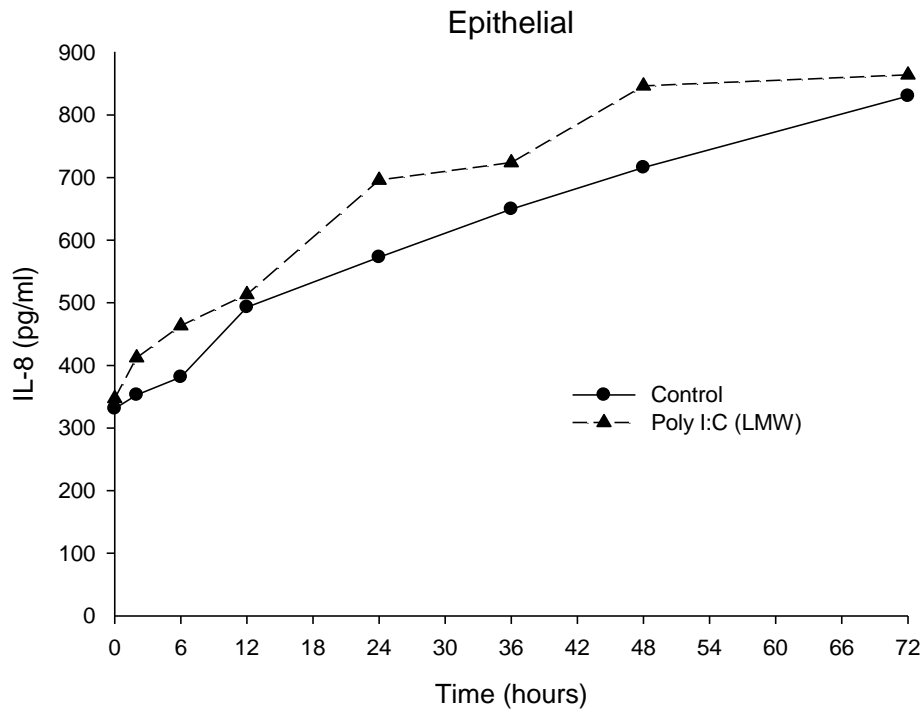
Figure 5. Cows endometrial epithelial cells cytokine production treated with the TLR3 ligand Poly I:C (LMW). Culture medium was collected following 0, 2, 6, 12, 24, 36, 48 and 72 hours of Poly I:C (LMW) stimulation and analyzed for IL-8 by ELISA. Cells were treated with Poly I:C (LMW) at a final concentration of 1μL/ml. The P values were calculated by a two way ANOVA and means were compared by Tukey test. (n= 3 cows)

The accumulation of IL-8 in stromal cells treated by Poly I:C (LMW) existed before, after and at 24 hours of treatment when compared to Control group (P< 0.05; Figure 6).

The fact was that the accumulation of IL-8 treated by Poly I:C (LMW) started to increase at 6 hours when compared to Control group (P< 0.001). This accumulation continued to be different at 12 hours (P< 0.05), 24 hours (P< 0.01); 36 hours (P < 0.01); 48 hours (P< 0.01) and at 72 hours (P< 0.001) when Poly I:C (LMW) stromal cells were compared to the cells from Control group (Figure 6).
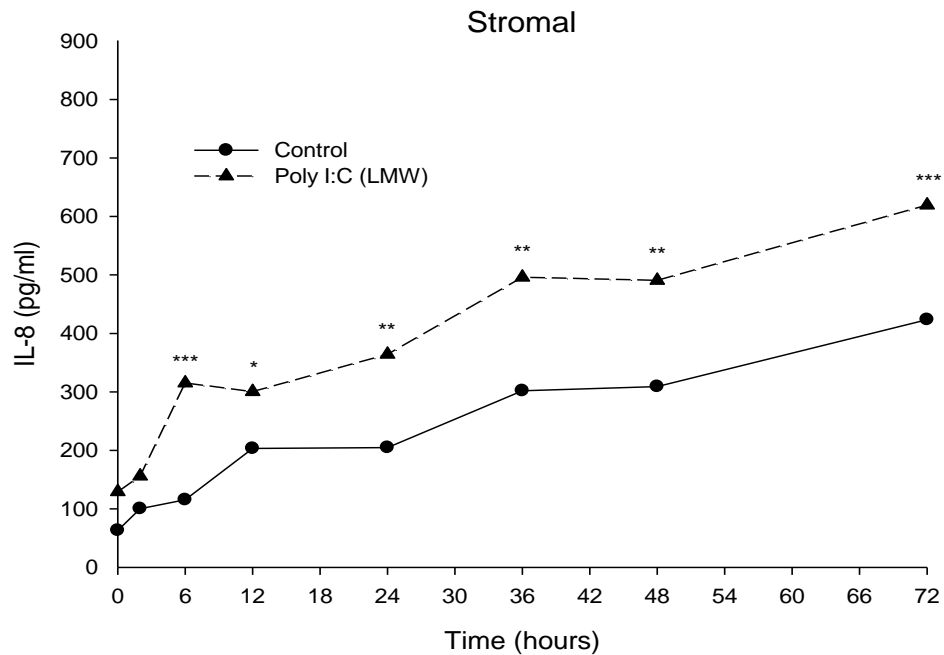
Figure 6. Cows endometrial stromal cells cytokine production treated with the TLR3 ligand Poly I:C (LMW). Culture medium was collected following 0, 2, 6, 12, 24, 36, 48 and 72 hours of Poly I:C (LMW) stimulation and analyzed for IL-8 by ELISA. Cells were treated with Poly I:C (LMW) at a final concentration of 1µL/ml. The P values were calculated by a two way ANOVA and means were compared by Tukey test. Significantly different from control: *$P< 0.05$; **$P< 0.01$; ***$P< 0.001$; (n=5 cows)

Discussion

Previous studies reported that the mammalian TLR genes are primarily expressed by antigen presenting cells, including macrophages, natural killer cells, and dendritic cells (AKIRA; TAKAEDA, 2004; McGUIRE et al., 2006). Even though, most of immune studies focused on defense by primary blood cells in this research the authors wanted to investigate how the local immunity of bovine endometrium cells play a role against viral sensing, as according to Davies et al. (2008), in the female genital tract the first line of defense against invading pathogens is considered to be the endometrium.

A panel of different PAMPs was treated directly to endometrial epithelial and stromal cells to stimulate the production of proinflammatory cytokines via innate

immune system defense. In this study LPS was chosen as a TLR4 binder, Poly I:C (LMW) and Poly I:C (HMW) as TLR3 binder, and Poly (U) as a TLR7 binder. The focus of this study was to explore specially TLR3 as it can recognize dsRNA produced in the course of a viral infection (ALEXOPOULOU et al., 2001; KARIKO et al.; 2004).

As observed, bovine endometrial epithelial cells from DOTAP positive group, accumulated IL-6 when treated with Poly I:C (HMW), compared to Control group (P< 0.05; Figure 1A), confirming that innate viral recognition depends on TLR3, in accordance with Lee et al. (2008) that revealed that bovine monocytes had IL-12 and IFN I up-regulated after treating the cells with TLR3 and TLR7 analogs, this last is a ssRNA and is this study Poly (U) was used as a ssRNA to stimulate TLR7, but no significant up regulated of cytokines was observed after endometrial cells were treated with this PAMP (P> 0.05).

Sivori et al. (2004) found that human dendritic cells responded to dsRNA by accumulating IL-12, suggesting that even in this work blood cells were not included, the authors confirmed that endometrial epithelial cells plays important role against dsRNA analogs. Some studies in human TLR3-transfected respiratory cells, concluded that the respiratory syncytial virus was able to activate cytokines in a time and dose-dependent manner compared to controls, still, this virus up regulated TLR3 in lung fibroblasts and epithelial cells (RUDD et al., 2005).

