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ORIGINAL ARTICLE

The Effect of *Escherichia coli* Lipopolysaccharide and Tumour Necrosis Factor Alpha on Ovarian Function

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Granulosa, infection, LPS, ovary, theca, TNFα

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Problem
Pelvic inflammatory disease and metritis are important causes of infertility in humans and domestic animals. Uterine infection with *Escherichia coli* in cattle is associated with reduced ovarian follicle growth and decreased estradiol secretion. We hypothesized that this effect could be mediated by the bacterial lipopolysaccharide (LPS) or cytokines such as tumour necrosis factor alpha (TNFα).

Method of study
In vitro, bovine ovarian theca and granulosa cells were treated with LPS or TNFα and steroid secretion measured. *In vivo*, the effect of LPS or TNFα intrauterine infusion was determined by ovarian ultrasonography and measurement of hormones in cattle.

Results
Lipopolysaccharide reduced granulosa cell estradiol secretion, whilst TNFα decreased theca and granulosa cell androstenedione and estradiol production, respectively. *In vivo*, fewer animals ovulated following intrauterine infusion with LPS or TNFα.

Conclusion
Lipopolysaccharide and TNFα suppress ovarian cell function, supporting the concept that pelvic inflammatory disease and metritis are detrimental for bovine ovarian health.

Introduction

Pelvic inflammatory disease (PID) and metritis are important causes of serious disease and infertility in humans and domestic animals. Each year in the USA, more than one million women experience an episode of acute PID, more than 100,000 become infertile as a result and more than 150 women die from PID or its complications.[1] Metritis affects 40% of dairy cattle after parturition, with each case costing an estimated $250 for reduced milk production, delayed conception and treatment.[2] Much of the infertility following a case of PID or metritis is associated with damage to the genital tract and reduced embryo survival.[3–5] However, there is increasing evidence that uterine disease also affects ovarian function.

Metritis is associated with slower growth of the dominant follicle in the ovary, fewer ovulations and lower peripheral plasma estradiol concentrations compared with clinically normal cattle.[6–8] The most numerous pathogenic bacterium in the bovine uterus is *Escherichia coli* and its presence is specifically

associated with ovarian dysfunction.[8] The effects of *E. coli* are likely mediated directly through the endotoxin, lipopolysaccharide (LPS), or indirectly through the inflammatory mediators associated with *E. coli* infection including cytokines such as tumour necrosis factor alpha (TNFα).[9] Indeed, there are increased concentrations of LPS and TNFα in the peripheral plasma of animals with uterine infection.[6,8,10,11] More importantly, LPS concentrations are increased in the ovarian follicular fluid of animals with uterine disease.[12]

Studies exploring the effect of LPS on reproductive biology in the whole animal have focused on suppression of gonodotrophin releasing hormone (GnRH) and luteinising hormone (LH) from the hypothalamus and pituitary, respectively, rather than on ovarian follicle function.[13–15] However, in sheep, there is evidence that LPS was associated with reduced estradiol secretion independently of LH pulse secretion.[16] There is *in vitro* evidence that theca and granulosa cell function may be perturbed by LPS in the rat.[17] Ovarian granulosa cells express the innate immune receptor complex for detection of LPS, and treatment with LPS modulates their endocrine function.[12] Alternatively, cytokines associated with uterine inflammation may affect ovarian function as they appear to suppress ovarian cell steroidogenesis, although serum-free culture methods were not always used in previous experiments.[18,19]

In the present study, cattle have been used to investigate the effect of uterine disease on ovarian function because the disease is biologically relevant and granulosa cells can be isolated free of immune cell contamination.[12] Furthermore, unlike humans, ovarian tissue is readily available from normal animals post-mortem and intervention studies can readily be preformed *in vivo*.[20,21] We use pure populations of ovarian cells *in vitro* and uterine infusion *in vivo* to test the hypothesis that LPS directly, or indirectly via TNFα, perturbs ovarian function.

Materials and methods

In vitro Study

Granulosa and theca cell culture

Granulosa cells were obtained and cultured separately in serum-free medium as previously described.[12,22] Briefly, bovine ovaries were collected at a local abattoir immediately post-mortem and returned to the laboratory within 1 hr. Follicles were isolated manually by dissection and selected for isolation of cells if they had a translucent appearance, a well-vascularized theca and clear follicular fluid with no visible debris or blood. Follicles were measured using a grid or calipers and classed by external diameter as small (<4 mm diameter), medium (4–8 mm diameter) or large (>8 mm diameter), reflecting their gonadotropin dependence and changes in the expression of steroidogenic enzymes and LH receptors.[23,24] At 4 mm diameter, follicles are recruited into follicle waves in cattle and become responsive to follicle stimulating hormone (FSH), with increased expression of aromatase.[25] From 8 mm diameter, granulosa cells express LH receptors and these selected dominant follicles require pulsatile LH stimulation to continue growing.[25] Follicles were cut in half and granulosa cells obtained by flushing the hemisected shells and collecting the cell-rich supernatant.[22] Theca cells were then obtained by manually peeling the basal lamina from the hemisected follicular shells and digesting the tissue in M199 (Sigma, Dorset, UK) containing 1 mg/mL collagenase (Sigma) and 100 μg/mL trypsin inhibitor, in a moving water bath for 45 min at 37°C and collecting the cell-rich supernatant.[26]

