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Effects of preimplantation factor on interleukin-6 and prostaglandin $F_{2\alpha}$ and E_2 in the bovine endometrium

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ABSTRACT

Preimplantation factor (PIF) is a pregnancy specific peptide with immune modulatory properties exerted on the human endometrium. Viable bovine embryos secrete PIF, but its effect on the bovine endometrial immune response is unknown, both in native and inflammatory stimulated endometrial tissue. An *ex vivo* bovine endometrial tissue culture model was used with lipopolysaccharide (LPS) as an inflammatory stimulant. The effect of synthetic PIF (sPIF) was assessed, in three separate experiments, on the secretion or mRNA expression of essential prostaglandins and cytokines. Radioimmunoassays were used to assess prostaglandin secretion and ELISA for IL-6 secretion from endometrial explants. mRNA expression of *IL6* and *IL8* was analysed from endometrial explants with real-time PCR. Synthetic PIF reduced native IL-6 secretion from explants when pre-treated for 24 h. There was no effect of sPIF on IL-6 secretion from LPS challenged explants; however, sPIF increased *IL6* mRNA expression when challenged with 500 ng/mL LPS. There was no effect of sPIF on prostaglandin secretion or mRNA expression of *IL8*. Therefore, sPIF is able to modulate the native IL-6 production pathway in the bovine endometrium, yet demonstrates no effect on prostaglandin secretion or *IL8* expression. Unlike in human studies, effects of sPIF were minimal, thus sPIF is not an effective modulator of the immune targets investigated in the bovine endometrium.

1. Introduction

Preimplantation factor (PIF) is a 15 amino acid peptide that is produced by viable embryos as early as the two-cell stage [1]. Bovine embryos produce PIF both pre- and post-implantation [1,2]. Preimplantation factor works through an immune tolerance pathway in human pregnancy to facilitate the acceptance of the embryo by the mother [3,4]. It is the action of this pathway that is of interest to studies of disease, as sPIF may have potential as an immune modulator. Applied to healthy human endometrium, sPIF is able to upregulate secretion of several interleukins, including IL-8 and IL-6 of decidualized stromal cells [5]. Interestingly, within a murine multiple sclerosis model, sPIF decreased the secretion of IL-6 from splenocytes in culture showing a tissue specific role of the peptide [6]. In a preliminary study, sPIF was investigated in an equine model of *E. coli* post-mating induced endometritis. It was shown that sPIF was able to reduce prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) secretion from LPS induced explants 24 h after challenge [7] but only in mares of the follicular stage of the oestrous cycle. More recently, in CD14 + cells, it has been demonstrated that sPIF does not directly interact with TLR-4, but specific downstream targets within the TLR-4 pathway [8]. As sPIF has been demonstrated to interact with the human endometrium in an immune modulatory manner, it was proposed that the peptide may act in a similar manner in the bovine endometrium. Furthermore, as uterine inflammation is a common cause of infertility in cattle through a dysregulation of endocrine function [9], it is of interest to investigate the role of sPIF as an immune modulator in an endometrial inflammatory environment.

Previous studies have utilised an *ex vivo* bovine model of normal and inflammatory endometrium, which shows responses similar to the whole cow [10,11] and so this model provides a basis for this initial investigation. Both prostaglandins and interleukins are secreted by cyclic, pregnant and inflammatory endometrial tissue and so have previously been used as targets to measure in *ex vivo* studies [10–12]. Prostaglandins are eicosanoid hormones produced by the endometrium and have essential functional roles in the

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bovine oestrous cycle and pregnancy [13,14]. Furthermore, both $PGF_{2\alpha}$ and prostaglandin E_2 (PGE_2) are involved in the endometrial inflammatory response. Following a challenge with *Escherichia coli*-derived lipopolysaccharide (LPS), there is an increase in secretion of $PGF_{2\alpha}$ and PGE_2 from endometrial tissue explants [12,15]. Interleukins, such as IL-6 and IL-8 are demonstrated to be expressed during the oestrous cycle [16]. Furthermore, in *ex* vivo studies, IL-6 and IL-8 have been shown to have key roles within the endometrial innate immune response [11,17].

The aim was to investigate the use of sPIF as a potential immune modulator within the bovine endometrium by assessing the role of the peptide using a previously developed endometrial tissue model, using LPS treatment to model an *E. coli* challenge [11,12,15]. It has already been demonstrated that sPIF does not bind to LPS [8]. It was hypothesised that sPIF would reduce key immune (IL-6 and IL-8) and endocrine ($PGF_{2\alpha}$ and PGE_2) factors in the bovine endometrium, at both a native and inflammatory level.

2. Materials and methods

2.1. Sample collection and endometrial explant culture

As these experiments used post-slaughter material, licencing through the Animals (Scientific Procedures) Act 1986 and ethical review were not necessary. Bovine uteri and corresponding blood samples were collected from cows presented for slaughter at a local abattoir. A total of 46 animals were used in the study. Uteri with stage I and IV ovaries were investigated to allow the study of sPIF on endometrial tissues that were not under the immune suppressive effects of progesterone [18,19]. Samples were staged by assessing ovarian morphology as previously described [20,21]. Briefly, stage I was defined as having a newly ruptured corpus luteum with a diameter of 0.5–1.5 cm and stage IV as having a regressing corpus luteum with a diameter of <1 cm [20].