In a study developed by Schaefer et al. (2005), uterine human epithelial cells demonstrated a TLR3-mediated stimulation also treated with a 24 hours of Poly (I:C) induction, what in fact resulted in the secretion of proinflammatory cytokines and chemokines as IL-6 and IL-8 that are known to facilitate the recruitment of immune cells to the site(s) of viral infection.

A very similar study conducted by Jorgenson et al. (2005) established that stimulation of TLR3-positive cell lines and primary human endometrial epithelial cells with dsRNA leads to TLR3-dependent expression of interleukin IL-6, IL-8, IFN inducible protein 10, RANTES, and IFN-β, indicating that the cytokine profile of endometrial epithelial cells can be modified through TLR3 stimulation, what is in accordance to this research when IL-6 from DOTAP positive group was increased after dsRNA stimulation in endometrial epithelial cells (P< 0.05; Figure 1A) while, on

the other hand, no accumulation of IL-8 was observed in this study in this same line of cells after treated them with dsRNA analog (P> 0.05; Figure 2A). These studies indicated that while bovine uterine epithelial cells express TLR1–TLR7, TLR9 only selected TLR agonists affect the expression of chemokines and proinflammatory cytokines.

In the present study the authors used LPS as a TLR4 binder, however, in endometrial epithelial cells neither IL-6 (P> 0.05; Figure 1A) nor IL-8 (P> 0.05; Figure 2A) were produced after this TLR4 binder was used, what is contrast of Yunhea et al. (2013), that observed that both TLR2 and TLR4 mRNA were up-regulated when bovine endometrial epithelial cells were stimulated by LPS. These authors also concluded that the up-regulation of TLR2 was lower when bovine endometrial epithelial cells were treated with heat-killed *Escherichia coli*. On the other hand, Crook et al (1998) suggested that the corneal epithelial do not express TLR2 and TLR4 at their cell surface.

An interesting fact in the present study was that even LPS did not stimulate endometrial epithelial cells to produce IL-6 neither IL-8, a good positive response of this PAMP was observed in endometrial stromal cells in production of IL-6 (DOTAP negative group) and IL-8 (DOTAP positive and negative group) when compared to Control groups (P< 0.05; Figure 1B and P< 0.01; Figure 2B respectively), suggesting that each cell type has a distinct role in the innate immune response to different pathogens.

Similar responses in cytokines production via LPS induction was reported in in studies in bovine granulosa cells. Price, Bromfield and Sheldon (2013) related that after supernatants were collected from granulosa cells from dominant follicles treated with LPS for 24 hours, they produced cytokines IL-1 β and IL-6 and chemokine IL-8. Also, other study conducted by Bromfield and Sheldon (2011), revealed that the presence of LPS produced IL-6 and IL-8 in supernatants in a dose-dependent manner, still the releasing of this both cytokines was further increased following 48 hours of culture with each PAMP compared to 24 hours.

In the present study the authors wanted to certify if the use of DOTAP Liposomal Reagent increased the PAMPs action in each cell type, so that more cytokines were supposed to be released after the treatments including DOTAP

Liposomal Reagent, as this tool was used as a transfection reagent. The use of DOTAP in this experiment increased the production of IL-8 in epithelial cells treated with Poly (I:C) LMW when compared to those epithelial cells also treated with Poly (I:C) but without DOTAP (Figure 2A, P< 0.05).

As revealed by Skoberne, Beignon and Bhardwak (2004), transfection of dendritic cells with synthetic ssRNA induced this cell type maturation and cytokine production through activation of TLR7 and/or TLR8, this unique characteristic could explain the differences seen in proinflammatory cytokine secretion following transfection with ssRNA compared with transfection with others PAMPs, since, only ssRNA results in dendritic cell maturation which will end up in greater proinflammatory cytokine response. In this present study, only bovine endometrial epithelial and stromal cells were transfected with synthetic ssRNA, and no transfection effect in this type of PAMP was observed when compared transfect and non-transfected cells (P> 0.05; Figure 1 A,B and Figure 2 A,B).

A research conducted by Ueta et al. (2004) did not find increase in cytokine production after transfecting PAMP in human corneal epithelial cells with DOTAP Liposomal Reagent, they noticed that even when LPS was artificially delivered intracellular it did not lead to the subsequent activation of NF-kB that is a mediated signaling for the induction of IL-6 and IL-8. One explanation is due the fact that epithelial cells have long been thought to protect the integrity of mucosal surfaces mainly by acting as a physical barrier to invading pathogens, serving as a critical immunological barrier against invasion by bacteria and viruses.

Interesting findings of Crook et al. (1998) suggested that DOTAP Liposomal Transfection Reagent alone is not an efficient tool at delivering DNA to cells. However, in their studies, when cholesterol was included in the complex in increasing amounts, the transfection level increased dramatically. The DOTAP: cholesterol ratios were tested at 1:2, 1:1 and 2:1 in the presence of 5–10% serum was much more efficient than with DOTAP complexes alone.

In the second experiment of the present study, a time-point was made in both endometrial epithelial and stromal cells to check if the production of proinflammatory cytokines existed before and after 24 hours of treatment.

Endometrial epithelial cells treated by Poly I:C (LMW) exposure did not exhibit difference at any time point in accumulation IL-6 when compared to Control group ($P > 0.05$; Figure 3). Also, for IL-8 in endometrial epithelial cells, no difference was observed at those different times treated with Poly I:C (LMW) when compared to Control group ($P > 0.05$; Figure 5).

A similar fact was observed in a study conducted by Schaefer et al. (2004), where human uterine epithelial cells at a 24 hour time point treated by Poly (I:C) did not affected the release of interleukins like IL-6 and IL-8. Although, studies conducted by Jorgenson et al. (2005) revealed that when endometrial epithelial human cells were stimulated by Poly (I:C) the secretion of IL-6 and IL-8 started 4 hours after stimulation and sustain production through 72 hours, still, the peak of production of this interleukins was 18 hours after treatment.

Kalali et al. (2008), examined the effects of Poly (I:C) in human epithelial keratinocytes cells, and observed that the production of IL-8 had it maximum up-regulation at 8 hours of treatment, suggesting that the early response from this dsRNA virus appeared to be together with the active production of proinflammatory and effector chemokines and cytokines, what is considered as another important aspect of the antiviral defense strategy of human epidermal keratinocytes.

A curious fact in this present study was that stromal cells produced IL-6 at 48 hours ($P < 0.05$) and 72 hours ($P < 0.001$) after Poly I:C (LMW) was induced in this cells when compare to Control group (Figure 4).

The authors in this present study observed that the production of IL-8 after stromal cells were treated by Poly I:C (LMW) existed before, after and 24 hours of treatment when compared to Control group (Figure 7).The fact less expected was that the accumulation of IL-8 treated by Poly I:C (LMW) starts to increase at 6 hours when compared to Control group ($P < 0.001$). This accumulation continued to be different at 12 hours ($P < 0.05$), 24 hours ($P < 0.01$); 36 hours ($P < 0.01$); 48 hours ($P < 0.01$) and at 72 hours ($P < 0.001$) (Figure 6).

Lee et al. (2008) working with bovine monocyte cells used two Bovine Viral Diarrhea Viruses strains (noncytopathic and cytopathic), which are ssRNA analog, and revealed that TLR3 was significantly up-regulated at one hour after treated with noncytopathic strain, moreover, TLR3 mRNA expression was dominant in early

infection and after 24 hours stimulation the TLR3 gene expression level was significantly down-regulated, on the other hand, this was not observed in the cytopathic one. Whereas, TLR7 expression dominated at 24 hours infection with both strains.