As determined by Trypan Blue exclusion, granulosa and theca cells were >80% viable and were seeded separately in 96-well plates (Nunc) at 1.5×10^6 cells/mL. Cells were incubated at 37°C in a 5% CO_2 atmosphere in serum-free medium, with granulosa cell medium supplemented with 10^{-7} M androstenedione.[22]

After an initial 48-hr establishment period, cell culture media was removed and replaced with fresh media containing treatments. In the first experiment, cells were treated with medium containing either 0, 0.1, 1, or 10 μg/mL LPS (*E.coli* serotype 055.B5; Sigma). These concentrations are similar to the concentrations of LPS in the follicular fluid of animals with clinical disease and are also similar to concentrations of LPS used in previous studies to explore immune cell function.[12,27–29] In a further experiment, cells were treated with 0, 1, 10, or 100 ng/mL TNFα, as these concentrations have been shown to affect steroidogenesis.[18] The media were changed after 48 hr and then collected at 96 hr to identify any longer term effects on ovarian cell steroidogenesis, reflecting the whole animal clinical context.[7,8] At the end of the incubation period, the number of viable cells was determined by neutral red dye uptake, as previously described.[30]

Hormone Assays

Culture supernatants were analyzed by radioimmunoassay as previously described,[31] adapted for estradiol or androstenedione. Samples were diluted in 0.05 M Tris buffer containing 0.1% gelatin and 0.01% sodium azide. Standards, antiserum and tritiated tracer were purchased from Sigma (UK), Biogenesis (UK) and Amersham International PLC (Amersham, UK), respectively. The limits of detection were 80 pg/mL for estradiol and 50 pg/mL for androstenedione. The respective intra- and inter-assay coefficients of variation were 8.8% and 9.9% for estradiol and 3.1% and 12.6% for androstenedione.

In vivo Study

Animals

Eight nulliparous post-pubertal Holstein heifers aged between 13 and 15 months at the start of the study were assigned to control or treatment groups in a randomized crossover design ($n = 8$ per treatment). Animals were housed in a straw-bedded yard in a naturally ventilated shed and fed a diet formulated according to standard guidelines.[32] The diet comprised *ad libitum* grass hay and concentrates (Growergrain Nuts; BOCM Pauls Ltd, Ipswich, UK) and the animals had *ad libitum* access to water. To limit any influence of infectious disease, the heifers were tested free of bovine viral diarrhoea (BVD), Leptospirosis, bovine herpes virus-1 (BHV-1), Tuberculosis, Brucella and enzootic bovine leukosis (EBL) before experiments began. Maiden heifers were chosen for the study as they were predicted to have a sterile uterine environment, which was confirmed by regular uterine bacterial swabs, collected as previously described.[33] No bacteria were isolated from any of the animals at any point during the study. In addition, before the study began, the heifers were clinically assessed to ensure normal reproductive anatomy and ovarian function, and animals were acclimatized to the handling and housing facilities for 3 weeks. All procedures were carried out under Home Office authorization in compliance with the Animals (Scientific Procedures) Act 1986 and experimental protocols were approved by the Royal Veterinary College Ethical Review Committee.

The experimental protocol was as previously described.[34] Briefly, oestrus cycles were synchronized in the heifers by two intramuscular injections of a prostaglandin $F_{2\alpha}$ analogue (cloprostenol, Estrumate, Scherring Plough Animal Health) 11 days apart [prostaglandins (PG1 and PG2)]. For 3 days following the PG2 injection, animals were observed for 30 min every 2 hr to detect oestrus, as determined by the first time an animal stood to be mounted. On the morning of the seventh day after first observed standing oestrus, each heifer was given a third injection of prostaglandin $F_{2\alpha}$ analogue (PG3) to induce luteal regression and permit ovulation of the expected dominant follicle.

Treatments

In the first experiment investigating the effects of LPS, animals were randomly administered a control infusion of 10 mL sterile phosphate-buffered saline (PBS) or 10 mL sterile PBS containing 3.0 μg/kg *E. coli* serotype 055:B5 LPS (Sigma-Aldrich). In the second experiment investigating the effects of TNFα, animals were randomly administered a control infusion of 10 mL sterile PBS, or 10 mL sterile PBS containing 0.1 μg rhTNFα (Sigma-Aldrich). Treatments were administered in a crossover design such that each animal received both control and LPS treatments in the first experiment, followed by control and TNFα treatments in the second experiment, with a recovery period of one spontaneous oestrous cycle between treatment periods to minimize carry-over effects from the previous treatment.

Infusions began 24 hr after first observed standing oestrus following PG2 and were administered every 6 hr for 9 days. Infusions were carried out by passing a sterile, disposable bovine uterine catheter (Arnolds, Shropshire, UK) through the cervix and into the uterine lumen guided by transrectal palpation. The treatment was drawn into a sterile 10-mL syringe (BD, Oxford, UK) which was then attached to the end of the catheter and the contents infused into the uterus.

