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Research paper

Expression profiling of selected miRNAs in equine endometrium in response to LPS challenge in vitro: A new understanding of the inflammatory immune response

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ABSTRACT

Bacterial infections of the genital tract are the major cause of reproductive failure in the mares. MiRNAs are important regulators of gene expression, mostly through transcriptional and translational regression. We hypothesized that LPS induced aberrant expression of miRNAs and their targets, which are involved in regulation of uterine homeostasis. Three groups of primary endometrial epithelial and stromal cells, and endometrial tissue explants were cultured. The 1st group was kept as control, while the 2nd and 3rd groups were challenged with low (0.5 μg/mL) or high (3.0 μg/mL) doses of Lipopolysaccharides (LPS). Cell pellets and tissue explants were collected after 24 and 48 h, for total RNA isolation and qRT-PCR of the selected miRNAs and their targets. Culture media and cell lysates were collected after 24 and 48 h, for cytokines (IL6 and TNF_{α}) and prostaglandins (PGE_2 & $PGF_{\alpha 2}$) measurement. Both endometrial cells expressed TLR4 and its accessory molecules (MyD88 & CD14) that are required for triggering inflammatory immune response after LPS, via up-regulation of TRAF6, TNF_{α}, IL6 and IL8, compared to the respective control. After both doses of LPS challenge, miR-155, miR-223 and miR-17 were significantly increased; miR-181b, miR-21 and let-7a were significantly decreased compared to respective controls. Interestingly, miR-24 and miR-532-5p were clearly up-regulated after only the low LPS dose. TNF_{α}, IL6 and PGs in culture media and from cell lysates revealed dose- and time-dependent patterns, after LPS. Results indicated that both epithelial and stromal cells have a primary role in innate immune response after LPS challenge, while this recognition occurred via TLR4 and its accessory molecules. Dysregulation of miRNAs and their targets expression after LPS might affect normal uterine function through perturbation of PG and cytokine secretion.

1. Introduction

Equine endometritis, a local inflammation of the superficial layers of the uterus, has been ranked as the third most common medical problem of adult horses by equine practitioners in the USA (Traub-Dargatz et al., 1991), with approximately 15% of all Thoroughbred mares showing signs of persistent mating-induced endometritis (PMIE) (Zent et al., 1998). It was reported that the estimated economic cost due to infertility in horses is 38% in broodmares in Ireland and the UK, which fail to produce a foal annually (http://www.weatherbys.co.uk, 2004) (Nash et al., 2010). Endometritis can be associated with bacterial infections, or can occur in response to semen. Additionally, it is often underdiagnosed especially in subclinical form (LeBlanc and Causey, 2009). The decreased pregnancy rates in affected mares cause significant losses to horse breeding industries (Marth et al., 2015; Riddle et al., 2007). Reproductive consequences of equine endometritis are represented in a shortened luteal phase, failure to conceive, early embryonic death, mid-gestational abortion, placentitis, post-partum metritis, and birth of a septic neonate. Furthermore, endometritis has a significant economic impact, which results from irregular estrous cycles, and the need of for intensive breeding management incurring additional costs to the owner (LeBlanc, 2010). Pathologists have classified endometritis according to; signs (clinical and subclinical), types of inflammation (acute and chronic), and causative agent (septic due to pathogens and aseptic after insemination) (Troedsson et al., 1993; Woodward and Troedsson, 2015). Microbiological studies on the mare's

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genital tract indicated that *Streptococcus equi* subspecies zooepidemicus, *Escherichia coli* (*E. coli*), Pseudomonas aeruginosa, and Taylorella equigenitalis are the main pathogens that cause infectious or septic endometritis (M, 2012).

The female genital tract responds to septic inflammation via innate immunity and mucosal defense systems (Cronin et al., 2012; Sheldon et al., 2010). In mammals, the immune response of endometrial tissue is performed not only by natural immune cells, but also through endometrial epithelial and stromal cells, which simulate natural immune cells to overcome infection, through different expressed receptors such as the Toll-like receptor 4 (TLR4) and its down-stream signaling pathway (Cronin et al., 2012; Sheldon et al., 2014). The expression pattern of the main molecules required for recognition and triggering inflammatory immune response pathways of the endometrium after bacterial infection has not yet been elucidated in equines.

A new era is emerging in diagnosis of endometritis and its therapy with the aid of unprecedented advances in molecular biological approaches during the past decade, which have led to the identification of many key players of gene regulation under both normal and diseased conditions (Pan and Chegini, 2008). Among these key players are microRNAs (miRNAs), which have emerged as important regulators of gene expression. MiRNAs are small, non-coding RNA molecules that act as post-transcriptional regulators of gene expression by inhibiting translation or degrading mRNA through partial or complete base pairing with the 3′-UTRs of the target mRNAs (Bartel, 2004; Lim et al., 2003). They also may enhance translation in certain biological scenarios such as starvation conditions or cellular stress (Nothnick, 2012; Raychaudhuri, 2012). Accumulative evidence suggests that alterations in the expression of pro-inflammatory mediators are responsible for inappropriate tissue regeneration, embryo implantation failure and other reproductive disorders (Chapwanya et al., 2009; Ibrahim et al., 2015; Jaiswal et al., 2006). A recent study it implicated clear alterations of the endometrial transcriptome (mRNA) and miRNome profiles in cows affected by subclinical or clinical endometritis, which had a significant effect on uterine homeostasis and receptivity (Salilew-Wondim et al., 2016). Until now, the post-transcription mechanisms of endometritis pathogenesis are not understood in mares. To our knowledge, there is little information about role of miRNAs during pathogenesis of endometritis in equines. Hence, further investigations are warranted for a better understanding of the molecular events during endometritis in mares. Previous studies in bovine (Herath et al., 2009) as well as equine species (Nash et al., 2008; Perrini et al., 2016) have shown that endometrial cell culture and/or tissue explants challenged with LPS can be a good model to study endometritis.