It is unknown how TLR function *in vivo* during viral infection or what role it plays in determining the defense of the endometrium. Future studies examining the function of this class of PRRs during *in vitro* viral infections that uses virus known to infect bovine endometrium could investigate the balance between beneficial and detrimental TLR-mediated immune responses in the uterus.

Conclusions

In conclusion, beyond all the PAMPs tested in this experiment, bovine endometrial cells sensed to dsRNA analogs; endometrial epithelial cells produced cytokine IL-6 after treated by Poly I:C (HMW) (DOTAP positive); while the presence of LPS induced the production of IL-6 (DOTAP negative) and IL-8 (DOTAP positive and negative) by bovine endometrial stromal cells. Still the use of a Liposomal Transfection Reagent (DOTAP) did affect the production of cytokine in bovine uterine endometrial cells treated by a dsRNA analog.

Furthermore, in response to dsRNA virus analog Poly (I:C), bovine endometrial stromal cells produced IL-6 at 48 and 72 hours, and IL-8 at 6, 12, 24, 36, 48 and 72 hours when compared to the Control.

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CHAPTER 3 – BOVINE ENDOMETRIAL CELLS DETECT dsRNA ANALOG MEDIATED BY RIG-I CYTOSOLIC PATHWAY

BOVINE ENDOMETRIAL CELLS DETECT dsRNA ANALOG MEDIATED BY RIG-I CYTOSOLIC PATHWAY

ABSTRACT – The aims of this study were to validate by a time point scale (12, 24, 48 or 72 hours) when endometrial stromal and epithelial cells produced RIG-I and the protein p65 from the transcription factor NF-κB after both stimulated by the PAMP Poly (I:C) selected as a dsRNA virus analog. Uterine samples from post pubertal mixed-breed beef cows were used. The samples were dissected using a protocol to obtain stromal and epithelial cells. The PAMP Poly (I:C) LMW and a negative control were used as treatments. A Western Blot test for RIG-I, p65 (NF-κB), and β- actin (as a control) were developed. Proteins were collected after 12, 24, 48 and 72 hours. In response to Poly (I:C) induction, bovine endometrial stromal cells activated RIG-I at 48 hours (P <0.05) compared to Control group. On the other hand, endometrial epithelial cells were not sufficient stimulated by Poly (I:C) to activate RIG-I at any time point evaluated (P >0.05). The protein p65 from the transcription factor NF-κB after stimulation by the PAMP Poly (I:C) was activated at 12 hours by stromal cells (P <0.05) and at 24 hours by epithelial cells (P <0.05) when compared to the Control group. It can be concluded that only endometrial stromal cells activated RIG-I cytosolic pathway at 48 hours after induction of Poly (I:C). Also, the subunit p65 from the transcription factor NF-κB was released by both stromal and epithelial cells at two different moments after stimulated by Poly (I:C). The activation from p65 originated from the RIG-I or TLR3 pathway was not clear. Further investigations in endometrial bovine cells immune pathways against viral infections need to be develop.

Keywords: proteins, stromal, toll like receptors, transcription factor NF-κB

Introduction

Innate immunity and reproduction are highly conserved and key drivers during evolution from flies and worms to humans. Thus, immune system is vastly integrated with reproduction and likely in sharing common pathways, the central paradigm is that innate rather than acquired immunity is the primary defense mechanism against pathogens invasion (BROMFIELD; SHELDON, 2011).

Recent studies have identified three major classes of pattern recognition receptors: RIG-I, NLRs, and TLRs (HONDA; TAKAOK; TANIGUCHI, 2006). TLRs are transmembrane proteins that localize either on plasma membrane or inside intracellular vesicles, indicating that TLRs can detect PAMPs in extra-cellular or luminal spaces (KATO et al., 2005).

On the other hand, in the cytosol, RIG-I and MDA5, discriminate different classes of RNA viruses (KATO et al.; 2006; TAKEUCHI; AKIRA, 2008). The most important action of PAMP recognition is the sensing of non-self-nucleic acids. Viruses for example are almost exclusively sensed via their nucleic acid genomes or as a result of their replicative or transcriptional activity (TAKEUCHI; AKIRA, 2009).

According to Chiu; Macmillan and Chen (2009), like RNA, the accumulation of foreign or self DNA in the cytosol also triggers potent innate immune responses. DNA can be introduced into the cytosol of mammalian cells following infection with DNA viruses or bacteria, and the detection of cytosolic DNA is important for mounting an immune response against these pathogens.

It is likely that in most cell types DNA viruses trigger type I IFN gene transcription via TLR-independent DNA sensing mechanisms. Although types I IFNs are best studied in antiviral immunity, still a role for these cytokines in bacterial, fungal and parasitic infections has also emerged (ABLASSER et al., 2009).

Not less important, dsRNA is also a potent inducer of type I IFN synthesis and is believed to be the primary viral gene product that causes IFN production by infected cells (VILCEK; SEN, 1996). The fact is that RLHs interacts with dsRNAs through their helicase domain, and dsRNA stimulation induces their ATP catalytic activity (TAKEUCHI; AKIRA, 2008). Moreover, induction of type I IFNs and

proinflammatory cytokines requires activation of transcription factors such as NF-κB (subunit p50; p65) and IRF3 (SATO et al., 2000; TANIGUCHI; TAKAOKA, 2002). Like other members of the NF-kB family, p65 resides in the cytoplasm in an inactive form bound to inhibitory proteins. The process of cellular activation results in the nuclear translocation of subunits p50:p65 for initiating gene transcription (DALLOT et al., 2005).

The hypothesis of the present study was that after a viral infection mediated by a dsRNA analog virus, epithelial and stromal cells from bovine endometrium stimulated the cytosol pathway mediated by RIG-I activation and also transcription factors specially p65 (NF-κB).

The aims of this study were to validate by a time point scale (12, 24, 48 or 72 hours) which moment endometrial stromal and epithelial cells produced RIG-I and the protein p65 from the transcription factor NF-κB after both stimulated by the PAMP Poly (I:C) selected as a dsRNA virus analog.

Material and Methods

Pre-treating the cells

Bovine uterine samples

Uterine samples from post pubertal mixed-breed beef cows over ten-month were collected from a local slaughterhouse. Before selection, samples were examined macroscopically and only uterus with no gross evidence of genital disease or infection content were selected to be part of the experiment.

The uterine samples were kept on ice (2-8°C) for one hour until further processing in the laboratory. When the samples arrived at the University they were washed with ethanol 70% (Appendix 1).

Bovine endometrial cell culture protocol

To obtain stromal and epithelial cells the steps (1, 2, 3, and 4) were based on a cell isolation protocol previously described (FORTIER, M.A.; GUILBAULT, L.A.; GRASSO, F., 1988; CHENG, Z. et al., 2003) with some modifications adjusted by Herath et al. (2006).

1) The ipsilateral horn of the uterus with active luteal or follicular structure was opened longitudinally with sterile scissors; the endometrium was exposed and washed with Endo Wash (Appendix 2).

2) The endometrium was dissected from the uterine horn as thinly as possible in order to avoid myometrium contamination. Strips of the dissected endometrium were removed and placed directly in a pot containing 25 ml of Endo Strip Wash (Appendix 3) and then transferred into a second 60 ml container with 25 ml of HBSS (Hanks Balanced Solution, HBSS, Sigma -Aldrich Ltd, Dorset, UK). The strips were cut into 3-5 mm^3 pieces and kept in a 50 ml falcon tube containing HBSS, then placed into a water bath for 10 minutes at 37°C.