Clinical Examination

Reproductive function was monitored as previously described.[7,8] Briefly, the genital tract of each cow was examined by transrectal palpation and ultrasonography using a 7.5-MHz linear array transducer (Honda HS-2000; Honda Electronics, Aichi, Japan). Ovarian follicles ≥4 mm in diameter and corpora lutea in each ovary were identified and the maximum diameter of each structure was estimated by averaging measurements made using the instrument's internal calipers in two dimensions at 90°.[7,35] A follicle

was defined as a non-echogenic structure with a clear demarcation between the follicular wall and the antrum; and, the day of dominance was defined as the day the largest follicle on the ovary achieved an internal diameter of ≥8.5 mm, which corresponds to the onset of deviation.[36] The day of ovulation was classed as the last day a dominant follicle was scanned before it disappeared and a corpus luteum (CL) subsequently formed in its place.[35] A CL was characterized by a grainy echogenic structure with a well-defined border within the ovarian stroma, often with a non-echogenic lacuna.

General health was monitored every 6 hr throughout the study period, including measurement of rectal temperature using a standard digital thermometer (National Veterinary Supplies, Stoke-on-Trent, UK). Body weight was estimated using a proprietary weigh-band (Ascott Ltd, Powys, UK) placed around the chest and shoulder area. Body condition was scored by visual assessment and palpation of areas of each animals body and assigning a score based on the fat distribution.[37]

Blood Sample Collection and Analysis

Blood samples were collected at regular intervals from the jugular vein. Blood samples were collected following PG2 every 3 hr for 6 days, every 12 hr for 5 days and finally every 3 hr until the end of the study. In the LPS experiment, only blood samples were collected via jugular catheter every 12 min during an 8-hr time period. This allowed measurement of pulsatile LH release from the pituitary as there is evidence that LPS can suppress LH pulsatility.[13]

Plasma concentrations of estradiol were analyzed using the Estradiol MAIA radioimmunoassay kit (Biogenesis, Poole, UK) by the method previously described by Prendiville et al.,[38] with some modifications as described by Williams et al.[8] The intra- and inter-assay coefficients of variation were 20.8 and 21.6%, respectively and the sensitivity was 0.24 pg/mL. Plasma concentrations of progesterone were measured in duplicate using a commercial ELISA kit (Ridgeway Science, Gloucester, UK) following the manufacturers guidelines. The intra- and inter- assay coefficients of variation were 2.7 and 12.2%, respectively and the sensitivity was 0.6 ng/mL.

The concentrations of LH and FSH were measured in duplicate in peripheral plasma as described previously.[35] Standards were purchased from the National Hormone and Pituitary Programme (NHPP, CA, USA) and iodinated tracers were purchased from Amersham International PLC (Amersham, UK). For FSH, the internal recovery was 95%, the intra-assay coefficient of variation was 14.0% and the inter-assay coefficient of variation was 10.5%. For LH, internal recovery was 95%, the intra-assay coefficient of variation was 14.0% and the inter-assay coefficient of variation was 10.4%. LH concentrations were analyzed using the computer algorithm PULSAR to determine the mean, maximum and minimum LH concentrations, number of pulses, mean pulse amplitude, mean pulse length, mean frequency and mean inter-pulse interval.[39] The onset of LH peak was defined as the time at which LH concentration exceeded 5 ng/mL.

Concentrations of the acute phase protein α_1-acid glycoprotein were measured using a previously described method adapted for 96-well plates (Life Technologies, Invitrogen, Renfrew, UK).[40,41] The intra-assay and inter-assay coefficients of variation were 12 and 18%, respectively and the sensitivity was 0.2 mg/mL.

Statistical Analysis

All data were analyzed using the statistics program SAS 9.1.[42] Results are reported as the arithmetic mean ± S.E.M., and significance ascribed when $P < 0.05$. The effects of treatments *in vitro* were explored using Mixed Model ANOVA as described previously.[7] *In vivo* data were normalized to first day of uterine infusion, which was defined as day 0. Data from control and treated animals were compared by ANOVA and post hoc tests performed with Dunnet's adjustment, except for proportions, which were compared using the Fisher exact test.

Results

Effect of LPS on Ovarian Cell Function *in vitro*

Treatment with LPS did not affect cell numbers or androstenedione production by theca cells isolated from small, medium or large ovarian follicles (Fig. 1a). However, LPS treatment decreased estradiol production by granulosa cells isolated from small ($P < 0.001$: mixed model analysis), medium ($P < 0.001$), or large ($P < 0.001$) ovarian follicles (Fig. 1b).