Uteri and blood samples were stored on ice during the 1-h transportation to the laboratory. Tissues were used for explant culture and blood serum for analysis of progesterone concentration via ELISA (DRG Diagnostics, Marburg, Germany). To support ovarian morphology staging, the blood sera were used for progesterone analysis. For stage I and IV uteri, samples were deemed to have high progesterone if serum concentrations were above 1 ng/mL [10]. Progesterone inter- and intra-assay CVs were 8.66% and 2.18%, respectively.

Tissue culture was established using the method previously described [11]. Briefly, tissue was sampled from the uterine horn ipsilateral to the staged ovary using an 8 mm biopsy punch. Samples were weighed and placed in 6 well plates (Corning, Amsterdam, The Netherlands) with 3 mL of RPMI 1640 media (Gibco, Life Technologies, Paisley, UK) supplemented with 50 IU/mL penicillin, 50 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and 2.5 μg/mL amphotericin B (Sigma-Aldrich). All treatments were run in duplicate or triplicate and described for each experiment. Explants were incubated in a sterile incubator at 37 °C and 5% CO_2 for up to 72 h. Ultra-pure LPS from *E. coli* 0111:B4 strain was used (InvivoGen, Toulouse, France). Synthetic PIF (MVRIKPGSANKPSDD) was synthesised with >95% purity by Bioincept (New Jersey, USA). The amino acid structure of the human 15 amino acid PIF has previously been analysed and the 3D structure predicted [22]. The sPIF used in the present study is utilised in all research investigating sPIF.

2.2. Experiment 1

The aim of experiment 1 was to investigate the effect of sPIF on prostaglandin and IL-6 secretion from bovine endometrial explants with and without an LPS challenge. Bovine stage IV uteri (n = 14) were utilised. Preliminary studies from our laboratory, utilising unidentified cattle breeds, showed varied results in terms of prostaglandin secretion following sPIF treatment. Therefore, cattle were separated into two groups at the abattoir through identification of being either: beef heifers (n = 7), unlikely to have been pregnant or; dairy type cows (n = 7) and having had one or more pregnancies. Tissues were sampled from the endometrium and challenged with the following treatments in triplicate: control (media alone); LPS (1 μg/mL) alone; sPIF at three concentrations (50, 100 or 500 nM); or LPS (1 μg/mL) combined with each of the three sPIF concentrations. The LPS concentration was chosen based on previous studies utilising the same endometrial tissue model [11,12]. Synthetic PIF concentrations were based on the previously described physiological range within the circulation during human pregnancy (50 and 100 nM) and one supra-physiological concentration (500 nM) [23]. Media supernatants were sampled 24, 48 and 72 h after challenge, from the same well at each time point. Time points of 24 and 48 h were chosen based on previous studies [11,12] and 72 h based on an equine endometrial explant study as a persistent infection time point [24]. Supernatant samples were stored at −20 °C until analysed for $PGF_{2\alpha}$, PGE_2 and IL-6.

2.3. Experiment 2

The aim of experiment 2 was to investigate the effect of a pre-treatment of sPIF on prostaglandin and IL-6 secretion from bovine endometrial explants with and without an LPS challenge and to ensure that there was no underlying inflammation in tracts that may cause variability in the results. Bovine stage I (n = 12) and stage IV (n = 12) uteri were utilised, as for experiment 1, split into heifer (n = 6 for each stage) or cow (n = 6 for each stage) groups. To ensure there was no presence of sub-clinical inflammation, endometrial swabs were collected post-mortem using a modified cytobrush technique [25] and stained and fixed with Kwik-diff (Shandon, Thermo Scientific, Loughborough, UK) to test for inflammation. Samples were assessed for percentage of polymorphonuclear cells (PMN) by counting a minimum of 100 cells at X 400 magnification on Zeiss Axiovert 200 M (Zeiss, Jena, Germany). Animals with a PMN percentage greater than 5% were excluded based on the guidelines for the detection of subclinical endometritis [26,27].

Based on the results of experiment 1, the treatment protocol was modified so that explants were pre-treated with sPIF (50, 100 or 500 nM) or media alone (if explants were not to receive sPIF for the main treatment protocol) for 24 h before challenging with LPS. At the end of the pre-treatment, the media supernatants were aspirated and replaced with fresh media alone or containing sPIF (50, 100 or 500 nM) or LPS (1 μg/mL) treatments as in experiment 1. Supernatant was sampled at 24 and 48 h from different explant wells. No samples were collected 96 h after the beginning of the pre-treatment (72 h after LPS challenge) because integrity of tissue is likely to be compromised in serum free culture beyond 72 h. Supernatant samples were stored at −20 °C until analysed for $PGF_{2\alpha}$, PGE_2 and IL-6.

2.4. Experiment 3

The aim of experiment 3 was to investigate the effect of a pre-treatment of sPIF on prostaglandin and IL-6 secretion and *IL6 and IL8* mRNA expression in bovine endometrial explants, with and without three low dose LPS challenges to induce a less severe inflammatory response. Bovine stage I (n = 4) and stage IV (n = 4) uteri were utilised. Only tracts from cows (at least 1 previous pregnancy) were used. As in experiment 2, all tracts were swabbed