We hypothesized that LPS induced aberrant expression of miRNAs and their targets (inflammatory immune response genes), which are involved in regulation of the uterine immune response as well as homeostasis. In this context, the objectives of the current study were (1) to investigate expression profiling of some candidate miRNAs in equine endometrial cells as well as tissue explants in response to LPS challenge in vitro; (2) to examine different scenarios between the selected miRNAs and their potential target genes; and (3) to study the TLR4 pathway after LPS challenge during induced equine infectious endometritis in vitro. For the latter, we selected candidate inflammatory immune response genes that are involved in the TLR4 pathway, namely: toll like receptor 4 (TLR4), myeloid differentiation primary response 88 (MyD88), cluster of differentiation 14 (CD14), TNF receptor associated factor 6 (TRAF6), tumor necrosis factor alpha (TNF_{α}), interleukin 6 (IL6), interleukin 8 (IL8) and interleukin 10 (IL10), which could have a crucial role in the normal uterine tissue immune response against bacterial infection. Furthermore, we investigated their potential regulatory miRNAs including: eca-let-7a, eca-miR-181b, eca-miR-21, eca-miR-155, eca-miR-223, eca-miR-17, eca-miR-24, and eca-miR-532-5p, in primary equine endometrial cells (both epithelial and stromal fibroblast) and tissue explants exposed to different doses of LPS.

2. Materials and methods

All chemicals and reagents for in vitro culture were purchased from Sigma Aldrich unless otherwise stated.

2.1. Endometrial tissue explants

Uteri (n˜56) from cyclic, nonpregnant healthy mares with no evidence of genital disease were collected during the breeding season (April-July) at local abattoirs immediately after slaughter and kept on ice until further laboratory processing. Mares were clinically healthy, as declared by official government veterinary inspection and individual veterinary histories of animal health. The physiological stage of the estrous cycle was determined by observation of ovarian morphology, and progesterone and 17b-estradiol concentrations as described previously (Kenney, 1978), in comparison to routine histopathological examination of the endometrial tissue. These collected tissues were divided into two portions: one portion was used for histological examination, and the second portion was used for tissue explantation as well as cell culture and bacterial challenge for 24 and 48 h in vitro. The histopathological examination of endometrial tissues was based on the grading scheme described in (Kenney, 1986). The presence of fibrotic nests, neutrophils, plasma cells and lymphoid follicles was examined. Moreover, the presence of inflammatory cells in the endometrium was graded as (0) if no inflammatory cells were present, (1) if there was low number of these cells, (2) if there were moderate numbers, and (3) if there was an abundance of inflammatory cells. The categorization was based primarily on the number of inflammatory cells and their distribution in the stratum compactum (D., 2007). Mares with endometrial tissues graded as 0 or 1 as well as being at the early luteal phase of the estrous cycle were selected for the current study.

The explant tissue is comprised of the epithelial layer together with stromal and resident leukocytes, as occuring in vivo. Thus, the explant is a functional representation of the endometrium (Nash et al., 2008). For tissue explants (at least four independent experiments each done in quadruplicate), endometrial tissue explants were cultured as described previously (Kozai et al., 2016), with a little modification. Briefly, endometrial tissues were washed once in 70% ethanol, then three times in sterile saline solution. Endometrial tissues were cut into small pieces (40–70 mg) with a scalpel, then placed into 24-well culture plates containing 2 ml Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12, supplemented with 10 µl/ml antibiotics and antimycotic solution, and 0.5 g per 500 ml bovine serum albumin (BSA). The tissue explants kept at 38.5 °C under 5% CO_2 in air for two h, and then explant were challenged separately with 3.0 µg/ml or 0.5 µg/ml of LPS according to **Nash** et al. (Nash et al., 2008) for 48 h. The supernatant and explants were collected at 24 and 48 h, and kept at − 80 °C until further use.

2.2. Endometrial cells isolation (epithelial and stromal fibroblast)

Isolation of endometrial epithelial and stromal cells, from mares in the early luteal phase of the estrous cycle (ipsilateral), was done according to (Szostek et al., 2012; Theuß et al., 2010), with a little modification. In brief, the endometrial samples (at least four independent experiments each done in quadruplicate) were cut into very small pieces and incubated in a sterile digestive solution comprised of: 50 mg dispase, 50 mg collagenase II, and 10 µl deoxyribonuclease I (Qiagen) in 100 ml phosphate buffer saline at 38.5 °C for 60–90 min with gentle shaking. Then the cell suspension was filtered through 70-µm, and then 40-µm meshes. The DMEM/F-12 medium was supplemented with 10% fetal bovine serum (FCS), and 10,000 U mL^{-1} penicillin G, 10 mg mL^{-1} streptomycin and 25 µg mL^{-1} amphotericin, added to the filtrate in order to stop the enzymatic digestion. Isolated cells were washed three times by centrifugation for 9–10 min at 200 × g at 4 °C. Afterwards, the cells were suspended in DMEM/F-12 containing