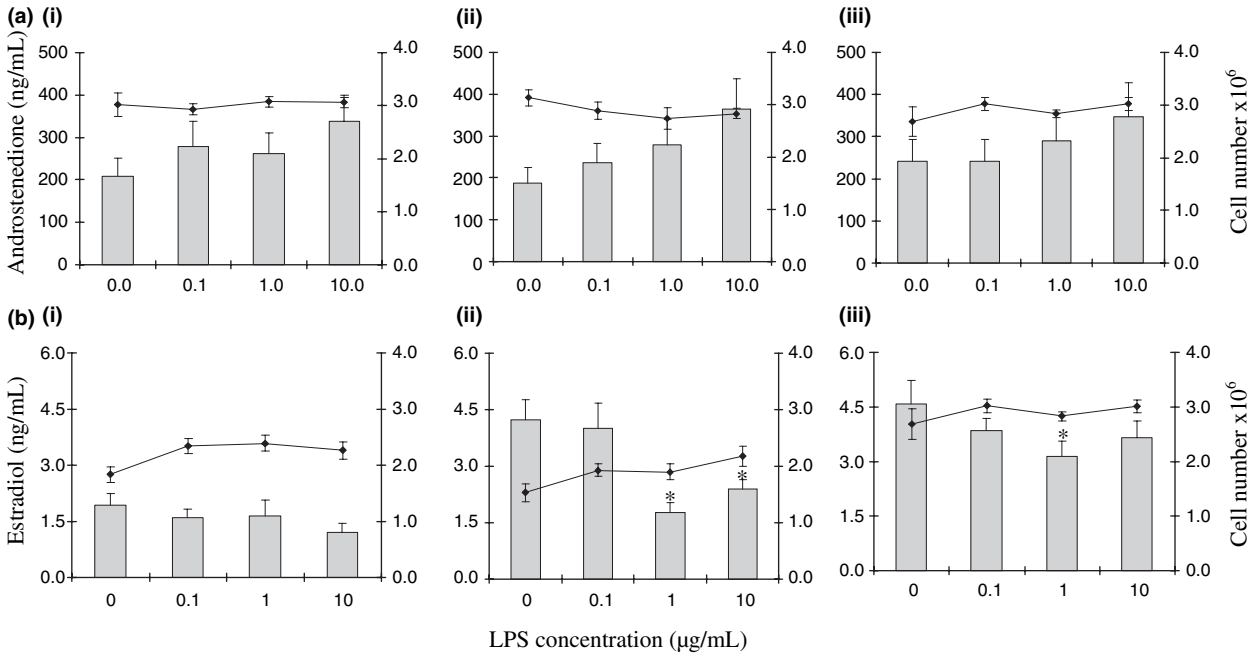

Fig. 1 Mean ± S.E.M. (a) androstenedione production by theca cells, and (b) estradiol production by granulosa cells, isolated from (i) small (<4 mm diameter), (ii) medium (4–8 mm diameter) or (iii) large (>8 mm diameter) bovine follicles. Cells were treated with lipopolysaccharide (LPS) at the concentrations indicated. Cell numbers are shown by the black line on the secondary axis. Steroid concentrations differ from control *$P < 0.05$ within follicle size.

Effect of TNFα on Ovarian Cell Function *in vitro*

Treatment with TNFα reduced androstenedione production by theca cells isolated from all follicle sizes despite increasing the number of cells in some cases ($P < 0.001$; Fig. 2a). In addition, TNFα suppressed estradiol production by granulosa cells isolated from small ($P < 0.01$), medium ($P < 0.001$), and large ($P < 0.01$) ovarian follicles supplied with 10^{-7}M androstenedione. Granulosa cell numbers were not affected by TNFα (Fig. 2b).

In vivo Studies

In the LPS and TNFα studies, rectal temperatures were consistently within the normal range of 38.0–38.5°C during each study period, and peripheral concentrations of the acute phase protein alpha-1 acid glycoprotein (AGP) were not different between treatments. In the LPS study, AGP concentrations ranged from 0.07 to 2.25 mg/ml and from 0.04 to 2.28 mg/mL for control and treated animals, respectively. In the TNFα study, AGP concentrations ranged from 0.30 to 4.35 mg/mL and from 0.33 to 4.34 mg/mL for control and treated animals, respectively. One animal was removed prior to the TNFα study because of an unrelated clinical disease.

All animals had an active corpus luteum at the time of induction of luteolysis (PG2), as determined by ultrasonography and peripheral plasma progesterone concentrations >1 ng/mL, displayed standing estrus and the dominant follicle ovulated. An LH surge was observed at the time of first standing oestrus ±6 hr and there was no significant difference between treatment groups. Similarly, FSH concentrations did not differ between treatment groups (Fig. 3).

Effect of LPS on Ovarian Function *in vivo*

Following oestrus, a new wave of ovarian follicles emerged in all animals and dominant follicle diameter increased over time ($P < 0.001$). No significant differences were observed in dominant follicle diameter or estradiol concentrations between treated and control animals (Fig 4). Plasma progesterone concentrations increased over time ($P < 0.001$) concomitant with formation of the CL after oestrus in all animals

