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Effect of sub-clinical endometritis on miRNAs expression profile of endometrial and oviductal epithelium and its implication of early embryonic development

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Dedicated to my beloved Mother, my lovely sisters Nihal & Basma, and my lovely brothers Mohammed & Abdel Rahman

In memory of my father

Effect of sub-clinical endometritis on miRNAs expression profile of endometrial and oviductal epithelium and its implication of early embryonic development

Understanding the molecular mechanisms associated with regulation of inflammatory responses during female genital tract infection is one step forward for development of diagnostic and therapeutic strategies in bovine reproduction. Therefore, the aim of this thesis was to investigate post-transcriptional regulation of inflammatory immune response genes during *LPS* treatment in bovine oviductal, endometrial cells and embryos. For this, two studies were conducted. In the first study, the mRNA expression analysis of inflammatory response genes (TNFα & IL1β) was performed in primary bovine oviductal cell culture and co-cultured blastocysts after minimum dose of *LPS* treatment *in vitro*. In the second study, the alterations of *let-7* miRNAs expression were addressed in primary bovine endometrial cells after *LPS* challenge (with clinical dose of 3.0 µg/ml or a sub-clinical dose of 0.5 µg/ml), as well as functional study of *let-7* miRNAs using gain and loss of function. While *LPS* treatment resulted in significantly up-regulation of pro-inflammatory cytokines (TNFα & IL1β) and stress response genes (SOD & CAT) in bovine oviductal cell and co-cultured blastocysts, the expression level of essential elements like OVGP1 and IGF2 was reduced in the challenged group compared to the untreated control. Interestingly, the over-expression of these pro-inflammatory cytokines in bovine oviductal cells was associated with aberrant expression of their potential regulatory miRNAs (miR-155, miR-146a, miR-223, miR-21, miR-16 and miR-215). Furthermore, blastocysts co-cultured with oviductal cells in the presence of *LPS* showed reduced mitochondrial distribution pattern, higher ROS and apoptotic cells. A minimum dose of *LPS* challenge resulted in changes in relative abundance of *let-7* miRNAs in a time dependent-manner, where the peak expression of *let-7a* reached at 6h, while *let-7e*, *let-f* and *let-7i* peaked at 24h post treatment. Overexpression of *let-7a* inhibited pro-inflammatory cytokines (TNFα & IL6) on mRNAs as well as protein levels, while the *let-7a* inhibitor (antagonist) resulted in an increase in the expression of the same genes. The mRNAs and protein levels of TNFα, IL6 have shown a clear suppression upon transfection with *let-7f* inhibitor. In conclusion, infections in endometrial or oviductal microenvironment resulted in aberrant expression of genes and miRNAs which support the role of regulatory *let-7* miRNAs during bovine uterine infection by fine-tuning inflammatory cytokines.

Einfluss der subklinischen Endometritis auf miRNA-Expressionsprofile im Epithel von Endometrium und Eileiter sowie die Konsequenzen für die embryonale Frühentwicklung

Die Klärung von molekularen Mechanismen, die die Regulation einer Entzündungsreaktion während Infektionen des weiblichen Genitaltraktes begleiten, ist entscheidend für die Entwicklung von diagnostischen und therapeutischen Behandlungsmethoden in der bovinen Reproduktion. Daher war das Ziel dieser Studie die post-transkriptionale Regulation von Genen der entzündungsbedingten Immunreaktionen zu untersuchen.

Dazu wurden innerhalb von zwei Forschungsansätzen bovine Zellen des Eileiters und des Endometriums sowie präimplantative Embryonen mit LPS behandelt. In der ersten Studie wurde die mRNA Expression von Genen der Entzündungsreaktion (TNFα & IL1β) in kultivierten primären bovinen Eileiterzellen und co-kultivierten Blastozysten *in vitro* gemessen, nachdem diese mit einer minimalen Menge an LPS behandelt wurden. In einem zweiten Experiment wurden die Veränderungen der *let-7* miRNAs Expression untersucht. Zu diesem Zweck wurden primäre bovine Zellen des Endometriums mit LPS behandelt (klinische Dosis (3.0 µg/ml) oder subklinische Dosis (0.5 µg/ml)) sowie eine funktionale Studie durchgeführt, um mögliche Funktionsnutzen und -verluste durch *let-7* miRNAs zu untersuchen.

Die Behandlung mit LPS führte zur signifikanten Hochregulierung von pro-inflammatorischen Zytokinen (TNFα & IL1β) und Genen der Stressantwort (SOD & CAT) in bovinen Eileiterzellen und co-kultivierten Blastozysten. Die Expressionsniveaus von essentiellen Genen wie OVGP1 und IGF2 waren im Vergleich zur unbehandelten Kontrolle reduziert. Interessanterweise ging die Überexpression dieser pro-inflammatorischen Zytokine in den Eileiterzellen mit einer abweichenden Expression ihrer potentiell regulierenden miRNAs (miR-155, miR-146a, miR-223, miR-21, miR-16 und miR-215) einher. Darüber hinaus zeigten die Blastozysten, die mit den Eileiterzellen co-kultiviert wurden, bei der Behandlung mit LPS veränderte Muster der mitochondrialen Verteilung sowie erhöhte Anteile an ROS und apoptotischen Zellen. Der Einsatz einer minimalen Menge an LPS führte zu einer zeitabhängigen Veränderung der relativen Abundanz der *let-7* miRNAs, wobei die höchste Expression von *let-7a* nach 6h erreicht war, während *let-7e*, *let-f* und *let-7i* eine maximale Abundanz 24h nach der Behandlung zeigten. Die Überexpression von *let-7a* hemmte das mRNA- sowie das Proteinniveau der pro-inflammatorischen Zytokine, wohingegen der *let-7a* Inhibitor (Antagonist) zu einer Steigerung der Expression der gleichen Gene führte. Die mRNA- und Proteinniveaus von TNFα und IL6 zeigten eine deutliche Suppression nach der Transfektion mit dem *let-7f* Inhibitor.

Abschließend lässt sich folgern, dass eine Infektion im Endometrium oder im Eileiter zu Veränderungen der Expressionen von Genen und miRNAs führte, welches die regulative Bedeutung von *let-7* miRNAs auf inflammatorische Zytokine während einer Infektion des bovinen Uterus unterstützt.

Contents

List of abbreviations

3´UTR	:	Three prime untranslated region
AA	:	Arachidonic acid
ACTB	:	β-actin
Ago	:	Argonaute protein
ARE	:	Adenylate-uridylate-rich elements
ARE-BPs	:	ARE-binding proteins
BOEC	:	bovine oviductal epithelial cell culture
C. elegans	:	*Caenorhabditis elegans*
CASP3	:	Caspase 3, poptosis-related cysteine peptidase
CD45	:	Pan-leukocyte marker
cDNA	:	Complementray DNA
CL	:	Corpus luteum
CLSM	:	Confocal laser scanning microscope
CO_2	:	Carbondioxide
COCs	:	Cumulus oocyte complexes
COX 1&2	:	Cyclooxygenases
Ct	:	Threshold cycle
DAPI	:	4',6-Diamidin-2'-phenylindoldihydrochlorid
DEGs	:	Differential expressed genes
DNA	:	Deoxyribonucleic acid
DNase	:	Deoxyribonuclease
dNTP	:	Deoxyribonucleoside triphosphate
DTT	:	Dithiothreitol
E. coli	:	*Escherichia coli*
EGF	:	Epidermal growth factor
ELISA	:	Enzyme-linked immunosorbent assay
FBS	:	Fetal bovine serum
FIG	:	Figure
FITC	:	Fluoresceinisothiocyanat
FSH	:	Follicle stimulating hormone
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase

GPX	:	Glutathione peroxidase
HRP	:	Horseradish peroxidase
ICC	:	Immunocytochemistry
ICM	:	Inner cell mass
IGF2	:	Insulin-like growth factor 2
IGFs	:	Insulin-like growth factor
IL6	:	Interleukin 6
IL8	:	Interleukin 8
INOS	:	Nitric oxide synthase, inducible
IPA	:	Ingenuity pathway analysis
IVF	:	In vitro fertilization
IVM	:	In vitro maturationRryanodine-receptor gene
IVP	:	In vitro production
kDa	:	Kilo Dalton
Let-7	:	*Lethal-7*
LPS	:	*Lipopolysaccharides*
MFE	:	Minimal free energy
MiRNAs	:	MicroRNAs
mRNA	:	Messenger RNA
NFκB	:	Nuclear factor-kappa B
OD	:	Optical density
OVGP1	:	Oviductal glycoprotein 1
PBL	:	Peripheral blood lymphocytes
PBS	:	Phosphate-buffered saline
PBS^{-}	:	Phosphate buffer saline Ca^{2+}/Mg^{2+} free
PCR	:	Polymerase chain reaction
PDGF	:	Platelet-derived growth factor
PGFS	:	Prostaglandin F synthase
PGs	:	Prostaglandins
PID	:	Pelvic inflammatory disease
PLA2	:	Phospholipase A2
pre-miRNA	:	Precursor miRNA
pri-miRNA	:	Primary transcript

PTGES	:	Prostaglandin E synthase
qRT-PCR	:	Quantitative real-time polymerase chain reaction
RISC	:	RNA-induced silencing complex
RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species
rpm	:	Revoulution per minute
RT-PCR	:	Reverse transcriptase polymerase chain reaction
S.D.	:	Standard deviation
SDPR	:	Serum deprivation response
SDS-PAGE	:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	:	Sub-clinical endometritis
SOD	:	Superoxide dismutase
SOF	:	Synthetic oviductal fluid
TGFB1I1	:	Transforming growth factor beta 1 induced transcript 1
TGFβ	:	Transforming growth factor-β
TGFβ1	:	Transforming growth factor, beta 1
Th1	:	Pro-inflammatory mediators
Th2	:	Anti-inflammatory mediators
TLRs	:	Toll-like receptors
TNF	:	Tumor necrosis factor
TRAF6	:	TNF receptor associated factor 6
TTP	:	Tristetraprolin
TUNEL	:	Terminal deoxinucleotil transferase uracil nick end labeling
V/V	:	Volume per volume
VEGF	:	Vascular endothelial growth factor
18S	:	18S ribosomal RNA

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2.1 Chapter 2

1.1 Introduction

Fertility is a multi-factorial trait and its deterioration has been caused by a network of genetic, environmental and managerial factors and their complex interactions make it difficult to determine the exact reason for this decline. The key for an optimal fertility in females is a healthy environment of reproductive tract. A healthy and a sterile reproductive milieu is the basis for high submission and conception rates and early embryonic development (Kaufmann et al. 2009, Kaufmann 2010, Walsh et al. 2011). Inflammatory process of uterine tissue is one of the most common reproductive disorders in bovine, which could hamper the implantation and development of the embryo. Innate immune responses to pathogens are the driving force for bacterial removal, regulation of inflammation, and keeping of uterine homeostasis (Herath et al. 2006, Herath et al. 2009, Sheldon et al. 2002).

Based on fertilization rates of 90%, embryonic and fetal mortality rates are approximately 40% and 56%, while calving rates are approximately 55% and 40% in moderate and high yielding dairy cows, respectively (Diskin and Morris 2008). The very early embryo remains in the oviduct for 4–5 days after ovulation before travelling into the uterus. It was found that the causes of very early embryo mortality (0–7 days) focus on the early embryos inability to develop as a consequence of poor oocyte quality or an inadequate oviduct/uterine environment (Walsh et al. 2011). In addition, uterine function is compromised by the presence of pathogenic bacteria which can cause embryonic death and abortion (Sheldon et al. 2006). Advances in knowledge about infection and immunity in the female genital tract should be exploited to develop new treatments and prevention strategies for female infertility.

However, the expression of molecules such as cytokines, proteases, chemokines, growth factors and extracellular matrix in female genital tract must be optimal, precise, and synchronized to generate a timely progression of their signalling pathways (Pan and Chegini 2008). It was demonstrated that the alterations in the expression of these mediators seem to be responsible for inappropriate tissue repairing, defect of implantation process, and other uterine or oviductal abnormalities (Abal et al. 2006, Makker and Singh 2006, Prat et al. 2007). Moreover, resolution of the endometrial inflammatory response is necessary for reparative mechanisms to take place (Jabbour et al. 2006).

MicroRNAs (miRNAs) may function at multiple hierarchical levels of gene regulatory networks, from targeting hundreds of effector genes incompatible with the differentiated state controlling the levels of global regulators of transcription and alternative pre-mRNA splicing. This multistage regulation can enable individual miRNA to profoundly affect the gene expression program of differentiated cells (Makeyev and Maniatis 2008). The product of some of these genes acting in an autocrine/paracrine and interactive manners are known to regulate many events such as inflammatory immune responses, cellular differentiation, apoptosis, and tissue remodeling. The fine-tuning regulation of the expression of these genes is fundamental in directing these processes for normal female fertility (Pan and Chegini 2008).

1.1.2 Endometritis

The mammalian uterus is usually a sterile environment but it is readily contaminated with bacteria during coitus or parturition (Herath et al. 2009). The prevalence of uterine infections range from pelvic inflammatory disease (PID) to chronic endometritis and infertility, also previous studies showed that prevalence of sub-clinical endometritis (SE) in the range between 12–94% (Gilbert et al. 2005, Hammon et al. 2006, Kasimanickam et al. 2004, Kasimanickam et al. 2005). In bovine, bacterial contamination of the uterine lumen is ubiquitous after parturition, and up to 40% of animals develop PID and 20% have endometritis. Infection of the endometrium with *Escherichia coli* (*E. coli*) precedes infection by other pathogens, and is associated with the severity of PID and the impact on female fertility (Sheldon et al. 2002, Williams et al. 2007).

Sub-clinical endometritis is characterized by inflammation of the endometrium, which associated with a remarkable reduction in reproductive performance, and the absence of signs of clinical endometritis (Sheldon et al. 2009a, Sheldon et al. 2009b). Sub-clinical disease is diagnosed by measuring the proportion of neutrophils present in a sample collected by flushing the uterine lumen, using a cytobrush or measuring uterine fluid by transrectal ultrasonography (Gilbert et al. 2005). A cow with sub-clinical endometritis had >18% neutrophils in uterine cytology samples collected 20–33 days postpartum, or >10% neutrophils at 34–47 days postpartum (Sheldon et al. 2009b), and the incidence is 37–74% (Gilbert et al. 2005, Lincke et al. 2007). Animals with sub-

clinical disease also have more days open, take longer to conceive, have lower conception rates and are culled more than normal animals. Typical conception rates are half that of normal animals (Kasimanickam et al. 2004). The endometrial cells appear to have a key role in innate immune defence of the female genital tract and that also lead to modulation of endocrine function and persistence of neutrophils in the endometrium in the absence of bacteria, which is the primary characteristic of sub-clinical endometritis (Gilbert et al. 2005, Kasimanickam et al. 2004, Sheldon et al. 2009b). The cost of uterine disease and the associated infertility was estimated to be $650 million per annum in the United States (Cronin et al. 2012, Sheldon et al. 2009a)

Bacterial infections of the endometrium provoke a marked and persistent inflammatory response, with secretion of chemokines & cytokines, an influx of neutrophils, and disruption of the integrity of the epithelium (Sheldon et al. 2006, Subandrio et al. 2000, Zerbe et al. 2003). Subsequently, these changes in uterine tissue perturb its function and influence the transport of spermatozoa and implantation of the zygote (Achache and Revel 2006). In previous study by (Hill and Gilbert 2008), they observed that the exposure to an inflamed environment has been reduced the number of trophectoderm cells around the embryo.

Resolution of the endometrial inflammatory response is necessary for reparative mechanisms to take place (Pan and Chegini 2008), where the endometrial regenerative process is initiated by an inflammatory reaction followed by a rapid cell proliferation, angiogenesis, differentiation and tissue remodelling (Girling and Rogers 2005, Jabbour et al. 2006, Makker and Singh 2006). The efficiency of the uterus to resolve an infection depends on its ability to recognize and respond to microbial ligands (Foldi et al. 2006, Williams et al. 2005). After infection, an immune response is initiated by cell surface receptors as toll-like receptors (TLRs) (Chapwanya et al. 2009), which detect pathogens and initiate signaling pathways that activate molecules as nuclear factor-kappa B (NFκB) (Cronin et al. 2012, O'Neill 2006). NFκB is a crucial transcription factor that controls the release of a pro-inflammatory immune response through activation of genes encoding cytokines like interleukin 6 (IL6), tumor necrosis factor (TNF) and chemokines as interleukin 8 (IL8), (Bonizzi and Karin 2004).

1.1.3 Oviduct

Bovine oviduct also referred to as bovine fallopian tube, which is a highly specialized organ and it assumes one of the most fundamental roles during different stages of female reproductivity (Abe and Hoshi 1997, Rottmayer et al. 2006). In mammals, oviduct function is more than a simple conduit for the transport of ova, spermatozoa or developing embryos between ovary and uterus (Georgiou et al. 2005). The functional oviduct is an active organ that maintains and modulates a dynamic fluid-filled milieu, whereas oviductal fluid provides a suitable environment for ovum, sperm and early embryonic development (Aviles et al. 2010, Leese et al. 2001). The epithelium of the oviduct consists of ciliated and secretory cells (Yaniz et al. 2000). The ciliated cells play an important role in the transport of oocytes and embryos, while the secretory cells produce and release specific secretory materials (Abe 1996, Hagiwara et al. 1997, Odor and Blandau 1973). Together with a selective transudate of serum (Hugentobler et al. 2008, Leese 1988), these secretions form the oviductal fluid. Some of these secretory products associate with the gametes and/or the embryo and may play important roles in embryonic development and sperm function (Hunter, 1994; Malette et al., 1995; Gandolfi, 1995). It has been reported that *in vitro* development of embryos is improved by co-culture with oviductal cells (Nagao et al. 1994, Tavares LM 2011). Therefore, it seems likely that oviductal epithelial cells are deeply involved in the reproductive and developmental events that occur in the oviduct.

In bovine species, the fertilization process occurs in ampulla, which is the second portion of the fallopian tube (Ellington 1991, Rizos et al. 2010). The first cleavage to the 2-cell stage occurs approximately 1 to 2 days after fertilization and between days 3–4 after fertilization (8- to 16-cell stage), the embryo moves from the oviduct to the uterus (Hunter 2012). Between days 5–6, the embryo reaches the 16- to 32-cell stage and the cells begin to form intimate junctions (Boni et al. 1999), forming a compact ball of cells termed the morula. During the early postfertilization period, several vital developmental events happen in the embryo including; the first cleavage division, the embryonic genome activation, and morula compaction. These events are started in the oviduct, and the absence of assistance from the oviduct may compromise the developmental ability of embryo in bovine (Ellington 1991, Lechniak et al. 2008, Rizos et al. 2010). Furthermore, gametes as well as the early embryo undergo epigenetic

changes in oviduct. These epigenetic changes involve histone modifications and DNA methylation, which play a crucial role in the regulation of both nonimprinted and imprinted genes. Therefore, environmental and metabolic stimuli from the oviduct could have a remarkable impact on further embryonic development and prenatal and postnatal phenotype (Ulbrich et al. 2010, Wrenzycki et al. 2005). It was indicated that oviduct secrets a plethora of essential elements such as growth factors [insulin-like growth factor (IGFs), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF)], (Lei and Rao 1992, Thibodeaux et al. 1993, Winger et al. 1997, Xia et al. 1996). In addition, other molecules that have precise role in the regulation of oviduct contractions include endothelin (Rosselli et al. 1994), nitric oxide synthase (Rosselli et al. 1996), vascular endothelial growth factor (VEGF), (Wijayagunawardane et al. 2005) and prostaglandins (Wijayagunawardane et al. 1998), which have been identified as important regulatory factors of oviductal motility and embryo transport. Moreover, it was demonstrated that oviduct expressed genes encoding antioxidant enzymes as superoxide dismutase (SOD) and glutathione peroxidase (GPX), (Harvey et al. 1995). Expressions of these genes prepare an optimal maternal environment as well as reduce the deleterious effect of free oxygen radicals as reactive oxygen species (ROS), and subsequently support early embryonic development (Lapointe and Bilodeau 2003, Ulbrich et al. 2010).

1.1.4 The role of immunomediators and other molecules in successful pregnancy

It is generally accepted that successful pregnancy requires a delicate balance between pro- and anti-inflammatory orchestra (Chen et al. 2012, Singh et al. 2011). This process is highly fascinating and sophisticated, which result from numerous interactions among the receptive uterus, blastocyst and immunohormonal mediators (Saito 2000, Thellin et al. 2000).

Cytokines are multifunctional water-soluble, signaling proteins and glycoproteins (Holloway et al. 2002), where their biological actions are mediated through specific cell surface receptors and work as potent intercellular signals regulating functions of female reproduction as well as embryo–maternal interactions (Singh et al. 2011). These cytokines are not only secreted by the embryo, but also by peripheral blood lymphocytes (PBL), macrophages, oviductal, and endometrial cells (Holloway et al. 2002, Schafer-Somi 2003, Singh et al. 2011). Cytokine expression may

be regulated by other cytokines and they may stimulate other cells as well (Schafer-Somi 2003). Moreover, cytokines could be directly/indirectly regulated by microRNAs (Asirvatham et al. 2009, McCoy 2011). In mammals, it was reported that the aberrant regulation of cytokines expression and their signaling resulting in an absolute or partial failure of implantation and abnormal placental formation (Guzeloglu-Kayisli et al. 2009). However, inflammation is necessary for successful implantation, but excessive inflammation can cause embryo resorption, so adequate balance for Th1/Th2 immunity is necessary for successful pregnancy (Saito et al. 2010). Furthermore, it was observed that Th1-type immunity in recurrent spontaneous abortion and pregnancy disorders (Piccinni et al. 1998, Raghupathy 1997). However, Th2-dominant immunity was also observed in recurrent abortion cases (Bates et al. 2002, Chaouat et al. 2003). Thus, Th1/Th2 paradigm is now enough to address mechanisms behind early embryonic deaths and unsuccessful pregnancy (Saito et al. 2010).

The term growth factor like IGFs, EGF, and transforming growth factor-β (TGFβ), stands for a family of secreted signaling molecules have ability to induce mammalian cells proliferation and differentiation (Singh et al. 2011). These factors bind to specific cell surface receptors and upon ligand binding, the receptors further initiate signaling cascade (Dey et al. 2004, Jones et al. 2006, Rechler and Nissley 1985).

Similarly, prostaglandins (PGs) play a crucial role in female reproduction (Poyser 1995, Swangchan-Uthai et al. 2012); they are synthesized from membrane phospholipids that release arachidonic acid (AA) via phospholipase A2 (PLA2) action (Godkin et al. 2008). Prostaglandin F synthase (PGFS) and prostaglandin E synthase (PTGES) produce $PGF_{2\alpha}$ and PGE_2, respectively (Smith et al. 1996). Both uterine epithelial and stromal fibroblast cells produce both PGE_2 and $PGF_{2\alpha}$ (Asselin et al. 1996). Cyclooxygenases (COX-1 and COX-2) are important enzymes, which are responsible for the synthesis of various PGs. The expression pattern of both Cox-1 & Cox-2 genes in mouse uterus during preimplantation stage reveals the crucial role of PGs in implantation and subsequently pregnancy process. Recently, it was reported that steroid hormones regulate the expression of COX-2 (St-Louis et al. 2010).

1.1.5 MiRNAs

MiRNAs term were formally introduced in 2001 (Lagos-Quintana et al. 2001, Lau et al. 2001). However, only in 1993 Lee et al. discovered that the *lin-14* gene is regulated by a short 22-nt RNA encoded by the *lin-4* gene in *C. elegans*. Hinske and colleagues found that 42.6% miRNAs are located within intronic regions, 5.3% within exonic regions and the remaining 52.1% are intergenic miRNAs by mapping miRNA genomic coordinates to genomic position of all annotated human genes. Intriguingly, genomic distribution of miRNAs in other surveyed species with well-annotated protein-coding genes was show to be similar to the one in humans (Hinske et al. 2010). Animal miRNAs are phylogenetically conserved (~55% of *C. elegans* miRNAs have homologs in humans), and mammalian miRNA genes also have multiple isoforms (paralogs), which are probably generated by duplication (the human *let-7* gene accounts for 8 different isoforms distributed across 11 genomic loci) (Hertel et al. 2012). On the basis of miRNAs genomic organization, miRNAs can be divided in two main classes; intergenic miRNAs are independent transcription units, while intragenic miRNAs are located inside a host gene and transcribed in the same orientation (Lee et al. 2004). Intergenic miRNA genes can be mono-, bi-, or polycistronic (Di Leva et al. 2014, Lee et al. 2002). Intragenic miRNAs are generally located in the introns of their host genes, but a small percentage is located in exons and the host genes can be coding or noncoding (Di Leva et al. 2014, Rodriguez et al. 2004). A new class of intronic miRNAs (called 'mirtrons') has been derived from short hairpin introns via a non-classical miRNA pathway and constitutes 5-10% of miRNA genes in invertebrates and vertebrates (Berezikov et al. 2007, Glazov et al. 2008). The discovery of miRNAs and their target mRNAs has revealed novel mechanisms regulating gene expression beyond the central dogma. MiRNAs belong to noncoding small RNAs, which are not translated into proteins (Sun et al. 2010). The biogenesis of miRNA is a multistep process that can be divided into transcription, nuclear cropping, export to the cytoplasm, and cytoplasmic dicing (Fatima et al. 2014, Kim 2005).

Genes encoding for miRNAs are transcribed from DNA to produce a primary transcript (pri-miRNA) that is processed into a shorter precursor miRNA (pre-miRNA), which is further processed into a mature, single-stranded miRNA that is 18 to 24 nucleotides long (Axtell et al. 2011, Sun et al. 2010). If the binding of the mature

miRNA to the three prime untranslated region (3´UTR) "seed sequence" exhibits perfect base pairing, the mRNA transcript is degraded and mRNA translation does not occur (Jackson and Standart 2007). When base pair binding homology between miRNA and 3´UTR of the mRNA is imperfect, mRNA translation is inhibited (Saxena et al. 2003). While the majority of the literature supports the notion that miRNAs inhibit translation, there is some evidence that miRNAs can actually enhance translation through alterations in the Argo component of the RNA-induced silencing complex (RISC), (Bhattacharyya et al. 2006, Vasudevan and Steitz 2007). Although miRNAs appear to primarily regulate translation in an inhibitory fashion, they also may enhance translation in certain biological scenarios such as starvation conditions or cellular stress (Nothnick 2012, Raychaudhuri 2012).

For animal miRNA-target prediction, most computational approaches depend on the algorithm match between seed-region and 3´UTR, which recognizes the high-complementarity region in the 3´UTR to score among different web-sites and enumerate putative gene targets (Krek et al. 2005, Sun et al. 2010). In addition, other biological features such as binding affinities via calculation of minimal free energy (MFE) of the miRNA/its target gene, accessibility of target sites, and conservation of target sites are incorporated into the computational methods for identifying miRNA targets to increase accuracy and to avoid non-random hits (John et al. 2004, Kiriakidou et al. 2004, Lewis et al. 2005, Sun et al. 2010).

Let-7 comprises 1-5% of mammalian genome, which is represented one of the most abundant classes of genes regulators (Bentwich et al. 2005, Berezikov et al. 2005, Jerome et al. 2007). There are 14 and 13 different *let-7* family members in mouse and human, respectively (Roush and Slack 2008). Whereas, *let-7a-1*, *let-7a-2*, *let-7a-3*, *let-7b*, *let-7c*, *let-7d*, *let-7e*, *let-7f-1*, *let-7f-2*, *let-7g*, *let-7i*, miR-98, and miR-202 are found on different chromosomes in human (He and Wang 2012, Ruby et al. 2006). *Let-7* family consists of eight essentially identical mature *let-7* miRNAs, which are co-expressed in many immune cell types (Kuchen et al. 2010). These findings are puzzling and suggest that *let-7* miRNAs have a crucial role in different biological activities (Chen et al. 2013). It has been reported that *let-7a*, *let-7b*, *let-7c*, *let-7d*, *let-7f,* and *let-7i* expressed in human endometrium (Pan et al. 2007). Some members of *let-7* miRNAs were down-regulated in response to different infections as *Salmonella Typhimurium*, *Cryptosporidium parvum*, *Helicobacter pylori* and *lipopolysaccharides* (*LPS*), either in

immune or non-immune cells (Chen et al. 2007, Eulalio et al. 2012, Teng et al. 2013). Thus, *let-7* miRNAs seem a common denominator in the response of both phagocytic (macrophages) and non-phagocytic (epithelial) cells (Eulalio et al. 2012, Schulte et al. 2011).