10% fetal bovine serum, and 10,000 U mL^{-1} penicillin G, 10 mg mL^{-1} streptomycin and 25 µg mL^{-1} amphotericin B and plated at a density of 1×10^5 cells/ml in 24-well plates. The viability of the cells was 90 to 95%, as determined by trypan blue. To obtain separate stromal and epithelial cell populations, the cell suspension was removed from seeding 3–4 h, which allowed selective attachment of stromal cells. The isolated cell suspension was then re-plated and incubated to allow epithelial cells to adhere. Stromal and epithelial cell populations were distinguished by cell morphology as previously described (Szostek et al., 2012), and by immunocytochemistry (ICC) for pan-cytokeratin and vimentin as markers for epithelial cells and stromal fibroblast, respectively (data not shown). The culture media were changed every 48 h until the cells have reached confluence. Cell cultures were maintained at 38.5 °C and 5% CO_2 in a humidified incubator. The absence of immune cells in endometrial cell cultures was confirmed by PCR, and qRT-PCR for the CD45 pan-leukocyte marker as previously described in bovine species (Harris et al., 2002; Herath et al., 2006), (data not shown). After the cells reached confluence, we challenged endometrial epithelial and stromal cells separately, with 3.0 µg/ml or 0.5 µg/ml of LPS according to Nash et al. (Nash et al., 2008) for 48 h. The cell culture supernatant and cell pellets were collected at 24 and 48 h, and kept at −80 °C, until isolation of total RNA and qRT-PCR of candidate genes and miRNAs, as well as measuring PGs and cytokines.

2.3. In-silico analysis for the selected candidate miRNAs

The differentially expressed genes in endometrial cells and explants 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 the miRNAs hits were filtered on the basis their potential relevance for physiological function and inflammatory immune response of uterine tissue at least in four different search algorithms. Results identified that eca-miR-223, eca-miR-155, eca-miR-181b, eca-miR-532-5p, eca-miR-24, eca-miR-17, eca-let-7a, and eca-miR-21, as potential targets. Interestingly, eca-miR-181b, eca-miR-21, eca-miR-223, and eca-let-7a were mentioned by (Feng and Tsao, 2016; Hailemariam et al., 2014; Sally, 2015; Zhang et al., 2015), whereas these miRNAs were differentially expressed due to endometritis in cows or immune cells after LPS challenge, while eca-miR-155 is well known as an endotoxin-responsive gene (Schulte et al., 2013; Tili et al., 2007). In addition, a functional annotation analysis was performed using DAVID Bioinformatics Resource (http://david.abcc.ncifcrf.gov/).

2.4. Statistical analysis

Raw data of fluorescence values (Rn) for the real-time RT-PCR analyses were imported into PCR Miner to calculate efficiency (Zhao and Fernald, 2005). NormFinder (Andersen et al., 2004) was used to select the most stable reference gene for mRNA as well as the miRNAs expression profile. All data were tested for homogeneity using Gaussian distribution, and then data obtained from all the experiments (except data for cytokine and PGs concentrations) were analyzed using one-way ANOVA and a post-hoc test was conducted using Dunnett's test. Only data for IL6, TNF_α and PGs concentrations (Fig. 12 and 13) were analyzed using two-way ANOVA. The values shown in graphs are presented as the mean ± standard error of the mean (S.E.M) of at least three independent experiments each done in quadruplicate, P values ≤ 0.05 were considered statistically significant. GraphPad Prism 5.0 (San Diego, CA, USA) was used to perform statistical analysis and for generating bar graphs.

3. Results

3.1. Assessment of cell viability after LPS challenge

The viability of mixed equine endometrial epithelial and stromal cells was determined after LPS challenge with low dose (0.5 µg/ml) and/or high dose (3.0 µg/ml) for 48 h. We found that the viability of LPS challenged cells with both doses was significantly decreased ($p <$ 0.001), compared to the control (untreated) cells after 24 and 48 h (Supplemental Fig. 1).

3.2. Expression of TLR4 and its accessory molecules after LPS challenge in equine endometrial tissue explants

Dose-dependent responses of LPS to *TLR4* and its accessory molecules (*MyD88/CD14*) in equine endometrial tissue explants were observed: the low concentration of LPS induced low expression levels of *TLR4*, *MyD88* and *CD14*, while the high LPS concentration induced high levels of expression. The expression profiles of *TLR4*, *MyD88* and *CD14* were significantly ($p <$ 0.001) up-regulated in challenged tissue explants compared to the untreated control group (Supplemental Fig. 2).

3.3. Response of TLR4 and its accessory molecules post LPS challenge in equine endometrial cultured cells (epithelial & stromal)

After LPS treatment, we assessed the expression levels of *TLR4* and its accessory molecules (*MyD88/CD14*) in equine primary endometrial epithelial and stromal cells. The relative abundance of *TLR4*, *MyD88*, and *CD14* was significantly ($p <$ 0.001) increased in challenged epithelial as well as stromal fibroblast cells, and that increase was dose-dependent: the higher LPS dose evoked higher expression profiles of *TLR4*, *MyD88* and *CD14* in challenged cells compared to control unchallenged cells (Fig. 1).

3.4. Up-regulation of pro-inflammatory mediators after LPS challenge in equine endometrial tissue explants

The expression patterns of inflammatory mediators; *TRAF6*, *TNFα*, *IL6*, *IL8* and *IL10* in equine endometrial tissue explants were quantified. The high dose of LPS (3.0 µg/ml) induced higher expression patterns ($p <$ 0.001) of *TRAF6*, *TNFα*, *IL6* and *IL8* mRNA, while the low dose (0.5 µg/ml) resulted in lower expression ($p <$ 0.001) of these inflammatory mediators, compared to the control untreated group (Supplemental Fig. 3). The relative abundance of *IL10* was significantly ($p <$ 0.001) down-regulated in tissue explants after LPS treatment compared to untreated control (Supplemental Fig. 3).