3) In cell culture hood supernatant from the tubes were removed and 25 ml of Digest Media (Appendix 4) were added. Following one hour incubation in a shaking water bath at 37°C, the cell suspension was filtered through a 40 μm cell strainer (Fisher Scientific, Loughborough, UK) into a falcon tube containing 5 ml of Stop Solution (Appendix 5). The suspension was centrifuged at 700 × g for 7 minutes at 25°C (VWR®, CT6E, Japan) and carefully supernatant was removed and a 5 ml of warm sterile water was added into the cell pellet and also tubes were vortexed.

4) Immediately, the cell water mixture was top up with Stop Solution (Appendix 5) until a total volume of 40 ml and another centrifuging (700 × g for 7 minutes at 25°C) (VWR®, CT6E, Japan) was performed. Supernatant decant was removed and the cells pellet were re-suspended in a 5 ml of fresh warm Complete Media (Appendix 6).

The cells were cultured in 75 cm^2 flasks (Greiner Bio-One, Gloucester, UK) for 18 hours to allow selective attachment of stromal cells (FORTIER, M.A.; GUILBAULT, L.A.; GRASSO, F., 1988), while the remaining was transferred to a new flask to obtain epithelial cells (KIM, J.J.; FORTIER, M.A, 1995). These cells were then incubated in a humidified atmosphere of air with 5% CO_2, and the medium changed every 48 hours.

Primary cultured cells were examined under a microscope (Axiovert 40 C, Carl Zeiss Microscopy, USA) every day to ensure sub-confluency. As both epithelial and stromal cells were present within the same culture, differentiation in splitting of epithelial and stromal cells was required 72 hours post-dissection.

The epithelial or stromal cells were detached from the flasks using a protocol detachment solution with Accutase (Millipore, Watford, UK), as follows: in each flask, 5 ml of PBS were added to wash the cells, then this PBS was discarded and 2 ml of Accutase was added to detached first stromal cells, after 3 minutes the cells were re-suspended in 13 ml of Complete Media (Appendix 6). For epithelial cells, 4 ml of Accutase were added after washed with PBS, and 7 minutes were waited to re-suspend in 8 ml of Complete Media (Appendidx 6).

For each flask 2 ml of Accutase (Millipore, Watford, UK) were added to adherent cells to suspend them. Once the cells were re-suspended, a Complete Media (Appedinx 6) was added to neutralize Accutase action. The cells were then centrifuged at 400 x g for 7 minutes (VWR®, CT6E, Japan); supernatant was rejected and the cells were re-suspended in 3 ml of Complete Media (Appendix 6). A hemocytometer FastRead102 (Immune Systems LTD, UK) was used for counting the samples in 7 µL of cell suspension.

Stromal and epithelial cells were plated at 1×10^4 cells/ml, in a 6 -well plates (TPP, Trasadingen, Switzerland), where 3 ml of re-suspended cells were added in each well respectively. Before any cellular treatment the plates were incubated during 24 hours to cellular adhesion and examined under a microscope (Axiovert 40 C, Carl Zeiss Microscopy, USA) to verify their normality and adhesion degree.

Treating the cells

Endometrial cells were exposed to dsRNA at a time course

In this study, endometrial cells were stimulated in two different groups, one containing Poly (I:C) [1 µL/ml (Poly (I:C) (LMW), Invivogen, Nottingham, UK)] diluted in Optimen Media (Appendix 9) transfected with DOTAP Liposomal Transfection Reagent (10- 20µg DOTAP cells/ml culture Media) (Roche, Brasileia, Switzerland)

and the other group (negative control) composed only with Optimen Media (Appendix 9) also with DOTAP Liposomal Transfection Reagent (10- 20μg DOTAP cells/ml culture Media) (Roche, Brasileia, Switzerland). To complete a final 3 ml/well treatment, both groups were diluted in a Complete Media (Appendix 6).

The groups were incubated with treatments at 12, 24, 48 and 72 hours before extracting proteins for future analyses using Western Blot test.

A total of 12 different bovine uterus (n=12) (in both groups) were used as follows: three uterus to evaluate RIG-I in stromal cells n=3); three to evaluate RIG-I in epithelial cells (n=3); three to evaluate p65 (NF-κB) in stromal cells (n=3) and finally other three to evaluate p65 (NF-κB) in epithelial cells (n=3).

Post-treating the cells

Proteins extraction

For proteins extraction, cells were washed with PBS (Sigma Aldrich, Dorset, UK) then, incubated with 200 μl of Phosphosafe™ Extraction Reagent (Novagen, UK) during 5 minutes. To collect the total proteins sterile cell scrapers were used, then proteins were stored in 0.5 ml eppendorfs at -20°C.

One day before using the proteins, they were defrosted overnight at 4°C then were quantified using a DC™ Protein Assay kit (Bio-Rad, Berkeley, USA) according to the manufacturer's instructions. Appropriate amounts of protein samples were then added to 6X Laemmli buffer (Appendix 10) and subsequently vortexed and heated to 95°C for 5 minutes.

Samples were loaded with 5 μl of Biotinylated Protein ladder (Cell Signalling) and molecular weight marker (Precision Plus Protein™ Dual Color Standards, Bio-Rad, Berkley, USA) in a 12% polyacrylamide pre-cast gel (mini-PROTEAN® TGX™, Bio-Rad, Berkley, USA).

Western Blots

Markers were run to the bottom of the gel at a constant 200 Volts (v) with running buffer (Appendix 11). After running the gel for 45 minutes the samples were transferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Little Chalfont, UK) in semi-dry conditions using Trans-Blot Turbo® (Bio-Rad, Berkeley, USA). The PVDF membrane was first soaked in methanol for 1 minute followed by transfer buffer (Appendix 12) where it stayed during 5 minutes. The semi-dry conditions consisted of sandwiching the gel and PVDF membrane between extra thick blot paper (Bio-Rad, Berkley, USA) wetted in transfer buffer (Appendix 12). This step lasted for 30 minutes at 250v/10Ampere (A) followed by one hour incubation in 5% bovine serum albumin (BSA) (Appendix 13) in Tris Buffer Saline Tween (TBST) 1X (Appendix 14).

Primary antibodies were appropriately diluted 1:1000 in 5% BSA (Appendix 13) in TBST 1X (Appendix 14) and incubated overnight at 4°C. On the next day, membranes were washed 3 times for 5 minutes in TBST 1X (Appendix 14) before one hour of incubation with the secondary antibodies.

After more three membrane washes the immunoreactive bands were visualized by chemiluminescence using a Chemi-Doc imaging system (Bio-Rad, Berkley, USA) and Clarity™ Western ECL Substrate (Bio-Rad, Berkley, USA). The quantification or densitometry of the bands was calculated used Quantity One software (Bio-Rad, Berkley, USA).

Proteins Target

Proteins targets for endometrial stromal and epithelial cells were: RIG-I (Rig-I D33H10 Rabbit mAb, Cell Signaling, Danvers, USA) and phosphorylated p65 (Phospho - NF-κB p65 Ser536/93H1 Rabbit mAb, Cell Signaling, Danvers, USA). In all Western Blots tests the protein β-actin (Anti-beta Actin [mAbcam 8226], (Mouse mAb, abcam®, Cambridge, UK) was used as a positive control.

As second antibody, rabbit immunoglobulin G (IgG) (Anti-rabbit IgG, ThermoFisher Scientific, Abigdon, UK) and mouse IgG (Anti-mouse IgG, ThermoFisher Scientific, Abigdon, UK) were inoculated (1:1000) in each membrane

immersed in 5% BSA (Appendix 13) according to respective target gene. Molecular weight and worked concentrations were described in Table 1.