1.2 Rationale and objectives

Here, we hypothesized that changes of oviductal milieu due to *LPS* challenge could perturb oviductal function as well as early embryonic development in bovine (chapter 2), and the aberrant expression of *let-7* family and mRNA of endometrium due to induced inflammation using *LPS*, could abrogate uterine function (chapter 3). Therefore, objectives were:

1. Address the changes in inflammatory response genes and their potential regulatory miRNAs expression profile in oviductal epithelial cells after *LPS* challenge, and to investigate the subsequent effect of inflamed environment on embryos viability (chapter 2).

2. Study the alterations in *let-7* miRNAs and their target (mRNA and its product) expression profile in endometrial cells (both primary epithelial and stromal fibroblast) due to *in vitro LPS* challenge, as well as to identify the role of *let-7* miRNAs in inflammatory immune response of uterine tissue against bacterial infection (chapter 3).

1.3 Materials and methods

In order to accomplish our goals, in the current thesis we conducted two different primary cell cultures; (I) bovine oviductal epithelial cell culture (BOEC) and (II) endometrial cells (both epithelium and stromal fibroblast cells). The details of materials and methods that have been used can be found in the relevant chapters of this thesis. Here, the main concept of methods and their approaches is addressed.

1.3.1.1 Total RNA isolation from BOEC and endometrial cells

Total RNA (involved large and small RNA) was isolated from BOEC and endometrial cells using the miRNeasy mini kit (Qiagen, Hilden, Germany). Genomic DNA contamination was removed by performing on column DNA digestion using RNase-free DNase (Qiagen, Hilden, Germany). At the end total RNA was eluted by adding RNase free water to the membrane of the spin colmun and total RNA concentration and purity were assessed using NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Schwerte, Germany).

1.3.1.2 Total RNA isolation from embryos (blastocyst stage)

RNA isolation has been done using the PicoPure^{TM} RNA isolation kit (Arcturs, Munich, Germany). Genomic DNA contamination was removed by performing on column DNA digestion using RNase-free DNase (Qiagen, Hilden, Germany). Later total RNA was eluted by adding 11 µl of elution buffer to the membrane of the spin colmun, then concentration and purity of total RNA were evaluated using NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Schwerte, Germany).

1.3.2 First strand cDNA synthesis for large and small RNA

The cDNA of small RNA was synthesized from the isolated total RNA using the miScript II RT kit (Qiagen, Hilden, Germany), where 5 µl of total RNA (with 50 ng of input RNA) samples were mixed with reverse-transcription master. Reaction incubation was performed at 37°C for 60 min followed by heating at 95°C for 5 min to inactivate miScript Reverse Transcriptase. According to the amount of small RNA used for RT-PCR, the resulting cDNA samples were diluted before use as a template for miRNA qPCR assay. The cDNA for large RNA was synthesized from the isolated total RNA

using SuperScript® II (Invitrogen, CA, USA), then thermocycler programmed was adjusted at 42°C, 90 minutes; 75°C 15 minutes and hold at 4°C. The synthesised cDNA was confirmed in a PCR reaction using GAPDH primer and kept at -20°C until use.

1.3.3 Quantitative real-time PCR (qRT-PCR)

The real-time PCR reaction were performed in a 20 µl reaction volume containing miScript SYBR® Green PCR kit or using SYBR Green/ ROX Mix (Qiagen, Hilden, Germany) for miRNAs or mRNAs, respectively. Real-time PCR was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Melting curve analysis was constructed to verify the presence of specific amplification and for the absence of primer dimer. The data were analyzed by the comparative threshold cycle (Ct) method and normalization was done using geometric mean of at least two endogenous controls, where GAPDH, β-actin (ACTB) and 18S were for mRNAs, and 5S, U6 and SNORD48 were for miRNAs as endogenous references.

1.3.4 Western immunoblotting

Proteins from lysates of cultured cells were normalized to 1 mg/ml using a NanoDrop ND-8000 spectrophotometer (Thermo scientific, Schwerte, Germany) and separated (10 µg/lane) using gradient gel 4-18% (vol/vol) SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membrane (Thermo Scientific, Schwerte, Germany) using the omniPAGE electroblotting system (Cleaver Scientific, Rugby, UK). Membranes were incubated with different antibodies separately (dilution rate and antibodies detailed can be found in the respective chapters). Membranes were then incubated in secondary horseradish peroxidase- conjugated antibody (Santa Cruz Biotechnology, INC, Germany). The immunoreactive protein bands were visualized using enhanced chemiluminescence Clarity Western ECL Substrate (Bio-Rad, Munich, Germany). Densitometric quantification of immunoreactive bands was carried out using Quantity One analysis software (Bio-Rad, Munich, Germany).

1.3.5 Luciferase reporter constructions and luciferase assay

The 3´ UTRs of TNFα, transforming growth factor beta 1 induced transcript 1 (TGFB1I1) and serum deprivation response (SDPR) sequences encompassing the predicted binding sites of both *let-7a* and *let-7f*, respectively, were designed using

SnapGene Viewer 2.3.5. Then, the amplified PCR products were cloned into pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, WI, USA). Cloning of the right sequence was confirmed by sequencing from the constructed plasmid vector. In addition, we used mutated sequence of 3´ UTRs of TNFα, TGFB1I1 and SDPR sequences encompassing the predicted binding sites of both *let-7a* and *let-7f*, respectively. Luciferase activity was determined using Dual-Luciferase® Reporter Assay System (Promega, WI, USA) and the Lmax microplate luminometer (Centro LB 960, Berthold Technologies, Germany). *Renilla* luciferase activity was used to normalize transfection efficiency.

1.3.6 Experimental design

Other sub-experiments have been done to:

1. Detect DNA fragmentation in blastocysts cultured either in normal SOF or co-cultured with BOEC and challenged with/without *LPS* by TUNEL assay (terminal deoxinucleotil transferase uracil nick end labeling).

2. Measure reactive oxygen species (ROS) production in bovine blastocyst stage embryos (day 7) cultured either in normal SOF or co-cultured with BOEC and challenged with/without *LPS*.

3. Check the pattern of mitochondrial distribution in bovine blastocyst on day 7 cultured either in normal SOF or co-cultured with BOEC and challenged with/without *LPS*.

1.3.7 Statistical analysis

Data of miRNAs and mRNAs expression profiling were analyzed using Student's t test. The values shown in graphs are presented as the mean ± standard deviation (SD) of at least three independent experiments each done in quadruplicate, *p-values* < 0.05 were considered statistically significant.

1.4 Results

The most important findings are briefly summarized below. Detailed explanations can be found in the respective chapters of this thesis.

1.4.1 Changes in expression of genes associated with inflammatory immune response and physiological function of BOEC after *LPS* challenge

In the first experiment (Chapter 2), the relative abundance of inflammatory immune response genes as IL1β, TNFα and transforming growth factor, beta 1 (TGFβ1) was significantly increased ($0.001 \leq p \leq 0.01$) and the expression level of secretory essential elements like oviductal glycoprotein 1 (OVGP1) and IGF2 was significantly decreased in the challenged group compared with the control group. Furthermore, the stimulation of primary BOEC with minimum dose of *LPS* significantly up-regulated the expression of TLR4 and its accessory molecules. These results revealed that minimum dose of *LPS* can have a profound effect on transcriptome profile of primary bovine oviduct epithelial cells.

1.4.2 Temporal pattern of miRNAs potentially targeting inflammatory immune response genes

We identified the potential regulatory miRNAs (miR-155, miR-146a, miR-223, miR-21, miR-16 and miR-215) targeting the candidate genes in oviductal epithelial cells, using bioinformatics tools. Then we checked alignment between seed region and 3´ UTR of selected candidate genes. Moreover, we observed the dynamics pattern of microRNAs expression level in BOEC after *LPS* challenge at different time points (0, 3, 6, 12, 24 and 48h). Interestingly, all miRNAs except miR-21 were significantly increased at 6h after *LPS* treatment. The expression level of some miRNAs was found to show a reciprocal pattern to their target genes (IL1β, TGFβ1 and TNFα), whereas the expression level of some miRNAs were found to have a similar pattern to their target genes (IGF2 and OVGP1). The overall results showed that miR-155, miR-146a, miR-223, miR-21, miR-16 and miR-215 have shown a clear suppression in the challenged group after BOEC treated with *LPS* for 24h (Chapter 2).

1.4.3 Effect of *LPS* treated BOEC on co-cultured embryos

In the second experiment (chapter 2), the cleavage rate of cultured or co-cultured embryos was not affected significantly among groups namely: embryo+*LPS* free media (83.75%), embryo+*LPS* (85.38%), BOECs+embryo (86.20%) and BOEC+embryo+*LPS* (88.00%). On the other hand, embryos challenged with *LPS* with or without BOEC, resulted in significantly lower blastocyst rates (15.66±7.78, 25.60±6.84, respectively), than unchallenged embryos (22.59±10.98, 37.51±9.47, respectively).

1.4.4 *LPS* challenge induced alterations in *let-7* miRNAs and their target gene expression in primary bovine endometrial cells *in vitro*

In the current thesis (chapter 3), *LPS* was shown to induce alterations in *let-7* miRNAs family expression profiling in both epithelial and stromal cells, whereas some *let-7* members were down-regulated in both cell types as *let-7a*, other members were expressed in an opposite pattern in both cell types as *let-7e*. Next, we examined the expression profile of candidate genes that are targeted by *let-7* miRNAs after *LPS* challenge. The expression of TNFα, IL6, IL1β, NFκB, caspase 3, apoptosis-related cysteine peptidase (CASP3), and inducible nitric oxide synthase (INOS) were significantly increased in a dose-dependant manner in challenged cells compared to untreated control and in both epithelial and stromal cells. In contrast, SDPR and TGFB1I1 were significantly reduced in *LPS* treated cells compared to untreated control cells, in epithelial and stromal endometrial cells as well.

1.4.5 Pathways interaction between center genes and targeting microRNA

The list of differential expressed miRNAs as well as the differential expressed genes (DEGs) of the *in vivo* study was uploaded into the Ingenuity Pathway Analysis (IPA) to uncover common pathways, and to identify the biological functions & canonical pathway between miRNAs and their potential target genes. Networks of the genes were then algorithmically generated based on their connectivity. The significance of the association between the data set and the canonical pathway was calculated as the ratio of the number of genes from the data set that mapped to the pathway divided by the

total number of genes that mapped to the canonical pathway. Thus, we proposed that contents resulting from functional interpretation of the correlation between a limited number of miRNAs and their top inversely correlated mRNA targets, could identify a distinct function of candidate molecules, with results comparable to the global analysis (chapter 3).

1.4.6 Effect of functional modulation of *let-7* miRNAs on pro-inflammatory cytokines in *LPS* challenged endometrial stromal cells

To assess the potential role of *let-7* miRNAs in inflammatory immune response of endometrial stromal cells, we examined the effect of *let-7a* on TNFα and IL6 in stromal cells using gain- and loss-of-function experiments. Overexpression of *let-7a* inhibited pro-inflammatory cytokines like TNFα and IL6 on mRNAs as well as protein levels. Interestingly, TGFβ1I1, SDPR and NFκB mRNAs show a reciprocal pattern upon transfection with *let-7a* mimic or inhibitor. Furthermore, luciferase activity of reporters containing either 3´ UTR of TNFα or TGFβ1I1 or SDPR were significantly decreased upon *let-7a* mimic transfection in stromal endometrial cells. So TNFα, TGFβ1I1 and SDPR were identified as novel *let-7* miRNAs targets. Moreover, we investigated whether down-regulation of the *let-7* miRNAs in *LPS* challenged primary endometrial stromal cells in turn may elevate the activities of the reporters. For this, TNFα, TGFβ1I1 and SDPR 3´ UTR reporters with the intact or a mutated *let-7* binding sites were transfected into primary endometrial stromal cells, and upon treatment with *LPS*; TNFα, TGFβ1I1 and SDPR reporter activities were elevated, while regulation was lost upon the mutated *let-7* binding-site. These findings indicated that *let-7a* may regulate the expression of the secretory pro-inflammatory cytokines (TNFα and IL6) in *LPS* challenged endometrial stromal cells (chapter 3).

1.5 Conclusions

The mechanisms that regulate the mucosal immune system against bacterial infection in the bovine oviduct and their subsequent effect on early embryonic development have received only little attention. This could be due to the difficulties of conducting *in vivo* experiments. Therefore, we established and optimized an *in vitro* model as a tool to investigate inflammatory immune response of primary bovine oviductal cells.

In this thesis, we have evidenced that BOEC immediately recognized low *LPS* dose through TLR4 and its accessory molecules, which displayed a clear dynamic pattern at different time points post *LPS* stimulation. Moreover, the minimum dose of *LPS* stimulated TLR4 and its downstream genes, NFκB, IL1β and TNFα expression, switched off $PGF_{2\alpha}$ production, and subsequently increased $PGE_2/PGF_{2\alpha}$ ratio. This increased PGE_2 production resulted in proliferation of infected epithelial cells (Fukata et al. 2006). In addition, all miRNAs were clearly reduced after *LPS* challenge. These results are similar to a recent report (Teng et al. 2013) that mentioned miRNA regulations in mammalian host cells challenged with various microbial pathogens, such as *let-7* miRNAs were significantly decreased in patients with *H.pylori* infection. Also, we observed dynamic pattern of miRNAs expression at different time points. Interestingly, all selected miRNAs except miR-21 reached to peak at 6h after *LPS* stimulation. So it seems that certain miRNA functions may only be revealed at a specific concentration of an environmental trigger. The aberrant expression of inflammatory cytokines as TNFα was observed in blastocysts either cultured or co-cultured with *LPS*. In addition, *LPS* potentiated the release of reactive oxygen through TNFα induced ROS production, which is known to activate NF-κB (Kastl et al. 2014). Beside the pro-inflammatory actions of TNFα, recently it was observed that the release of TNFα is associated with an increased oxidative stress (An et al. 2012, Manna et al. 1998) and it serves a role of ROS as second messenger to activate signaling pathways and lead to alterations in gene expression (Droge 2002, Weinberg et al. 2010). We noticed a clear suppression of IGF1 expression in groups challenged with *LPS*, and this could be related to increased apoptosis and decreased blastocyst quality. Similarly, the previous studies demonstrated that the perturbed IGF signalling pathway within the oviduct affects embryo development and blastocyst cell number (Neira et al. 2010, Yilmaz et al. 2012). Collectively, we concluded that the minimum dose of *LPS* had a

clear effect on pro-inflammatory mediators expression profiles and their potential regulatory targeting miRNAs, which may disturb oviduct function. These alterations in pattern of mitochondrial distribution were associated with higher ROS generation and apoptotic cells in blastocysts. Moreover, the aberrant changes in blastocyst transcriptome profile after *LPS* treatment may lead to a defective genomic imprinting and subsequent a less viable embryo.

Additionally, we addressed a comprehensive investigation of the *let-7* miRNAs in bovine endometrial cells after *LPS* challenge with two doses as model for clinical and sub-clinical endometritis. We found that the evolutionarily conserved *let-7* miRNAs family were aberrant regulated in endometrial cells after *LPS* challenge. Furthermore, *LPS* stimulation activated NFκB signaling and led to the release of a plethora of pro-inflammatory cytokines like TNFα, IL1β and IL6. Also, these changes in *LPS* challenged endometrial cells were associated with an increased $PGE_2/PGF_{2\alpha}$ ratio, and all these alterations contributed to induced inflammatory immune response in endometrial cells post *LPS* treatment. Our findings were similar to the findings of previous studies (Herath et al. 2009, Xu et al. 2014). Notably, the dysregulation of *let-7* miRNAs family expression were associated with over-expression of pro-inflammatory cytokines as IL1β and obvious changes in other genes expression that might be potentially regulated by *let-7* miRNAs family as SDPR, TGFβ1I1, CASP3, NFκB, TLR4 and INOS. Persistent inflammation is linked clinically and epidemiologically to bovine infertility (Cheong et al. 2011), and the proper regulation of pro-inflammatory cytokines appears to play an important role in maintaining uterine function and uterine homeostasis as well. So far, little is known about the role of *let-7* miRNAs family in bovine endometritis. Therefore, we suppose that there is an intimate link between aberrant regulation of *let-7* miRNAs and persistent inflammation in bovine endometrial cells through post-transcriptional regulation of genes related to inflammatory immune response. Our results provided primary evidence that *let-7* miRNAs family is involved in the regulation of pro-inflammatory cytokines as TNFα and IL6, which are key modulators of the local inflammatory immune response in *LPS* challenged endometrial cells, either in direct or indirect manner, using gain and loss of function.

In particular, this thesis highlights for the first time that *let-7* miRNAs family have a precise role in bovine endometrium, where *LPS* induced a remarkable suppression of

let-7 miRNAs expression, and subsequently resulted in an increased in pro-inflammatory cytokines level as TNFα, IL6, NFκB, TGFβ1I1 and SDPR either in direct or indirect manner. To our knowledge, this is the first study showing that TNFα, TGFβ1I1 and SDPR were identified as novel *let-7* miRNAs family targets and may have vital role in inflammatory immune response in *LPS* challenged bovine endometrial cells.

1.6 References

Abal M, Planaguma J, Gil-Moreno A, Monge M, Gonzalez M, Baro T, Garcia A, Castellvi J, Ramon YCS, Xercavins J, Alameda F, Reventos J (2006): Molecular pathology of endometrial carcinoma: transcriptional signature in endometrioid tumors. Histol Histopathol 21, 197-204

Abe H (1996): The mammalian oviductal epithelium: regional variations in cytological and functional aspects of the oviductal secretory cells. Histol Histopathol 11, 743-768

Abe H, Hoshi H (1997): Bovine oviductal epithelial cells: their cell culture and applications in studies for reproductive biology. Cytotechnology 23, 171-183

Achache H, Revel A (2006): Endometrial receptivity markers, the journey to successful embryo implantation. Hum Reprod Update 12, 731-746

An L, Wang X, Cederbaum AI (2012): Cytokines in alcoholic liver disease. Arch Toxicol 86, 1337-1348

Asirvatham AJ, Magner WJ, Tomasi TB (2009): miRNA regulation of cytokine genes. Cytokine 45, 58-69

Asselin E, Goff AK, Bergeron H, Fortier MA (1996): Influence of sex steroids on the production of prostaglandins F2 alpha and E2 and response to oxytocin in cultured epithelial and stromal cells of the bovine endometrium. Biol Reprod 54, 371-379

Aviles M, Gutierrez-Adan A, Coy P (2010): Oviductal secretions: will they be key factors for the future ARTs? Mol Hum Reprod 16, 896-906

Axtell MJ, Westholm JO, Lai EC (2011): Vive la difference: biogenesis and evolution of microRNAs in plants and animals. Genome Biol 12, 221

Bates MD, Quenby S, Takakuwa K, Johnson PM, Vince GS (2002): Aberrant cytokine production by peripheral blood mononuclear cells in recurrent pregnancy loss? Hum Reprod 17, 2439-2444

Bentwich I, Avniel A, Karov Y, Aharonov R, Gilad S, Barad O, Barzilai A, Einat P, Einav U, Meiri E, Sharon E, Spector Y, Bentwich Z (2005): Identification of hundreds of conserved and nonconserved human microRNAs. Nat Genet 37, 766-770

Berezikov E, Chung WJ, Willis J, Cuppen E, Lai EC (2007): Mammalian mirtron genes. Mol Cell 28, 328-336

Berezikov E, Guryev V, van de Belt J, Wienholds E, Plasterk RH, Cuppen E (2005): Phylogenetic shadowing and computational identification of human microRNA genes. Cell 120, 21-24

Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W (2006): Relief of microRNA-mediated translational repression in human cells subjected to stress. Cell 125, 1111-1124

Boni R, Tosti E, Roviello S, Dale B (1999): Intercellular communication in in vivo- and in vitro-produced bovine embryos. Biol Reprod 61, 1050-1055

Bonizzi G, Karin M (2004): The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol 25, 280-288

Chaouat G, Ledee-bataille N, Zourbas S, Dubanchet S, Sandra O, Martal J, Ostojojic S, Frydman R (2003): Implantation: can immunological parameters of implantation failure be of interest for pre-eclampsia? J Reprod Immunol 59, 205-217

Chapwanya A, Meade KG, Doherty ML, Callanan JJ, Mee JF, O'Farrelly C (2009): Histopathological and molecular evaluation of Holstein-Friesian cows postpartum: toward an improved understanding of uterine innate immunity. Theriogenology 71, 1396-1407

Chen CZ, Schaffert S, Fragoso R, Loh C (2013): Regulation of immune responses and tolerance: the microRNA perspective. Immunol Rev 253, 112-128
Chen SJ, Liu YL, Sytwu HK (2012): Immunologic regulation in pregnancy: from mechanism to therapeutic strategy for immunomodulation. Clin Dev Immunol 2012, 258391
Chen XM, Splinter PL, O'Hara SP, LaRusso NF (2007): A cellular micro-RNA, let-7i, regulates Toll-like receptor 4 expression and contributes to cholangiocyte immune responses against Cryptosporidium parvum infection. J Biol Chem 282, 28929-28938
Cheong SH, Nydam DV, Galvao KN, Crosier BM, Gilbert RO (2011): Cow-level and herd-level risk factors for subclinical endometritis in lactating Holstein cows. J Dairy Sci 94, 762-770
Cronin JG, Turner ML, Goetze L, Bryant CE, Sheldon IM (2012): Toll-like receptor 4 and MYD88-dependent signaling mechanisms of the innate immune system are essential for the response to lipopolysaccharide by epithelial and stromal cells of the bovine endometrium. Biol Reprod 86, 51
Dey SK, Lim H, Das SK, Reese J, Paria BC, Daikoku T, Wang H (2004): Molecular cues to implantation. Endocr Rev 25, 341-373
Di Leva G, Garofalo M, Croce CM (2014): MicroRNAs in cancer. Annu Rev Pathol 9, 287-314
Diskin MG, Morris DG (2008): Embryonic and early foetal losses in cattle and other ruminants. Reprod Domest Anim 43 Suppl 2, 260-267
Droge W (2002): Free radicals in the physiological control of cell function. Physiol Rev 82, 47-95
Ellington JE (1991): The bovine oviduct and its role in reproduction: a review of the literature. Cornell Vet 81, 313-328
Eulalio A, Schulte L, Vogel J (2012): The mammalian microRNA response to bacterial infections. RNA Biol 9, 742-750
Fatima A, Waters S, O'Boyle P, Seoighe C, Morris DG (2014): Alterations in hepatic miRNA expression during negative energy balance in postpartum dairy cattle. BMC Genomics 15, 28
Foldi J, Kulcsar M, Pecsi A, Huyghe B, de Sa C, Lohuis JA, Cox P, Huszenicza G (2006): Bacterial complications of postpartum uterine involution in cattle. Anim Reprod Sci 96, 265-281
Fukata M, Chen A, Klepper A, Krishnareddy S, Vamadevan AS, Thomas LS, Xu R, Inoue H, Arditi M, Dannenberg AJ, Abreu MT (2006): Cox-2 is regulated by Toll-like receptor-4 (TLR4) signaling: Role in proliferation and apoptosis in the intestine. Gastroenterology 131, 862-877
Georgiou AS, Sostaric E, Wong CH, Snijders AP, Wright PC, Moore HD, Fazeli A (2005): Gametes alter the oviductal secretory proteome. Mol Cell Proteomics 4, 1785-1796
Gilbert RO, Shin ST, Guard CL, Erb HN, Frajblat M (2005): Prevalence of endometritis and its effects on reproductive performance of dairy cows. Theriogenology 64, 1879-1888
Girling JE, Rogers PA (2005): Recent advances in endometrial angiogenesis research. Angiogenesis 8, 89-99
Glazov EA, Cottee PA, Barris WC, Moore RJ, Dalrymple BP, Tizard ML (2008): A microRNA catalog of the developing chicken embryo identified by a deep sequencing approach. Genome Res 18, 957-964

Godkin JD, Roberts MP, Elgayyar M, Guan W, Tithof PK (2008): Phospholipase A2 regulation of bovine endometrial (BEND) cell prostaglandin production. Reprod Biol Endocrinol 6, 44
Guzeloglu-Kayisli O, Kayisli UA, Taylor HS (2009): The role of growth factors and cytokines during implantation: endocrine and paracrine interactions. Semin Reprod Med 27, 62-79
Hagiwara H, Aoki T, Ohwada N, Fujimoto T (1997): Development of striated rootlets during ciliogenesis in the human oviduct epithelium. Cell Tissue Res 290, 39-42
Hammon DS, Evjen IM, Dhiman TR, Goff JP, Walters JL (2006): Neutrophil function and energy status in Holstein cows with uterine health disorders. Vet Immunol Immunopathol 113, 21-29
Harvey MB, Arcellana-Panlilio MY, Zhang X, Schultz GA, Watson AJ (1995): Expression of genes encoding antioxidant enzymes in preimplantation mouse and cow embryos and primary bovine oviduct cultures employed for embryo coculture. Biol Reprod 53, 532-540
He Y, Wang ZJ (2012): Let-7 microRNAs and Opioid Tolerance. Front Genet 3, 110
Herath S, Fischer DP, Werling D, Williams EJ, Lilly ST, Dobson H, Bryant CE, Sheldon IM (2006): Expression and function of Toll-like receptor 4 in the endometrial cells of the uterus. Endocrinology 147, 562-570
Herath S, Lilly ST, Fischer DP, Williams EJ, Dobson H, Bryant CE, Sheldon IM (2009): Bacterial lipopolysaccharide induces an endocrine switch from prostaglandin F2alpha to prostaglandin E2 in bovine endometrium. Endocrinology 150, 1912-1920
Hertel J, Bartschat S, Wintsche A, Otto C, Stadler PF (2012): Evolution of the let-7 microRNA family. RNA Biol 9, 231-241
Hill J, Gilbert R (2008): Reduced quality of bovine embryos cultured in media conditioned by exposure to an inflamed endometrium. Aust Vet J 86, 312-316
Hinske LC, Galante PA, Kuo WP, Ohno-Machado L (2010): A potential role for intragenic miRNAs on their hosts' interactome. BMC Genomics 11, 533
Holloway AF, Rao S, Shannon MF (2002): Regulation of cytokine gene transcription in the immune system. Mol Immunol 38, 567-580
Hugentobler SA, Humpherson PG, Leese HJ, Sreenan JM, Morris DG (2008): Energy substrates in bovine oviduct and uterine fluid and blood plasma during the oestrous cycle. Mol Reprod Dev 75, 496-503
Hunter RH (2012): Components of oviduct physiology in eutherian mammals. Biol Rev Camb Philos Soc 87, 244-255
Jabbour HN, Kelly RW, Fraser HM, Critchley HO (2006): Endocrine regulation of menstruation. Endocr Rev 27, 17-46
Jackson RJ, Standart N (2007): How do microRNAs regulate gene expression? Sci STKE 2007, re1
Jerome T, Laurie P, Louis B, Pierre C (2007): Enjoy the Silence: The Story of let-7 MicroRNA and Cancer. Curr Genomics 8, 229-233
John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS (2004): Human MicroRNA targets. PLoS Biol 2, e363
Jones RL, Stoikos C, Findlay JK, Salamonsen LA (2006): TGF-beta superfamily expression and actions in the endometrium and placenta. Reproduction 132, 217-232
Kasimanickam R, Duffield TF, Foster RA, Gartley CJ, Leslie KE, Walton JS, Johnson WH (2005): The effect of a single administration of cephapirin or cloprostenol on the reproductive performance of dairy cows with subclinical endometritis. Theriogenology 63, 818-830

