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Preimplantation factor modulates acute inflammatory responses of equine endometrium
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Original Article

Preimplantation factor modulates acute inflammatory responses of equine endometrium

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Abstract

Persistent mating-induced endometritis (PMIE) is a significant cause of mare infertility hence its treatment would advance the management of susceptible mares. Preimplantation factor (PIF) is secreted by viable embryos, including human, mouse and cattle, and is essential for maternal immune-tolerance without immune-suppression by modulating inflammation. This preliminary study aimed to test whether PIF exerts inflammatory-modulating properties upon equine endometrium challenged with *Escherichia coli*-derived lipopolysaccharide (LPS) using endometrial explant culture. Follicular (n = 3), luteal (n = 4), anoestrous (n = 4) and transitional (n = 4) stage endometrial explants were established and cultured in triplicate in either serum-free medium alone (control) or medium with; 50 or 100 nmol/L synthetic PIF (sPIF); 3 μg/mL LPS; LPS and 50 or 100 nmol/L sPIF; or scrambled PIF (PIFscr; same amino acid composition arranged in a different order). Media samples were collected at 24 and 72 h, representing acute and chronic inflammatory response. Radioimmunoassay determined Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) as an inflammatory marker. The only significant observation was the abrogation of $PGF_{2\alpha}$ response to LPS challenge by 100 nmol/L PIF for follicular stage tissue, 24 h after treatment. Further studies are therefore, warranted to realise PIF potential in managing PMIE.

Keywords: equine endometritis, inflammation, treatment, preimplantation factor.

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Introduction

In mares, the acute but transient, mating-induced uterine inflammation (MIE) observed after insemination is a ubiquitous innate immune response to semen and/or bacteria (Allen & Pycock 1989; Katila 2001; LeBlanc 2010). Bacteria are frequently recovered from the uterus after mating; particularly, *Streptococcus zooepidemicus* and *Escherichia coli* (Hughes & Loy 1975; Wingfield Digby & Ricketts 1982) and bacterial antigens stimulate an innate immune response (Christoffersen & Troedsson 2017). However, in up to 40% of mares, inflammation persists (persistent mating-induced endometritis; PMIE) causing embryonic loss (Pycock & Newcombe 1996). In practice, treatment may only commence after PMIE is established (LeBlanc 2010), therefore there is interest in agents which may

modulate the innate immune response before inflammation becomes established. Steroids, Mycobacterium cell wall extract and N-acetylcysteine have shown potential (Fumuso *et al.* 2007; Bucca *et al.* 2008; Gores-Lindholm *et al.* 2013; Melkus *et al.* 2013); however, with each there are important drawbacks (LeBlanc 2010; Gores-Lindholm *et al.* 2013; Woodward *et al.* 2015). Therefore, a need exists to discover novel anti-inflammatories which may complement the innate immune response.

Preimplantation factor (PIF) is a protein secreted exclusively by viable embryos shortly after fertilisation, inducing maternal acceptance through immune tolerance without maternal immune-suppression (Barnea 2004; Paidas *et al.* 2010; Stamatkin *et al.* 2011). PIF exerts its immune function on the maternal system directly on endometrial and immune cells; PIF up-regulates human decidual cell genes

including interleukin 6 (IL-6) and IL-1β and changes expression at the protein level (Paidas *et al.* 2010). Due to its immune-modulatory function, PIF is therefore a potential novel anti-inflammatory.

An optimised and validated tissue explant culture system using equine endometrium provides an exemplar for bacteria-induced MIE (Nash *et al.* 2008). Explants respond to *E. coli*-derived lipopolysaccharide (LPS) challenge by secreting the inflammatory marker prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) similarly to the whole animal and is integral to physiological reproductive function as well as inflammation (Nash *et al.* 2008, 2010).

The aim of this preliminary study was to determine the effect of synthetic PIF (sPIF) on the innate immune response to LPS challenge using equine endometrial explants collected from mares at different stages of the oestrous cycle, using $PGF_{2\alpha}$ as the inflammatory marker.

Materials and methods

All experiments used uteri from mares sent to slaughter at a commercial abattoir for reasons unrelated to the study. Therefore, ethics approval was not required.