Table 1. RIG-I, phosphorylated p65 and β-actin molecular weight, worked concentrations and second antibodies treated in bovine endometrial stromal and epithelial cells culture.

Protein target	Molecular weight (kDa)	Concentration
Primary Antibodies		
RIG-I	105 kDa	2 µg/ml
Phosphorylated p65	65 kDa	2 µg/ml
β- actin	42 kDa	1 µg/ml
Second Antibodies		
Rabbit IgG	N/A*	1 µg/ml
Mouse IgG	N/A*	1 µg/ml
Biotinylated ladder	N/A*	0.33 µg/ml

*N/A – means not applicable

Statistical analyses

The effect of the variables RIG-I and p65 (NF-κB) in both epithelial and stromal cells at different time-points (12, 24, 48, 72 hours) were analyzed using a two-way ANOVA and means were compared by Tukey test analysis [AgroEstat (BARBOSA; MALDONADO, 2015)]. Differences were considered significant when $P < 0.05$. Protein levels of RIG-I and p65 (NF-κB) were compared with the corresponding β-actin level of those treated cells.

Results

Apart from endosome signaling pathway, the authors tested a very specialized pathway localized in the cytosol to start immune defense mediated by RIG-I. Stromal cells treated with Poly (I:C) showed higher accumulation of RIG-I at 48 hours (P<

0.01) and 72 hours (P< 0.05) when compared with the Control group without any treatment (Figure 1).
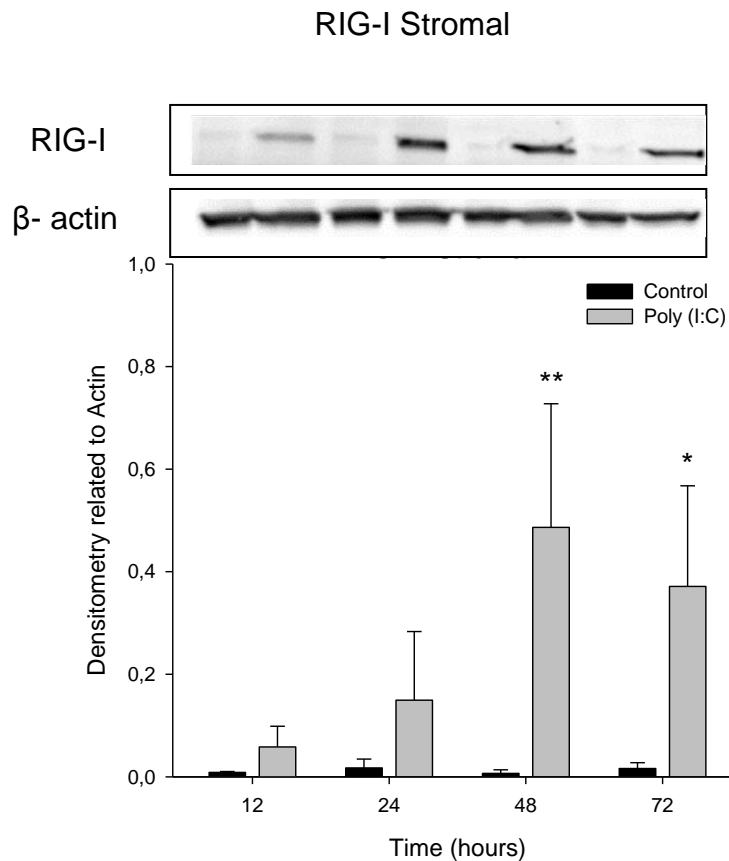
RIG-I Stromal

Figure 1. Stimulation of cultured bovine endometrial stromal cells with Poly (I:C) induced by RIG-I at 12, 24, 48 and 72 hours of treatment and by a Control group (no Poly (I:C)). Proteins levels were compared with the corresponding β-actin levels of each cell performed by a Western Blot test. Cells were treated with Poly (I:C) at a final concentration of 1µL/ml. Statistical significances were calculated using a two-way ANOVA with Tukey test. *P <0.05; **P <0.01.

On the other hand, endometrial stromal cells cultured with Poly (I:C) did not shown any RIG-I stimulation at 12 and 24 hours when compared to the control group (P> 0.05) (Figure 1).

A time-point effect could not be observed at any moment tested with Poly (I:C) in endometrial epithelial cells as the accumulation of RIG-I did not differ at any time when compared to the control group (P> 0.05; figure 2).
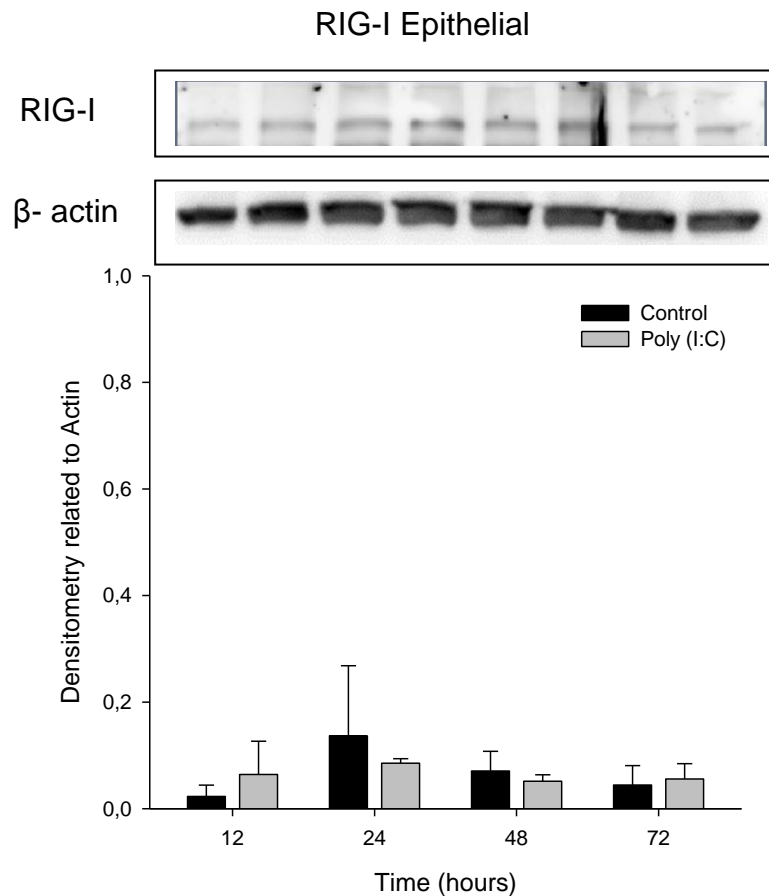
RIG-I Epithelial

Figure 2. Stimulation of cultured bovine endometrial epithelial cells with Poly (I:C) induced by RIG-I at 12, 24, 48 and 72 hours of treatment and by a Control group (no Poly (I:C)). Proteins levels were compared with the corresponding β-actin levels of each cell performed by a Western Blot test. Cells were treated with Poly (I:C) at a final concentration of 1µL/ml. Statistical significances were calculated using a two-way ANOVA with Tukey test. *P <0.05; **P <0.01.

The protein phosphorylated p65 was also evaluated in this experiment. This protein is a key product of RIG-I stimulation and also from both MyD88 dependent and independent pathways to end up in the final production of proinflammatory cytokines and type I interferon during an immune response.