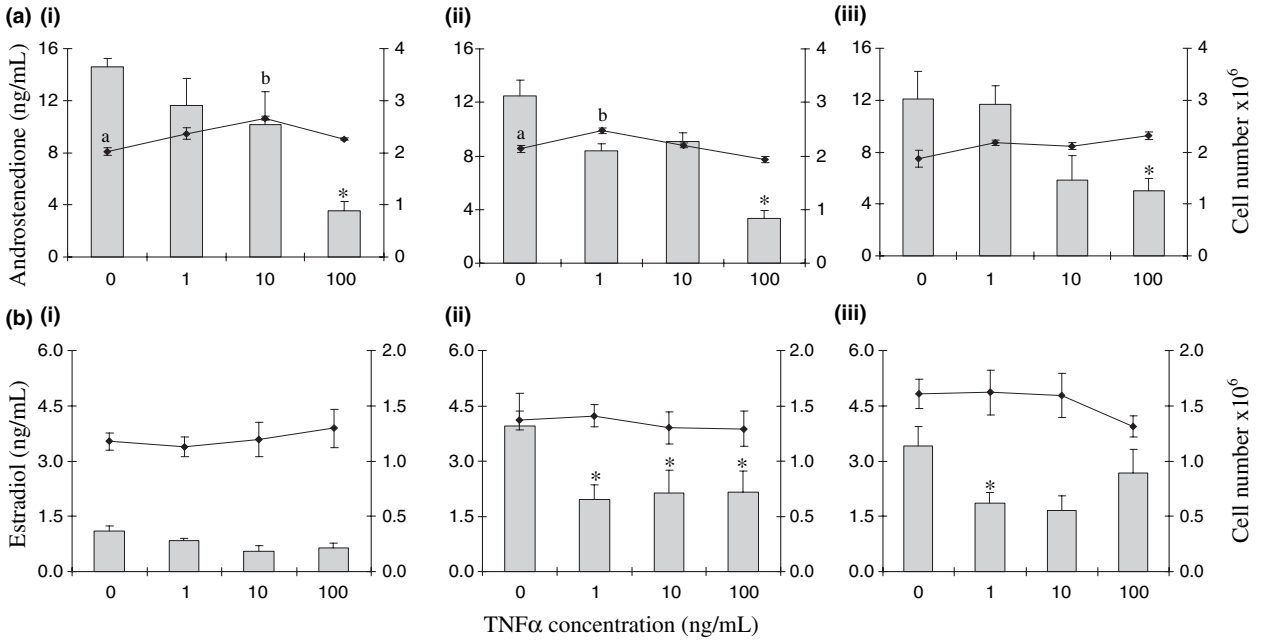

Fig. 2 Mean ± S.E.M. (a) androstenedione production by theca cells, and (b) estradiol production by granulosa cells, isolated from (i) small (<4 mm diameter), (ii) medium (4–8 mm diameter) or (iii) large (>8 mm diameter) bovine follicles. Cells were treated with tumour necrosis factor alpha (TNFα) at the concentrations indicated. Cell numbers are shown by the black line on the secondary axis. Steroid concentrations differ from control $*P < 0.05$ and cell numbers differ $^{ab}P < 0.05$, within follicle size.

(Fig. 5a(i)). Progesterone concentrations were lower in animals treated with LPS between days 3 and 9 ($P < 0.05$: mixed model analysis). A prostaglandin $F_{2\alpha}$ analogue injection was administered on day 6 to permit ovulation of the new dominant follicle (PG3), but fewer LPS-treated animals did ovulate (3/8 versus 7/8, $P < 0.05$).

Following PG3, pulsatile LH secretion was observed in all animals and no difference in mean, maximum, or minimum LH concentrations was observed between LPS or control treatments. The number of peaks observed was the same for each treatment and there was no difference in peak amplitude, peak frequency, peak length, or inter-peak interval between treatments (Table I). Representative patterns of pulsatile LH secretion during the control or LPS infusion are shown in Fig. 6.

Effect of TNFα on Ovarian Function *in vivo*

Following oestrus, a new wave of ovarian follicles emerged in all animals and as expected, dominant follicle diameter increased over time ($P < 0.001$). Dominant follicle diameter and plasma estradiol concentrations did not differ significantly between treatments (Fig. 4). Plasma progesterone concentrations increased over time ($P < 0.001$) concomitant with formation of the CL after oestrus in all animals, but did not differ significantly between treatments (Fig. 5). A prostaglandin $F_{2\alpha}$ analogue injection was administered on day 6 to permit ovulation of the new dominant follicle (PG3), but fewer animals ovulated when treated with TNFα as compared with control (4/7 versus 6/7, $P = 0.09$).

Following PG3, an LH surge was observed in all animals at the time of oestrus ±9.1 hr (corresponding to 41.4 ± 1.7 hr after PG3); LH concentration was 14.1 ± 1.7 ng/mL with no significant difference between control and TNFα treatment.