Kasimanickam R, Duffield TF, Foster RA, Gartley CJ, Leslie KE, Walton JS, Johnson WH (2004): Endometrial cytology and ultrasonography for the detection of subclinical endometritis in postpartum dairy cows. Theriogenology 62, 9-23
Kastl L, Sauer SW, Ruppert T, Beissbarth T, Becker MS, Suss D, Krammer PH, Gulow K (2014): TNF-alpha mediates mitochondrial uncoupling and enhances ROS-dependent cell migration via NF-kappaB activation in liver cells. FEBS Lett 588, 175-183
Kaufmann T (2010): Clinical and subclinical endometritis in dairy cattle: Prevalence, Indicators, and Therapy.
Kaufmann TB, Drillich M, Tenhagen BA, Forderung D, Heuwieser W (2009): Prevalence of bovine subclinical endometritis 4h after insemination and its effects on first service conception rate. Theriogenology 71, 385-391
Kim VN (2005): MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol 6, 376-385
Kiriakidou M, Nelson PT, Kouranov A, Fitziev P, Bouyioukos C, Mourelatos Z, Hatzigeorgiou A (2004): A combined computational-experimental approach predicts human microRNA targets. Genes Dev 18, 1165-1178
Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, Rajewsky N (2005): Combinatorial microRNA target predictions. Nat Genet 37, 495-500
Kuchen S, Resch W, Yamane A, Kuo N, Li Z, Chakraborty T, Wei L, Laurence A, Yasuda T, Peng S, Hu-Li J, Lu K, Dubois W, Kitamura Y, Charles N, Sun HW, Muljo S, Schwartzberg PL, Paul WE, O'Shea J, Rajewsky K, Casellas R (2010): Regulation of microRNA expression and abundance during lymphopoiesis. Immunity 32, 828-839
Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001): Identification of novel genes coding for small expressed RNAs. Science 294, 853-858
Lapointe J, Bilodeau JF (2003): Antioxidant defenses are modulated in the cow oviduct during the estrous cycle. Biol Reprod 68, 1157-1164
Lau NC, Lim LP, Weinstein EG, Bartel DP (2001): An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science 294, 858-862
Lechniak D, Pers-Kamczyc E, Pawlak P (2008): Timing of the first zygotic cleavage as a marker of developmental potential of mammalian embryos. Reprod Biol 8, 23-42
Lee RC, Feinbaum RL, Ambros V (1993): The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75, 843-845
Lee Y, Jeon K, Lee JT, Kim S, Kim VN (2002): MicroRNA maturation: stepwise processing and subcellular localization. EMBO J 21, 4663-4670
Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, Kim VN (2004): MicroRNA genes are transcribed by RNA polymerase II. EMBO J 23, 4051-4060
Leese HJ (1988): The formation and function of oviduct fluid. J Reprod Fertil 82, 843-856
Leese HJ, Tay JI, Reischl J, Downing SJ (2001): Formation of Fallopian tubal fluid: role of a neglected epithelium. Reproduction 121, 339-346
Lei ZM, Rao CV (1992): Expression of epidermal growth factor (EGF) receptor and its ligands, EGF and transforming growth factor-alpha, in human fallopian tubes. Endocrinology 131, 947-957
Lewis BP, Burge CB, Bartel DP (2005): Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 15-20

Lincke A, Drillich M, Heuwieser W (2007): Subclinical endometritis in dairy cattle and its effect on fertility a review of recent publications. Berl Munch Tierarztl Wochenschr 120, 245-250
Makeyev EV, Maniatis T (2008): Multilevel regulation of gene expression by microRNAs. Science 319, 1789-1790
Makker A, Singh MM (2006): Endometrial receptivity: clinical assessment in relation to fertility, infertility, and antifertility. Med Res Rev 26, 699-746
Manna SK, Zhang HJ, Yan T, Oberley LW, Aggarwal BB (1998): Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. J Biol Chem 273, 13245-13254
McCoy CE (2011): The role of miRNAs in cytokine signaling. Front Biosci (Landmark Ed) 16, 2161-2171
Nagao Y, Saeki K, Hoshi M, Kainuma H (1994): Effects of oxygen concentration and oviductal epithelial tissue on the development of in vitro matured and fertilized bovine oocytes cultured in protein-free medium. Theriogenology 41, 681-687
Neira JA, Tainturier D, Pena MA, Martal J (2010): Effect of the association of IGF-I, IGF-II, bFGF, TGF-beta1, GM-CSF, and LIF on the development of bovine embryos produced in vitro. Theriogenology 73, 595-604
Nothnick WB (2012): The role of micro-RNAs in the female reproductive tract. Reproduction 143, 559-576
O'Neill LA (2006): How Toll-like receptors signal: what we know and what we don't know. Curr Opin Immunol 18, 3-9
Odor DL, Blandau RJ (1973): EGG transport over the fimbrial surface of the rabbit oviduct under experimental conditions. Fertil Steril 24, 292-300
Pan Q, Chegini N (2008): MicroRNA signature and regulatory functions in the endometrium during normal and disease states. Semin Reprod Med 26, 479-493
Pan Q, Luo X, Toloubeydokhti T, Chegini N (2007): The expression profile of micro-RNA in endometrium and endometriosis and the influence of ovarian steroids on their expression. Mol Hum Reprod 13, 797-806
Piccinni MP, Beloni L, Livi C, Maggi E, Scarselli G, Romagnani S (1998): Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions. Nat Med 4, 1020-1024
Poyser NL (1995): The control of prostaglandin production by the endometrium in relation to luteolysis and menstruation. Prostaglandins Leukot Essent Fatty Acids 53, 147-195
Prat J, Gallardo A, Cuatrecasas M, Catasus L (2007): Endometrial carcinoma: pathology and genetics. Pathology 39, 72-87
Raghupathy R (1997): Th1-type immunity is incompatible with successful pregnancy. Immunol Today 18, 478-482
Raychaudhuri S (2012): MicroRNAs overexpressed in growth-restricted rat skeletal muscles regulate the glucose transport in cell culture targeting central TGF-beta factor SMAD4. PLoS One 7, e34596
Rechler MM, Nissley SP (1985): The nature and regulation of the receptors for insulin-like growth factors. Annu Rev Physiol 47, 425-442
Rizos D, Ramirez MA, Pintado B, Lonergan P, Gutierrez-Adan A (2010): Culture of bovine embryos in intermediate host oviducts with emphasis on the isolated mouse oviduct. Theriogenology 73, 777-785

Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A (2004): Identification of mammalian microRNA host genes and transcription units. Genome Res 14, 1902-1910
Rosselli M, Dubey RK, Rosselli MA, Macas E, Fink D, Lauper U, Keller PJ, Imthurn B (1996): Identification of nitric oxide synthase in human and bovine oviduct. Mol Hum Reprod 2, 607-612
Rosselli M, Imthurn B, Macas E, Keller PJ (1994): Endothelin production by bovine oviduct epithelial cells. J Reprod Fertil 101, 27-30
Rottmayer R, Ulbrich SE, Kolle S, Prelle K, Neumueller C, Sinowatz F, Meyer HH, Wolf E, Hiendleder S (2006): A bovine oviduct epithelial cell suspension culture system suitable for studying embryo-maternal interactions: morphological and functional characterization. Reproduction 132, 637-648
Roush S, Slack FJ (2008): The let-7 family of microRNAs. Trends Cell Biol 18, 505-516
Ruby JG, Jan C, Player C, Axtell MJ, Lee W, Nusbaum C, Ge H, Bartel DP (2006): Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in C. elegans. Cell 127, 1193-1207
Saito S (2000): Cytokine network at the feto-maternal interface. J Reprod Immunol 47, 87-103
Saito S, Nakashima A, Shima T, Ito M (2010): Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. Am J Reprod Immunol 63, 601-610
Saxena S, Jonsson ZO, Dutta A (2003): Small RNAs with imperfect match to endogenous mRNA repress translation. Implications for off-target activity of small inhibitory RNA in mammalian cells. J Biol Chem 278, 44312-44319
Schafer-Somi S (2003): Cytokines during early pregnancy of mammals: a review. Anim Reprod Sci 75, 73-94
Schulte LN, Eulalio A, Mollenkopf HJ, Reinhardt R, Vogel J (2011): Analysis of the host microRNA response to Salmonella uncovers the control of major cytokines by the let-7 family. EMBO J 30, 1977-1989
Sheldon IM, Cronin J, Goetze L, Donofrio G, Schuberth HJ (2009a): Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. Biol Reprod 81, 1025-1032
Sheldon IM, Lewis GS, LeBlanc S, Gilbert RO (2006): Defining postpartum uterine disease in cattle. Theriogenology 65, 1516-1530
Sheldon IM, Noakes DE, Rycroft AN, Pfeiffer DU, Dobson H (2002): Influence of uterine bacterial contamination after parturition on ovarian dominant follicle selection and follicle growth and function in cattle. Reproduction 123, 837-845
Sheldon IM, Price SB, Cronin J, Gilbert RO, Gadsby JE (2009b): Mechanisms of infertility associated with clinical and subclinical endometritis in high producing dairy cattle. Reprod Domest Anim 44 Suppl 3, 1-9
Singh M, Chaudhry P, Asselin E (2011): Bridging endometrial receptivity and implantation: network of hormones, cytokines, and growth factors. J Endocrinol 210, 5-14
Smith WL, Garavito RM, DeWitt DL (1996): Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem 271, 33157-33160
St-Louis I, Singh M, Brasseur K, Leblanc V, Parent S, Asselin E (2010): Expression of COX-1 and COX-2 in the endometrium of cyclic, pregnant and in a model of pseudopregnant rats and their regulation by sex steroids. Reprod Biol Endocrinol 8, 103

Subandrio AL, Sheldon IM, Noakes DE (2000): Peripheral and intrauterine neutrophil function in the cow: the influence of endogenous and exogenous sex steroid hormones. Theriogenology 53, 1591-1608
Sun W, Julie Li YS, Huang HD, Shyy JY, Chien S (2010): microRNA: a master regulator of cellular processes for bioengineering systems. Annu Rev Biomed Eng 12, 1-27
Swangchan-Uthai T, Lavender CR, Cheng Z, Fouladi-Nashta AA, Wathes DC (2012): Time course of defense mechanisms in bovine endometrium in response to lipopolysaccharide. Biol Reprod 87, 135
Tavares LM FW, Oliveira VP, Nichi M, Assumpcao ME, Visintin JA (2011): In vitro development of bovine embryos cultured under different fetal calf serum concentrations and cell types. Braz. J. Vet. Res. Anim. Sci., São Paulo 48, 38-45
Teng GG, Wang WH, Dai Y, Wang SJ, Chu YX, Li J (2013): Let-7b is involved in the inflammation and immune responses associated with Helicobacter pylori infection by targeting Toll-like receptor 4. PLoS One 8, e56709
Thellin O, Coumans B, Zorzi W, Igout A, Heinen E (2000): Tolerance to the foeto-placental 'graft': ten ways to support a child for nine months. Curr Opin Immunol 12, 731-737
Thibodeaux JK, Del Vecchio RP, Hansel W (1993): Role of platelet-derived growth factor in development of in vitro matured and in vitro fertilized bovine embryos. J Reprod Fertil 98, 61-66
Ulbrich SE, Zitta K, Hiendleder S, Wolf E (2010): In vitro systems for intercepting early embryo-maternal cross-talk in the bovine oviduct. Theriogenology 73, 802-816
Vasudevan S, Steitz JA (2007): AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2. Cell 128, 1105-1118
Walsh SW, Williams EJ, Evans AC (2011): A review of the causes of poor fertility in high milk producing dairy cows. Anim Reprod Sci 123, 127-138
Weinberg F, Hamanaka R, Wheaton WW, Weinberg S, Joseph J, Lopez M, Kalyanaraman B, Mutlu GM, Budinger GR, Chandel NS (2010): Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. Proc Natl Acad Sci U S A 107, 8788-8793
Wijayagunawardane MP, Kodithuwakku SP, Yamamoto D, Miyamoto A (2005): Vascular endothelial growth factor system in the cow oviduct: a possible involvement in the regulation of oviductal motility and embryo transport. Mol Reprod Dev 72, 511-520
Wijayagunawardane MP, Miyamoto A, Cerbito WA, Acosta TJ, Takagi M, Sato K (1998): Local distributions of oviductal estradiol, progesterone, prostaglandins, oxytocin and endothelin-1 in the cyclic cow. Theriogenology 49, 607-618
Williams EJ, Fischer DP, Noakes DE, England GC, Rycroft A, Dobson H, Sheldon IM (2007): The relationship between uterine pathogen growth density and ovarian function in the postpartum dairy cow. Theriogenology 68, 549-559
Williams EJ, Fischer DP, Pfeiffer DU, England GC, Noakes DE, Dobson H, Sheldon IM (2005): Clinical evaluation of postpartum vaginal mucus reflects uterine bacterial infection and the immune response in cattle. Theriogenology 63, 102-117
Winger QA, de los Rios P, Han VK, Armstrong DT, Hill DJ, Watson AJ (1997): Bovine oviductal and embryonic insulin-like growth factor binding proteins: possible regulators of "embryotrophic" insulin-like growth factor circuits. Biol Reprod 56, 1415-1423
Wrenzycki C, Herrmann D, Lucas-Hahn A, Gebert C, Korsawe K, Lemme E, Carnwath JW, Niemann H (2005): Epigenetic reprogramming throughout preimplantation

development and consequences for assisted reproductive technologies. Birth Defects Res C Embryo Today 75, 1-9
Xia P, Han VK, Viuff D, Armstrong DT, Watson AJ (1996): Expression of insulin-like growth factors in two bovine oviductal cultures employed for embryo co-culture. J Endocrinol 149, 41-53
Xu Y, Jin H, Yang X, Wang L, Su L, Liu K, Gu Q, Xu X (2014): MicroRNA-93 inhibits inflammatory cytokine production in LPS-stimulated murine macrophages by targeting IRAK4. FEBS Lett 588, 1692-1698
Yaniz JL, Lopez-Gatius F, Santolaria P, Mullins KJ (2000): Study of the functional anatomy of bovine oviductal mucosa. Anat Rec 260, 268-278
Yilmaz O, Calka J, Bukowski R, Zalecki M, Wasowicz K, Jaroszewski JJ, Markiewicz W, Bulbul A, Ucar M (2012): Nitric oxide in the bovine oviduct: influence on contractile activity and nitric oxide synthase isoforms localization. Theriogenology 77, 1312-1327
Zerbe H, Schuberth HJ, Engelke F, Frank J, Klug E, Leibold W (2003): Development and comparison of in vivo and in vitro models for endometritis in cows and mares. Theriogenology 60, 209-223

Chapter 2 (*Plos One PONE-D-14-35766R2 - [EMID:1d643e9e6a99d05b]*)

Expression pattern of inflammatory response genes and their regulatory microRNAs in bovine oviductal cells in response to *lipopolysaccharide*: Implication for early embryonic development

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Abstract

In the present study, we used an *in vitro* model to investigate the response of the oviduct with respect to inflammatory mediators and their regulatory microRNAs in case of bacterial infection and subsequent association with embryo survival. For this, we conducted two experiments. In the first experiment, cultured primary bovine oviductal cells (BOECs) were challenged with *lipopolysaccharide* (*LPS*) for 24h and the temporal expression pattern of inflammatory mediators and their regulatory microRNAs were measured at 0, 3, 6, 12, 24 and 48h after *LPS* treatment. Intriguingly, the temporal patterns of all miRNAs except miR-21 were a significantly up-regulating at 6h after *LPS* treatment. Whereas, we observed significantly overexpression of pro-inflammatory mediators as tumor necrosis factor alpha (TNFα) and interleukin-1 beta (IL1β) after *LPS* challenge for 24h. On the other hand, the expression level of essential element like oviductal glycoprotein 1 (OVGP1) and insulin-like growth factor 2 (IGF2) was significantly decreased in challenged groups compared with control. Moreover, miR-155, miR-146a, miR-223, miR-21, miR-16 and miR-215 have shown a clear suppression in the challenged group after *LPS* treatment. In the 2nd experiment there were four groups of blastocysts produced namely: embryo+*LPS* free media, embryo+*LPS*, BOECs+embryo and BOECs+embryo+*LPS*. The suboptimal oviduct environment due to *LPS* challenge is found to have a significant influence on the expression of inflammatory response genes (TNFα & CSF1), stress response genes (SOD and CAT), mitochondrial activity, reactive oxygen species (ROS) accumulation and apoptotic level either in cultured or co-cultured blastocysts. Collectively, *LPS* challenge led to aberrant changes in oviductal transcriptome profile, which could lead to a suboptimal environment for embryo development.

Key words: bovine oviduct, lipopolysaccharide, microRNAs, embryo co-culture

Introduction

In cattle, bacterial contamination of the uterine lumen is ubiquitous after parturition, and up to 40% of animals develop pelvic inflammatory disease (PID) and 20% have endometritis [1,2]. Infection of the endometrium with *Escherichia coli* (*E. coli*) precedes infection by other pathogenic bacteria and viruses, which paves the way for other pathogens to cause endometrial damage and disrupt ovarian cycles, including extended luteal phases [3]. The cost of female reproductive disorders and the associated infertility was estimated to be $650 million per annum in the United States [4,5].

Oviduct is the female genital organ at which oocyte maturation, sperm capacitation, fertilization and transport of gametes and embryos is occurring (maturation, capacitation etc do not occur in other organ) [6]; the disturbance of oviduct function due to pathogenic infection could result early embryonic loses and infertility. The surface of the oviduct and other female reproductive systems are lined with mucosa which can act as a physical barrier against the outside environment and participates in both innate and acquired immune defence [7,8]. The mucus faces the challenge of different antigenic/inflammatory stimuli arising during mating or parturition [9,10]. The effect of endometritis on ovarian function is a well documented fact in which higher and moderate level of *LPS* concentrations were found in follicular fluid collected from post partum cows with clinical and subclinical endometritis, respectively compared with the normal ones [9]. As an intermediate organ between the uterus and the ovary, there is a higher chance that the oviduct will be under the influence of *LPS*. Despite the mucosal role in immune defence of the genital tract, little is known about the mechanism of bovine oviduct epithelial cell activation by pathogens or about the receptors and secondary mediators involved in this response. It is well established that Toll-like receptors (TLRs) have important roles in detecting pathogens and in initiating

inflammatory responses that subsequently prime specific adaptive immune responses during infection [11]. It has also been recognized that dysregulation of this process is a hallmark of inflammatory and autoimmune diseases [12]. TLR4, in association with accessory molecules MD-2 and CD14 are required for Gram-negative bacterial *LPS* detection. Binding of TLR4 to *LPS* initiates a cascade of signaling events that evokes the production of cytokines and pro-inflammatory mediators [13]. Although the functional role of TLR4 in the intact endometrium and oviduct of human, mouse and in the intact endometrium of bovine has been explored, less is known about its role in bovine oviduct [10,14,15].

To date, the early embryonic death in bovine is a poorly understood phenomenon and this is attributed to the paradoxical biological and immunological cellular activities involved in this process. It was estimated that up to 40% of total embryonic losses occur between days 0–7 of pregnancy in cattle [16-18]. In addition, in laboratory and domestic farm animals, it is becoming increasingly clear that the oviducts play a critical role in the development of the zygote and during the early cleavage stages–a phase that embraces the transition from maternal to embryonic regulation of the genome. In some manner yet to be explained, the exposure to the oviduct milieu acts to promote an integrated unfolding and expression of the embryonic gene programme [19].

Here, we report the establishment and optimization of an *in vitro* model, as a tool to test whether TLR4 and its accessory molecules (CD14/MyD88) are essential for the response to minimum dose of *LPS* by bovine oviduct epithelial cells. In addition to decipher the molecular & cellular changes mediated by *LPS* on oviduct function and to investigate subsequent effects on early embryonic development in bovine will enable us to draw the association between oviductal infection and early embryonic death in

bovine. The aim of the current study was (I) to address the changes in inflammatory response mRNAs and their targeting miRNAs expression profile in oviductal epithelial cells after *LPS* challenge, (II) to investigate the subsequent effect of challenged oviductal cells on co-cultured embryos viability. For this, we have selected candidate inflammatory and stress response genes namely: tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL1β), TNF receptor associated factor 6 (TRAF6), insulin-like growth factor 2 (IGF2), oviductal glycoprotein 1 (OVGP1), caspase 3, apoptosis-related cysteine peptidase (CASP3), transforming growth factor, beta 1 (TGFβ1), superoxide dismutase 1 (SOD) and glutathione peroxidase 4 (GPX4), which could have a crucial role in oviductal function as well as immune response against bacterial infection. Moreover, we have investigated their potential regulatory miRNAs including miR-16, miR-21, miR-223, miR-215, miR-146a and miR-155 in primary bovine oviductal epithelial cells exposed to minimum dose of *LPS*.

Material and methods

All chemicals and reagents for *in vitro* culture were purchased from Sigma Aldrich unless otherwise stated. Plastic dishes, four-well plates, and tubes were obtained from Nunc (Thermo Scientific). All chemicals for reverse transcription were acquired from Invitrogen (Life Technologies). The whole experiment was replicated at least three independent biological replicates each done in quadruplicate.

The use of *in vitro* produced bovine embryos for this experiment was approved by the Animal Welfare committee of the University of Bonn with proposition number 84-02.05.20.12.075. But all the other experiments were conducted using oviductal cells and ovaries collected as by-products of the local abattoirs (Bernhard Frenken GmbH

Vieh- und Fleischhandel Schlachthof Düren and EFM Euskirchener Fleisch MarktGmbH) which are slaughtering animals for local meat consumption.

Experiment 1: Effect of in vitro LPS challenge on bovine oviductal epithelial cells

Bovine oviductal epithelial cell culture (BOEC)

The collection and culture of oviductal cells were performed as described previously [20]. Briefly, oviductal tissue ipsilateral to corpus luteum (CL) was collected from five cows after slaughter and transported to the lab in phosphate buffer saline without Ca^{2+}/Mg^{2+} (PBS^{-}) supplemented with 2% Penicillin-Streptomycin (Gibco®, Karlsruhe; Germany). Upon arrival in the laboratory, the oviducts were rinsed in 70% ethanol followed by three times washing in PBS^{-}. The oviduct ampulla was gently squeezed in a stripping motion with forceps to obtain epithelial cells. After repeated pipetted, the cell suspension was passed twice through a 23 gauge (G) syringe needle and then incubated for 30 min to allow the fibroblast cells to adhere to the culture dish. The cells were then washed with fresh culture medium and allowed to sediment for 25 min. Then cells were seeded in a 24–well culture plate in a concentration of 10^6 cells per well in 800 µl culture medium. An aliquot of the cell suspension was used for cell count. The proportion of alive cells prior seeding was assessed by Trypan blue staining (Gibco®, Karlsruhe; Germany). The cells were cultured at 38°C in a humidified atmosphere with 5% CO2 in air. Culture medium was changed every 2–3 days, until the cells attained 85–90% confluency.

Cell culture treatment

After cells reached confluency, one group of cells were challenged with *LPS* and untreated groups were used as a control. The cells were challenged with arachidonic

acid (AA, 100 µM), (Sigma, Munich; Germany) and with 0.5 µg/ml of Ultra-pure *LPS* (InvivoGen, San Diego; USA), the *LPS* concentrations at a level found in the uterine lumen during sub-clinical infected animals [9]. The cells were harvested at one time point (after 24h) from *LPS* challenge as well as at different time points (0, 3, 6, 12, 24 and 48h) from *LPS* challenge to observe temporal expression pattern of miRNAs. The supernatant and harvested cells were kept in -80^{o}C for molecular analysis. In addition, the cell culture supernatant was collected for measurement of TNFα and prostaglandins (PGE_2 & $PGF_{2\alpha}$) by using specific bovine ELISA kits. The absence of immune cells in the oviduct epithelial cell cultures was confirmed by PCR for the CD45 pan-leukocyte marker as previously described [21,22] (data not shown). Cell viability was assessed by using WST-1 Cell Proliferation Assay Kit (Cayman Chemical, Michigan; USA).

RNA isolation and cDNA synthesis

Total RNA was isolated from the cells using the miRNeasy® Mini kit (Qiagen, Hilden; Germany) according to manufacture's protocol. In order to remove possible contaminations of genomic DNA, the extracted RNA was subjected to on-column DNA digestion by using RNase free DNase set on-column (Qiagen, Hilden; Germany). The cDNA for gene expression analysis was synthesised from the isolated total RNA using SuperScript® II (Invitrogen, CA; USA). Briefly, maximum volume of 10 µl RNA from each replicate of the treatment was co-incubated with 0.5 µl of 100 µM $Oligo(dT)_{15}$ (Promega, WI; USA) and 0.5 µl of Random Primer (Promega, WI; USA) at 70°C for 3 min then chilled on ice for 2 min. Reverse transcription was performed in a 20 µl mixture of 4 µl 5x first strand buffer, 2 µl 0.1 M DTT, 1 µl 10 mM dNTP, 0.7 µl Superscript II reverse transcriptase (Invitrogen, CA; USA), 0.3 µl RNasin (Promega, WI; USA) and RNase free water was added to the RNA mixture in a PCR strip and run

in a thermocycler programmed at 42°C, 90 minutes; 75°C 15 minutes and hold at 4°C. The cDNA was synthesis for small RNA from the isolated total RNA using the miScript II RT kit (Qiagen, Hilden; Germany) following manufacturer's protocols. Briefly, 2 µl of total RNA samples were mixed with reverse-transcription master, which composed of 4 µl 5x miScript HiSpec, 2 µl 10x Nucleics Mix, 2 µl miScript Reverse Transcriptase Mix in a 20 µl reaction volume. Reaction incubation was performed at 37°C for 60 min followed by heating at 95°C for 5 min to inactivate miScript Reverse Transcriptase. Depending on the amount of total RNA used for RT-PCR, the resulting cDNA samples were diluted before use as a template for miRNA quantitative real-time PCR assay.

Quantitative real-time PCR analysis of selected candidate genes

Gene specific primers were designed using Primer3 Program version 4.0 (http://primer3.ut.ee/) [23]. Details of primers are described in (Table 1). The specificity of each primer amplicon was evaluated by sequencing the PCR products using GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter). Quantitative real-time PCR of mRNAs was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City; CA), using SYBR Green/ ROX Mix (Qiagen, Hilden; Germany), with the following program: 95°C for 3 min, 40 cycles at 95°C for 15 s, 60°C for 1 min and 95°C for 1 min. Melting curve was evaluated at the end of the run to observe the specificity of the amplification. The data was analyzed by the comparative threshold cycle (ΔCt) method and normalization was performed using geometric mean of GAPDH, β-actin (ACTB) and 18S.

Quantitative real-time PCR analysis of selected candidate miRNAs

The differential expressed genes in BOECs due to *LPS* challenge were uploaded into miRNA prediction tools namely: DIANA-microT v3.0 (http://diana.cslab.ece.ntua.gr/microT/) and miRecords (http://mirecords.biolead.org/). Then we filtered the miRNA hits on the basis their potential relevance for physiological function and immune response of oviduct at least in four different search algorithms, and thus, miR-16, miR-21, miR-223, miR-215, miR-146a and miR-155 were identified as a potential miRNAs targeting our genes of interest. Interestingly, miR-16, miR-21, miR-223 and miR-215 were reported by [24], where these miRNAs are differentially expressed between healthy and sub-clinical endometritis cows, but miR-146a and miR-155 are indicated to be endotoxin-responsive genes [25,26]. Sequence specific miRNA primers were used to quantify the candidate miRNAs with the corresponding mature sequences listed in (Table 2). In addition, a functional annotation analysis was performed using DAVID Bioinformatics Resource (http://david.abcc.ncifcrf.gov/).

Quantitative real-time PCR of these miRNAs was performed using sequence specific miRNA primer sets and miScript SYBR® Green PCR kit (Qiagen, Hilden; Germany). Prior to real-time PCR profiling, 200 µl RNase-free water was added to each 20 µl reverse-transcription reaction. A PCR master mix was prepared using 2 µl of cDNA template, 12.5 µl of 2x QuantiTect SYBR Green PCR Master mix, 2.5 µl of 10x miScript Universal primers, 2.5 µl of 10x miScript Primer Assay. RNase-free water was added to final volume 25 µl. Quantitative real-time PCR was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City; CA). The thermal cycling conditions were initial activation step 15 min at 95°C followed by 3-step cycling; denaturation 15 s at 94°C, annealing 30 s at 55°C and extension 30 s at 70°C (40 cycles). Melting curve analysis was constructed to verify the presence of

specific amplification. The data was analysed by the comparative threshold cycle method and normalization was done using geometric mean of the two endogenous controls [5S and U6 snRNA (Exiqon, Vedbaek; Denmark)].

ELISA for TNFα and PGs (PGE_2 and $PGF_{2\alpha}$) concentrations in the culture media

The concentration of TNFα and PGs were measured in the cell culture supernatant from *LPS* challenge and control cell groups at different time points using commercially available bovine specific TNFα (Bethyl Laboratories, Montgomery; USA) and PGE_2 &$PGF_{2\alpha}$ (Oxford Biomedical Research, Oxford; USA), respectively, following the manufacturer's instructions. The optical density (OD) value was detected using ELISA microplate reader (Labequip Ltd, Ontario; Canada) at 450 nm wavelengths for TNFα and at 650 nm for PGs using an ELISA microplate reader (Labequip Ltd, Ontario; Canada).

Immunoblotting

Proteins from lysates of cultured cells were normalized to 1 mg/ml using a NanoDrop ND-8000 spectrophotometer and separated (10 µg/lane) using gradient gel 4–18% (vol/vol) SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membrane (Whatman- Protran®, Rodgau; Germany). Membranes were incubated with antibodies for TNFα (1:400), (LifeSpan Biosciences; North America), OVGP1 (1:200), and TGFβ1 (1:200), (Santa Cruz Biotechnology, CA; USA) separately for the same membrane by using stripping buffer (mild stripping) according to Abcam's protocol (http://www.abcam.com/ps/pdf/protocols/Stripping%20for%20reprobing.pdf). Protein loading was evaluated and normalized by examining GAPDH protein levels using a GAPDH antibody (Santa Cruz Biotechnology, CA; USA). Densitometric quantification

of immunoreactive bands was carried out using Quantity One analysis software (Bio-Rad, Munich; Germany).