Strips of endometrium were collected from 17 slaughtered mares post-mortem (aged 10.3 years ± 1.25) at follicular (n = 3), luteal (n = 6), anoestrous (n = 4) and transition (n = 4) stages of cycle, assessed as previously described (Nash *et al.* 2008). Uteri were harvested from mares presented at a commercial abattoir for reasons unrelated to this study. Uteri were placed on ice and transported to the laboratory. The uterine body was opened with a scalpel and the endometrium dissected from underlying myometrium using sterile scissors and forceps. Strips were chopped to 1 mm^3 and washed in HBSS (Hank's buffered salt solution). Sterile lens tissue-covered aluminium mesh platforms were placed into each well of a six-well plate (Corning Costar). A quantity of 150 mg endometrial tissue was weighed onto each platform and 4.25 mL of supplemented William's medium (Sigma) added as previously described (Nash *et al.* 2008). Tissues were incubated for 24 h in a humidified atmosphere at 37°C and 5%

carbon dioxide (CO_2). After 24 h, the medium was replaced with fresh, growth factor-free medium alone (control) or treatment medium (designated time point 0 h). Explants from each horse were cultured in triplicate for each treatment. Treatments were: medium alone (control); 50 nmol/L sPIF; 100 nmol/L sPIF; 3.0 μg/mL LPS from *E. coli* strain 0111:B4 (Source Bioscience); 3.0 μg/mL LPS with 50 or 100 nmol/L sPIF; 100 nmol/L scrambled PIF (PIFscr; same amino acid composition arranged in a different order, routinely used as a control measure in other published work in this field (Barnea *et al.* 2012; Roussev *et al.* 2013). Culture supernatants were collected 24 and 72 h after treatment, representing the acute and persistent inflammatory periods respectively. The sPIF concentrations chosen were those found to affect human endometrium in culture (Paidas *et al.* 2010). Culture supernatants were radio immunoassayed for $PGF_{2\alpha}$.

Serum progesterone concentrations were measured from blood collected at exsanguination was measured using a solid phase ELISA kit (Immunodiagnostic Systems) to retrospectively confirm the stage of oestrous cycle as previously described (Nash *et al.* 2008). Briefly, coupled with morphological assessment of the ovarian and cervical characteristics, stage of cycle was defined where progesterone concentrations were: <1 ng/mL, transition and anoestrous stage; <2 ng/mL, follicular stage; >2 ng/mL luteal stage.

$PGF_{2\alpha}$ secretion by treated explants was expressed as percentage change from control ($\pm$SEM) to account for individual mare variation. Data were square root transformed to achieve normality. A univariate general linear model was used to compare $PGF_{2\alpha}$ secretion between control and treated explants and Dunnett's post hoc test was used in SPSS v22. Significance was attributed when $P < 0.05$.