Discussion

Animals with metritis, particularly those associated with *E. coli* infection, have reduced ovarian follicle growth and function and are less likely to ovulate.[7,8,43] These effects could be mediated directly by *E. coli* LPS or indirectly by cytokines such as TNFα in response to uterine infection. In this study, ovarian cell steroidogenesis was inhibited by treatment with LPS or TNFα *in vitro*. Granulosa cell estradiol secre-

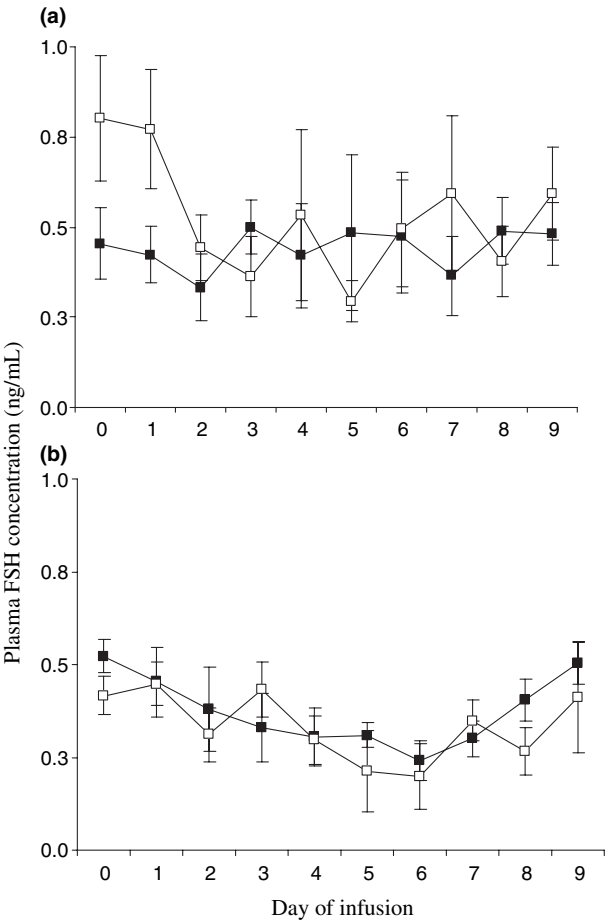

Fig. 3 Mean ± S.E.M. plasma FSH concentrations for control (■) and treated (□) animals infused with (a) LPS (n = 8 per treatment) or (b) TNFα (n = 7 per treatment).

tion was reduced by LPS treatment, whilst TNFα reduced theca and granulosa cell androstenedione and estradiol production, respectively. The effects of LPS or TNFα on ovarian function were more subtle *in vivo* when infused into animals during a defined period of ovarian follicle and corpus luteum development. However, the dominant follicle formed during LPS or TNFα treatment was less likely to ovulate than in control animals. Taken together, these studies support the hypothesis that LPS or TNFα can directly impair ovarian function. Thus, it is important for clinicians treating patients with PID or metritis to be aware that uterine infection can affect ovarian as well as uterine health.

Microbial infection of the uterus is a common and costly cause of disease and infertility in humans and domestic animals. Dairy cows appear to have a particular propensity for uterine disease after parturition, with up to 40% of animals affected.[2] Uterine disease and other systemic diseases are associated with perturbation of ovarian follicle function in cattle.[7,44,45] In metritis, the most important pathogen is *E. coli*.[8,10] Many of the effects of *E. coli* in disease processes are mediated via the endotoxin, LPS, or by cytokines such as TNFα associated with the inflammation that occurs during infection.[9] These products are found in the peripheral plasma and ovarian follicular fluid during uterine infection.[6,8,10,12]

Ovarian follicle growth and function are characterized by secretion of estradiol which is produced in the ovary by granulosa cell aromatisation of theca-derived androstenedione.[24] Estradiol regulates two positive feedback loops to maintain the dominant follicle and induce ovulation. At ovarian level, estradiol enhances gonadotropic induction of LH receptors and more FSH receptors in granulosa cells and in synergy with FSH, increases its own production by stimulating aromatase activity and the expression of its own receptors in granulosa and theca cells.[46–49] At pituitary level, estradiol secreted by the dominant follicle feeds back in the absence of progesterone to enhance gonadotropin secretion, thus ensuring the pre-ovulatory LH surge which induces ovulation.[50]

The cell surface receptor that mediates most of the effects of LPS is Toll-like receptor 4 (TLR4) in association with CD14 and MD-2,[9,27] and this receptor complex is present in the ovary of mice and cattle.[12,51] In this study, estradiol production by granulosa cells in an immune cell-free culture was inhibited in response to LPS, despite FSH and androstenedione concentrations remaining constant. Furthermore, in the theca cell cultures where LH remained constant, androstenedione production did not change in response to LPS. Together these results indicate that rather than affecting pituitary FSH production or secretion, LPS reduces the ability of granulosa cells to respond to FSH or to aromatize androstenedione to estradiol. Similarly, in the rat, LPS decreases the LH-induced aromatization of androgens to oestrogen resulting in an inhibition of estradiol production.[52] Following 48-hr LPS treatment, no difference was seen in LH receptor expression of bovine granulosa cells[12]; alternatively, a dose-dependant decrease in LH receptor formation was observed in rat granulosa cells treated with LPS. It could be postulated that longer exposure to LPS reduces LH receptors on