Experiment 2: Effect of LPS challenge of BOECs on embryo development and quality

In vitro production of embryos

Bovine ovaries were collected from local slaughterhouse and transported to the laboratory in 0.9% physiological saline solution 37°C within 1–3 h of slaughter. Ovaries were dipped in 70% ethanol then washed 2–3 times in PBS^{-}. Cumulus oocyte complexes (COCs) were then aspirated from antral follicles (2–8 mm in diameter), only COCs with a homogenous cytoplasm and surrounded by at least three layers of compact cumulus cells were used for *in vitro* maturation, in a group of 50 in modified TCM199 culture media supplemented with 4.4 mM HEPES, 33.9 mM $NaCHO_3$, 0.2 mM sodium pyruvate, 50 mg/ml gentamicin, 10 µl/ml FSH (Folltropin, Vetrepharm) and 12% ECS. After maturation, COCs were co-incubated with concentration of 2×10^6 sperms/ml for *in vitro* fertilization in F-TALP as indicated previously [27]. Subsequently, presumptive zygotes were denuded by repeated pipetting and transferred to SOF culture medium [28,29] supplemented with 10% ECS. Thereafter, presumptive zygotes were transferred into wells containing 400 µl of SOF medium in four-well dishes (Thermo Fisher Scientific, Roskilde; Denmark) alone or with a BOECs (isolation and seeding mentioned before at experiment (1) of material and methods), twenty-five presumptive zygotes (n=25–30/group) were allocated according to the experimental design. Each group of sample namely; embryo+*LPS* free media, embryo+*LPS*, BOECs+embryo and BOEC+embryo+*LPS*, were cultured under mineral oil at 38.7°C until the blastocyst stage. The maturation, fertilization and cultural procedures were performed under 20% oxygen level. Then blastocysts were kept at -80°C for further analysis.

RNA isolation, cDNA synthesis and real-time-PCR of selected genes from different embryo groups

Expression of some candidate genes related to inflammation, growth factor, apoptosis, embryo quality & competence, and stress response in bovine blastocyst cultured either in SOF media or co-culture with BOECs with or without *LPS* was measured by qRT-PCR. For this, total RNA was isolated from three independent biological replicates from each group of blastocysts, using a picopure® RNA isolation kit (Arcturs, CA; USA) according to methods recommended by the manufacturer. Purified RNA was transcribed into cDNA immediately using First Strand cDNA Synthesis Kit (Thermo scientific, Schwerte; Germany). Then real-time PCR was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City; CA), using SYBR Green/ ROX Mix (Qiagen, Hilden; Germany). The thermocycler program was: 95°C for 3 min, 40 cycles at 95°C for 15 s, 60°C for 1 min and 95°C for 1 min. The data was analyzed by the comparative threshold cycle (ΔCt) method and normalization was done using geometric mean of the GAPDH and β-actin (ACTB).

Measurement of ROS

ROS level in blastocyst stage embryos in experiment 2 was performed using the H_2DCFDA fluorescent probe (6-carboxy- 2',7'-dichlorodihydrofluorescin diacetate), (Life technologies, Darmstadt; Germany). Fifteen blastocysts from each group were incubated with 400 µl of 5 µM H_2DCFDA for 20 minutes in dark at 37°C. Then embryos were washed twice in PBS and the images were captured immediately under inverted microscope (Leica DM IRB, Germany) using green fluorescence filter.

Analysis of mitochondrial distribution

We visualized the pattern of mitochondrial distribution in bovine blastocyst on day 7 cultured either in normal SOF or co-cultured with BOECs, challenged with or without *LPS*, using MitoTracker® Red CMXRos (Invitrogen, Darmstadt;, Germany). Ten blastocysts from each group were incubated with 200nM of MitoTracker red dye for 45 minutes followed by twice washing with PBS^- and fixed with 4% formaldehyde in 4°C for overnight. Fixed specimens were mounted on the slide with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame; CA). The stained embryos were examined under a confocal laser scanning microscope, CLSM LSM-780 (Carl Zeiss, Germany), at 40 x magnification.

Detection of DNA fragmentation by TUNEL assay

In situ cell death was detected using Tunel assay (Roche®, Mannheim; Germany), as described by Paula-Lopes and Hansen [29,30]. Briefly, after embryos were fixed and permeabilized, all specimens were incubated in micro-drops of the TUNEL Kit containing 10% of the enzymatic solution (deoxinucleotidil terminal transferase enzyme) with 90% of the marking solution (2´-deoxyuridine 5´-triphosphate-dUTP+fluorescein isothiocyanate-conjugated-FITC) for 1h in a humid chamber at 37°C in the dark. Whereas, a positive control was performed by treating samples with 1 IU/µL of DNase (Promega, WI; USA) and negative control was incubated in micro-drops containing only marking solution. After washing, control and experimental samples were stained with Hoechst 33342 (Sigma, Munich; Germany) and mounted in glycerol on histological slides and observed under a fluorescence microscope. Nuclei with green fluorescence (FITC) were considered TUNEL positive (fragmented DNA). Hoechst 33342 stained all healthy and apoptotic cells.

Data analysis

Statistical analysis of expression data was performed using Student's t test. The values shown in graphs are presented as the mean ± standard deviation (SD) of at least three independent experiments each done in quadruplicate, *p-values* < 0.05 were considered statistically significant. GraphPad Prism 5.0 was used for data plotting.

Results

Effect of LPS on viability of oviduct epithelial cells

The oviduct epithelial cells viability was determined after the cells were challenged with minimal dose of *LPS* for 24h. We found the cells viability were significantly higher ($p < 0.001$) in control group compared with the challenged group (Fig. 1).

Changes in expression of genes associated with inflammation and immunological response of BOECs after LPS challenge

The relative abundance of IL1β, TRAF6, TNFα, CASP3, TGFβ1 and SOD was increased significantly ($0.001 \leq p \leq 0.01$) and the expression level of OVGP1 and IGF2 was decreased significantly in the challenged group compared with control (Fig. 2). Furthermore, stimulation of primary BOECs with minimum dose of *LPS* significantly increased the expression of TLR4 and its accessory molecules (CD14/MyD88). Moreover, the expression level of the stress response genes; SOD was significantly up-regulated (Fig. 3), whereas the level of GPX4 was reduced after *LPS* challenge at 24h (data not shown). These results revealed that minimum dose of *LPS* can have a profound effect on transcriptome profile of primary bovine oviduct epithelial cells.

Temporal pattern of miRNAs potentially targeting inflammatory response genes

We identified the potential regulatory miRNAs (miR-155, miR-146a, miR-223, miR-21, miR-16 and miR-215) targeting the candidate genes in oviductal epithelial cells, using bioinformatics tools. Then we checked alignment between seed region and 3´ UTR of selected candidate genes (Supplemental Fig. 1). Furthermore, we observed the dynamics pattern of microRNAs expression level in BOECs after *LPS* challenge at different time points (0, 3, 6, 12, 24 and 48h). Surprisingly, all miRNAs except miR-21 were significantly increased at 6h after *LPS* treatment. The expression level of some miRNAs was found to show a reciprocal pattern to their target genes (TRAF6, IL1β, TGFβ1 and TNFα), whereas the expression level of some miRNAs were found to have a similar pattern to their target genes (IGF2, OVGP1 and INOS), (Supplemental Fig. 2–4). The overall results showed that miR-155, miR-146a, miR-223, miR-21, miR-16 and miR-215 have shown a clear inhibition in challenged group after BOECs treated with *LPS* for 24h (Fig. 4).

Protein level in primary BOECs in response to LPS

We have determined the level of TNFα and $PGE_2/PGF_{2\alpha}$ ratio in cell culture supernatant at different time points post-*LPS* treatment. TNFα level was significantly increased in *LPS* treated group compared with untreated control and the $PGE_2/PGF_{2\alpha}$ ratio was significantly higher in the challenged group (Fig. 5A). The OVGP1 immunoreactive protein was lower after *LPS* challenge compared with untreated controls. On the other hand, as evidenced by a clear band, TGFβ1 and TNFα were found to be higher in treated group compared with the control (Fig. 5B).

Effect of LPS treated BOECs on co-cultured embryos

The cleavage rate of cultured or co-cultured embryos was not affected significantly among groups namely; embryo+*LPS* free media (83.75%), embryo+*LPS* (85.38%), BOECs+embryo (86.20%) and BOEC+embryo+*LPS* (88.00%). On the other hand, embryos challenged with *LPS* with or without BOECs, resulted in significantly lower blastocyst rates (15.66±7.78, 25.60±6.84, respectively), than those unchallenged embryos (22.59±10.98, 37.51±9.47, respectively) (Table 3).

Alterations in relative abundance of mRNA in co-cultured bovine blastocyst

Here we quantified some candidate genes related to inflammation (NFκB, LIF, CSF1 and TNFα), growth factor (IGF1), apoptosis (CASP3), marker for embryo quality & competence (CTSB) and stress response (SOD and CAT), in bovine blastocyst cultured either in SOF media or co-culture with BOECs with or without *LPS*. The inflammatory response genes (NFκB, LIF, CSF1 and TNFα) were significantly increased in challenged embryos with *LPS*. Notably, stress response genes as SOD and CAT were significantly higher expressed in *LPS* treated groups compared with untreated. Furthermore, embryos quality gene (CTSB) and apoptotic gene (CASP3) were up-regulated in embryos challenged with *LPS*. Only IGF1 was up-regulated in untreated groups (Fig. 6).

Mitochondrial distribution and ROS accumulation in co-cultured bovine blastocyst

In order to gain insight whether *LPS* could alter the distribution pattern of mitochondria in bovine blastocyst, day 7 bovine blastocysts were produced either from SOF media or co-culture with BOECs with/without *LPS*, then incubated with MitoTracker red. We observed inadequate distribution of mitochondria and decreased mitochondrial

functional efficiency, which was associated with higher ROS production in *LPS* treated groups compared with untreated controls. So we suggest that *LPS* has a deteriorated effect on mitochondria, which could compromise further embryonic development (Fig. 7 and supplemental Fig. 5).

Detection and quantification of apoptosis

Distributions of the TUNEL-positive nuclei were higher in *LPS* challenged compared with unchallenged groups (Fig. 8A). In addition to control embryos, embryos co-cultured with BOECs in the absence of *LPS* displayed very few apoptotic nuclei per embryo (5.6% and 2.8%, respectively). In contrast, blastocysts cultured or co-cultured with BOECs in the presence of *LPS* displayed a significant increase in the percentage of apoptotic nuclei per embryo (11.01% and 4.81%, respectively), (Fig. 8B). Therefore, *LPS* induces apoptosis in preimplantation embryos.

Discussion

The oviduct is a sterile milieu in its nature but it is readily contaminated with pathogens via uterus, peritoneal cavity and follicular fluid [9,19,31]. For this, the oviduct should be equipped with an efficient and strictly controlled immune system that would maintain optimal conditions for fertilization and early embryo development. Local immune responses, regulated by the secretions of epithelial cells, form a part of the mucosal innate immunity, recently are termed “epimmunome” [32]. Despite extensive studies demonstrating the negative effect of clinical or sub-clinical endometritis on bovine fertility with respect to molecular genetic aspects of uterine tissue in *in vivo* or *in vitro* models, the functional understanding of bacterial infection on oviductal function has remained elusive.

In the current study, the *in vitro* approach has been used to challenge bovine oviductal epithelial cells with minimum dose of *LPS* (0.5 μg/ml), to elucidate the effect of oviductal infection on expression of inflammation related genes and their regulatory miRNAs, and subsequent influence on embryo development and quality. Here we have evidenced that BOECs immediately recognized low *LPS* dose through TLR4 and its accessory molecules (MyD88 and CD14), which displayed a clear dynamic pattern at different time points post *LPS* stimulation. In addition to stimulate TLR4 and its downstream genes CD14, MyD88, nuclear factor kappa B (NFκB), IL1β and TNFα expression, *LPS* switched off $PGF_{2\alpha}$ production, thus leading to an increase of the $PGE_2/PGF_{2\alpha}$ ratio. This increased PGE_2 production resulted in proliferation of infected epithelial cells [33].

LPS treatment blocked oviductal function by suppression the expression of OVGP1 and IGF2; these genes similar to components of the maternal environment that are necessary for optimal embryonic development, increased blastocyst cell number and birth of a healthy calf [34,35]. In contrast, pro-inflammatory mediators such as TNF, IL1β, TGFβ1, apoptotic gene (CASP3) and stress response genes (SOD & GPX4) were up-regulated in challenged cells [36]. Taken together, these results suggest the existence of an early signaling system to respond to infection in the BOECs.

Accumulative evidences suggest that alterations in the expression of pro-inflammatory mediators seem to be responsible for inappropriate tissue regeneration, embryo implantation failure and other reproductive disorders [37,38]. Also, resolution of the inflammatory response is necessary for reparative mechanisms to take place [39]. It has become clear that miRNAs are instrumental players in the arena of mammalian inflammatory responses. So far, the role miRNAs in bovine oviduct against bacterial infection especially Gram negative bacteria, is unknown. Therefore, we checked some

selected miRNAs, which are targeting most of expressed genes in BOECs in response to *LPS* stimulation. Herein, all miRNAs were decreased after *LPS* challenge. These results are similar to a recent report [40] that mentioned miRNA regulations in mammalian host cells challenged with various microbial pathogens, such as *let-7* were significant decreased in patients with *H.pylori* infection. Moreover, we observed dynamic pattern of miRNAs expression at different time points. Intriguingly, all selected miRNAs except miR-21 reached to peak at 6h after *LPS* stimulation. So it seems that certain miRNA functions may only be revealed at a specific concentration of an environmental trigger. Furthermore, this might hold true for miRNA controlled pathways that are related to immune response [41].

Cytokines are important immunoregulatory mediators at the mammalian maternal-fetal interface. An improper balance of the pro- and anti-inflammatory cytokines (Th1 and Th2, respectively) is known to play a role in the intrauterine infection pathway [42,43]. Here, we checked embryonic development and quality in terms of cleavage rate, blastocyst rate, mitochondrial activity, ROS accumulation and apoptosis, after *LPS* treatment. No significant differences were observed in cleavage rate among groups, but blastocyst rate was obviously decreased in challenged groups. These results are consistent with previous reports [18,44,45], which showed that exposure of embryos to improper surrounding environment lead to accumulation of free radicals, thus resulting in lower embryo quality, survival and a delay in embryonic development.

In the present study, we found that NFκB, LIF, CSF1, TNFα were over-expressed in blastocysts produced in the presence of *LPS*. Aberrant expression of these inflammatory cytokine and increased NFκB expression are some of the molecular factors that contribute to immune response disorders [46]. Notably, *LPS* showed

modulation the expression of different cytokines like TNFα and growth factors like CSF1 [37]. In addition, *LPS* potentiated the release of reactive oxygen through TNFα-induced ROS production, which is known to activate NF-κB [47]. Beside the pro-inflammatory actions of TNFα, recently it was observed that the release of TNFα is associated with an increased oxidative stress [48,49] and it serves a role of ROS as second messenger to activate signaling pathways and lead to alterations in gene expression [50,51].

A recent study by [52] suggested that mitochondrial dynamics are an important constituent of cellular quality control and function. Moreover, mitochondrial ROS are important for modulating immunoreactions as part of the innate immune system through NFκB [53,54]. Furthermore, maintaining mitochondrial functions with respect to energy production and apoptosis is crucial for cellular quality and development [55]. Similarly, here we demonstrated significant alterations in mitochondrial distribution patterns in embryos challenged with *LPS*. Moreover, these alterations were associated with higher ROS production in *LPS* treated groups. Also, we examined expression of the stress response genes (SOD and CAT) in blastocysts. The produced blastocyst in *LPS* treated groups showed higher abundance of SOD and CAT and this was accompanied by higher ROS generation. So we suggested *LPS* induced a remarkable increase in SOD and CAT mRNA levels, which were insufficient to scavenge the whole produced ROS, whereas *LPS* and cytokines could act synergistically to evoke more ROS [56].

Apoptosis is known to be associated with the quality and viability of mammalian embryos at preimplantation stages and it may more likely occur because of suboptimal conditions [57-59]. In the current study, *LPS* elicited a series of signal transduction events that evoke numerous biochemical mediators, including cytokines (TNFα and CSF1) and toxic free radicals. Successful pregnancy requires a delicate balance between

pro-inflammatory (Th1) and anti-inflammatory molecules (Th2), to maintain maternal immune system integrity, while preventing rejection of the embryo [60]. Therefore, the disturbance of these mediators showed an inhibitory effect on cell growth or proliferation and enhanced apoptosis in *LPS* treated groups. In agreement with previous studies [61,62], we found that CTSB and CASP3 expression increased in blastocysts challenged with *LPS* and this was associated with inferior embryos quality. Furthermore, we observed a clear suppression of IGF1 expression in groups challenged with *LPS* and this could be related to increased apoptosis and decreased blastocyst quality. Similarly, the previous studies demonstrated that the perturbed IGF signalling pathway within the oviduct affects embryo development and blastocyst cell number [8,35].

Taken together the previous mentioned mechanisms could be implicated in female infertility and early embryonic death, we illustrated it through a schematic drawing model (Fig. 9). These findings shed a new light on relevance of inflammatory condition induced by *LPS* in the oviduct milieu and subsequent early embryo development. It indicated a balance among immune mediators, mother and embryo that could act dependently and synergistically, and is one of the most elegant and fascinating interactions in first cross-talk, which takes place in oviduct between mother and embryo to initiate and maintain the embryonic development and subsequent implantation process. Meanwhile, disturbance of that delicate balance between pro-inflammatory mediators (Th1) and anti-inflammatory mediators (Th2) may be reflected on dynamic function of mitochondria through over-production of ROS and subsequently increased apoptosis during early embryonic development.

References

1. Williams EJ, Fischer DP, Noakes DE, England GC, Rycroft A, et al. (2007) The relationship between uterine pathogen growth density and ovarian function in the postpartum dairy cow. Theriogenology 68: 549-559.
2. Herath S, Lilly ST, Fischer DP, Williams EJ, Dobson H, et al. (2009) Bacterial lipopolysaccharide induces an endocrine switch from prostaglandin F2alpha to prostaglandin E2 in bovine endometrium. Endocrinology 150: 1912-1920.
3. Sheldon IM, Noakes DE, Rycroft AN, Pfeiffer DU, Dobson H (2002) Influence of uterine bacterial contamination after parturition on ovarian dominant follicle selection and follicle growth and function in cattle. Reproduction 123: 837-845.
4. Cronin JG, Turner ML, Goetze L, Bryant CE, Sheldon IM (2012) Toll-like receptor 4 and MYD88-dependent signaling mechanisms of the innate immune system are essential for the response to lipopolysaccharide by epithelial and stromal cells of the bovine endometrium. Biol Reprod 86: 51.
5. Sheldon IM, Cronin J, Goetze L, Donofrio G, Schuberth HJ (2009) Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. Biol Reprod 81: 1025-1032.
6. Hunter RH (2003) Reflections upon sperm-endosalpingeal and sperm-zona pellucida interactions in vivo and in vitro. Reprod Domest Anim 38: 147-154.
7. Herath S, Lilly ST, Santos NR, Gilbert RO, Goetze L, et al. (2009) Expression of genes associated with immunity in the endometrium of cattle with disparate postpartum uterine disease and fertility. Reprod Biol Endocrinol 7: 55.
8. Yilmaz O, Calka J, Bukowski R, Zalecki M, Wasowicz K, et al. (2012) Nitric oxide in the bovine oviduct: influence on contractile activity and nitric oxide synthase isoforms localization. Theriogenology 77: 1312-1327.
9. Herath S, Williams EJ, Lilly ST, Gilbert RO, Dobson H, et al. (2007) Ovarian follicular cells have innate immune capabilities that modulate their endocrine function. Reproduction 134: 683-693.
10. Carletti MZ, Christenson LK (2009) MicroRNA in the ovary and female reproductive tract. J Anim Sci 87: E29-38.
11. O'Neill LA (2006) How Toll-like receptors signal: what we know and what we don't know. Curr Opin Immunol 18: 3-9.
12. Cook DN, Pisetsky DS, Schwartz DA (2004) Toll-like receptors in the pathogenesis of human disease. Nat Immunol 5: 975-979.
13. Murphy AJ, Guyre PM, Pioli PA (2010) Estradiol suppresses NF-kappa B activation through coordinated regulation of let-7a and miR-125b in primary human macrophages. J Immunol 184: 5029-5037.
14. Horne AW, Stock SJ, King AE (2008) Innate immunity and disorders of the female reproductive tract. Reproduction 135: 739-749.
15. Wira CR, Fahey JV, Sentman CL, Pioli PA, Shen L (2005) Innate and adaptive immunity in female genital tract: cellular responses and interactions. Immunol Rev 206: 306-335.
16. Humblot P (2001) Use of pregnancy specific proteins and progesterone assays to monitor pregnancy and determine the timing, frequencies and sources of embryonic mortality in ruminants. Theriogenology 56: 1417-1433.
17. Diskin MG, Morris DG (2008) Embryonic and early foetal losses in cattle and other ruminants. Reprod Domest Anim 43 Suppl 2: 260-267.

18. Walsh SW, Williams EJ, Evans AC (2011) A review of the causes of poor fertility in high milk producing dairy cows. Anim Reprod Sci 123: 127-138.
19. Hunter RH (2012) Components of oviduct physiology in eutherian mammals. Biol Rev Camb Philos Soc 87: 244-255.
20. Rottmayer R, Ulbrich SE, Kolle S, Prelle K, Neumueller C, et al. (2006) A bovine oviduct epithelial cell suspension culture system suitable for studying embryo-maternal interactions: morphological and functional characterization. Reproduction 132: 637-648.
21. Herath S, Fischer DP, Werling D, Williams EJ, Lilly ST, et al. (2006) Expression and function of Toll-like receptor 4 in the endometrial cells of the uterus. Endocrinology 147: 562-570.
22. Harris SG, Padilla J, Koumas L, Ray D, Phipps RP (2002) Prostaglandins as modulators of immunity. Trends Immunol 23: 144-150.
23. Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132: 365-386.
24. Hailemariam D, Ibrahim S, Hoelker M, Drillich M, Heuwieser W, et al. (2013) MicroRNA-regulated molecular mechanism underlying bovine subclinical endometritis. Reprod Fertil Dev: 898-913.
25. Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, et al. (2007) Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol 179: 5082-5089.
26. Schulte LN, Westermann AJ, Vogel J (2013) Differential activation and functional specialization of miR-146 and miR-155 in innate immune sensing. Nucleic Acids Res 41: 542-553.
27. Parrish JJ, Krogenaes A, Susko-Parrish JL (1995) Effect of bovine sperm separation by either swim-up or Percoll method on success of in vitro fertilization and early embryonic development. Theriogenology 44: 859-869.
28. Holm P, Booth PJ, Schmidt MH, Greve T, Callesen H (1999) High bovine blastocyst development in a static in vitro production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology 52: 683-700.
29. Sudano MJ, Paschoal DM, Rascado Tda S, Magalhaes LC, Crocomo LF, et al. (2011) Lipid content and apoptosis of in vitro-produced bovine embryos as determinants of susceptibility to vitrification. Theriogenology 75: 1211-1220.
30. Paula-Lopes FF, Hansen PJ (2002) Heat shock-induced apoptosis in preimplantation bovine embryos is a developmentally regulated phenomenon. Biol Reprod 66: 1169-1177.
31. Kowsar R, Hambruch N, Liu J, Shimizu T, Pfarrer C, et al. (2013) Regulation of innate immune function in bovine oviduct epithelial cells in culture: the homeostatic role of epithelial cells in balancing TH1/TH2 response. J Reprod Dev 59: 470-478.
32. Swamy M, Jamora C, Havran W, Hayday A (2010) Epithelial decision makers: in search of the 'epimmunome'. Nat Immunol 11: 656-665.
33. Fukata M, Chen A, Klepper A, Krishnareddy S, Vamadevan AS, et al. (2006) Cox-2 is regulated by Toll-like receptor-4 (TLR4) signaling: Role in proliferation and apoptosis in the intestine. Gastroenterology 131: 862-877.
34. Killian GJ (2004) Evidence for the role of oviduct secretions in sperm function, fertilization and embryo development. Anim Reprod Sci 82-83: 141-153.

35. Neira JA, Tainturier D, Pena MA, Martal J (2010) Effect of the association of IGF-I, IGF-II, bFGF, TGF-beta1, GM-CSF, and LIF on the development of bovine embryos produced in vitro. Theriogenology 73: 595-604.
36. Hvid M, Baczynska A, Deleuran B, Fedder J, Knudsen HJ, et al. (2007) Interleukin-1 is the initiator of Fallopian tube destruction during Chlamydia trachomatis infection. Cell Microbiol 9: 2795-2803.
37. Jaiswal YK, Chaturvedi MM, Deb K (2006) Effect of bacterial endotoxins on superovulated mouse embryos in vivo: is CSF-1 involved in endotoxin-induced pregnancy loss? Infect Dis Obstet Gynecol 2006: 32050.
38. Chapwanya A, Meade KG, Doherty ML, Callanan JJ, Mee JF, et al. (2009) Histopathological and molecular evaluation of Holstein-Friesian cows postpartum: toward an improved understanding of uterine innate immunity. Theriogenology 71: 1396-1407.
39. Pan Q, Chegini N (2008) MicroRNA signature and regulatory functions in the endometrium during normal and disease states. Semin Reprod Med 26: 479-493.
40. Teng GG, Wang WH, Dai Y, Wang SJ, Chu YX, et al. (2013) Let-7b is involved in the inflammation and immune responses associated with Helicobacter pylori infection by targeting Toll-like receptor 4. PLoS One 8: e56709.
41. Eulalio A, Schulte L, Vogel J (2012) The mammalian microRNA response to bacterial infections. RNA Biol 9: 742-750.
42. McGregor JA, French JI (2000) Bacterial vaginosis in pregnancy. Obstet Gynecol Surv 55: S1-19.
43. Raghupathy R, Kalinka J (2008) Cytokine imbalance in pregnancy complications and its modulation. Front Biosci 13: 985-994.
44. Guerin P, El Mouatassim S, Menezo Y (2001) Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum Reprod Update 7: 175-189.
45. Rizos D, Lonergan P, Boland MP, Arroyo-Garcia R, Pintado B, et al. (2002) Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: implications for blastocyst quality. Biol Reprod 66: 589-595.
46. Schetter AJ, Heegaard NH, Harris CC (2010) Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways. Carcinogenesis 31: 37-49.
47. Kastl L, Sauer SW, Ruppert T, Beissbarth T, Becker MS, et al. (2014) TNF-alpha mediates mitochondrial uncoupling and enhances ROS-dependent cell migration via NF-kappaB activation in liver cells. FEBS Lett 588: 175-183.
48. Manna SK, Zhang HJ, Yan T, Oberley LW, Aggarwal BB (1998) Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. J Biol Chem 273: 13245-13254.
49. An L, Wang X, Cederbaum AI (2012) Cytokines in alcoholic liver disease. Arch Toxicol 86: 1337-1348.
50. Droge W (2002) Free radicals in the physiological control of cell function. Physiol Rev 82: 47-95.
51. Weinberg F, Hamanaka R, Wheaton WW, Weinberg S, Joseph J, et al. (2010) Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. Proc Natl Acad Sci U S A 107: 8788-8793.

52. Park J, Choi H, Min JS, Park SJ, Kim JH, et al. (2013) Mitochondrial dynamics modulate the expression of pro-inflammatory mediators in microglial cells. J Neurochem 127: 221-232.
53. Kasahara E, Sekiyama A, Hori M, Hara K, Takahashi N, et al. (2011) Mitochondrial density contributes to the immune response of macrophages to lipopolysaccharide via the MAPK pathway. FEBS Lett 585: 2263-2268.
54. West AP, Brodsky IE, Rahner C, Woo DK, Erdjument-Bromage H, et al. (2011) TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. Nature 472: 476-480.
55. Chan DC (2006) Mitochondria: dynamic organelles in disease, aging, and development. Cell 125: 1241-1252.
56. Sugino N, Telleria CM, Gibori G (1998) Differential regulation of copper-zinc superoxide dismutase and manganese superoxide dismutase in the rat corpus luteum: induction of manganese superoxide dismutase messenger ribonucleic acid by inflammatory cytokines. Biol Reprod 59: 208-215.
57. Makarevich AV, Markkula M (2002) Apoptosis and cell proliferation potential of bovine embryos stimulated with insulin-like growth factor I during in vitro maturation and culture. Biol Reprod 66: 386-392.
58. Hardy K (1999) Apoptosis in the human embryo. Rev Reprod 4: 125-134.
59. Makarevich AV MP, Lukac N, Pivko J (2008) Apoptosis detection as a tool for the determination of animal embryo quality. Slovak J Anim Sci 41: 153 - 159.
60. Walker CG, Meier S, Littlejohn MD, Lehnert K, Roche JR, et al. (2010) Modulation of the maternal immune system by the pre-implantation embryo. BMC Genomics 11: 474.
61. Altmae S, Reimand J, Hovatta O, Zhang P, Kere J, et al. (2012) Research resource: interactome of human embryo implantation: identification of gene expression pathways, regulation, and integrated regulatory networks. Mol Endocrinol 26: 203-217.
62. Balboula AZ, Yamanaka K, Sakatani M, Hegab AO, Zaabel SM, et al. (2010) Intracellular cathepsin B activity is inversely correlated with the quality and developmental competence of bovine preimplantation embryos. Mol Reprod Dev 77: 1031-1039.
63. Oron E, Ivanova N (2012) Cell fate regulation in early mammalian development. Phys Biol 9: 045002.