Stromal cells induced with Poly (I:C), demonstrated higher production at 12 hours of p65 (NF-κB) treatment when compared to the Control group (P< 0.05) (Figure 3).
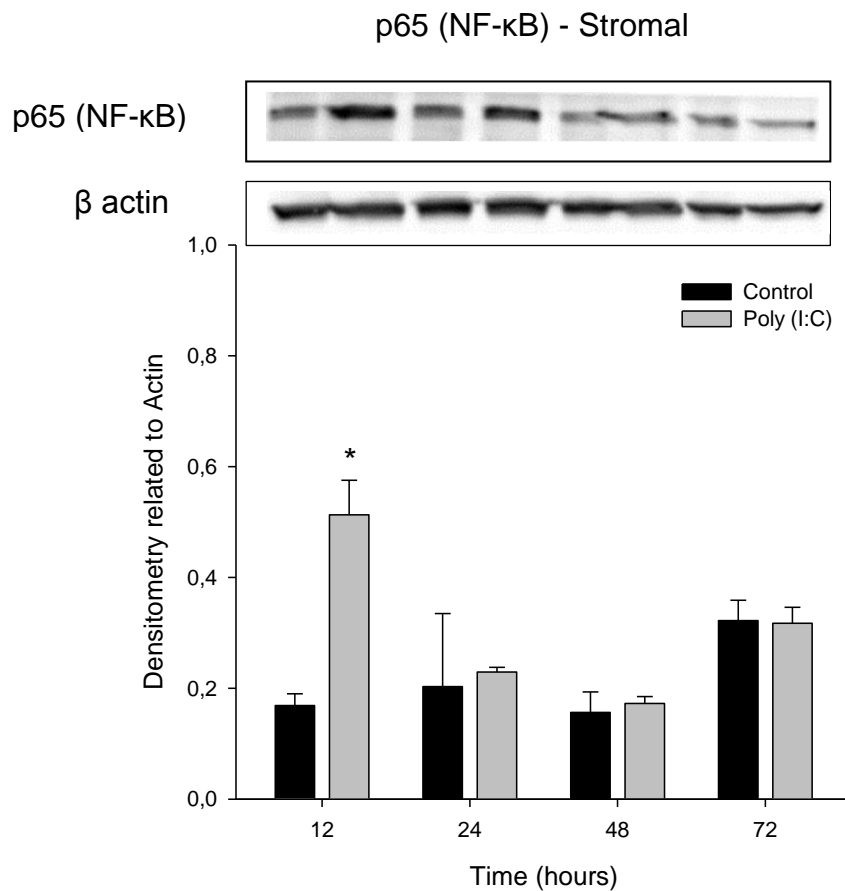
Figure 3. Stimulation of cultured bovine endometrial stromal cells with Poly (I:C) induced by p65 (NF-κB) at 12, 24, 48 and 72 hours of treatment and by a Control group (no Poly (I:C)). Proteins levels were compared with the corresponding β-actin levels of each cell performed by a Western Blot test. Cells were treated with Poly (I:C) at a final concentration of 1µL/ml. Statistical significances were calculated using a two-way ANOVA with Tukey test. *P <0.05; **P <0.01.

Different from stromal cells, epithelial endometrial cells had higher accumulation at 24 hours of p65 (NF-κB) after treated by dsRNA analog than Control group (P< 0.05). Although, at 12, 48, and 72 hours of treatment no difference was observed in p65 (NF-κB) production when compared to the group without treatment (P> 0.05) (Figure 4).

p65 (NF-κB) - Epithelial

Figure 4. Stimulation of cultured bovine endometrial epithelial cells with Poly (I:C) induced by p65 (NF-κB) at 12, 24, 48 and 72 hours of treatment and by a Control group (no Poly (I:C)). Proteins levels were compared with the corresponding β-actin levels of each cell performed by a Western Blot test. Cells were treated with Poly (I:C) at a final concentration of 1µL/ml. Statistical significances were calculated using a two-way ANOVA with Tukey test. *P <0.05; **P <0.01.

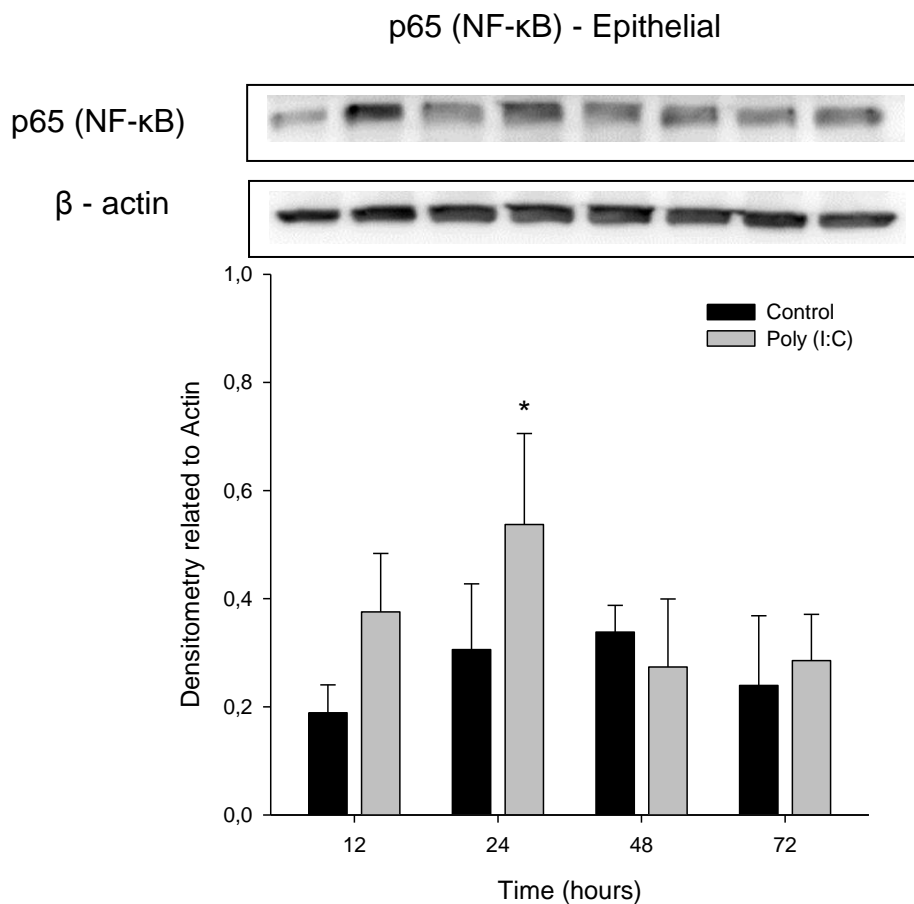It was observed that endometrial epithelial cells increased production of p65 (NF-κB) at least during 24 hours after a dsRNA induction.

Discussion

In innate immunity, functions of lymphocytes, macrophages, neutrophils, and dendritic cells have been characterized (CARROL; JANEWAY, 1999). In contrast, the

role of endometrial cells such as stromal and epithelial is less understood. Much is known that the endometrium lining the uterus of mammals is often infected by Gram-negative and Gram-positive bacteria that are able to ascend through the cervix, around the time of coitus or parturition (SHELDON et al., 2009; SHELDON; BROMFIELD, 2011). Following this idea, most studies in immunity against bacterial contamination are provided and few are known about how virus infections in endometrial cells activate immune pathways to protect the host.

Based on that, this research simulated a viral infection by using dsRNA analog and checked cytosolic pathways related to immune function. Three major classes of PRRs have already identified: TLRs, RLHs and NLRs. Since TLRs are transmembrane proteins, they are not able to detect viral components present in the cytoplasm of a cell (TAKEUCHI; AKIRA, 2008). The authors of this study choose RIG-I (part of RLHs family) to explore the cytoplasm defense as according to Yoneyama et al. (2004), RIG-I was identified as a candidate for a cytoplasmic viral RNA detector.