3.5. Kinetic patterns of inflammatory mediators after LPS challenge in equine endometrial cultured cells

The expression pattern of *TRAF6*, *TNFα*, *IL6*, *IL8* and *IL10* mRNA were determined in equine endometrial cultured primary epithelial and stromal cells after LPS challenge. The relative abundance of *TRAF6*, *TNFα*, *IL6* and *IL8* mRNA was significantly ($p <$ 0.001) increased in both types of challenged cells and that increase was dose-dependent (Fig. 2). The expression of *IL10* mRNA was significantly ($p <$ 0.001) decreased after LPS challenge in both epithelial and stromal cells compared to the control group (Fig. 2). Both epithelial and stromal cells responded similarly to LPS treatment. Moreover, the challenged epithelial and stromal cells showed the same expression pattern of these mediators as the challenged tissue explant. Collectively, these findings revealed that both doses of LPS have a profound effect on transcriptome profile of primary equine endometrial cells and tissue explants.

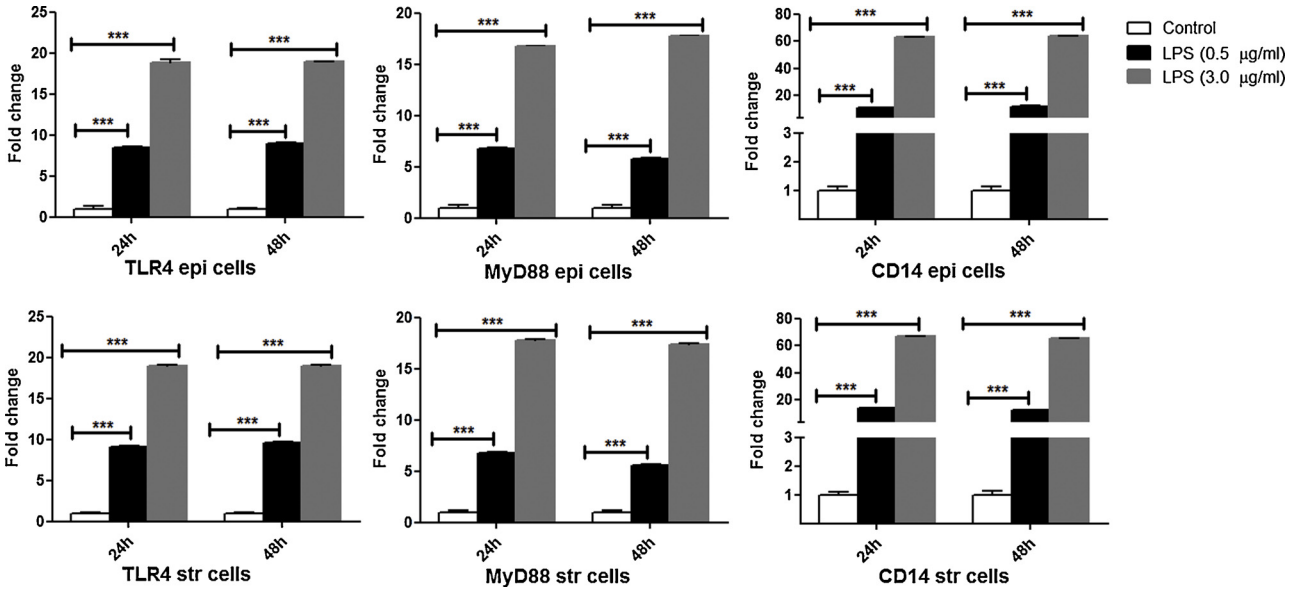

Fig. 1. Response of *TLR4* and its accessory molecules (*MyD88* &*CD14*) in equine primary endometrial epithelial (epi) and stromal (str) cells at 24 & 48 h after LPS challenge. Bars are presented as mean ± SEM. ***: statistical significant at $p < 0.001$.

3.6. LPS induced clear alterations in miRNAs expression profiles in equine primary endometrial cells and tissue explants

The in-silico analysis revealed that eca-miR-223, eca-miR-155, eca-miR-181b, eca-miR-532-5p, eca-miR-24, eca-miR-17, eca-let-7a, and eca-miR-21 could be potential regulatory miRNAs that target the inflammatory immune response genes after LPS challenge in equine primary endometrial cells and tissue explants. In addition the alignment between the seed region and 3´ UTR of selected candidate genes was examined. After LPS challenge, the dynamic patterns of miRNAs expression levels in equine primary endometrial cells and tissue explants were observed at 24 and 48 h. The expression profiles of eca-let-7a, eca-miR-181b and eca-miR-21 were significantly ($p < 0.001$) down-regulated at 24 and 48 h after LPS in challenged tissue explants (Supplemental Fig. 4) and in cells (epithelial & stromal) compared to the control untreated group (Fig. 3). The expression profiles of eca-miR-155, eca-miR-223, and eca-miR-17 were significantly ($p < 0.001$) up-regulated at 24 and 48 h of LPS challenge in tissue explants (Supplemental Fig. 5) and in both epithelial and stromal cells (Fig. 4), compared to the control group. The eca-miR-532-5p and eca-miR-24 miRNAs showed a unique expression pattern in response to LPS, where eca-miR-532-5p and eca-miR-24 were significantly ($p < 0.001$) over-expressed after the lower dose of LPS (0.5 µg/ml) both at 24 or 48 h. In contrast, the high dose of LPS (3.0 µg/ml) resulted in a significant ($p < 0.001$) inhibition of eca-miR-532-5p and eca-miR-24 expression profiles at both time points (24 and 48 h) in challenged tissue explants (Supplemental Fig. 6) and in cultured cells (epithelial and stromal) (Fig. 5), compared to the control unchallenged group.