Table 1. List of primers that were used for semi-quantitative PCR analysis of target genes

Gene name	Accession no.	Primer sequence (5′→3′)	Annealing temperature (°C)
CAT	NM_001035386	F: AGCCAGAAGAGAAACCCTCA R: CTGCCTCTCCATTTGCATTA	53
TGFβ1	NM_000660	F: CACGTGGAGCTGTACCAGAA R: GCGAAAGCCCTCTATTTCCT	55
IGF1	NM_001077828	F: TTGCACTTCAGAAGCAATGG R: ACTGGAGAGCATCCACCAAC	54
INOS	NM_001076799	F: TGTTCAGCTGTGCCTTCAAC R: AAAGCGCAGAACTGAGGGTA	55
CASP3	NM_001077840	F: TGCCACTGTATCAGGGAACA R: TGCTCAGCACAAACATCACA	52
TRAF6	NM_001034661	F: CCCAGGCTGTTCAGACTTTA R: CATACATGCTCTGGGTTTCC	53
CD14	NM_174008	F: TATCGTGGACAACAGGAGGT R: GCGTAGCGCTAGATATTGGA	54
TLR4	NM_174198	F: AGAGCCACTTCTGGTCACAG R: TAAAGCTCAGGTCCAGCATC	55
MYD88	NM_001014382	F: CCTCTCATCTGCCTTTTTGA R: GCCCCAGAAAGAAAGACTTC	53
CD45	NM_001206523	F: CAAAGAGCCCAGGAAGTACA R: GTTGATCTCCACAATCACA	51
IL1β	NM_174093	F: CCTTGGGTATCAAGGACAAG R: CGATTTGAGAAGTGCTGATG	53
SOD	NM_174615	F: CCTTGGGTATCAAGGACAAG R: CGATTTGAGAAGTGCTGATG	50
TNFα	NM_173966	F: CTTCCACCCCCTTGTTCCT R: AGGCGATCTCCCTTCTCCA	55
IGF2	XM_005227270	F: GCCCTGCTGGAGACTTACTG R: GGTGACTCTTGGCCTCTCTG	54
OVGP1	NM_001080216	F: CTCTGCACCCACCTGGTATT R: GCGATCACTGAACTGACGAA	54
GAPDH	NM_001034034	F: ACCCAGAAGACTGTGGATGG R: ACGCCTGCTTCACCACCTT	57
18S	NR_036642	F: CGCAGCTAGGAATAATGGAA R: TCTGATCGTCTTCGAACCTC	53
ACTB	NM_173979	F: GGCATTCACGAAACTACCTT R: CAATCCACACGGAGTACTTG	53
NFκB1	NM_001076409	F: AATTTGGGAAGGATTTGGAG R: CTGTCGTTTCCTTTGCACTT	55
CTSB	NM_174031	F: TCCCCATAGACGAACTGTGT R: TCTCAGATCTGTCCCACTCC	55
LIF	NM_173931	F: AGACCAGAAGGTCCTCAACC R: ACAGCCCAGCTTCTTCTTCT	55

CSF1	XM_005204070	F: CTTTGTCACTGGGATGGAAG R: TTTCCTGATCCAGAGAGTGC	55

Table 2. The list of miRNAs with the corresponding mature sequences amplified using quantitative real time PCR.

MiR name	Accession no.	Mature miRNA sequence (5′→3′)
bta-miR-16b	MIMAT0003525	UAGCAGCACGUAAAUAUUGGC
bta-miR-21	MIMAT0003528	UAGCUUAUCAGACUGAUGUUGACU
bta-miR-223	MIMAT0009270	UGUCAGUUUGUCAAAUACCCCA
bta-miR-215	MIMAT0003797	AUGACCUAUGAAUUGACAGACA
bta-miR-146a	MIMAT0009236	UGAGAACUGAAUUCCAUAGGUUGU
bta-miR-155	MIMAT0009241	UUAAUGCUAAUCGUGAUAGGGGU

Table 3. Developmental rates of presumptive zygotes which were cultured either in SOF or co-cultured with BOECs, with/without *LPS*

Group	**Matured oocytes**	**Embryonic development**		
	n	**Cleavage rate**		**Blastocyst rate**
		n	**%**	**Mean±SD**
Embryo culture in SOF	**517**	**433**	**83.75**	**22.59±10.98**[a]
Embryo culture in SOF+LPS	**520**	**444**	**85.38**	**155.66±7.78**[b]
Co-culure BOECs+ embryo	**522**	**450**	**86.20**	**37.51±9.47**[c]
Co-culure BOECs+ embryo+LPS	**525**	**462**	**88.00**	**25.60±6.84**[d]

[a,b,c,d] Values with different superscripts within columns differ significantly.

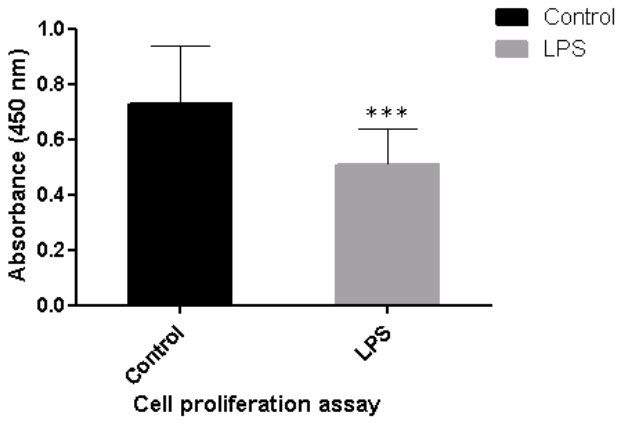


Figure 1. The viability of oviduct epithelial cells 24 hours after *LPS* challenge. ***: $p < 0.001$.

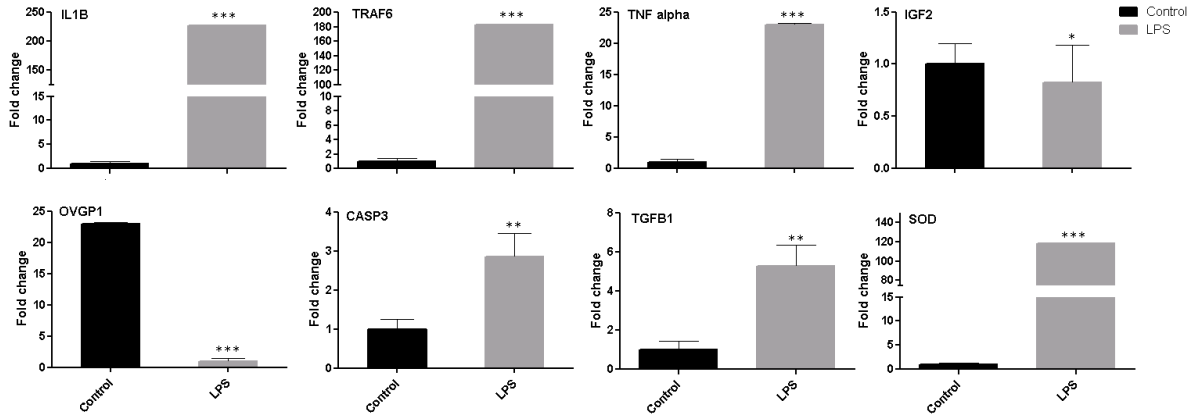


Figure 2. Real-time PCR analysis of candidate inflammatory response genes in oviduct epithelial cells after *LPS* challenge. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

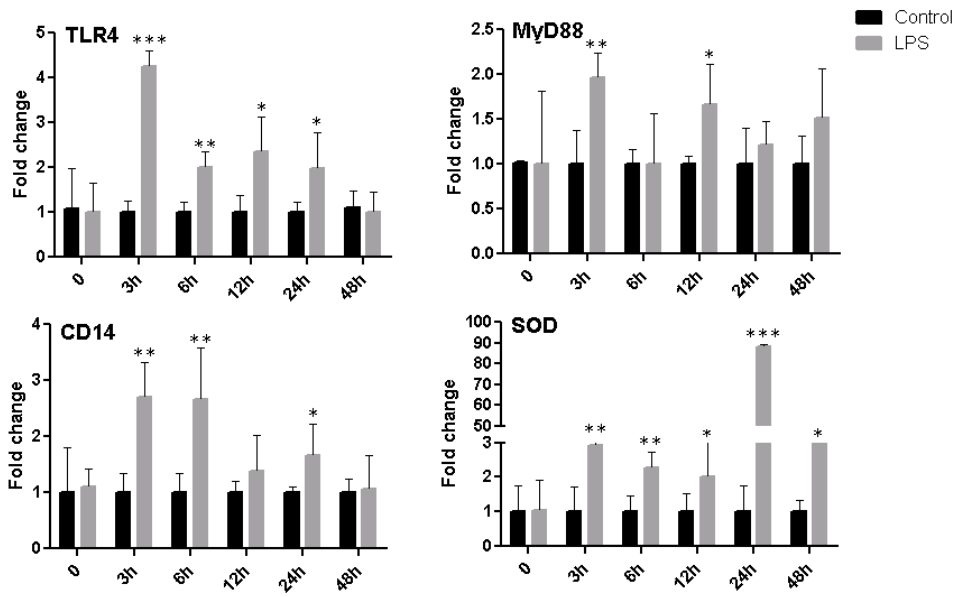


Figure 3. Temporal pattern of immune and stress response genes in BOEC at different time points after *LPS* challenge. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$.

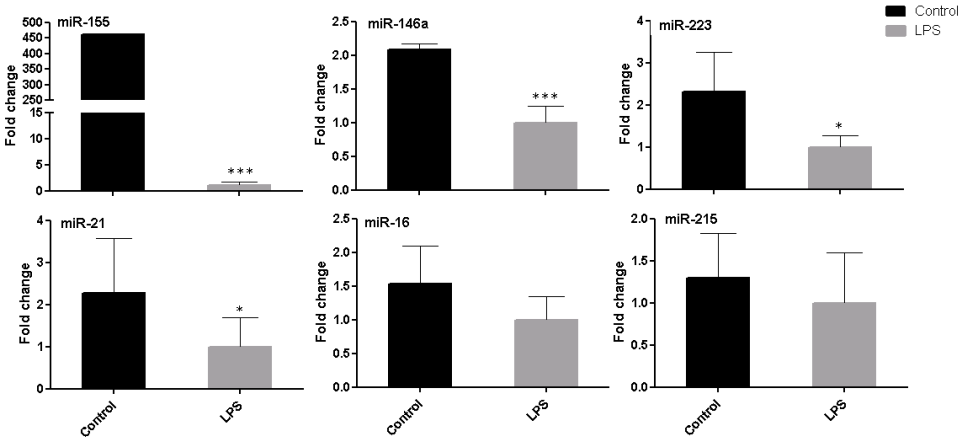


Figure 4. The relative abundance of selected regulatory miRNAs in BOEC after challenge by *LPS*. *; $p < 0.05$, ***; $p < 0.001$.

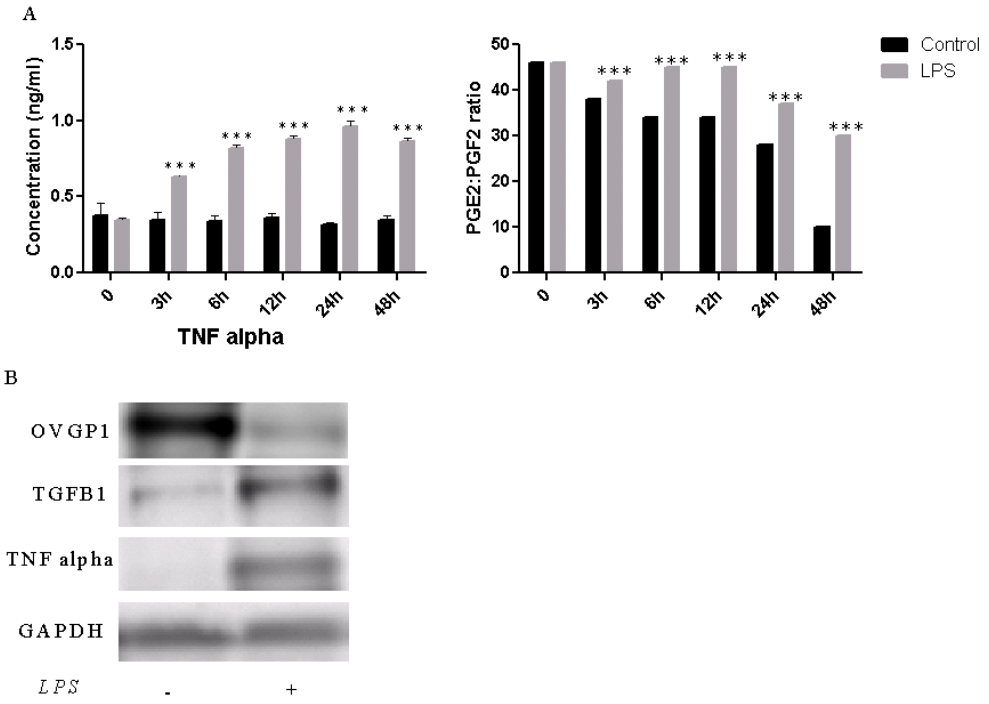


Figure 5. Protein expression analysis in BOEC after 24 hours *LPS* challenge. Enzyme-linked immunosorbent assay (ELISA) of TNFα and prostaglandin E_2 (PGE_2) to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) ratio in cell culture supernatant (A). The level of OVGP1, TGFβ1 and TNFα immunoreactive proteins in oviduct epithelial cells (B). ***; $p < 0.001$.

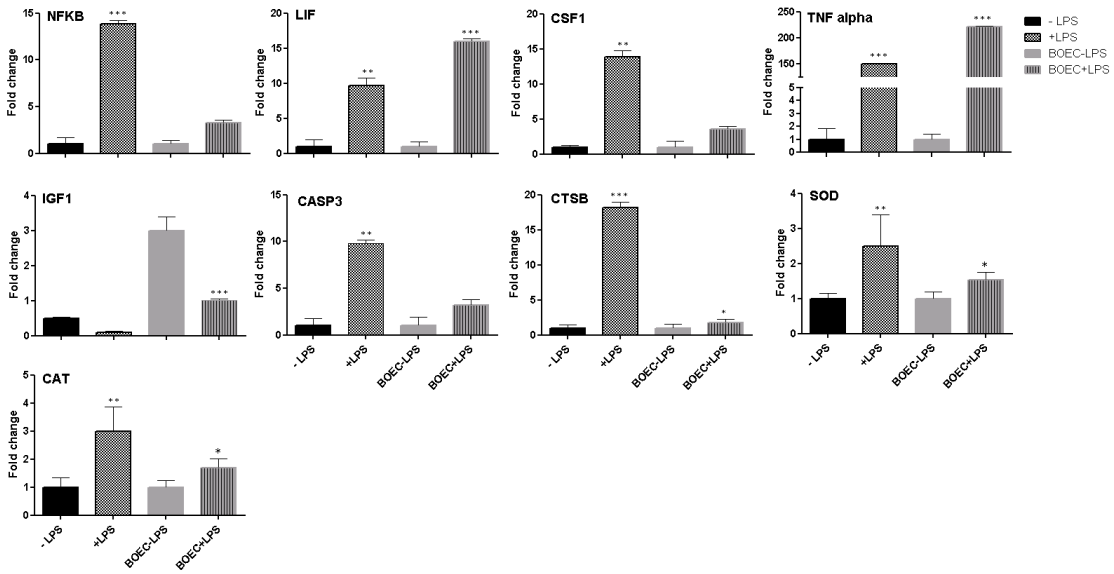


Figure 6. Quantitative expression analysis of genes related to inflammation (NFκB, LIF, CSF1 and TNFα), growth factor (IGF1), apoptosis (CASP3), marker for embryo quality & competence (CTSB) and stress response (SOD and CAT) in bovine blastocysts produced after culture in SOF media or co-culture BOEC with/without *LPS*. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$.

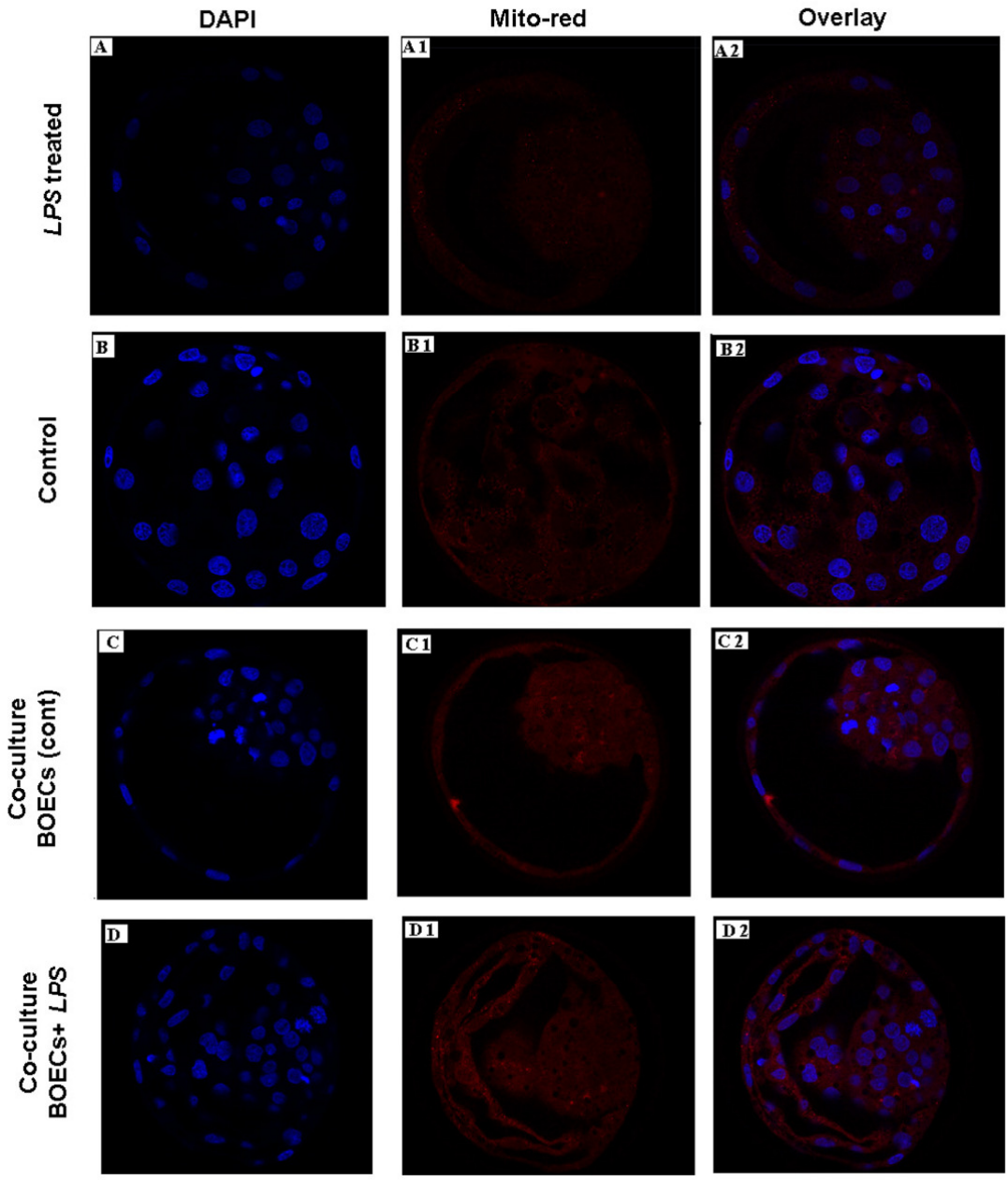


Figure 7. Pattern of active mitochondria as detected by staining with MitoTracker red in bovine blastocyst produced after co-culture *LPS*. A, B, C & D show stained nuclei using DAPI with blue fluorescence. A1, B1, C1& D1 show mitochondria stained with MitoTracker red and A2, B2, C2 & D2 indicate merged images. Original magnification 40×

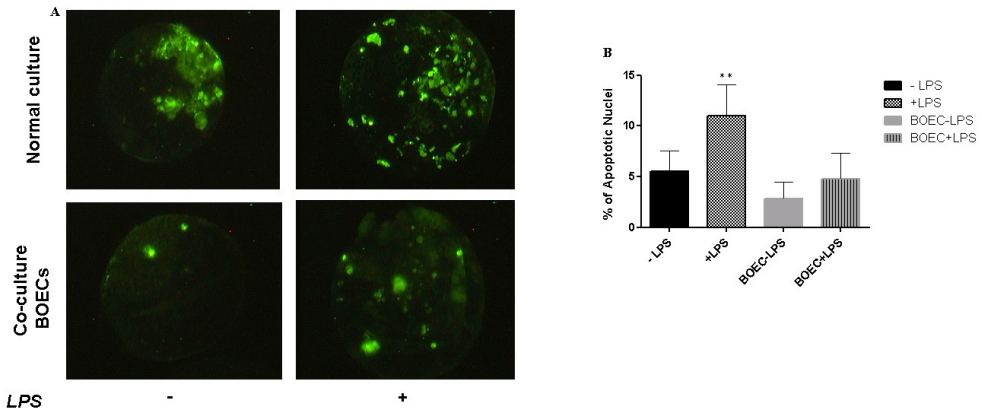


Figure 8. Detection of apoptotic nuclei in bovine blastocysts using TUNEL assay. Representative images of TUNEL assay to assess the level of apoptosis in blastocyst, which were produced either from SOF media or co-culture with BOEC with/without *LPS* (A). The number of individual cells that were TUNEL positive was counted in each blastocyst and is represented as the average number of cells that are TUNEL positive per blastocyst (B). **; $p < 0.01$.

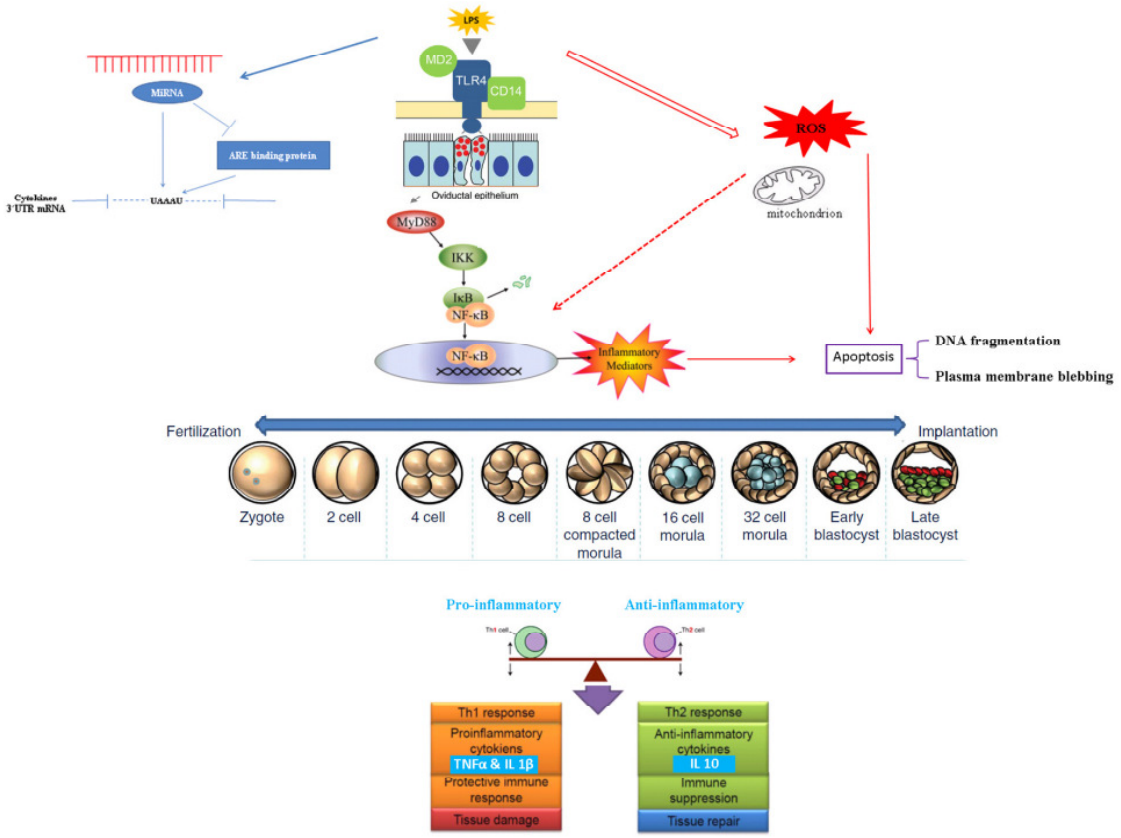
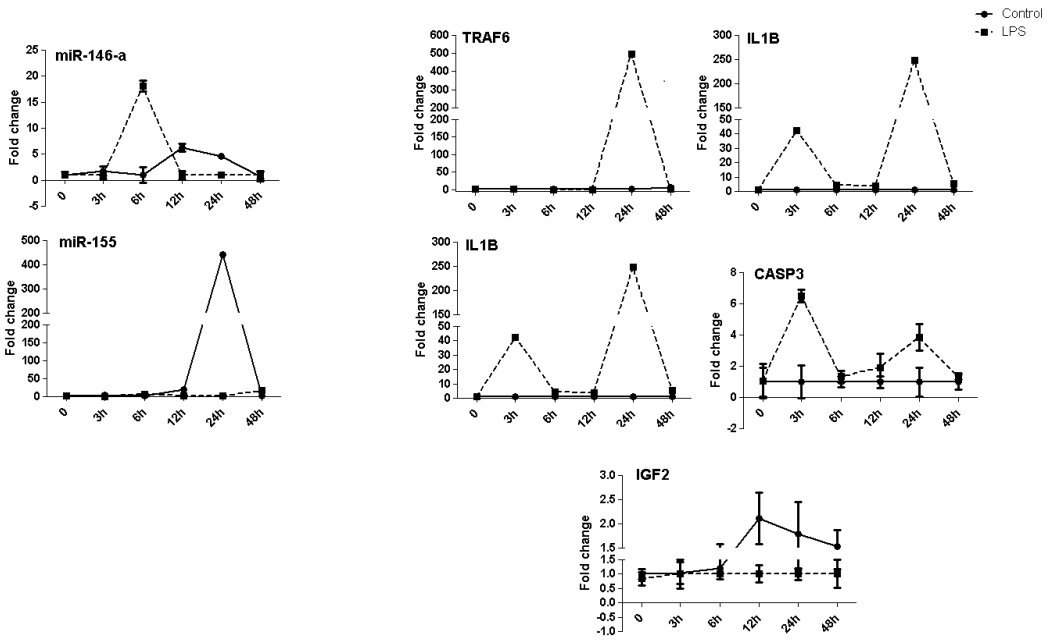
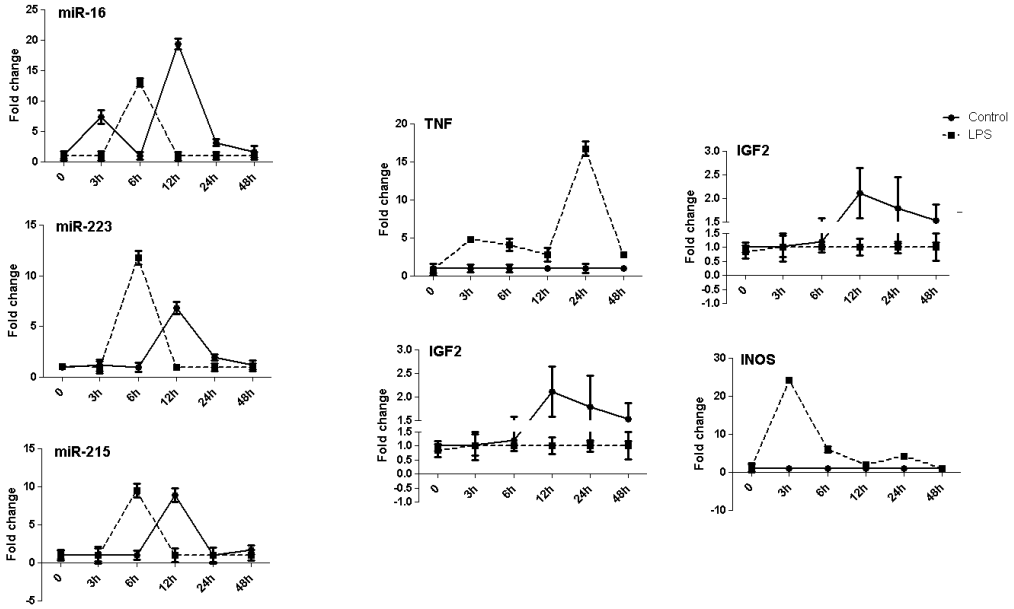


Figure 9. Hypothetical presentation of mechanisms of response during exposure of bovine oviduct to *LPS* and subsequent influence on early embryo development during pregnancy. Hypothetical embryo images are taken from [63]. BOEC are sensitive to minimum dose of *LPS*. They initiated innate immune response after TLR4 recognized *LPS*, then BOEC secreted a plethora of cytokines and chemokines like TNFα and IL1β. *LPS* could have a direct or indirect effect on mitochondria dynamic function in embryo at blastocyst stage, which were associated with higher ROS production as well as higher apoptotic cells. Aberrant regulation of miRNAs may perturb a delicate balance between Th1 and Th2 and subsequently abrogate first cross-talk between mother and embryo in oviduct.