Immunoblotting with an antibody to the RIG-I protein was performed in this study to evaluate if there was up-regulation of this protein before and after 24 hours of treatment with a dsRNA analog [Poly (I:C)], that may suggest which moment the RLH pathway was used in endometrial stromal and epithelial cells to induce proinflammatory cytokines and type I interferons production.

Stromal cells treated with Poly (I:C) accumulated RIG-I at 48 hours (P< 0.01; Figure 1) and 72 hours (P< 0.05; Figure 1) when compared with Control group. Suggesting that RIG-I was up-regulated after 24 hours with a dsRNA viral analog. Whether RIG-I up-regulation is due to the presence of dsRNA or TLR3 signaling would have to be further investigated.

On the other hand, epithelial cells with the same treatment did not accumulate RIG-I neither before nor after 24 hours (P> 0.05; Figure 2), the authors proposed that bovine epithelial endometrial cells might not be sufficient stimulated by a dsRNA analog to start a RIG-I accumulation as stromal cells were able to, probably because epithelial cells could be stimulated at an earlier or later period. More studies should be developed with different time-point (before 12 and after 72 hours) to check RIG-I function in endometrial epithelial cells.

In a study conduct by Jorgenson et al. (2005), they explored human endometrial epithelial cells also treated with dsRNA analog. Different from this study, epithelial human cells had a good response (P< 0.05) in the production of interleukins after treated by Poly (I:C). In their study, the cells started secreting proinflammatory cytokines 4 hours after treatment and they sustain the production through 72 hours.

In contrast from this study, in normal human epithelial keratinocytes, Kalali et al. (2008) found that RIG-I/MDA5 were expressed and regulated after 36 hours of dsRNA stimulation and that the recognition of Poly (I:C) by these molecules promoted mainly IFN-β production through a TBK-1 and IRF3-dependent pathway and less activation of NF-κB-regulated genes.

A time course of RIG-I expression was explored in HeLa endometrial cells by Yuzawa et al. (2008). They observed that this protein expression reached a maximal level 4-8 hours after stimulation still, endometrial epithelial cells were intensely stained; and moderate immunoreactivity was also found in the stromal cells treated not with a dsRNA analog, but with IFN-γ that is also responsible to induce RIG-I expression in endometrial cells (IMAIZUMI et al., 2004).

According to Kato et al. (2005), in a study with mice cells, they revealed that RIG-I was critical for RNA virus-mediated IFN response in fibroblasts and dendritic cells by generating RIG-I$^{-/-}$ mice, suggesting that RIG-I is essential for activating transcription factors initially activated after a viral infection.

An important transcription factor activated by PRRs is the NF-κB, which is responsible for the induction of proinflammatory cytokines. Moreover, the main activated form of NF-κB is a heterodimer of the protein p65 subunit associated with either subunits from p50 or p52 (LI; VERMA, 2002).

Based on these ideas the authors of this study also explored the action of subunit p65 in endometrial stromal and epithelial cells treated with a dsRNA analog. An immunoblotting with an antibody of p65 (NF-κB) protein was performed to check if there was up-regulation of this protein before and after 24 hours of treatment compared to a Control group, that may suggest which moment the NF-κB was activated in endometrial stromal and epithelial cells to coordinate the expression of a wide variety of genes that control immune responses.

The authors observed that stromal cells accumulated p65 (NF-κB) at 12 hours after treated by Poly (I:C) (P <0.05; Figure 3) when compared to Control group. Although, the authors expected to have it increased only after 48 hours, as it seems to be a RIG-I product activated after a viral infection. Proposing that the up-regulation of p65 (NF-κB) started earlier than the one represented by the RIG-I and that this is a very important member in the activation of transcription factors after a viral infection.

It was expected that p65 (NF-κB) started to be produced after not before RIG-I accumulation, what in fact this earlier release could came from different sources apart from RIG-I. One hypothesis was that one cellular pathway mediated by TLR response specifically by TLR3, as it also recognizes dsRNA virus. According to Kalali et al. (2008), dsRNA can be sensed in three different pathways, one mediated by the dsRNA-dependent protein kinase R (PKR) as crucial cytosolic; the other from the TLR3 as dominant pathway of NF-κB-induced gene activation; and the one mediated by RIG-I/MDA5 as signaling molecules with preferential IRF3 activation.

In contrast with RIG-I, endometrial epithelial cells accumulated p65 (NF-κB) 24 hours (P <0.05; Figure 4), after treated with a dsRNA virus analog. What in fact seems to be a curious fact that epithelial cells accumulated only p65 (NF-κB) as no RIG-I was observed at any time point in this type of cell, suggesting that both stromal and endometrial cells sensed to activation of p65 (NF-κB) each type of cell in a particular moment.

A study conducted by Dallot et al. (2005), revealed that in human endometrial myometrial cells, LPS via TLR4 was able to stimulate the nuclear translocation of NF-kB p65 subunit. Moreover, they concluded that the nuclear translocation of NF-kB p65 was complete at 120 min. In this study the authors did not use LPS, but is noticed that this PAMP is also able to stimulate the same pathway activated by dsRNA analogs, resulting in production of immune factors. Still, according to Li et al. (2001), the stimulation of cells with dsRNA or LPS results in rapid nuclear translocation of NF-kB proteins and induction of NF-kB target gene expression.

It was explored that dsRNA can be generated during infection with virtually any kind of virus (JACOBS; LANGLAND, 1996). When NF-kB is activated by dsRNA in infected cells it allows activation of antiviral gene expression to limit further infection (HAINES; STRAUSS; GILLESPIE, 1991). Nonetheless, in a study

conducted by Li et al. (2001), in mouse embryonic fibroblasts infected with dsRNA, observed that the expression of NF-kB may be important for inhibiting apoptosis of infected cells and may thus enhance viral infection and virulence, suggesting that It will thus be interesting to determine whether NF-kB-mediated inhibition of dsRNA-induced apoptosis is a mechanism important for enhancing viral virulence.

Interestingly, is that in cows cells not only dsRNA virus can stimulate the expression of NF-kB. Baravalle et al. (2015) demonstrated that intramammary inoculation of *Panax ginseng* stimulated the activation of NF-κB in mammary epithelial and stromal cells, still, the activation was confirmed by immunohistochemical nuclear staining of the NF-κB-p65 subunit in regulating the expression of pro- and anti-inflammatory cytokines.

For future prospects, it is important to elucidate the mechanisms of activation for transcription factors in virus-infected cells. Considering the emerging interest in the immune system induction by non-viral pathogens in antigen-presenting cells, it will also be important to clarify the role of the proinflammatory cytokines in linking the innate and adaptive immune systems (TANIGUCHI; TAKAOKA, 2002).

Conclusions

In conclusion, bovine endometrial stromal cells were able to activated the RIG-I cytosolic pathway at 48 and 72 hours after induction of a dsRNA virus analog (Poly I:C) on the other hand, endometrial epithelial cells were not sufficient stimulated by dsRNA analog (Poly I:C) to activate RIG-I at any time point.

In addition, the subunit p65 from the transcription factor NF-κB was released at 12 hours by stromal cells and at 24 hours by epithelial cells after stimulated by a dsRNA virus analog (Poly I:C). While the activation from p65 originated from the RI-G or TLR3 pathway was not clear. Further investigations in endometrial bovine cells immune pathways against viral infections need to be performed.

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Appendix

Appendix 1
Ethanol 70%: (EtOH, Sigma –E7023) diluted with sterile water (Sigma;W3500).