3.7. Dynamic patterns of cytokines (IL6 and TNF_α) and PGs (PGE_2 and $PGF_{2\alpha}$) concentrations after LPS treatment

The concentrations of PGE_2 and $PGF_{2\alpha}$ in culture media from the endometrial cultured cells (epithelial and stromal), as well as from tissue explants and from cell lysates were significantly ($p < 0.001$) increased after both LPS treatment doses at 24 and 48 h compared to control untreated group. The effect of different time points on PGs concentrations was analyzed by two-way ANOVA, and it was revealed that PGs concentrations were higher at 24 h, and then started to decline at 48 h of LPS treatment, compared to the control unchallenged group. Thus, different time points as well as LPS treatment revealed a clear effect ($p < 0.001$) on PGs concentrations (Figs. 6 A and B).

The of concentrations of IL6 and TNF_α in the culture media from the cultured endometrial cells, as well as from the tissue explants and from cell lysates were significantly ($p < 0.001$) increased after either LPS treatment doses at 24 and 48 h compared to the control untreated group. There was a profound effect of different time points as well as of LPS treatment on secretion of cytokines (IL6 and TNF_α), where IL6 and

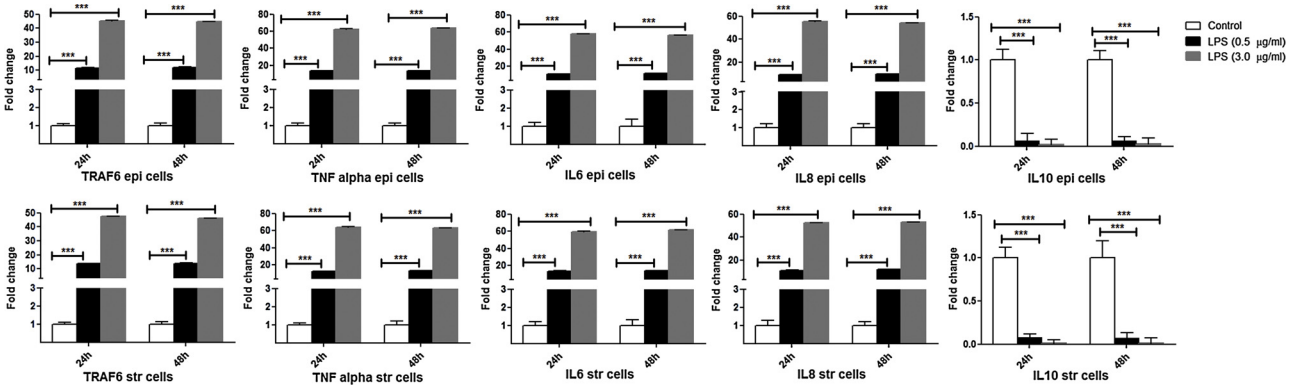

Fig. 2. Relative abundance of inflammatory response genes (*TRAF6*, *TNF alpha*, *IL6*, *IL8*, and IL10) in equine primary endometrial epithelial (epi) and stromal (str) cells at 24 & 48 h after LPS challenge. Bars are presented as mean ± SEM. ***: statistical significant at $p < 0.001$.

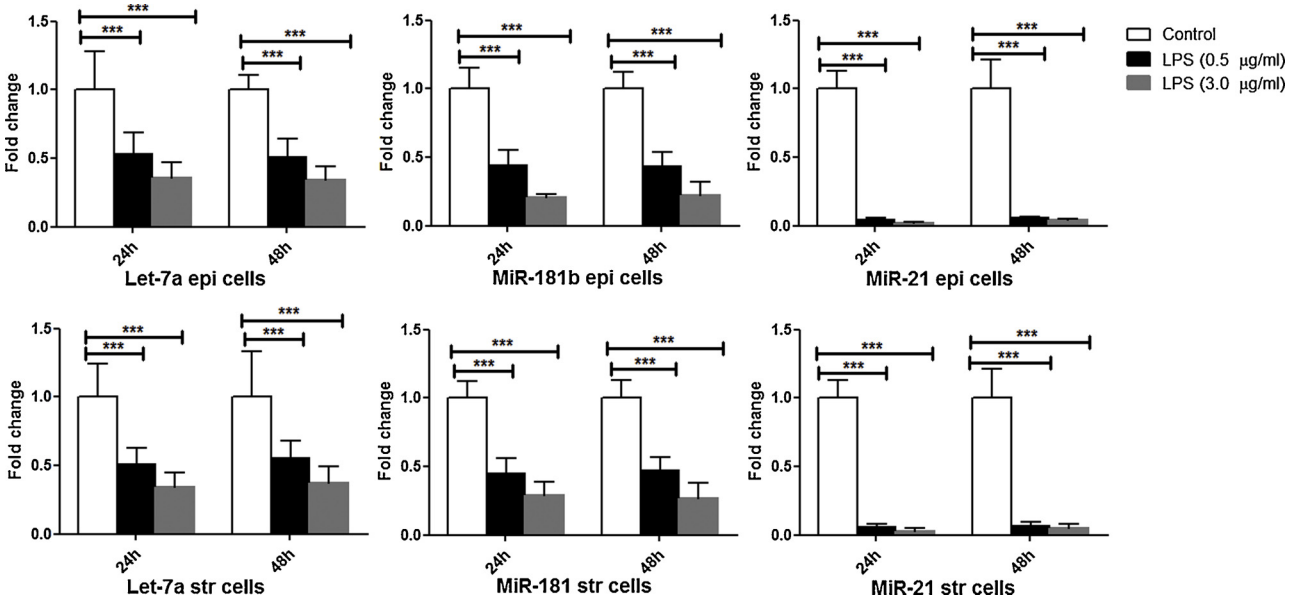

Fig. 3. LPS resulted in reduction of let-7a, miR-181b and miR-21 expression in equine primary endometrial epithelial (epi) and stromal (str) cells at 24 & 48 h. Bars are presented as mean ± SEM. ***: statistical significant at $p < 0.001$.