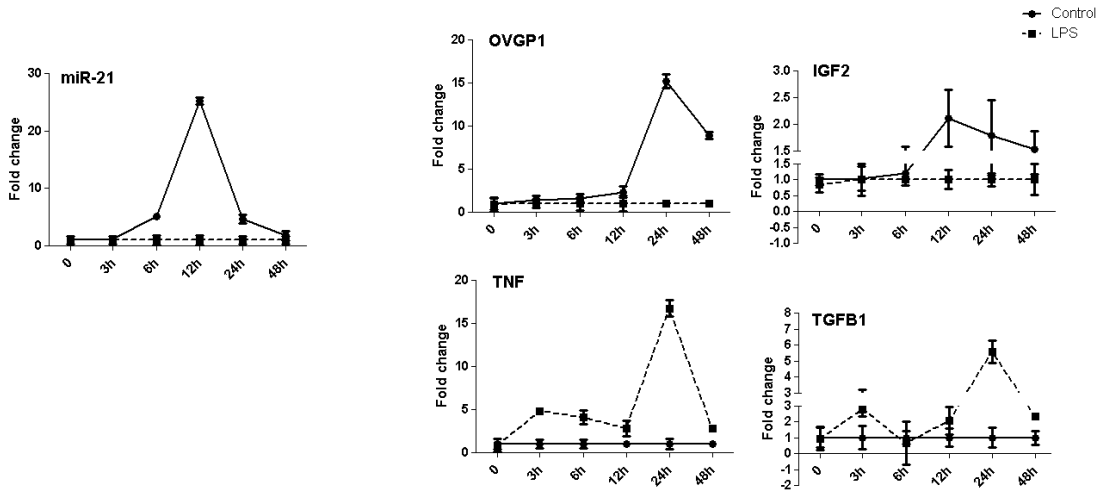
Supplementary Figure 1. The potential regulatory miRNAs and their sequence alignment with the binding sites of 3´ UTR of candidate genes.



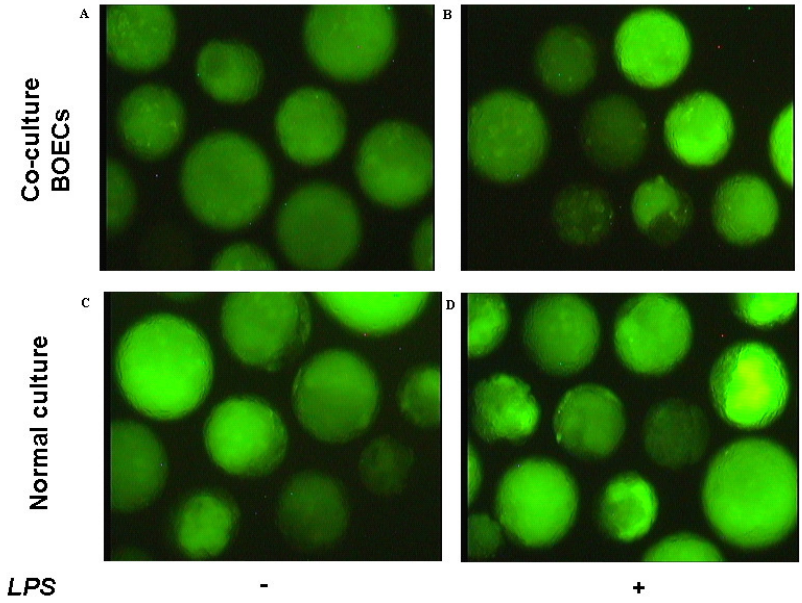
Supplementary Figure 2. Expression profile of miR-146a and its target genes (TRAF6 and IL1β). RT-PCR of miR-155 and its target genes (IL1β, CASP3 and IGF2) in BOEC after *LPS* challenge for 48 hours. Both miRNAs and their target genes show different dynamic patterns at different time points, where the peak of both miRNAs was at 6h after *LPS* stimulation then gradually decrease.



Supplementary Figure 3. Real-time PCR of miR-16 and its target genes (TNFα and IGF2), miR-223 and its target gene (IGF2), and miR-215 and its target gene (INOS) in BOEC after *LPS* challenge for 48 hours. All miRNAs reached their peaks at 6h after *LPS* stimulation. On the other hand, some genes revealed the same trend and/or reciprocal of miRNAs.



Supplementary Figure 4. Pattern of miR-21 expression profiling and its target genes (TNFα, TGFβ1, OVGP1 and IGF2) in BOEC after *LPS* challenge for 48 hours. MiR-21 reached to peak at 12h then gradually reduced post *LPS* challenge. Both OVGP1 and IGF2 shown peaks only in untreated groups. In contrast, TNF and TGFβ1 provide clear peaks in challenged groups.



Supplementary Figure 5. ROS generation was detected by fluorescent probe H_2DCFDA in bovine blastocyst on day 7. ROS production in embryo co-cultured with BOEC without or with *LPS*, (A & B) respectively. ROS production in bovine blastocysts which were cultured in SOF media without or with *LPS*, (C & D) respectively. Scale bars represent 100 μm.

Chapter 3

The regulatory role of *let-7* miRNAs family during bovine clinical and sub-clinical endometritis

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Abstract

The proper function of uterine tissue is regulated by multiple types of transcriptome, which are activated in time of physiological condition dependant manner. The post-transcriptional control of these transcripts is recently getting research attention. Among these the *let-7* miRNAs are potential regulatory non-coding RNA involved in post-transcriptional regulation of genes in various physiological processes. Here, we conducted two experiments. In the first experiment, we challenged primary bovine endometrial cells with clinical (3.0 µg/ml) and sub-clinical (0.5 µg/ml) doses of *lipopolysaccharide* (*LPS*) for 24h. In the 2nd experiment, we have investigated the potential role of *let-7* miRNAs (*let-7a* and *let-7f*) using gain and loss of function and validated some selected genes (TNFα, TGFB1I1 and SDPR) by reporter assay. *Let-7* miRNAs were dysregulated during inflammatory condition induced by *LPS*. Intriguingly, targeting *let-7* miRNAs, which act as rheostats for inflammatory immune response coincided with down-regulation of pro-inflammatory cytokines. Moreover, *let-7a* and *let-7f* directly target novel genes (TNFα, TGFβ1I1 and SDPR). Our results confirmed the role of *let-7* miRNAs in bovine endometritis and generates a novel biological hypothesis that *LPS* could alter *let-7* expression and subsequently was associated with an increase of pro-inflammatory cytokine level by directly/indirectly targeting the TNFα, IL6. Therefore, these alterations could abrogate uterine homeostasis.

Running title: Involvement of *let-7* miRNAs in uterine infection

Introduction

The mammalian uterine milieu is usually a sterile environment, but in cattle bacterial contamination of the uterine lumen is ubiquitous after parturition and up to 40% of animals develop pelvic inflammatory disease (PID) and 20% have endometritis. Infection of the endometrium with *Escherichia coli* (*E. coli*) is a predisposing factor for infection by other pathogens and is associated with the severity of PID as well as a clear impact on female fertility [1,2]**.** The costs of female reproductive disorders and the associated infertility were estimated to be $650 million per annum in the United States [3,4]. Innate immune responses to pathogens are the driving force for eradication of pathogens, regulation of inflammation, and maintenance of uterine homeostasis [5]**.** The assignment of the immune system is to provide protection from invading pathogens, and this system appears to be a pivotal for successful implantation and maintenance of pregnancy [6]. The endometrial cells appear to have a key role in innate immune defence of the female genital tract, and this lead to modulation of endocrine function [7]**.** The innate immune system, including epithelial cells plays a vital role in the first line of defense against foreign infection by recognizing the conserved components of microorganisms through toll-like receptors (TLRs) [8].

Inflammation is highly regulated and dampening inflammation is crucial to maintain homeostasis, where persistent and unhalted inflammation explains the pathogenesis of many inflammatory diseases [9,10]. Resolution of inflammation is partly achieved by regulating mRNA stability of pro-inflammatory cytokines at the post-transcriptional level. Whereas, these transcripts often contain adenylate-uridylate-rich elements (AU-rich) sequences within the 3′-untranslated region (3′-UTR) that are regulated and recognized either by AU-rich element (ARE)-binding proteins like tristetraprolin (TTP), which is one of the best-characterized ARE-binding proteins, or

by microRNAs [11-13]. Recently, it has been demonstrated that the aberrant expression of TTP and *let-7* often result in human disease [14]. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) represents a central transcriptional factor, which is involved in inflammatory immune response, stress, cellular differentiation & proliferation and apoptosis. The activation of NF-κB can be induced by different stimuli and subsequently initiated complex signaling pathways [15].

Lin-4 and the *lethal-7* (*let-7*) are the first two known miRNAs, which were originally discovered in the nematode *Caenorhabditis elegans* and control the timing of cell division and differentiation. *Let-7* and its family members are polycistronic, highly conserved in animal phylogenic tree [16], and misregulation of *let-7* leads to a less differentiated cellular state and the development of cell-based diseases such as cancer [17]. Recently in humans, 10 mature subtypes of the *let-7* family have been identified namely; *let-7a*, *let-7b*, *let-7c*, *let-7d*, *let-7e*, *let-7f*, *let-7g*, *let-7i*, miR-98 and miR-202, in which mature *let-7a* and *let-7f* were produced by precursor sequences (*let-7a-1*, *let-7a-2*, *let-7a-3*; *let-7f-1*, *let-7f-2*), [18]. *Let-7* miRNAs have the potential to regulate IL-10 [19], also *let-7i* regulates toll-like receptor 4 (TLR4) expression [20,21]**.** In normal physiology, *let-7* is primarily involved in development, cell adhesion and gene regulation. The *let-7* family members can differ from each other by as little as a single nucleotide, making the ability to differentiate between forms with single-nucleotide differences important [22].

Recently, we have investigated both miRNAs and genes transcriptome profile in clinically and sub-clinically endometritis cows compared to their healthy counterparts Salilew-Wondim et al. (unpublished data). In that study, a global suppression of *let-7* family members (*let-7a*, *let-7b*, *let-7d*, *let-7e*, *let-7f* and *let-7i*) was observed in the uterine biopsy of clinical and sub-clinical endometritis cows compared to healthy ones.

Potential target genes of *let-7* miRNAs were screened using miRecords (http://mirecords.biolead.org/) and common genes from the results of array experiment and in silico analysis were selected for further investigation. We found that tumor necrosis factor alpha (TNFα), interleukin 6 (IL6), transforming growth factor beta 1 induced transcript 1 (TGFβ1I1) and serum deprivation response (SDPR), which were differentially expressed in clinical and sub-clinical diseased cows, were found to be potentially targeted by *let-7* family miRNAs.

However, how *let-7* family modulates this robust immune response of the bovine endometrium to bacterial inflammation induced by *E. coli* is poorly understood. We hypothesized that aberrant regulation of *let-7* miRNAs could account as a functional link between perturbed transcriptome of endometrial cells with respect to inflammatory immune response between disease and healthy uterine environment. The aim of the present study was (i) to investigate the response of cultured endometrial cells to bacterial *LPS* with respect to the expression of *let-7* family miRNAs, and (ii) to elucidate the role of *let-7* miRNAs in modulation the expression of inflammatory response genes post *LPS* stimulation in bovine endometrium. This study evidenced the aberrant expression of *let-7* miRNAs during bacterial infection and subsequently elucidated the fine-tuning role of *let-7* family members.

Materials and methods

The current study was conducted two parts. Part 1 of the experiment has been carried out to investigate the expression pattern of *let-7* family members in primary bovine endometrial cells (both epithelial and stromal fibroblast cells) after *LPS* stimulation with two different doses, which simulated clinical and sub-clinical inflammatory conditions under *in vitro* conditions. For better understanding the role of *let-7* miRNAs in

inflammatory immune response of uterine tissue, part 2 was designed for functional study, as well as to check temporal pattern of *let-7* family members in response to *LPS* treatment. The whole experiment was replicated in three or more biological pools for further analysis.

Experiment 1

Isolation and culture of primary endometrial cell culture

As described previous by [23] with simple modification, bovine uteri were collected from postpubertal nonpregnant animals with no evidence of genital disease or microbial infection at a local abattoir and kept on ice until further processing in the laboratory. We determined the stage of the reproductive cycle by observation of ovarian morphology, and genital tracts with an ovarian stage I corpus luteum were selected for endometrial culture [24]. The endometrium from the horn ipsilateral to the corpus luteum was cut into smaller pieces and placed into PBS without Ca^{2+}/Mg^{2+} (PBS^{-}), tissue was digested in 25 ml sterile digestive solution, made by dissolving 50 mg trypsin III (Gibco®, Karlsruhe; Germany), 50 mg collagenase II (Sigma, Munich; Germany), and 10 µl deoxyribonuclease I (Qiagen, Hilden; Germany) in 100 ml PBS^{-}. After 1.5 h incubation in a shaking water bath at 37°C, the cell suspension was filtered through a 40-µm mesh to remove undigested material, then the filtrate was resuspended in washing medium comprised of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12), (Gibco®, Karlsruhe; Germany) with 10% fetal bovine serum (Gibco®, Karlsruhe; Germany). The suspension was centrifuged at 100 ×g for 10 min then washed two times in washing medium. The cells were resuspended in DMEM/F-12 containing 10% fetal bovine serum, 10 µl/ml penicillinstreptomycin (Gibco®, Karlsruhe; Germany) and 10 µl/ml Fungizol (Gibco®, Karlsruhe; Germany). The cells were plated at a density of 1 ×

10^5 cells/ml in 24-well plates. To obtain separate stromal and epithelial cell populations, the cell suspension was removed after 18h from seeding, which allowed attachment of stromal cells selectively [23]. The removed cell suspension was then replated and incubated allowing epithelial cells to adhere [25]. Stromal and epithelial cell populations were distinguished by cell morphology as previously described [23], and by immunocytochemistry (ICC) for cytokeratin 18 and vimentin, as markers for epithelial cells and stromal fibroblast, respectively (data not shown) [26,27]. The culture media were changed every 48h until the cells have reached confluency. Cell cultures were maintained at 37°C, 5% CO2 in air, in a humidified incubator.

Experimental treatment

Confluent stromal and epithelial cells were challenged with arachidonic acid (AA, 100 µM), (Sigma, Munich; Germany) and two doses of ultra pure *LPS* from *E. coli* 0111:B4 strain- TLR4 ligand [clinical dose (3.0) and sub-clinical dose (0.5) µg/ml], (InvivoGen, San Diego; USA) for 24h. The *LPS* concentrations reflected those found in the uterine lumen of clinical and sub-clinical infected animals, respectively [28,29], whereas 24h time points were selected on the basis of PGE_2 maximal concentrations according to [5]. Cell viability was assessed by using WST-1 Cell Proliferation Assay Kit (Cayman Chemical, Michigan; USA). The cell culture supernatant was collected from each well for measurement of PGE_2, $PGF_{2\alpha}$, TNFα and IL6 by ELISA. The supernatant and harvested cells were kept in -80°C until further use. The absence of immune cells in the primary epithelial cell and stromal cultures were confirmed by absence of the pan-leukocyte marker (CD45) using PCR as previously described [5], (data not shown).

Experiment 2

Temporal pattern of let-7 family members post LPS treatment

To observe temporal expression pattern of *let-7* family in primary endometrial stromal cells, the confluent cells were stimulated with *LPS* (0.5 µg/ml), then cells were harvested at different time points 0, 3, 6, 12, 24 and 48h. The harvested cells were kept in -80^{o}C till further analysis.

Target prediction and selection of candidates

The target genes of *let-7* miRNAs family were predicted using miRecords (http://mirecords.biolead.org/), and the corresponding target hits were further filtered on the basis of their score and free energy as well as their potential relevance for physiological function and immune response of endometrium at least in four different search algorithms. A functional annotation analysis was performed using DAVID Bioinformatics Resource (http://david.abcc.ncifcrf.gov/) and Ingenuity® Pathway Analysis (http://www.ingenuity.com/). In addition, an online resource to investigate AU-rich elements in mRNA UTR sequences (http://rna.tbi.univie.ac.at/cgi-bin/AREsite.cgi) was used.

Validation of miRNAs: mRNAs interaction

Plasmids containing 3´ UTR of TNF alpha, TGFβ1I1 and SDPR sequences encompassing the predicted binding sites of both *let-7a* and *let-7f*, respectively, were designed using SnapGene Viewer 2.3.5. Primer sequences of TNF 3´ UTR (Forward: 5´- GCGAGCTCTTTATTATTTATTTATTTACTAA-3´ and Reverse: 5´-GCTCTAGATCAGCATCATTTAGACAACTCGA-3´), TGFβ1I1 3´ UTR (Forward: 5´-TCTAGAAAAAAAAAAAAAAAAGAGCTCCACTTTGTTCCCA-3´ and Reverse:

5´-TCTAGATTTTTTTTTTTTTTTTTTTTTTTTGACGTGGAATGTGT-3´) and SDPR 3´ UTR (Forward: 5´-GAGCTCCATTTAGAAATCCAAGACTCATTCTTT -3´ Reverse: 5´-TCTAGAACATGCAGATAGTTTCGCTCCT-3´). The mutant sequence was 5´-…CAAGCGGCCGCAAGGAGACACAAACTAAGGGCTTAATTGTG…-3´. The luciferase assay was performed by transfecting primary bovine endometrial stromal cells with 100 ng of plasmid and 10 nM relevant miRNA mimics or negative-control using Lipofectamine® RNAiMAX Reagent (Invitrogen, CA; USA). Luciferase activity was determined using Dual-Luciferase® Reporter Assay System (Promega, WI; USA) and the Lmax microplate luminometer. Renilla luciferase activity was used to normalize transfection efficiency.

Modulation of let-7 miRNAs by overexpression or inhibition

Confluent primary endometrial stromal fibroblast cells were transfected in 6-well plates using Lipofectamine® RNAiMAX Reagent (Invitrogen, CA; USA). About 240,000 cells were transfected with 150 pmole *let-7* family inhibitors oligonucleotides *hsa-let-7* FI-1 and *hsa-let-7* FI-3 (Exiqon, Vedbaek, Denmark) or 5 nmole *Syn-bta-let-7a-5p* mimic (Qiagen, Hilden; Germany) or 15 pmole *hsa-let-7f-5p* mimic (Exiqon, Vedbaek; Denmark) or their irrelevant control (Exiqon, Vedbaek; Denmark) or BLOCK-iT™ Alexa Fluor® Red Fluorescent Oligo (Invitrogen, CA; USA) and cultured for 48h, following the manufactures instructions. Then transfected cells were challenged with *LPS* (0.5 µg/ml), and further incubated for 24h. Cells were harvested for further analysis.

RNA isolation and cDNA synthesis

Total RNA was isolated from the cells using the miRNeasy® Mini kit (Qiagen, Hilden; Germany) according to manufacture's instructions. On-column genomic DNA digestion was performed using RNase free DNase set on-column (Qiagen, Hilden; Germany). The cDNA was synthesised for small RNA from the isolated total RNA using the miScript II RT kit (Exiqon, Vedbaek; Denmark) following manufacturer's instructions, as well as cDNA was synthesised for large RNA from the isolated total RNA using SuperScript® II (Invitrogen, CA; USA) according to manufacture's instructions.

Quantitative real-time PCR analysis of miRNAs

The quantification of *let-7* family members (*let-7a*, *let-7c*, *let-7d*, *let-7e*, *let-7f* and *let-7i*) was performed using sequence specific miRNA qPCR primer sets and miScript SYBR® Green PCR kit (Exiqon, Vedbaek; Denmark). Quantitative real-time PCR of miRNAs were performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City; CA), the data were analyzed by the comparative threshold cycle (ΔCt) method and normalization was done using the geometric mean of the three endogenous controls 5S, U6 and SNORD48.

Quantitative real-time PCR analysis of target mRNAs

Here, we have selected candidate inflammatory immune response gene as interleukin 6 (IL6), and other genes TNFα, TGFβ1I1, SDPR, NF-κB and caspase 3, apoptosis-related cysteine peptidase (CASP3), which could be potentially targeted by *let-7* miRNAs and may have a crucial role in inflammatory immune response of bovine endometrial cells against bacterial infection. Primers used for this study, were designed using Primer3 Program version 4.0.0 (http://primer3.ut.ee/), [30]. Details of the primers are described in the table 1. Quantitative real-time PCR of mRNAs were performed in a

StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City; CA), the data were analyzed by the comparative threshold cycle (ΔCt) method and normalization was done using the geometric mean of the housekeeping genes GAPDH, β-actin (ACTB) and 18S.

ELISA for TNFα, IL6 and prostaglandins (PGs) concentrations in the supernatant

The concentrations of cytokines (TNFα and IL6) were measured in the cultured supernatant from control and *LPS* stimulated groups using commercially available specific Bovine ELISA (Bethyl Laboratories, Montgomery; USA and Uscn Life Science Inc., Wuhan Hubei; China, respectively). Additionally, the concentrations of PGs were measured in the cultured supernatant from control and *LPS* treated groups using commercially available specific Bovine ELISA, PGE_2 & $PGF_{2\alpha}$ (Oxford Biomedical Research, Oxford; USA), following the manufacturer's instructions. The optical density (OD) value was detected using ELISA microplate reader (Labequip Ltd, Ontario; Canada), using 450 nm for both cytokines and 650 nm for PGE_2 & $PGF_{2\alpha}$.

Analysis of target proteins

Protein was isolated from lysates of cultured cells according to Qiagen User-Developed Protocol. Next protein was normalized to 1 mg/ml using a NanoDrop ND-8000 spectrophotometer and separated (10 µg/lane) using gradient gel 4–18% (vol/vol) SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membrane (Whatman- Protran®, Rodgau; Germany). Membranes were incubated with antibodies for TNFα (1:400), IL6 (1:500) (LifeSpan Biosciences; North America), P65 (1:500) and P50 (1:400), (Abcam, Cambridge; UK), TTP (1:400) and IKKβ (1:400), (Santa Cruz Biotechnology, CA; USA) separately for the same membrane by using stripping buffer

(mild stripping) according to Abcam's protocol (http://www. abcam.com/ ps/ pdf/ protocols/ Stripping%20for%20reprobing.pdf). Protein loading was evaluated and normalized by examining GAPDH protein levels using a GAPDH antibody (Santa Cruz Biotechnology, CA; USA). Densitometric quantification of immunoreactive bands was carried out using Quantity One analysis software (Bio-Rad, Munich; Germany).

Data analysis

Statistical analysis of expression data was performed using Student's t test. The values shown in graphs are presented as the mean ± standard deviation (SD) of at least three independent experiments each done in quadruplicate, *p-values* < 0.05 were considered statistically significant. GraphPad Prism 5.0 was used for data plotting.

Results

LPS challenge induced alterations in expression of let-7 miRNAs in primary bovine endometrial cells in vitro

To gain insight into the biological activity of *let-7* miRNAs, we assessed the expression of *let-7* miRNAs after *LPS* challenge using quantitative real-time PCR. In primary endometrial epithelial cells challenged with clinical dose of *LPS* (3 µg/ml), *let-7a* was significantly decreased, but *let-7c* was significantly increased. The challenged primary endometrial epithelial cells with sub-clinical dose of *LPS* (0.5 µg/ml) showed significantly decreased in the expression of *let-7a*, *let-7e* and *let-7i*, while *let-7d* was significantly increased. In primary endometrial stromal fibroblast cells challenged with clinical dose of *LPS* (3 µg/ml), *let-7a*, *let-7c, let-7d* and *let-7e* were significantly down-regulated. The challenged primary endometrial stromal fibroblast cells with sub-clinical dose of *LPS* (0.5 µg/ml) showed a significant suppression of *let-7a* and *let-7c*

expression level, and an increased level of *let-7e*, *let-7f* and *let-7i* (Fig.1). So, *LPS* was shown to induce clear alterations in *let-7* miRNAs expression profile in both epithelial and stromal cells.

Inflammatory immune response of primary endometrial epithelial and stromal fibroblast cells after LPS challenge

In order to confirm the response of cells to *LPS* treatment, we measured some inflammatory mediators in cell culture supernatant. We found that the challenged endometrial cells exhibited an increased level of pro-inflammatory cytokines namely; TNFα, IL6 in dose-dependent manner, where higher *LPS* dose evoked higher release of pro-inflammatory cytokines. Moreover, PGE_2: $PGF_{2\alpha}$ ratio was higher in the challenged cells compared to untreated control cells (Fig. 2).

Temporal pattern of let-7 miRNAs in response to LPS at different time points

To elucidate time-dependant expression of *let-7* miRNAs in primary bovine endometrial stromal fibroblast cells, we monitored the expression of the *let-7* family after *LPS* challenge at different time points. The whole *let-7* members have been increased upon *LPS* stimulation, only *let-7a* peaked at 6h, and then gradually decreased, while *let-7e*, *let-f* and *let-7i* peaked at 24h (Fig. 3).

Let-7a and let-7f regulate the expression of TNFα, TGFβ1I1 and SDPR

We hypothesized that the 3´ UTR regions of TNFα, TGFβ1I1 and SDPR are susceptible to be targeted by *let-7a* and *let-7f* in a direct or an indirect manner. To identify the potential target genes we used miRecords (http://mirecords.biolead.org/). Once, the genes were selected, the alignment between the seed region and 3´ UTR of selected

candidate genes were checked, using FindTar3 Online Prediction (http://bio.sz.tsinghua.edu.cn/). Bovine TNFα harbored three putative *let-7a* & two *let-7f* target sites within its 3´ UTR, bovine TGFβ1I1 harbored one putative *let-7a* & one *let-7f* target site within its 3´ UTR, and bovine SDPR harbored two putative *let-7a* & three *let-7f* target sites within its 3´ UTR. The sequence alignment of the seed regions of the binding sites for *let-7a* and *let-7f* is shown in (Supplemental Fig. 1A, B & C). When the whole *let-7* seed-complementary sites of the TNFα, TGFβ1I1 and SDPR reporters were mutated, the regulation in response to the *let-7a* and *let-7f* mimics were abrogated. Upon scrambling of all predicted *let-7* seed-complementary sites within the TNFα, TGFβ1I1 and SDPR reporters sequence regulation were lost as well. In particular, targeting of TNFα, TGFβ1I1 and SDPR mRNAs by *let-7* seemed to be specific in primary endometrial stromal cells, where *let-7* over-expression was followed by a clear repression in TNFα, TGFβ1I1 and SDPR 3´ UTR reporter activity (Fig. 4A & B). Thus, TNFα, TGFβ1I1 and SDPR were identified as novel *let-7* miRNAs targets. Moreover, we investigated whether down-regulation of the *let-7* miRNAs family in *LPS* challenged primary endometrial stromal cells in turn may elevate the activities of the reporters. For this, TNFα, TGFβ1I1 and SDPR 3´ UTR reporters with the intact or a mutated *let-7* binding sites were transfected into primary endometrial stromal cells. Upon treatment with *LPS*, TNFα, TGFβ1I1 and SDPR reporter activities were elevated, but regulation was lost upon mutated *let-7* binding-site (Supplemental Fig. 3A & B).