Appendix 2
Endo Wash: Dulbecco's phosphate-buffered saline solution (Sigma-Aldrich Ltd, Dorset, UK) supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin and 2.5 µg/ml amphotericin B (all Sigma-Aldrich).

Appendix 3
Endo Strip Wash: 500 mL Hanks Balanced Solution (HBSS, Sigma -Aldrich Ltd, Dorset, UK), supplemented with 5 ml penicillin/streptomycin (Sigma -Aldrich Ltd Dorset, UK) and 5 ml of amphotericin B (Sigma -Aldrich Ltd Dorset, UK).

Appendix 4
Digest media: 50 mg trypsin* (Sigma-Aldrich), 50 mg collagenase II (Sigma-Aldrich), 100 mg BSA (Sigma-Aldrich) and 10 mg of 4% DNAse I** (Sigma-Aldrich) in 100 ml HBSS. *Current Trypsin EDTA bacth (#9B0063), use 140µl in 1860µl HBSS. So, add 1 ml of this to 99 ml of Digest media. **4% DNAse I: add 2.5 of water (Sigma-W3500) to 100 mg of DNAse I. Filter sterilize and aliquot into sterile vials and store at -20°C.

Appendix 5
Stop Solution: 500 ml of Hanks balanced salt solution (HBSS, Sigma-Aldrich, Ltd, Dorset, UK), supplemented with 10% of Fetal Bovine Serum (FBS, Biosera) heat inactivated.

Appendix 6
Complete media: 500 ml of RPMI 1640 medium with Glutamax (Gibco 61870-044) supplemented with 10% of FBS, heat inactivated (FBS, Biosera S1810) supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin and 2.5 µg/ml amphotericin B (all Sigma-Aldrich).

Appendix 7

Bovine IL-6 Elisa Protocol: Coating antibody was diluted in 1:100 in carbonate buffer by adding 110 µl coating antibody to 11 ml of carbonate buffer. A total of 100 µl was added of coating antibody to each well, the plate was covered with plate sealer and it stayed overnight at room temperature. On the other day coating antibody was aspirated and 150 µl of blocking buffer was added to each well. The plate was covered and incubated for 1 hour at room temperature. Blocking buffer was aspirate and standard with reagent diluent was prepared. The concentration of reconstituted standard was 10,000 pg/ml and it was diluted 1:2 in reagent diluted to prepare top standard (500 pg/ml). Using reagent diluent of top standard in 1:2 serial dilutions were made. Samples were added (100 µl) and plate was incubated for 1 hour at room temperature with moderating shaking. Plate was aspirated and washed three times with wash buffer (300 µl/well). Detection antibody was diluted 1:100 in reagent diluent and 100 µl was added to each well and the plate was incubated for 1 hour with moderating shaking. Plate was aspirated and washed three times with wash buffer (300 µl/well) and streptavidin –HRP was diluted in reagent diluent (1:400), then 100 µl/well was added and plate was incubated for 30 minutes at room temperature with moderate shaking. After that, plate was aspirated and washed three times with wash buffer (300 µl/well). Substrate solution was added (100 µl/well) and plate was incubated in the dark for 20 minutes at room temperature. At the end, the reaction was stopped by adding 100 µl/well of stop solution and finally the absorbance was read with a plate reader (Omega Polarstar®, BMG, Ortenberg, Germany).

D-PBS: 0.008M sodium phosphate, 0.002M potassium phosphate, 0.14M sodium chloride, 0.01M potassium chloride, pH 7.4, 0.2µm filtered.

Carbonate-bicarbonate Buffer: 0.2M sodium carbonate-bicarbonate buffer, pH 9.4, 0.2µm filtered.

Blocking Buffer: 4% BSA, 5% sucrose in D-PBS, 0.2µm filtered.

Reagent Diluent: 4% BSA in D-PBS (pH 7.4), 0.2µm filtered.

Wash Buffer: 0.05% Tween™-20 Detergent (e.g., 0.5% Thermo Scientific™ Surfact-Amps™ 20 Detergent Solution, Product No. 28320) in D-PBS, pH 7.4

Substrate: TMB (3,3',5,5'-Tetramethylbenzidine) substrate.

Stop solution: 0.18 M sulphuric acid.

Appendix 8

IL-8 Elisa Protocol: Coating antibody was diluted in 1:400 in carbonate. A total of 50 µl were added of coating antibody to each well the plate was covered with plate sealer and it stayed overnight at room temperature. On the other day coating antibody was aspirated and 150 µl of reagent diluted was added to each well. The plate was covered and incubated for 1 hour at room temperature. Reagent diluted was aspirate and standard with reagent diluent was prepared. The concentration of reconstituted standard was 1:250 and then 1:50 in reagent diluted to prepare top standard (4000 pg/ml). Using reagent diluent of top standard in 1:2 serial dilutions were made. Samples were added (50 µl) and plate was incubated for 1.5 hour at room temperature with moderating shaking. Plate was aspirated and washed three times with wash buffer (300 µl/well). Detection antibody was diluted 1:700 in reagent diluent and 100 µl was added to each well and plate was incubated for 2 hours with moderating shaking. Plate was aspirated and wash three times with wash buffer (300 µl/well) and tertiary antibody was diluted in reagent diluent (1:6000), then 50 µl/well was added and plate was incubated for 1 hour at room temperature with moderate shaking. After that, plate was aspirated and washed three times with wash buffer (300 µl/well). Substrate solution was mixed (1:1) added 50 µl/well and plate was incubated in the dark for 10 minutes at room temperature. At the end, the reaction was stopped by adding 50 µl/well of stop solution and finally the absorbance was read with a plate reader (Omega Polarstar®, BMG, Ortenberg, Germany).

D-PBS: 0.008M sodium phosphate, 0.002M potassium phosphate, 0.14M sodium chloride, 0.01M potassium chloride, pH 7.4, 0.2µm filtered.

Carbonate-bicarbonate Buffer: 0.2M sodium carbonate-bicarbonate buffer, pH 9.4, 0.2µm filtered.

Reagent Diluent: 4% Fish-skin Gelatin (G7765) in D-PBS, 0.2µm filtered.

Wash Buffer: 0.05% Tween™-20 Detergent (e.g., 0.5% Thermo Scientific™ Surfact-Amps™ 20 Detergent Solution, Product No. 28320) in D-PBS, pH 7.4

Substrate: TMB (3,3',5,5'-Tetramethylbenzidine) substrate.

Stop solution: 0.18 M sulphuric acid.

Appendix 9

Optimem Media: is an Eagle's minimum Essential Media Modificated containing insulin, transferrin, hypoxanthine, thymidine, and trace elements (Gibco, Thermo Fisher, Whaltham, USA).

Appendix 10

Laemmli buffer: 0.65 ml of TRIS Solution (pH:6.8); 2 ml 10% SDS (25 g Sodium dodecyl sulphate in 250 ml distilled water); 1 ml of glycerol in 6.375 ddH2O.

Appendix 11

Running Buffer: 25mM TRIS solution, 192mM Glycine, 0.1% Sodium dodecyl sulphate (pH 8.3) diluted in 1 liter of distilled water.

Appendix 12

Transfer Buffer: 200 ml/L methanol, 100 ml/L 10x transfer buffer [25 mM TRIS, 192 mM Glycine pH 8.3, 10% (w/v) Sodium dodecyl sulphate], and 700 ml/L of distilled water.

Appendix 13

5% BSA (Blocking Buffer): 12.5g BSA in 250 ml of TBS/T.

Appendix 14

TBST 1X: Tris buffered saline, (20 mM Tris, 500 mM NaCl) with 0.1% (w/v) Tween.