TNF_α levels were higher at 24 h and then gradually decreased at 48 of LPS treatment compared to the control untreated group (Supplemental Fig. 7 A and B). Taken together, both LPS doses as well as different time points had a clear effect ($p < 0.001$) on dynamic patterns of PGs and cytokine concentrations in the challenged groups compared to the untreated control group.

4. Discussion

Infectious endometritis, either before or after breeding, remains a major threat to mare fertility and to the equine industry, where the incidence of infectious (bacterial form) endometritis ranges from 25 to 60% (Frontoso et al., 2008;Nielsen et al., 2010 Rasmussen et al., 2015; Riddle et al., 2007). Microbiological studies on genital tract of mares showed that *Streptococcus equi subspecies zooepidemicus,* and *E. coli* are the strains most frequently isolated from septic endometritis in mares (Katila, 2016; M, 2012). LPS is the main glycolipid component of the outer membrane of Gram-negative bacteria, and is a potent secretagogue for a variety of inflammatory mediators and immunoregulatory cytokines from endometrial cells and leukocytes (G.S., 2010; Oguejiofor et al., 2015). A better understanding of the underlying mechanisms of uterine inflammation is necessary, in order to develop new strategies for control and/or treatment of endometritis in mares. In the current study, we have simulated clinical and/or subclinical endometritis using different challenge doses of LPS, in our well-defined in vitro models (cultured primary endometrial cells, and tissue explants).

Our data revealed that *TLR4* and its accessory molecules (*MyD88/CD14*) could be the main molecules that recognized LPS and subsequently triggered inflammatory down-stream mediators, via rapid over-expression of *TRAF6*, *TNFα*, *IL6* and *IL8* mRNAs, while the main anti-inflammatory molecule *L10* was clearly suppressed after LPS challenge. Interestingly, both primary endometrial epithelial and stromal

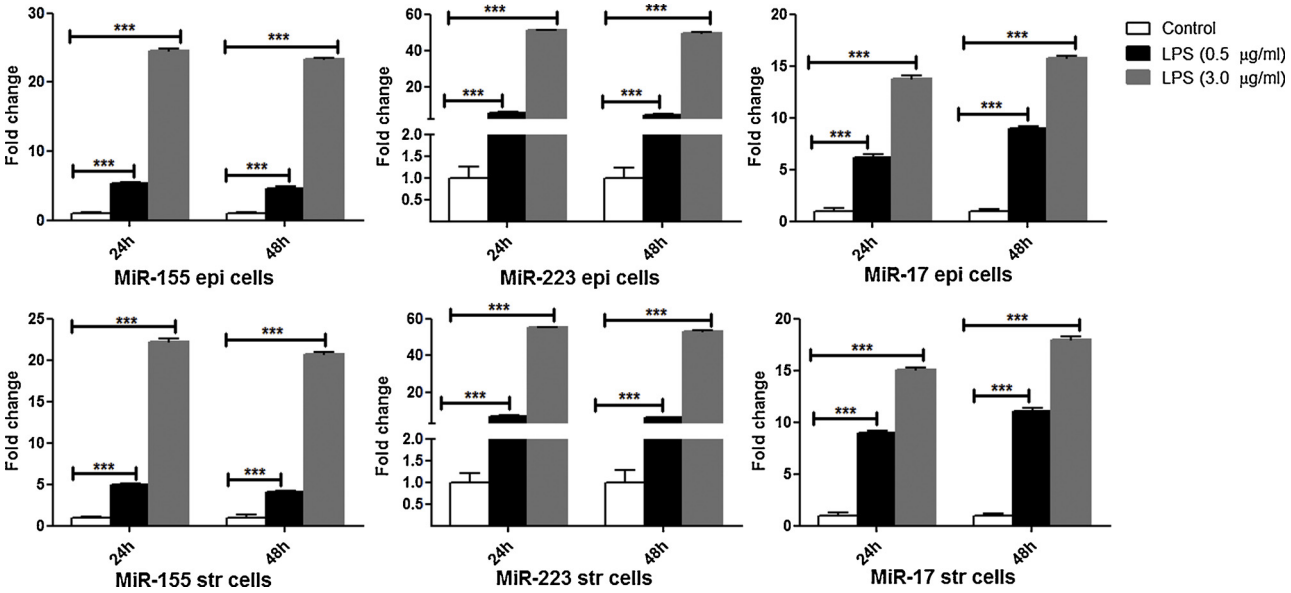

Fig. 4. Over-expression of miR-155, miR-223 and miR-17 after different doses of LPS challenge in equine primary endometrial epithelial (epi) and stromal (str) cells at 24 & 48 h. Bars are presented as mean ± SEM. ***: statistical significant at $p < 0.001$.