Inhibition and over-expression of let-7 miRNAs in primary endometrial stromal fibroblast cells for 48 hours

In an attempt to identify how *let-7* family regulates pro-inflammatory cytokines like TNFα and IL6 and other target genes related to apoptosis and pathogen recognition

pattern, we used gain-and loss-of-function approaches. Successful transfection during overexpression or inhibition studies was confirmed by visual fluorescent analysis, where transfection efficiency was > 85% of all cells (data not shown). Here, we used family inhibitors to target large number of *let-7* family members namely: *let-7a*, *let-7c*, *let-7e*, *let-7f* and *let-7i*, and we observed a clear suppression of all *let-7* members (*let-7a*, *let-7c*, *let-7e*, *let-7f* and *let-7i*) after transfection of primary endometrial stromal cells by *let-7* family inhibitors for 48h compared to untreated control cells and negative control of miR-inhibitor (scramble), (Fig. 5). Furthermore, overexpression or inhibition of both *let-7a* and *let-7f* were confirmed by real-time PCR (Fig. 6A & B).

Regulation of pro-inflammatory cytokines (TNFα and IL6) and other potential targets of let-7a and let-7f during LPS treatment

To assess the potential role of *let-7a* and *let-7f* in inflammatory immune response of endometrial stromal cells, we examined the effect of *let-7a* and *let-7f* transfection on TNFα and IL6 in *LPS* challenged cells. Overexpression of *let-7a* inhibited TNF-α and IL6 on mRNAs as well as protein levels, but *let-7a* inhibitor (antagonist) transfection resulted in an increase of these pro-inflammatory cytokines. Furthermore, TGFβ1I1, SDPR and NFκB mRNAs have shown a reciprocal pattern in response to *let-7a* over-expression or inhibition, but CASP3 significantly decreased upon transfection with *let-7a* mimic or inhibitor compared to scramble (Fig. 7A & B). On the other hand, the mRNAs and protein levels of TNFα and IL6 have shown a clear suppression only upon transfection with *let-7f* inhibitor, while TGFβ1I1 and SDPR mRNAs have shown a reciprocal expression pattern after transfection with *let-7f* mimic or inhibitor. Interestingly, NFκB and CASP3 mRNAs reduced significantly after transfection with *let-7f* mimic or inhibitor compared to scramble (Fig. 8A & B). Thus, these findings

demonstrate that *let-7a/let-7f* may regulate the expression of the secretory pro-inflammatory cytokines (TNFα and IL6) in *LPS* challenged endometrial stromal cells (Supplemental Fig. 2A & B).

Cytokines directly and indirectly regulated by let-7 miRNAs

To explore whether *let-7a* and *let-7f* may directly or indirectly target pro-inflammatory mediators, primary endometrial stromal fibroblast cells were transfected with miR-inhibitor and mimic (*let-7a* and *let-7f*) for 48h, then transfected cells were challenged by *LPS* for 24h. The main regulatory molecules involved in NFκB signaling pathway as P65, P50 and IKKβ, were demonstrated by immunoblotting. Notably, protein bands of P65, P50 and IKKβ were reduced upon transfection with *let-7a* mimic, while TTP was not affected upon transfection with *let-7a* inhibitor or mimic. On the other hand, P65, P50 and IKKβ were not affected after *let-7f* inhibitor or mimic transfection. Surprisingly, the immunoreactive band of TTP was clearly reduced after transfection with *let-7f* mimic compared with *let-7f* inhibitor transfection and scramble (Fig. 9A & B). Our findings indicated that cytokines are directly and indirectly regulated by *let-7* miRNAs.

Alteration in prostaglandins (PGs) ratio in primary endometrial stromal fibroblast cells after transfection with let-7a mimic or inhibitor and LPS challenge for 24h

The kinetics of PGs secretion in cell culture media shows a reciprocal pattern after cell transfection by *let-7a* mimic or *let-7a* inhibitor and *LPS* challenge, where PGE_2 to $PGF_{2\alpha}$ ratio was significantly lower after transfection with *let-7a* mimic. In contrast, PGE_2 to $PGF_{2\alpha}$ ratio was higher after transfection with *let-7a* inhibitor compared to scramble (Supplemental Fig. 2C).

Discussion

E. coli is the first and the most common pathogenic bacteria isolated from the uterine infection and it causes infertility not only by perturbing uterine function but also affecting ovarian cycles after parturition in cattle [29]. Infection of the endometrium with *E. coli* precedes infection by other pathogens, and is associated with the severity of PID and the impact on fertility (32, 41). The endometrial cells appear to have a key role in innate immune defence of the female genital tract. This also leads to modulation of endocrine function and persistence of neutrophils in the endometrium in the absence of bacteria, which is the primary characteristic of subclinical endometritis (8, 18). To tackle the continuing fertility problems associated with uterine inflammation, understanding the molecular mechanisms associated with the local inflammatory immune response is crucial. Molecular changes in miRNAs and their target genes expression may also identify reliable prognostic indicators for cows that will resolve inflammation and resume cyclicity. Our central hypothesis was whether *let-7* family has a primary role in the innate immune defence of the endometrium against bacterial infection, which is partly achieved by regulating mRNA stability of pro-inflammatory cytokines at the post-transcriptional level.

Here, we addressed a comprehensive investigation of *let-7* miRNAs in bovine endometrial cells after *LPS* challenge with two doses that resembled in clinical and sub-clinical endometritis. We found that the evolutionarily conserved *let-7* family was aberrant regulated in endometrial cells after *LPS* challenge. Moreover, *LPS* stimulation activated NFκB signalling, led to release of cytokines such as TNFα & IL6, and increased the PGE_2 to $PGF_{2\alpha}$ ratio. That all contributes to induce a distinct inflammatory immune response in endometrial cells. Our findings are similar to findings of previous studies [29,31]. Notably, the dysregulation of *let-7* family expression was associated

with over-expression of pro-inflammatory mediators as interleukin-1 beta (IL1β), and obvious alterations in other genes expression that might be potentially regulated by *let-7* family as SDPR, TGFβ1I1, CASP3, NFκB, toll-like receptor 4 (TLR4) and inducible nitric oxide synthase (INOS), (data not shown).

It is believed that stromal cells are much more abundant than epithelial cells in the endometrium after parturition, where all the epithelial cells sloughed leading to expose stromal cells to ascending bacteria; furthermore stromal cells are closer to the circulation and mononuclear cells. Therefore, the stromal cells may have equal importance in the immune response to pathogen in the endometrium [3,32]. So in the current study, we focused on the role of *let-7* in primary endometrial stromal fibroblast cells. In line with previous reports [33,34], the expression of miRNAs is subject to temporal and spatial regulation in different tissues. For this, we analysed the temporal pattern of *let-7* miRNAs at different time points in stromal endometrial fibroblast after *LPS* treatment. Interestingly, *let-7a* was increased post *LPS* challenge with a clear peak at 6h and then gradually decreased. On the other hand, *let-7e*, *let-7f* and *let-7i* were up-regulated after *LPS* stimulation and showed a clear peak at 24h; these findings suggested that *let-7* miRNAs might be required for inflammatory immune response in *LPS* challenged bovine endometrial cells. Our data are in agreement with previous studies, which have indicated that miRNA may act as a crucial regulator in epithelial immune responses [35,36]. Recently, up-regulation of *let-7i* in infected human epithelial cells with *Cryptosporidium parvum* was reported [37]. Also, it was demonstrated that *E. coli* initiated an earlier regulation of six miRNAs within the first 6h post challenge as compared to a one miRNA for *Staphylococcus aureus*, which was presented as delayed response [36]. In addition, miRNAs may modulate epithelial immune responses at every

step of the innate immune pathways, involving production of pro- and anti-inflammatory mediators [38].

Using the bioinformatic prediction tools, dozens of genes which are either involved in normal or disease conditions could be identified as potential targets of *let-7* family. This shows that *let-7* miRNAs are involved in sophisticated mechanisms to ensure appropriate regulation of inflammatory cytokines genes during immune response against infection. Based on the in silico analysis and wet lab experiment; TNFα, TGFβ1I1 and SDPR were found to be targeted by the *let-7* miRNAs. Another key point from this study was noticed that the luciferase activity was significantly elevated upon miR-inhibitors (*let-7a* and *let-7f*) transfection and *LPS* challenge; these findings revealed that *LPS* was triggered down-regulation of *let-7* expression in endometrial cells and were similar to a previous study [39].

Persistent inflammation is linked clinically and epidemiologically to bovine infertility [40], and the proper regulation of pro-inflammatory cytokines appears to play an important role in maintaining uterine function. However, these mechanisms are poorly understood. Thus, we supposed that there is an intimate link between aberrant regulation of *let-7* miRNAs and persistent inflammation in bovine endometrial cells through post-transcriptional regulation of genes related to inflammatory immune response. To proof this hypothesis, we examined the potential contribution of *let-7* miRNAs in immune response of bovine endometrial stromal fibroblast cells, following *LPS* challenge. Our results provided primary evidence that *let-7a* and *let-7f* are involved in the regulation of pro-inflammatory cytokines as TNFα and IL6, which are the key modulators of the local inflammatory immune in *LPS* challenged endometrial cells, either in direct or indirect manner. The functional manipulation of *let-7a* revealed distinct alterations in pro-inflammatory cytokines expression (TNFα and IL6) at the

mRNA and protein levels either over-expression or inhibition. *Let-7f* inhibitor caused a clear down-regulation of TNFα and IL6 at both mRNAs and protein levels in challenged endometrial cells. This could be to the fact that all *let-7* family members have conserved GU-rich 3´ sequences, but not the exact GUUGUGU motif. Furthermore, it was found that some sequence of the *let-7* miRNAs evoked TNFα production in a dose- and time-dependent pattern, which was similar to previous findings [41].

Previously, it was recorded that the steady-state switch in prostaglandin concentrations from the luteolytic F series to the luteotropic E series provided a mechanism to explain the pathomechanism associated with uterine disorders and female infertility in cattle [29]. Here, the over-expression *let-7a* in *LPS* challenged endometrial stromal cells revealed a remarkable reduction in PGE_2 to $PGF_{2\alpha}$ ratio.

In the same train of thought, it was observed that the widespread mode of miRNA action in animal cells shows a reciprocal pattern with their targets mRNAs [42], only a few miRNAs (for example, *let-7*) were demonstrated to activate their targets mRNAs [43]. Interestingly, in our data the pro-inflammatory mediators (TNFα and IL6) showed the same pattern of miRNA expression after cell transfection either with *let-7f* inhibitor or mimic, but the level of pro-inflammatory mediators after *let-7f* inhibitor or mimic transfection was lower compared to scramble. Furthermore, NFκB expression did not show any change after *let-7f* inhibitor or mimic transfection. So we supposed that *let-7f* could indirectly regulate pro-inflammatory cytokines. To profile this, we checked some regulatory molecules that are involved in canonical NF-κB signaling in response to inflammatory signals and we observed that *let-7f* inhibitor or mimic transfection did not have any effect on protein profile of P65, P50 and IKKβ. Previously, it was demonstrated that post-transcriptional regulation of cytokines

expression can be mediated by the AREs that are located in the 3´ UTR. The AREs are recognized by either ARE-binding proteins (ARE-BPs) such as TTP or by miRNAs and subsequently promotes degradation of ARE-containing transcripts [12]. Moreover, it was reported that microRNAs can regulate the expression of inflammatory cytokine by either directly binding to a seed region sequence in cytokine mRNAs or indirectly regulating ARE-BPs [44]. In our study, we observed that *let-7f* was targeting TTP, and TTP was negatively regulated after endometrial stromal fibroblast cells were transfected with *let-7f* inhibitor or mimic. Our results are supported by previous studies that have indicated that miRNAs can potentially regulate cytokine expression by directly binding to target sites in the 3′ UTRs of mRNAs or indirectly by targeting ARE-binding proteins like TTP [44]. Taken together, our data demonstrated that *let-7* miRNAs control distinct targets in uterine tissue immune pathways. The selective control of signaling components involved in various pro-inflammatory pathways by *let-7* miRNAs constitute a pervasive regulator of endometrium immune response via direct or indirect controlling of pro-inflammatory cytokines.

In summary, the better understanding of the mechanisms of improper immune response (persistent of pro-inflammatory cytokines) in bovine endometrial cells may provide new insights for controlling of the expression the *let-7* miRNAs. Here we addressed for the first time that *let-7* miRNAs have a precise role in bovine endometrium, where *LPS* dysregulated *let-7* miRNAs expression, and subsequently was associated with an increased pro-inflammatory cytokine level by directly/indirectly targeting the TNFα, IL6, NFκB, TGFβ1I1 and SDPR genes. To our knowledge, this is the first study showing that TNFα, TGFβ1I1 and SDPR were identified as novel *let-7* miRNAs targets and may have distinct role in inflammatory immune response of *LPS* challenged bovine endometrial cells. Our data represent a novel finding by which

uterine homeostasis is maintained through functional manipulation of *let-7a* that was subsequently influenced down-regulation of pro-inflammatory cytokines expression (TNFα and IL6) at the mRNA and protein levels. These findings suggest that *LPS* serves as a negative regulator of *let-7* miRNAs expression and provide a mechanism for the widespread increase in pro-inflammatory cytokines, which observed in bovine sub-clinical endometritis.

Acknowledgements

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Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Sheldon IM, Noakes DE, Rycroft AN, Pfeiffer DU, Dobson H (2002) Influence of uterine bacterial contamination after parturition on ovarian dominant follicle selection and follicle growth and function in cattle. Reproduction 123: 837-845.
2. Williams EJ, Fischer DP, Noakes DE, England GC, Rycroft A, et al. (2007) The relationship between uterine pathogen growth density and ovarian function in the postpartum dairy cow. Theriogenology 68: 549-559.
3. Cronin JG, Turner ML, Goetze L, Bryant CE, Sheldon IM (2012) Toll-like receptor 4 and MYD88-dependent signaling mechanisms of the innate immune system are essential for the response to lipopolysaccharide by epithelial and stromal cells of the bovine endometrium. Biol Reprod 86: 51.
4. Sheldon IM, Cronin J, Goetze L, Donofrio G, Schuberth HJ (2009) Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. Biol Reprod 81: 1025-1032.
5. Herath S, Fischer DP, Werling D, Williams EJ, Lilly ST, et al. (2006) Expression and function of Toll-like receptor 4 in the endometrial cells of the uterus. Endocrinology 147: 562-570.
6. Mor G, Cardenas I, Abrahams V, Guller S (2011) Inflammation and pregnancy: the role of the immune system at the implantation site. Ann N Y Acad Sci 1221: 80-87.

7. Sheldon IM, Price SB, Cronin J, Gilbert RO, Gadsby JE (2009) Mechanisms of infertility associated with clinical and subclinical endometritis in high producing dairy cattle. Reprod Domest Anim 44 Suppl 3: 1-9.
8. Kumar H, Kawai T, Akira S (2009) Toll-like receptors and innate immunity. Biochem Biophys Res Commun 388: 621-625.
9. Serhan CN (2008) Systems approach with inflammatory exudates uncovers novel anti-inflammatory and pro-resolving mediators. Prostaglandins Leukot Essent Fatty Acids 79: 157-163.
10. Serhan CN, Chiang N, Van Dyke TE (2008) Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. Nat Rev Immunol 8: 349-361.
11. Jennewein C, von Knethen A, Schmid T, Brune B (2010) MicroRNA-27b contributes to lipopolysaccharide-mediated peroxisome proliferator-activated receptor gamma (PPARgamma) mRNA destabilization. J Biol Chem 285: 11846-11853.
12. Stoecklin G, Anderson P (2006) Posttranscriptional mechanisms regulating the inflammatory response. Adv Immunol 89: 1-37.
13. Lykke-Andersen J, Wagner E (2005) Recruitment and activation of mRNA decay enzymes by two ARE-mediated decay activation domains in the proteins TTP and BRF-1. Genes Dev 19: 351-361.
14. Lee JY, Kim HJ, Yoon NA, Lee WH, Min YJ, et al. (2013) Tumor suppressor p53 plays a key role in induction of both tristetraprolin and let-7 in human cancer cells. Nucleic Acids Res 41: 5614-5625.
15. Hoesel B, Schmid JA (2013) The complexity of NF-kappaB signaling in inflammation and cancer. Mol Cancer 12: 86.
16. Hertel J, Bartschat S, Wintsche A, Otto C, Stadler PF (2012) Evolution of the let-7 microRNA family. RNA Biol 9: 231-241.
17. Roush S, Slack FJ (2008) The let-7 family of microRNAs. Trends Cell Biol 18: 505-516.
18. Wang X, Cao L, Wang Y, Liu N, You Y (2012) Regulation of let-7 and its target oncogenes (Review). Oncol Lett 3: 955-960.
19. Swaminathan S, Suzuki K, Seddiki N, Kaplan W, Cowley MJ, et al. (2012) Differential regulation of the Let-7 family of microRNAs in CD4+ T cells alters IL-10 expression. J Immunol 188: 6238-6246.
20. Altuvia Y, Landgraf P, Lithwick G, Elefant N, Pfeffer S, et al. (2005) Clustering and conservation patterns of human microRNAs. Nucleic Acids Res 33: 2697-2706.
21. Wang Y, Hu X, Greshock J, Shen L, Yang X, et al. (2012) Genomic DNA copy-number alterations of the let-7 family in human cancers. PLoS One 7: e44399.
22. Pritchard CC, Cheng HH, Tewari M (2012) MicroRNA profiling: approaches and considerations. Nat Rev Genet 13: 358-369.
23. Fortier MA, Guilbault LA, Grasso F (1988) Specific properties of epithelial and stromal cells from the endometrium of cows. J Reprod Fertil 83: 239-248.
24. Ireland JJ, Murphee RL, Coulson PB (1980) Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. J Dairy Sci 63: 155-160.
25. Kim JJ, Fortier MA (1995) Cell type specificity and protein kinase C dependency on the stimulation of prostaglandin E2 and prostaglandin F2 alpha production by oxytocin and platelet-activating factor in bovine endometrial cells. J Reprod Fertil 103: 239-247.

26. Xiao CW, Goff AK (1998) Differential effects of oestradiol and progesterone on proliferation and morphology of cultured bovine uterine epithelial and stromal cells. J Reprod Fertil 112: 315-324.
27. Takahashi H KI, Sato T, Takahashi M , Okano A (2001) Isolation and Culture of Bovine Endometrial Epithelial Cells in a Serum-Free Culture System. Journal of Reproduction and Development Vol. 47
28. Herath S, Williams EJ, Lilly ST, Gilbert RO, Dobson H, et al. (2007) Ovarian follicular cells have innate immune capabilities that modulate their endocrine function. Reproduction 134: 683-693.
29. Herath S, Lilly ST, Fischer DP, Williams EJ, Dobson H, et al. (2009) Bacterial lipopolysaccharide induces an endocrine switch from prostaglandin F2alpha to prostaglandin E2 in bovine endometrium. Endocrinology 150: 1912-1920.
30. Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132: 365-386.
31. Xu Y, Jin H, Yang X, Wang L, Su L, et al. (2014) MicroRNA-93 inhibits inflammatory cytokine production in LPS-stimulated murine macrophages by targeting IRAK4. FEBS Lett 588: 1692-1698.
32. Chapwanya A, Meade KG, Doherty ML, Callanan JJ, Mee JF, et al. (2009) Histopathological and molecular evaluation of Holstein-Friesian cows postpartum: toward an improved understanding of uterine innate immunity. Theriogenology 71: 1396-1407.
33. Taganov KD, Boldin MP, Chang KJ, Baltimore D (2006) NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A 103: 12481-12486.
34. Wang XW, Heegaard NH, Orum H (2012) MicroRNAs in liver disease. Gastroenterology 142: 1431-1443.
35. O'Neill LA, Sheedy FJ, McCoy CE (2011) MicroRNAs: the fine-tuners of Toll-like receptor signalling. Nat Rev Immunol 11: 163-175.
36. Jin W, Ibeagha-Awemu EM, Liang G, Beaudoin F, Zhao X, et al. (2014) Transcriptome microRNA profiling of bovine mammary epithelial cells challenged with Escherichia coli or Staphylococcus aureus bacteria reveals pathogen directed microRNA expression profiles. BMC Genomics 15: 181.
37. Chen XM, Splinter PL, O'Hara SP, LaRusso NF (2007) A cellular micro-RNA, let-7i, regulates Toll-like receptor 4 expression and contributes to cholangiocyte immune responses against Cryptosporidium parvum infection. J Biol Chem 282: 28929-28938.
38. Zhou R, O'Hara SP, Chen XM (2011) MicroRNA regulation of innate immune responses in epithelial cells. Cell Mol Immunol 8: 371-379.
39. Schulte LN, Eulalio A, Mollenkopf HJ, Reinhardt R, Vogel J (2011) Analysis of the host microRNA response to Salmonella uncovers the control of major cytokines by the let-7 family. EMBO J 30: 1977-1989.
40. Cheong SH, Nydam DV, Galvao KN, Crosier BM, Gilbert RO (2011) Cow-level and herd-level risk factors for subclinical endometritis in lactating Holstein cows. J Dairy Sci 94: 762-770.
41. Lehmann SM, Kruger C, Park B, Derkow K, Rosenberger K, et al. (2012) An unconventional role for miRNA: let-7 activates Toll-like receptor 7 and causes neurodegeneration. Nat Neurosci 15: 827-835.

42. Olsen PH, Ambros V (1999) The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev Biol 216: 671-680.
43. Jerome T, Laurie P, Louis B, Pierre C (2007) Enjoy the Silence: The Story of let-7 MicroRNA and Cancer. Curr Genomics 8: 229-233.
44. Asirvatham AJ, Magner WJ, Tomasi TB (2009) miRNA regulation of cytokine genes. Cytokine 45: 58-69.

Table 1. List of primers that were used for real-time PCR analysis of target genes

Gene name	Accession no.	Primer sequence (5′→3′)	Annealing temperature (°C)
SDPR	XM_610845	F: GAAAACAAGTCCCTGGA R: GTCCCCAGCTTGTTCATC	55
TGFβ1I1	NM_001035313	F: TCTCTGACTTCCGTGTCC R: TTATTGCAGGAGCCACAG	55
INOS	NM_001076799	F: TGTTCAGCTGTGCCTTCAAC R: AAAGCGCAGAACTGAGGGTA	55
CASP3	NM_001077840	F: TGCCACTGTATCAGGGAACA R: TGCTCAGCACAAACATCACA	52
TLR4	NM_174198	F: AGAGCCACTTCTGGTCACAG R: TAAAGCTCAGGTCCAGCATC	55
CD45	NM_001206523	F: CAAAGAGCCCAGGAAGTACA R: GTTGATCTCCACAATCACA	51
IL1β	NM_174093	F: CCTTGGGTATCAAGGACAAG R: CGATTTGAGAAGTGCTGATG	53
TNFα	NM_173966	F: CTTCCACCCCCTTGTTCCT R: AGGCGATCTCCCTTCTCCA	55
GAPDH	NM_001034034	F: ACCCAGAAGACTGTGGATGG R: ACGCCTGCTTCACCACCTT	57
18S	NR_036642	F: CGCAGCTAGGAATAATGGAA R: TCTGATCGTCTTCGAACCTC	53
ACTB	NM_173979	F: GGCATTCACGAAACTACCTT R: CAATCCACACGGAGTACTTG	53
NFκB1	NM_001076409	F: AATTTGGGAAGGATTTGGAG R: CTGTCGTTTCCTTTGCACTT	55

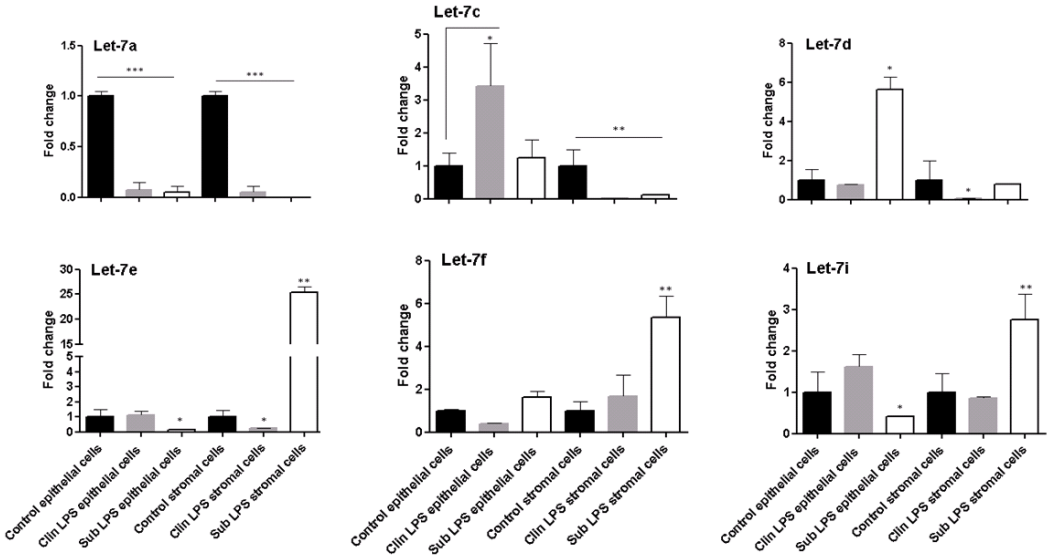


Figure 1. Relative expression of *let-7* miRNAs in primary bovine endometrial epithelial and stromal fibroblast cells after *LPS* (3.0 & 0.5 µg/ml) challenge for 24h. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$.

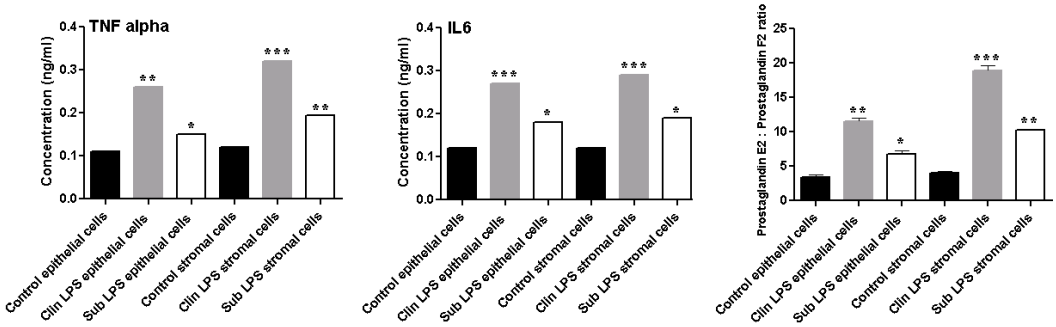


Figure 2. Enzyme-linked immunosorbent assay (ELISA) analysis of elaborated TNFα, IL6 and the prostaglandin E_2: prostaglandin $F_{2\alpha}$ ratio in the cell culture supernatant in primary endometrial cells challenged by clinical and subclinical doses of *LPS*. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$.

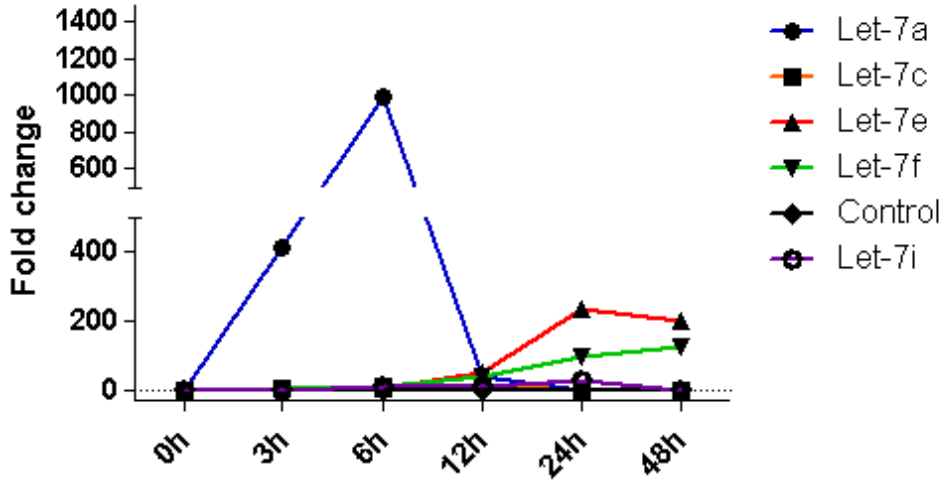


Figure 3. Temporal pattern of *let-7* miRNAs in primary endometrial stromal cells after *LPS* (0.5 µg/ml) challenge for 48h. Characterization of the time- and dose-dependent response of *let-7a*, *let-7c*, *let-7e*, *let-7f* and *let-7i* were determined by real-time PCR.