Results

Synthetic sPIF alone and PIFscr did not stimulate $PGF_{2\alpha}$ secretion by explants from any stage of the oestrous cycle after 24 or 72 h. After 24 h, LPS treated explants secreted significantly more $PGF_{2\alpha}$ than control tissue (follicular and luteal stages, $P < 0.001$; Figs 1a and 2a). The inflammatory response to LPS
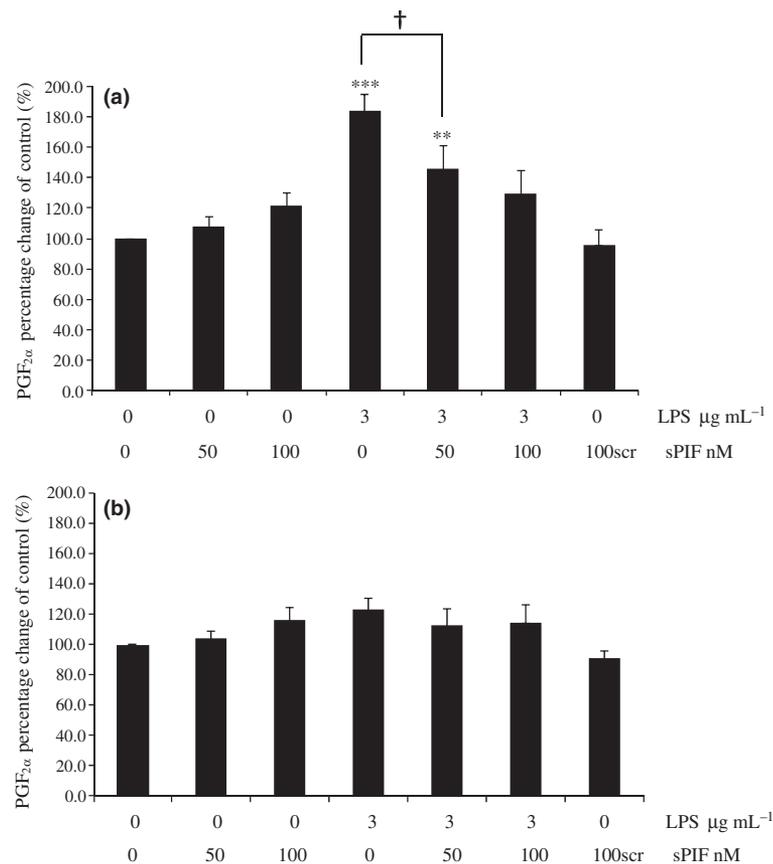
Fig. 1. Secretion of $PGF_{2\alpha}$ by follicular stage equine endometrial explants treated with control (media only), 50 nmol/L PIF, 100 nmol/L sPIF, 3.0 μg/mL LPS, 3.0 μg/mL LPS with 50 or 100 nmol/L PIF or 100 nmol/L scrambled PIF (PIFscr). The $PGF_{2\alpha}$ concentrations were measured (a) 24 h (acute inflammatory period) or (b) 72 h (chronic inflammatory period) after treatment. Values differ from control, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Values differ between treatments, $^{\dagger}P < 0.05$.

by follicular stage explants was abrogated in the presence of 100 nmol/L sPIF at 24 h ($PGF_{2\alpha}$ concentrations were not significantly different to the control; Fig. 1a). There was a significant difference between $PGF_{2\alpha}$ secreted by explants treated with LPS alone compared to LPS and 50 nmol/L PIF ($P < 0.05$; Fig. 1a). There was no effect of any treatment on explants collected from follicular or luteal mares after 72 h (Figs 1b and 2b), or after 24 or 72 h for transition or anoestrous explants (data not shown).

Discussion

This preliminary study demonstrated that 100 nmol/L sPIF abrogated $PGF_{2\alpha}$ in follicular stage explants 24 h after challenge with bacterial LPS. The ability

of PIF to abrogate $PGF_{2\alpha}$ during the follicular, but not luteal, stage of the cycle is biologically important as this is the time when mating, and therefore, bacterial challenge occurs. Therefore, PIF may interact with the native steroid milieu of endometrium; endogenous progesterone promotes immune-suppression but concentrations are low during the follicular stage (Watson *et al.* 1987). Neither PIF alone (either 50 or 100 nmol/L) nor PIFscr caused an increase of $PGF_{2\alpha}$ compared to controls in explants derived from all stages of the oestrous cycle, demonstrating that PIF itself did not inadvertently cause inflammation, at least in terms of prostaglandin response.