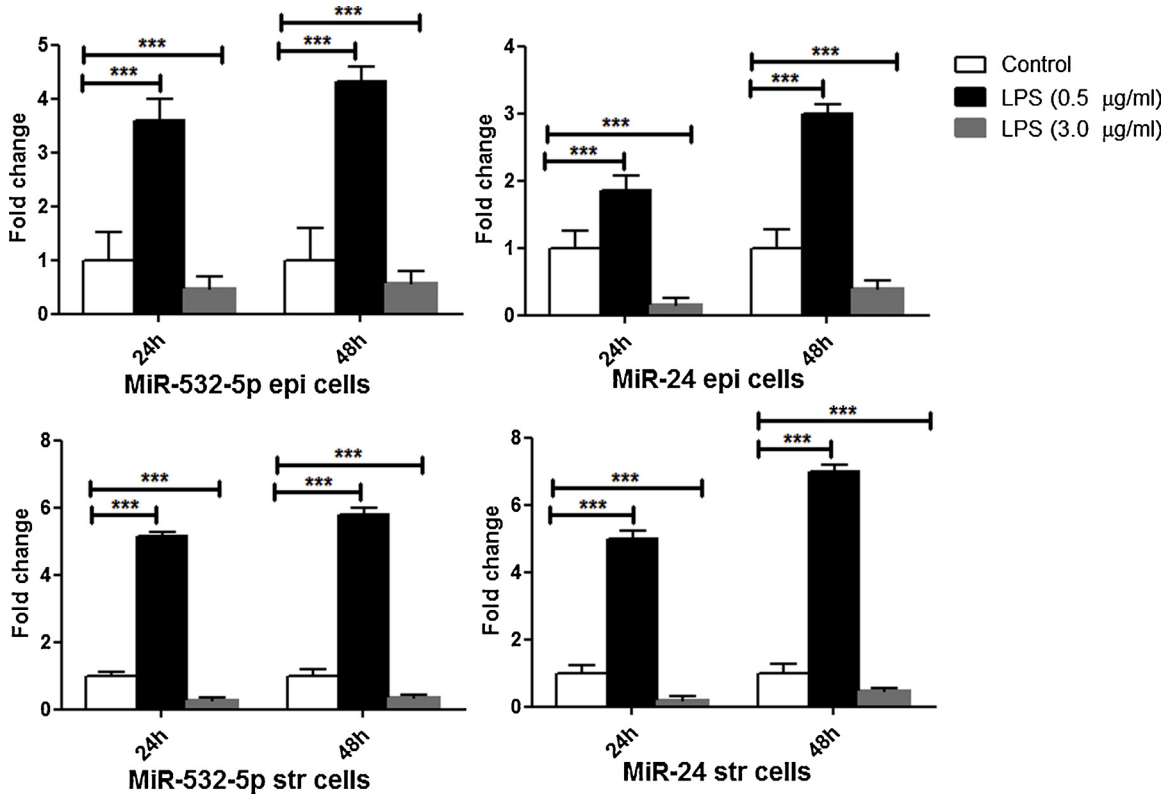

Fig. 5. Aberrations in miR-532-5p, and miR-24 expression after different doses of LPS challenge in equine primary endometrial epithelial (epi) and stromal (str) cells at 24 & 48 h. Bars are presented as mean ± SEM. ***: statistical significant at $p < 0.001$.

fibroblast cells responded similarly to LPS challenge, and this may indicate that both cell types have a pivotal role in the inflammatory immune response pathway in mares, through up-regulation the expression of *TLR4* and its regulatory molecules (*MyD88/CD14*). The response of these cells to LPS was dose dependent, where the higher dose (3.0 μg/ml) provoked higher expression and release of down-stream inflammatory cytokines such as *TRAF6*, *TNFα*, *IL6* and *IL8*, but the low dose of LPS (0.5 μg/ml) induced their lower expression and release of the previously mentioned down-stream inflammatory cytokines, compared to the control untreated group. We also investigated the protein profiles of some cytokines (IL6 and TNFα), and both of them showed a similar pattern as their mRNA expression levels. Endometrial tissue explants, which are composed of epithelial and stromal and resident leukocytes, showed the same pattern of expression for the molecules (*TLR4*, *CD14*, *MyD88, TRAF6*, *TNFα*, *IL6*, *IL8* and *IL10*), as observed in the cultured primary endometrial epithelial and stromal cells. Thus, these findings indicated the success and the functionality of our well-defined model, being were in accordance with previously reported

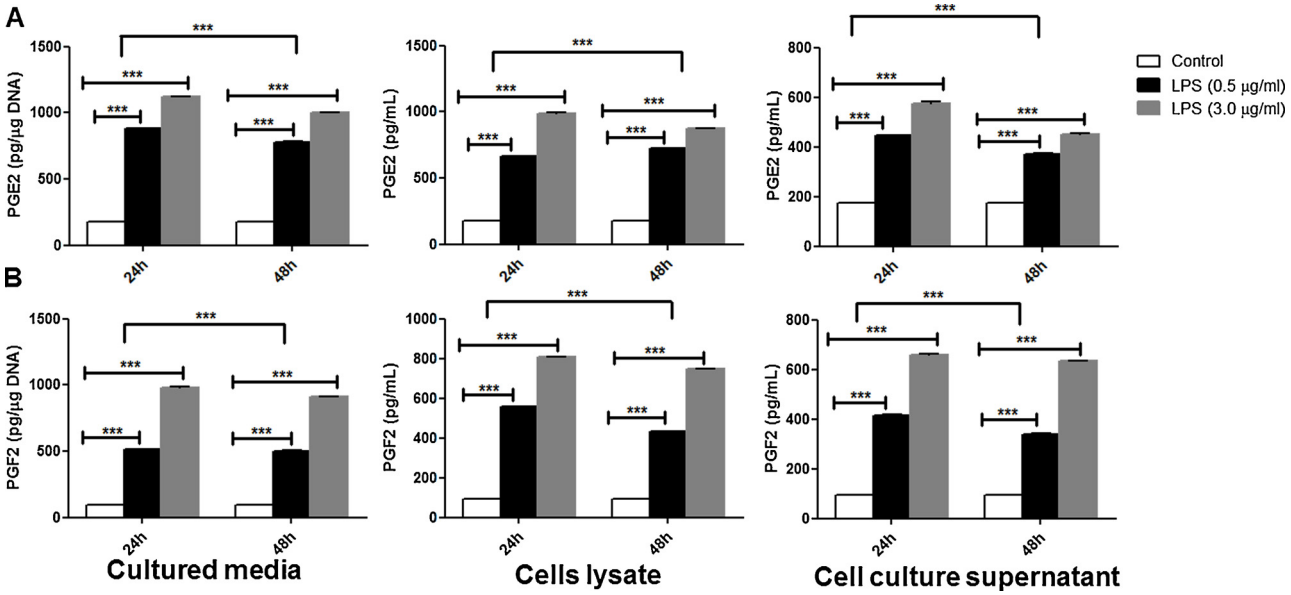

Fig. 6. Effect of LPS doses on PGs level in cultured media of cultured cells as well as tissue explants, and from cells lysate at 24 & 48 h. (A) Dynamic patterns of PGE_2 secretion after LPS treatment. (B) Level of $PGF_{2\alpha}$ secretion post LPS challenge. Results are presented as mean ± SEM. ***: statistical significant at $p < 0.001$.