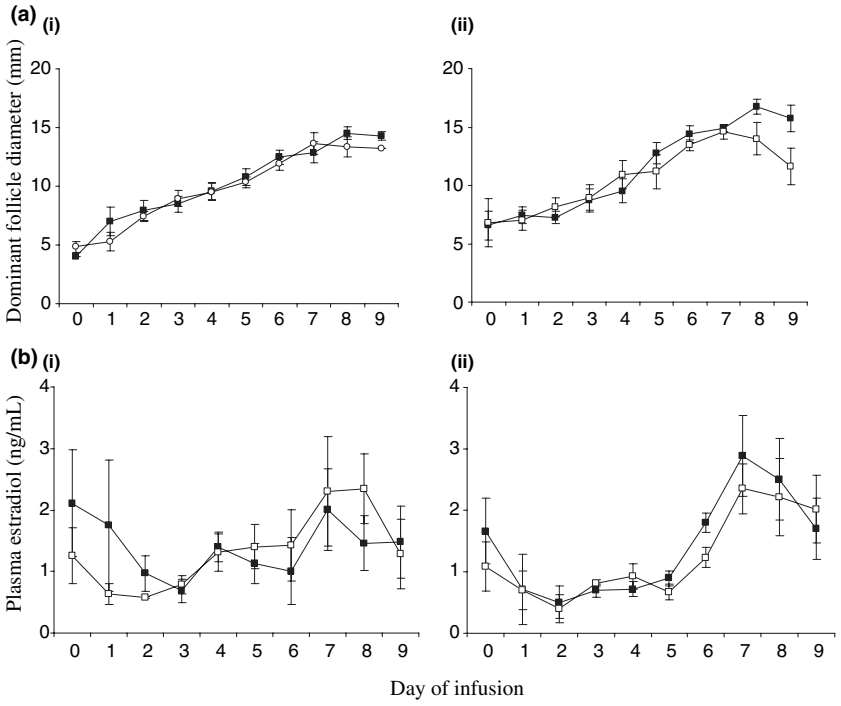

Fig. 4 Mean ± S.E.M. (a) dominant follicle diameter, and (b) plasma estradiol concentrations, for control (■) and treated (□) animals infused with (i) LPS (n = 8 per treatment) or (ii) TNFα (n = 7 per treatment).

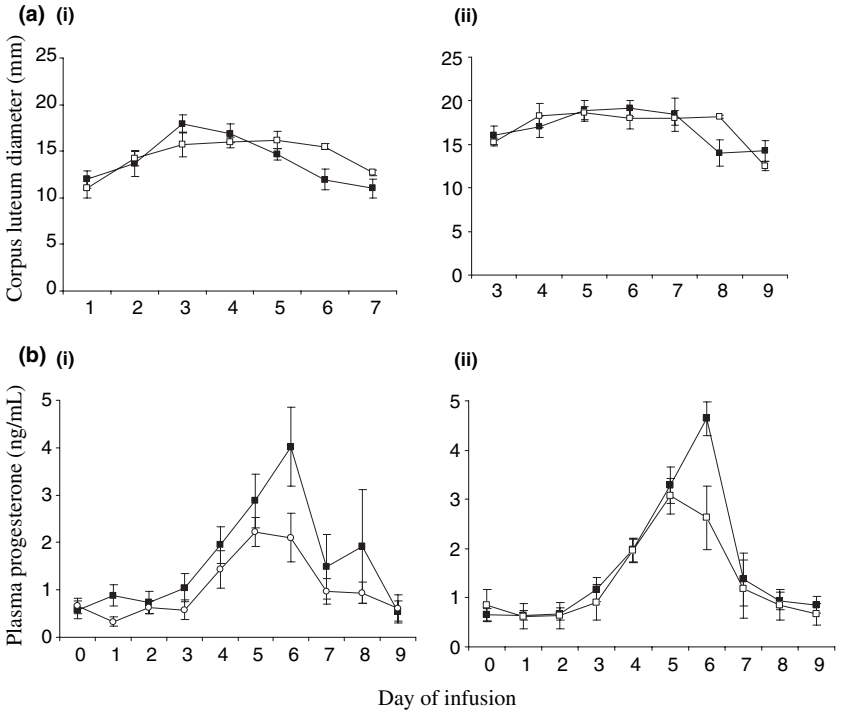

Fig. 5 Mean ± S.E.M. (a) corpus luteum diameter, and (b) plasma progesterone concentrations, for control (■) and treated (□) animals infused with (i) LPS (n = 8 per treatment) or (ii) TNFα (n = 7 per treatment).

granulosa cells thus inhibiting the response to LH and thereby blocking ovulation. This may explain why, in this study, animals treated with LPS failed to ovulate in the presence of normal LH concentrations, although further work is required to validate this.

Table I Pulsatile LH data for heifers infused in utero with LPS or control

LPS treatment	Mean LH concentration (ng/mL)	Maximum LH concentration (ng/mL)	Minimum LH concentration (ng/mL)	Number of pulses per 480 min	Pulse amplitude	Pulse length	Pulse frequency	Inter-pulse interval
Control	0.7 ± 0.1	1.5 ± 0.2	0.3 ± 0.2	8.4 ± 1.1	0.6 ± 0.1	28.3 ± 2.5	0.02 ± 0.0	57.1 ± 6.4
LPS treated	0.7 ± 0.1	1.2 ± 0.1	0.3 ± 0.1	7.3 ± 1.3	0.4 ± 0.0	22.9 ± 3.2	0.02 ± 0.0	58.4 ± 7.2

LPS, lipopolysaccharide; TNFα, tumour necrosis factor alpha; LH, luteinising hormone
Values are mean ± S.E.M.