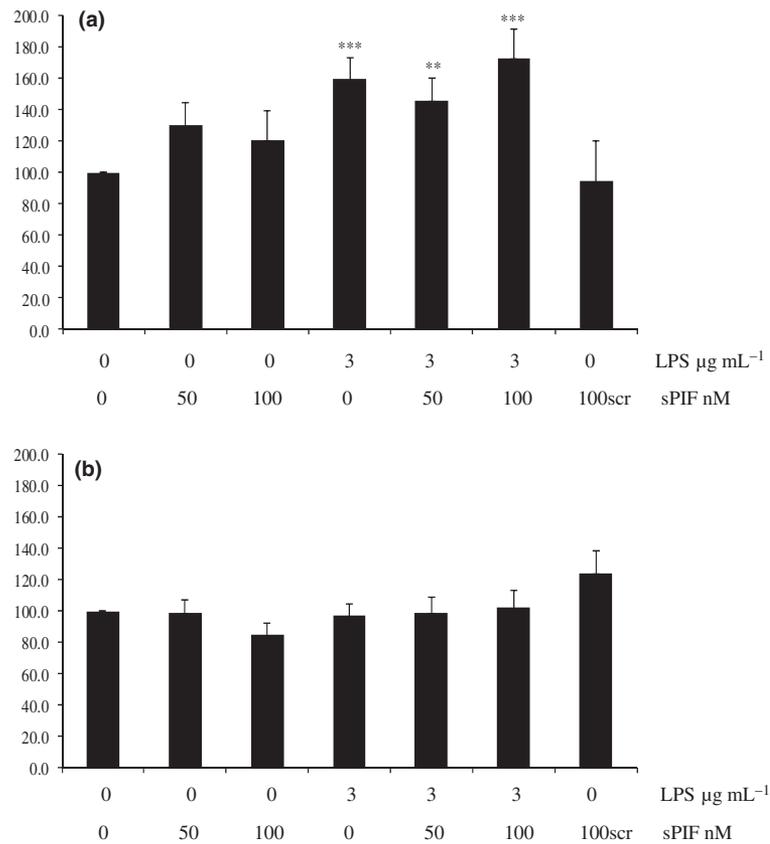A treatment of LPS combined with PIFscr as a further control was not included in this study. A $PGF_{2\alpha}$

Fig. 2. Secretion of PGF$_{2\alpha}$ by luteal stage equine endometrial explants treated with control (media only), 50 nmol/L PIF, 100 nmol/L sPIF, 3.0 μg/mL LPS, 3.0 μg/mL LPS with 50 or 100 nmol/L PIF or 100 nmol/L scrambled PIF (PIFscr). The PGF$_{2\alpha}$ concentrations were measured (a) 24 h (acute inflammatory period) or (b) 72 h (chronic inflammatory period) after treatment. Values differ from control, **$P < 0.01$; ***$P < 0.001$.

response greater than the concentrations observed for the LPS treatment alone was unlikely to be secreted in response to an LPS-PIFscr combination without the addition of arachidonic acid to the medium. Arachidonic acid is a substrate for prostaglandin synthesis and has been shown in work by colleagues to be necessary to add to culture medium when PGF$_{2\alpha}$ secretion may otherwise reach a plateaux and so maximal concentrations not achieved (Herath *et al.* 2006). The present study was confident that the LPS concentrations used would not exceed this plateaux based on previous optimisation studies and yet would still enable an effect of LPS and in turn its abrogation to be observed (Nash *et al.* 2008), and so arachidonic acid was not necessary to achieve the aim of this study. Furthermore, to maintain

explants in a manner that was as close to the whole animal as possible, avoiding further medium supplementation was desirable.

It is interesting that 100 nmol/L sPIF was effective at 24 h and not 50 nmol/L. Approximately 100 nmol/L PIF is the physiological concentration measured during normal pregnancy in women (Duzyj *et al.* 2010). sPIF of either concentration was not effective at 72 h. The medium was not replaced after initial treatment, so PIF activity may have been effective up to 24 h but not maintained thereafter. PIF stability is unconfirmed in tissue culture. However, there was also no PGF$_{2\alpha}$ response to LPS alone at the chronic, 72 h time point following challenge, which disagrees with Nash *et al.* (2008) where the model was reported as functional in response to LPS

up to 72 h. Additionally, LPS induced inflammation was evident at 24 h only in follicular stage explants, disagreeing with Nash *et al.* (2008) who reported an effect only in transition mares. The disparity between studies may be partly explained by the possibility that the response may have finished by 72 h and that improvements in laboratory practice since the model was first validated and optimised have generated data in the present study that are more representative of the whole animal response (Nash *et al.* 2008). Nonetheless, PIF has demonstrated some anti-PGF$_{2\alpha}$ properties, but its wider anti-inflammatory potential to manage PMIE in horses requires further study.

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Conflict of interest

The authors declare they have no competing interests.

Ethical statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as endometrial tissue was harvested from animals sent to commercial slaughter, for reasons unrelated to this study.

Contributions

DMN participated in study design, data analysis and wrote the manuscript. JP conducted laboratory work. MCGD contributed to writing the manuscript. ERB instigated the study.

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