results in the bovine and equine species (Cronin et al., 2012; Nash et al., 2008; Siemieniuch et al., 2016; Swangchan-Uthai et al., 2012). To focus only on the role of primary endometrial epithelia and stromal fibroblast cells, and exclude the effects of immune cells during LPS challenge in our in vitro model, we confirmed absence of the immune cells, using conventional PCR as well as qRT-PCR analysis of CD45, which is a gene encoding for a panleukocyte marker; this finding was similar to data reported previously in studies with cattle (Cronin et al., 2012; Swangchan-Uthai et al., 2012).

MiRNAs have emerged as important regulators of gene expression, and it has become clear that they are instrumental in the area of mammalian inflammatory responses (Ibrahim et al., 2015; Makeyev and Maniatis, 2008). Recently, it was clearly indicated that endometrial transcriptome (mRNA) and miRNome profiles were altered in cows affected by subclinical or clinical endometritis, which had a significant effect on uterine homeostasis and receptivity (Salilew-Wondim et al., 2016; Sally, 2015). Until now, nothing is known about the post-transcriptional regulation of inflammatory immune response genes in mares with endometritis. Therefore, in the current study, we examined the temporal expression patterns of selected miRNAs that could potentially be targeting most of the inflammatory immune response genes in the equine endometrium after LPS challenge. It was interesting that some candidate miRNAs, such as; eca-let-7a, eca-miR-181b and eca-miR-21, were inhibited but eca-miR-155, eca-miR-223, and eca-miR-17 were over-expressed at 24 and 48 h after LPS challenge in cells and tissue explants. These results are similar to recent studies that investigated regulation of miRNAs in mammalian host cells challenged with different pathogens (Salilew-Wondim et al., 2016; Sally, 2015; Teng et al., 2013). Intriguingly, eca-miR-532-5p and eca-miR-24 revealed a unique expression pattern in response to LPS, in which eca-miR-532-5p and eca-miR-24 were up-regulated after a low dose of LPS (0.5 μg/ml) but the high dose of LPS (3.0 μg/ml) down-regulated eca-miR-532-5p and eca-miR-24 expression profiles at both 24 and 48 h time points, and these patterns were observed in both challenged cultured primary cells and tissue explants, compared to the control unchallenged group. The explanation could be that certain miRNAs functions may only be revealed at a specific concentration of an environmental trigger (LPS dose), and also that miRNAs could control pathways associated with the immune response (Eulalio et al., 2012). It is essential to keep a delicate balance between the pro- and anti-inflammatory immune responses, in order to maintain homeostasis of uterine tissues. The different scenarios between miRNAs and their potential targets (inflammatory immune response genes), such as IL6, TNF_{α}, IL8 and IL10, revealed that some of these inflammatory mediators showed the same and/or the reciprocal pattern to their potential targeting miRNAs, in which the up-regulation of miR-17 was clearly associated with an increased in mRNA expression of IL6, 1L8 and TLR4, but the down-regulation of miR-181b was accompanied by significant up-regulation of IL6 and TLR4 mRNA expression. These findings indicate that aberrant expression of regulatory molecules such as miRNAs could be associated with an improper balance between pro- and anti-inflammatory mediators, and leading to disturbance of uterine functions and homeostasis. Thus, we can exploit these findings to modulate the expression of inflammatory mediators, and maintain a delicate balance between pro- and anti-inflammatory mediators, as well as avoiding excessive tissue damage.

Basal secretion of endometrial PGs (PGE_2 and $PGF_{2\alpha}$) was obviously increased after LPS challenge, when dynamic patterns of PGs secretion were dose-and time-dependent. Therefore, we could say that LPS stimulates PGs secretion. Furthermore, secretion of PGs in our in vitro model indicated its functionality, because prostaglandins play an important role in the equine endometrium during different stages of the estrous cycle, as well as in inflammatory processes. Moreover, any aberrations in secretion PGs could perturb normal physiological functions of uterine tissue, through premature luteolysis of the corpus luteum, subsequently causing early embryonic death, which is in agreement with findings of earlier studies (Nash et al., 2008; Pycock and Allen, 1990).

Our research elucidated the interactions among selected miRNAs and their potential inflammatory immune response target genes. These events could be necessary to support a tight balance between multiple pro- and anti-inflammatory mediators, which are required for rapid and efficient elimination of bacteria and subsequently maintain receptivity and cyclicity in mares. Moreover, further studies are required to investigate the precise role of miRNAs, and for better understanding of the mechanisms underlying interactions between innate immunity/inflammation responses and reproductive physiology in mares.

5. Conclusions

Epithelial and stromal cells have a primary role in innate immune responses in equine endometrium after *LPS* challenge, in which this recognition occurs via *TLR4* and its regulatory molecules (*MyD88* and *CD14*), and triggers down-stream inflammatory genes. Furthermore, aberrations of miRNAs expression after LPS challenge might affect normal uterine physiological function and homeostasis, by perturbing concentrations of PGs, IL6 and TNF_{α} in the affected cells and tissues.

Conflicts of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2019.02.006.

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