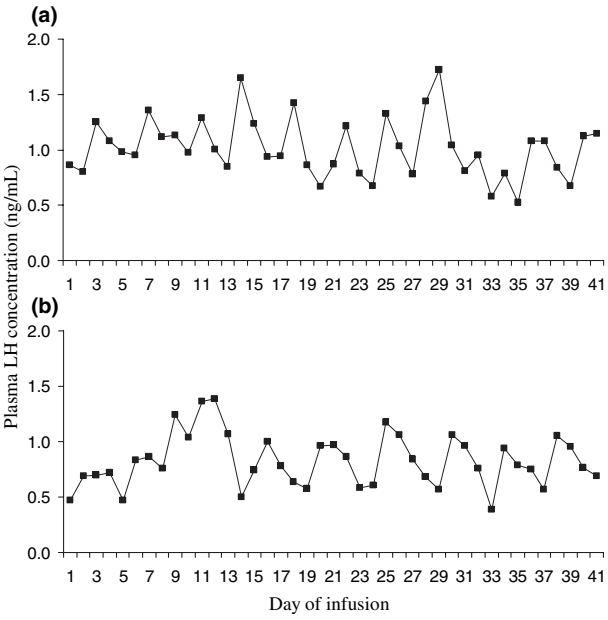

Fig. 6 Representative peripheral plasma LH concentration profiles for an animal infused with (a) control or (b) LPS.

It is well established that granulosa cells have receptors for TNFα.[53–55] Estradiol secretion is suppressed following treatment of granulosa cells with TNFα in the rat and human[52,53]; and the expression of 17βHSD and P450arom mRNA is decreased.[56] In the cow, FSH-induced estradiol production in granulosa cells from small follicles, and LH-induced androstenedione production by theca cells from large follicles, are both inhibited following treatment with TNFα.[19] In this study, TNFα treatment also inhibited FSH-induced estradiol production by bovine granulosa cells from small, medium, and large follicles. Furthermore, LH-induced androstenedione production by cells from small, medium and large follicles was also inhibited. This inhibition of ovarian cell steroidogenesis was observed 96-hr after treatment; thus, the results of this study suggest that it may be prolonged exposure to TNFα, which results in decreased steroidogenic function of these cells.

Infusion of LPS into the uterine lumen blunted the pre-ovulatory LH surge in heifers leading to failure of ovulation and the formation of cystic follicles.[43] Administration of LPS intravenously results in the disruption of neuroendocrine activity and interference with the oestrous cycle of sheep. Hypothalamic GnRH secretion is suppressed, pulsatile LH secretion from the pituitary is inhibited and there is a reduction in pituitary responsiveness to GnRH.[16] However, these effects are seen at concentrations of LPS, which induce systemic illness in the animals treated. In contrast to these previous studies, a feature of this study was the administration of treatment at concentrations, which do not cause systemic sickness in the animals. Indeed, the animals were clinically normal; rectal temperature and acute phase protein concentrations were in the normal range. Furthermore, there was no evidence that LPS or TNFα affected the secretion of LH allowing the direct effect on the ovary to be evaluated. Thus, our results provide evidence of a direct utero-ovarian pathway via which LPS or TNFα directly modulate ovarian function. The *in vivo* responses were subtle perhaps because of the limited systemic effect. However, treatment was associated with fewer ovulations even in the face of normal LH pulse profiles, as observed in sheep.[16]

Smaller ovulatory follicles result in less effective luteal structures;[57] however, in this study, the CL that was affected by LPS or TNFα developed from a follicle, which ovulated prior to treatment. This suggests that LPS or TNFα may affect luteal cells directly. Indeed, TNFα has recently been shown to play bifunctional roles in the regulation of CL function during the oestrus cycle.[58] In heifers infused with TNFα, peak progesterone concentrations tended to be lower, although differences were not significant. Receptors

for TNFα are present in the bovine CL and low concentrations of TNFα similar to those used in this study, cause luteolysis.[58,59] Additionally, TNFα stimulates the release of PGF2α from CL cells and also endometrial cells, which may also result in luteolysis.[58] Therefore, intrauterine infusion of TNFα may affect progesterone production directly via actions on luteal cells or indirectly via the induction of prostaglandin synthesis in the bovine endometrium and CL.

In conclusion, ovarian cell steroidogenesis was decreased by treatment with LPS or TNFα *in vitro*. Granulosa cell estradiol secretion was reduced by LPS treatment, whilst TNFα reduced theca and granulosa cells androstenedione and estradiol production, respectively. Although the effects of LPS or TNFα on ovarian structures and steroidogenesis were more subtle *in vivo*, ovulation of the dominant follicle was less likely than in control animals. The response was localized as peripheral immune markers were not increased and pituitary gonadotropin concentrations did not differ between treatment groups. Taken together, this study supports the hypothesis that LPS or TNFα can directly impact ovarian function. Thus, it is important to be aware that PID or metritis can affect ovarian as well as uterine health.

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