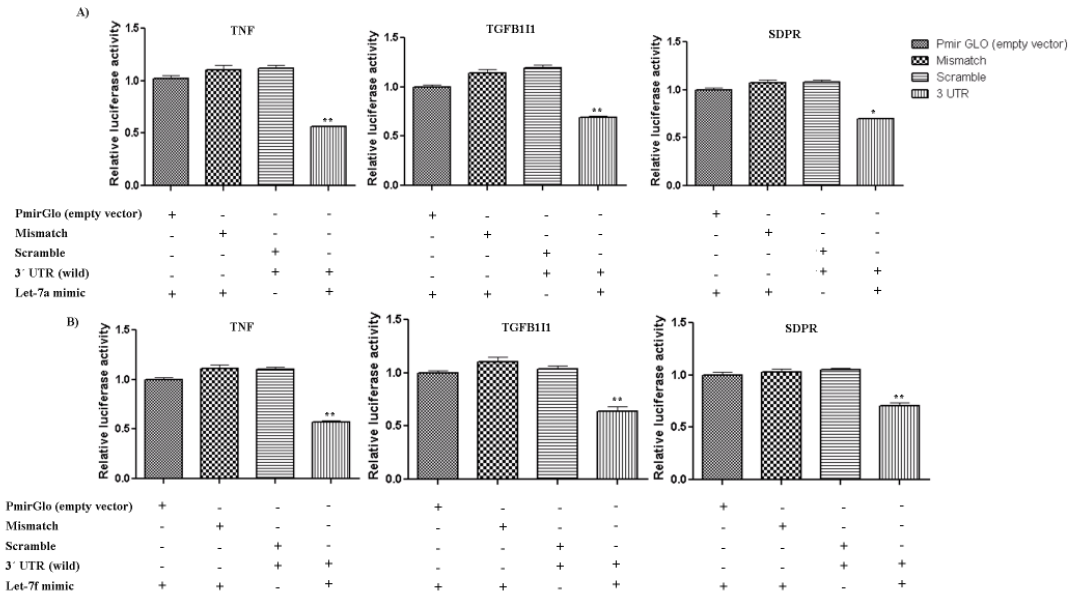


Figure 4. *Let-7a* and *let-7f* are directly targeting TNFα, TGFβ1I1 and SDPR genes. (A) Luciferase activity of reporters containing either 3´ UTR of TNFα or TGFβ1I1 or SDPR in stromal endometrial cells transfected with miRNA

negative control (scramble) or *let-7a* mimics. (B) Luciferase activity of reporters containing either 3´ UTR of TNFα or TGFβ1I1 or SDPR in stromal endometrial cells transfected with miRNA negative control (scramble) or *let-7f* mimics. *; $p < 0.05$, **; $p < 0.01$.

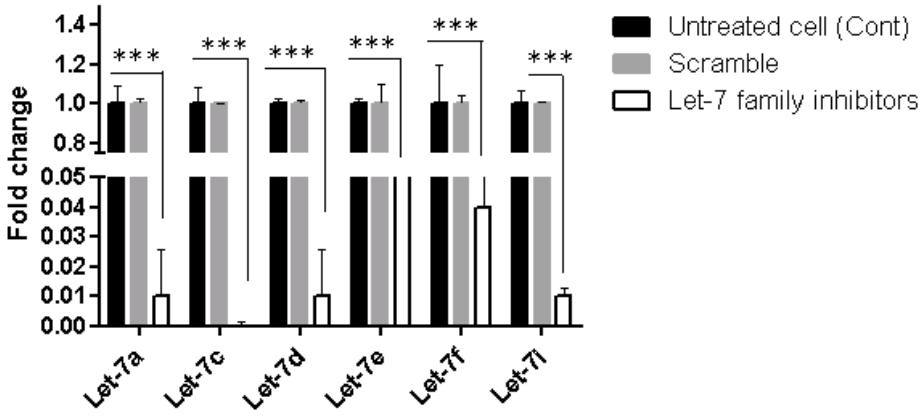


Figure 5. Relative abundance of *let-7* miRNAs after family inhibitors transfection. ***; $p < 0.001$.

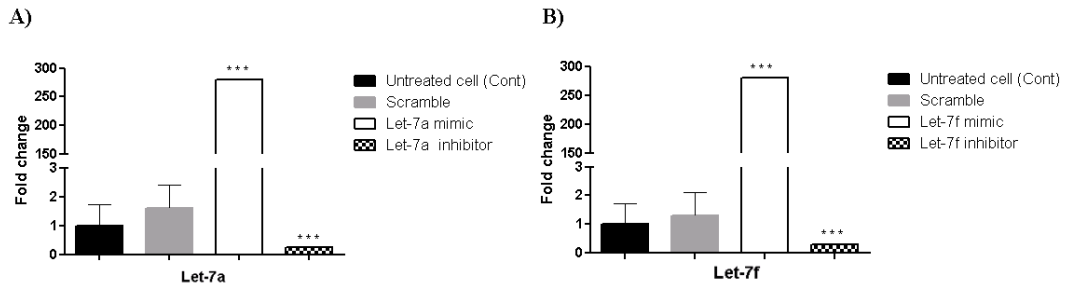


Figure 6. The expression pattern of *let-7* miRNAs after inhibition or over-expression. (A) Expression profiling of *let-7a* miRNA in primary stromal fibroblast endometrial cells transfection of stromal cells with inhibitor or mimic or scramble for 48h. (B) Characterization of *let-7f* after transfection of stromal cells with inhibitor or mimic or scramble for 48h. ***; $p < 0.001$.

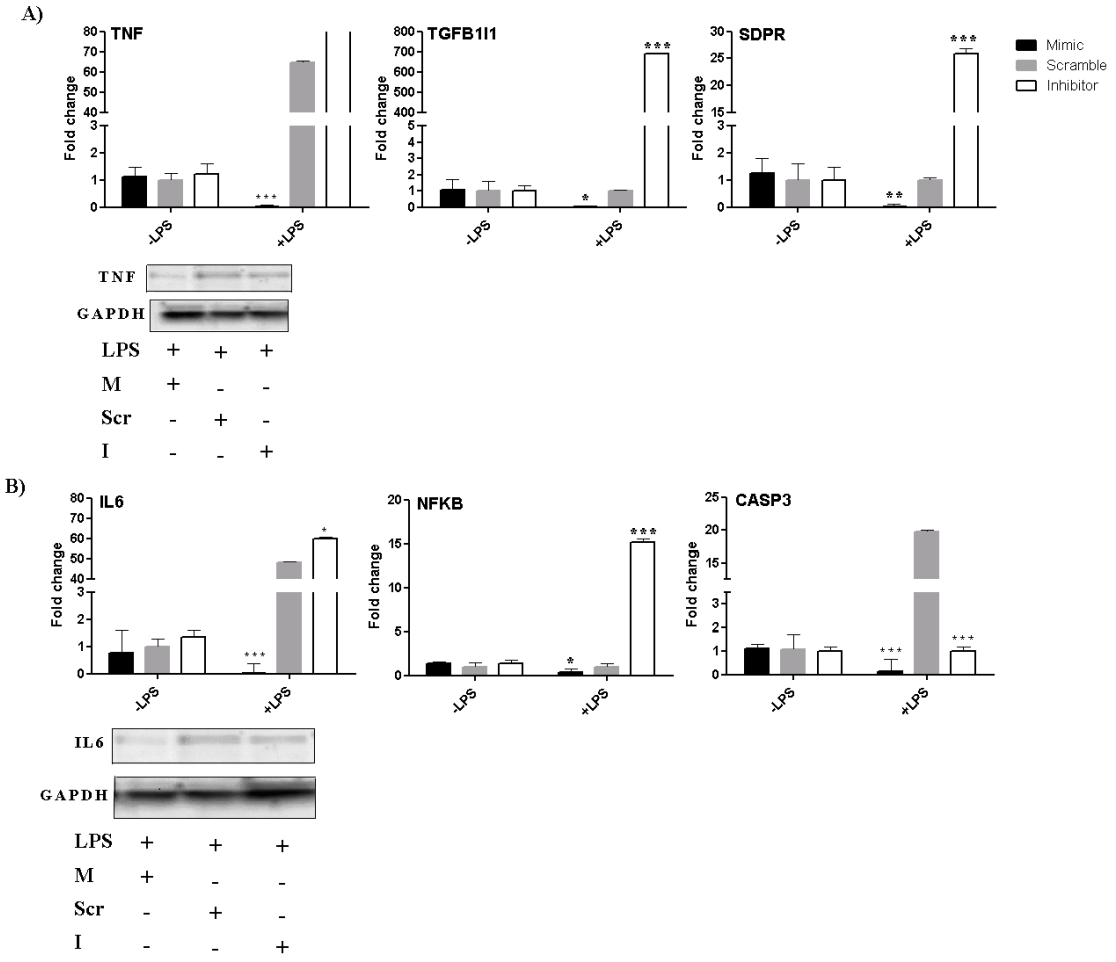


Figure 7. Functional modulation of *let-7a* revealed clear alterations in selected inflammatory cytokines and other target genes expression in *LPS* treated cells. (A) TNFα, TGFβ1I1 and SDPR expression showed significantly reciprocal pattern upon transfection with *let-7a* mimic (M) or inhibitor (I) or scramble (Scr). (B) The changes in IL6, NFκB and CASP3 expression profile after transfection with *let-7a* mimic or inhibitor or scramble. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$.

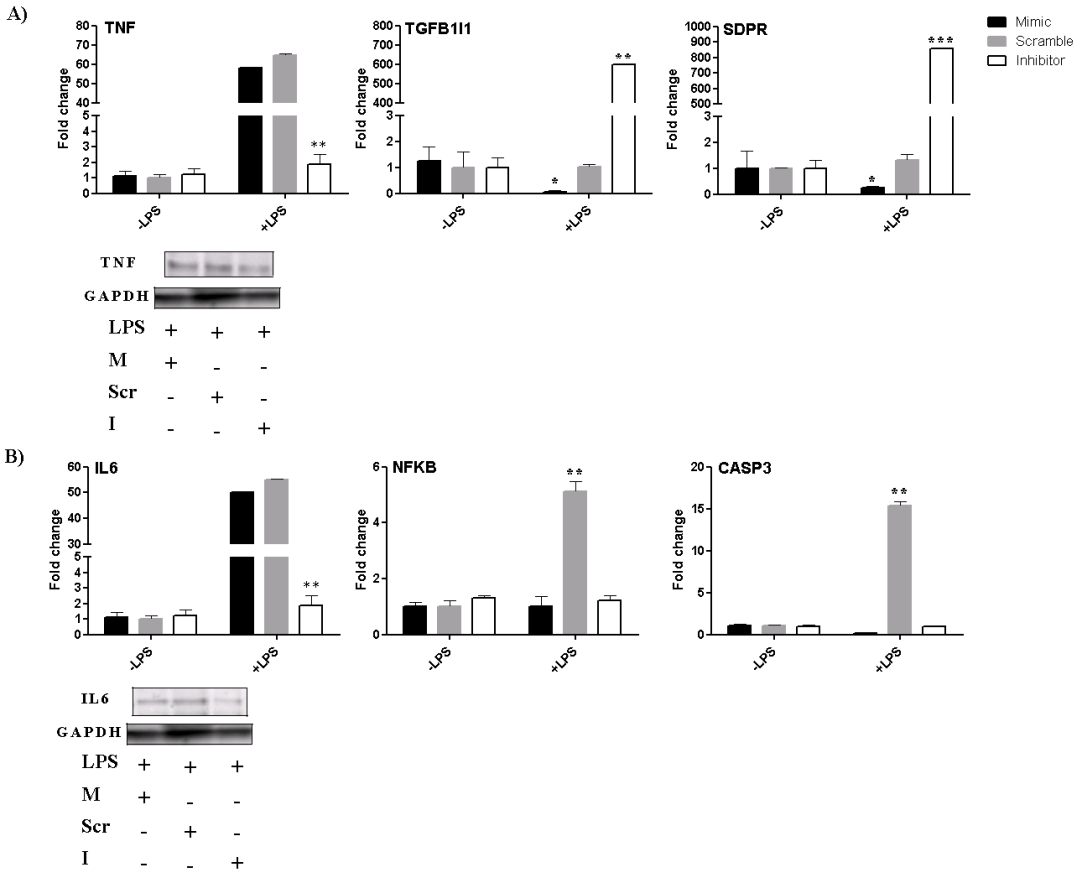


Figure 8. Effect of *let-7f* modulation on expression of candidate inflammatory cytokines and other target genes in *LPS* challenged stromal cells. (A) Alterations of TNFα, TGFβ1I1 and SDPR expression profile upon transfection with *let-7f* mimic (M) or inhibitor (I) or scramble (Scr). (B) Relative abundance of IL6, NFκB and CASP3 mRNAs after transfection with *let-7f* mimic or inhibitor or scramble. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$.

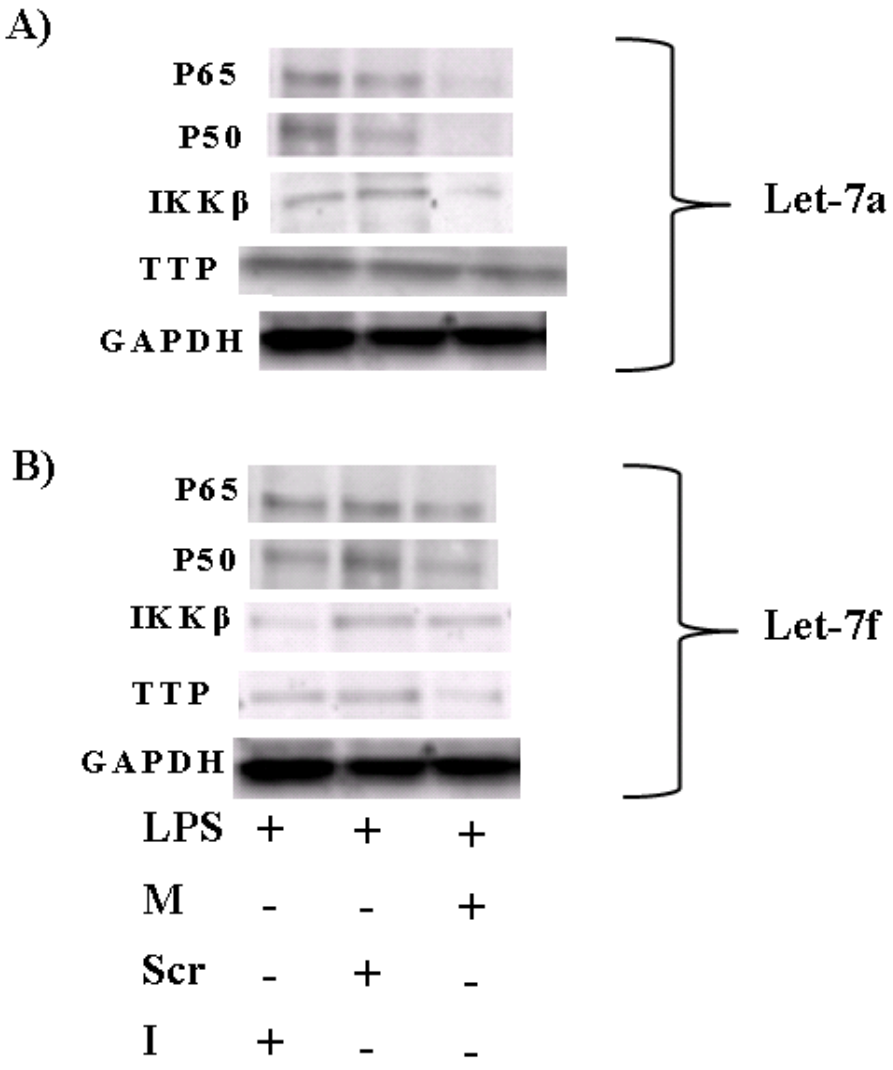
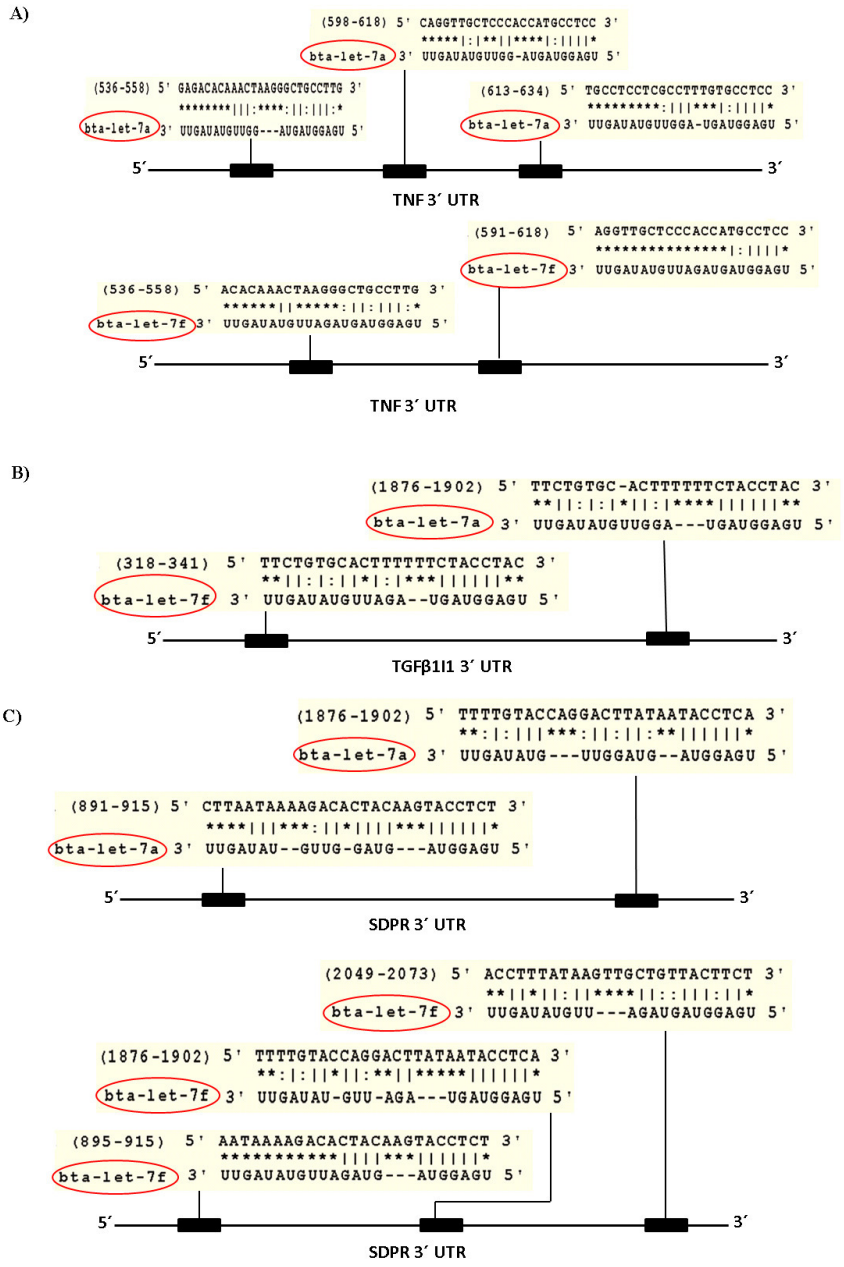
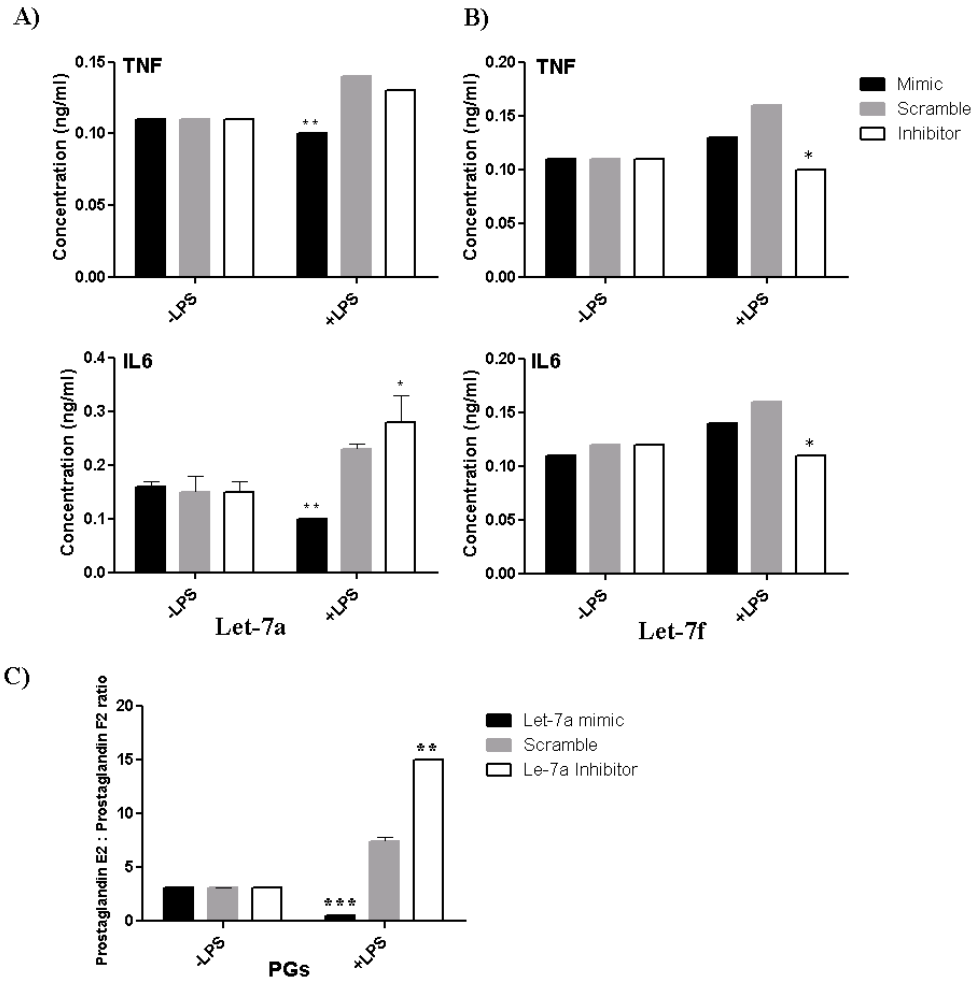


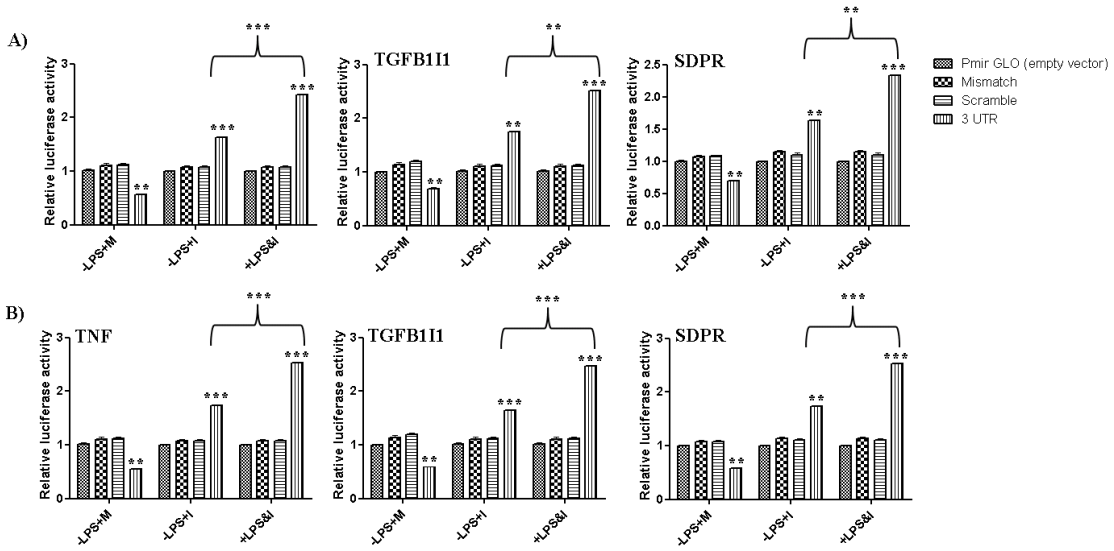
Figure 9. Direct/indirect effect of *let-7*a or *let-7f* transfection on NFκB and TTP proteins. (A) The indicated proteins were detected by Western blot from cell lysate of *LPS* challenged stomal cells after transfection with *let-7a* mimic (M) or inhibitor (I) or scramble (Scr). (B) Immunoreactive bands of indicated protein from cell lysate of *LPS* treated stomal cells after transfection with *let-7f* mimic or inhibitor or scramble, using Western blot.



Supplementary Figure 1. The potential regulatory *let-7a* and *let-7f* miRNAs and their sequence alignment with the binding sites of 3′ UTR (A) TNFα, (B) TGFβ1I1 and (C) SDPR candidate genes.



Supplementary Figure 2. Protein expression analysis in *LPS* challenged stromal cells. (A) ELISA analysis of TNFα and IL6 after transfection with *let-7a* mimic or inhibitor or scramble. (B) ELISA analysis of TNFα and IL6 after transfection with *let-7f* mimic or inhibitor or scramble. (C) prostaglandin E_2 (PGE_2) to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) ratio in cell culture supernatant upon transfection with *let-7a* mimic or inhibitor or scramble. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$.



Supplementary Figure 3. *LPS* regulates *let-7* miRNAs expression in primary endometrial stomal cells. (A) Luciferase activity of reporters containing either 3´ UTR of TNFα or TGFβ1I1 or SDPR in cells transfected with miRNA negative control (scramble) or *let-7a* mimics (M) or *let-7a* inhibitors (I) or *let-7a* inhibitors+*LPS*. (B) Luciferase activity of reporters containing either 3´ UTR of TNFα or TGFβ1I1 or SDPR in cells transfected with miRNA negative control (scramble) or *let-7f* mimics or *let-7f* inhibitors or *let-7f* inhibitors+*LPS*. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$.

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Curriculum vitae

4. Research publications

a) Peer reviewed published articles

1) W.M. Ahmed, M.M. Bashandy, A.K. Ibrahim, S.I.A Shalaby, Sherein I. Abd El-Moez, Faragalla M. El Moghazy and **Sally Ibrahim** (2010): Investigations on Delayed Puberty in Egyptian Buffalo-Heifers with Emphasis on Clinicopathological Changes and Treatment Using GnRH (Receptal®). Global Veterinaria 4, 78–85.
2) D. Hailemariam, **S. Ibrahim**, M. Hoelker, M. Drillich, W. Heuwieser, C. Looft, M. U. Cinar, E. Tholen, K. Schellander and D. Tesfaye (2013): MicroRNA-regulated molecular mechanism underlying bovine subclinical endometritis. Reproduction, Fertility and Development 26, 898–913.

b) Article under review

1) **Sally Ibrahim**, Dessie Salilew-Wondim, Franca Rings, Michael Hoelker, Christiane Neuhoff, Ernst Tholen, Christian Looft, Karl Schellander, Dawit Tesfaye (2014): Expression pattern of inflammatory response genes and their regulatory miRNAs in bovine oviductal cells in response to lipopolysaccharide: Implication for early embryonic development. Plos One (PONE-D-14-35766R2 - [EMID:1d643e9e6a99d05b]).

c) Thesis

1) M.V.SC. Thesis: **Sally Ibrahim** (2008): Some Clinicopathological Studies on Delayed Puberty Phenomenon in Buffalo-heifers. Faculty of Veterinary Medicine, Department of Clinical Pathology, Cairo University, Egypt

d) Abstract / posters

1) D Hailemariam, **S Ibrahim**, M Hoelker, M Drillich, W Heuwieser, C Looft, M U Cinar, E Tholen, K Schellander, D Tesfaye (2013): Sub-clinical endometritis induced changes on miRNA expression profile in bovine endometrium. DGfZ/GfT-Gemeinschaftstagung, 4./5.9.2013, Göttingen, Deutschland, Tagungsband: B2 (Proc).
2) **S Ibrahim**, D Salilew-Wondim, F Rings, M Hoelker, C Neuhoff, E Tholen, C Looft, K Schellander, D Tesfaye (2014): Transcriptomic profile of bovine oviduct epithelium after lipopolysaccharide (LPS) challenge in vitro and its implication on embryonic development. Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR), 10.-13.9.2014, Helsinki, Finland, Book of Abstracts (Abstr).
3) **S Ibrahim**, D Salilew-Wondim, F Rings, M Hoelker, C Neuhoff, E Tholen, C Looft, K Schellander, D Tesfaye (2014): Expression profiling of inflammatory response mRNAs and their potential regulatory miRNAs in bovine oviduct after lipopolysaccharide challenge and its implication on early embryonic development. DGfZ/GfT-Gemeinschaftstagung, 17./18.9.2014, Dummerstorf, Deutschland, Tagungsband: A23 (Proc).