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Title: Detrimental effects of uterine disease and lipopolysaccharide on luteal angiogenesis.

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Abstract

Reproductive tract inflammatory disease (RTID) commonly occurs after the traumatic events of parturition and adversely affects follicular function. This study is the first to describe the cellular and steroidogenic characteristics of corpora lutea from cattle with RTID and the effects of pathogen-associated molecular patterns (PAMPs) on luteal angiogenesis and function *in vitro*. Luteal weight ($P<0.05$) and progesterone content ($P<0.05$) were reduced (1.2-fold) in cows with RTID, accompanied by reduced CYP11A ($P<0.05$), HSD3B ($P<0.01$) and STAR ($P<0.01$) protein expression. Immunohistochemistry revealed that luteal vascularity (VWF) and pericyte (ACTA2) coverage were >3-fold lower in RTID cows ($P<0.05$). To link these observations to bacterial infection and determine specificity of action, a physiologically-relevant luteal angiogenesis culture system examined the effects of PAMPs on endothelial cell (EC) network formation and progesterone production, in the presence of pro-angiogenic factors. Luteal EC networks were reduced ≤95% ($P<0.05$) by lipopolysaccharide (LPS, toll-like receptor [TLR] 4 agonist) but not by TLR2 agonists lipoteichoic acid or peptidoglycan. Conversely, progesterone production and steroidogenic protein expression were unaffected by PAMPs ($P>0.05$). Moreover, the adverse effect of LPS on luteal EC networks was dose-dependent and effective from 1ng/ml ($P<0.05$), while few EC networks were present above 10ng/ml LPS ($P<0.001$). LPS reduced proliferation ($P<0.05$) and increased apoptosis of EC ($P<0.001$). The specific TLR4 inhibitor TAK242 reversed the effects of LPS on EC networks. In conclusion, luteal vasculature is adversely sensitive to LPS acting via TLR4, therefore ovarian exposure to LPS from any Gram-negative bacterial infection will profoundly influence subsequent reproductive potential.

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Introduction

Parturition is a traumatic event where the endometrium is disrupted and exposed to bacterial contamination. Consequently, uterine bacterial infections are a common consequence of parturition across a number of species and post-partum uterine disease affects up to 40% of dairy cattle (Gilbert, et al. 2005; Sheldon, et al. 2009). Reproductive tract inflammatory disease (RTID) is associated with decreased fertility, manifested as an increased time to conception, increased number of inseminations per conception and reduced pregnancy rates (Giuliodori, et al. 2013; McDougall 2001). These negative effects result from delayed or abnormal post-partum ovarian cyclicity (Mohammed, et al. 2019), reduced dominant follicle growth and function (Sheldon, et al. 2002; Williams, et al. 2007), or a failure to support early embryo growth including reduced progesterone production (Mateus, et al. 2002; Williams et al. 2007). The secretion of progesterone by the corpus luteum (CL) is crucial for pregnancy with even a slight delay in the post-ovulatory rise in progesterone markedly reducing embryonic development and fertility (Mann and Lamming 2001). Fundamental to luteal development is the intense proliferation of endothelial cells and activation of pericytes which enables the CL to develop rapidly and receive the necessary high blood flow for its critical function (Robinson, et al. 2009; Woad and Robinson 2016).

The bacteria that cause endometritis (persistent uterine inflammation) include Gram-negative (e.g. *Escherichia coli*) and Gram-positive (e.g. *Trueperella pyogenes, Fusobacterium necrophorum* and *Staphylococcus aureus*) organisms (Sheldon et al. 2002; Sheldon et al. 2009). In the endometrium, these bacteria induce an innate immune response, disrupt endometrial prostaglandin production and cause tissue damage (Borges, et al. 2012; Gabler, et al. 2009; Sheldon et al. 2009). The effects are not however limited to the endometrium, since the cell wall component of Gram-negative bacteria, lipopolysaccharide (LPS), accumulates in

follicular fluid to concentrations greater than those detected in peripheral plasma (Mateus, et al. 2003), despite bacteria being absent from the ovary itself. Indeed, mean follicular LPS concentrations of ~~up to~~ 176ng/ml are reported in cattle with (sub)clinical endometritis (Herath, et al. 2007) demonstrating that LPS released during endometritis reaches and could affect the ovary. Furthermore, there is the potential for specialised vascular transfer of uterine LPS to the ovary, in a manner similar to PGF2a (Bonnin, et al. 1999; Knickerbocker, et al. 1988). In contrast, less is known about the impact of Gram-positive bacteria on ovarian function, although *S. aureus*-induced mastitis was associated with decreased follicular steroidogenesis (Lavon, et al. 2011).

Pathogen-associated molecular patterns (PAMPs) are microbial components that are detected by pattern recognition receptors as part of the innate immune response (Akira, et al. 2006). The toll-like receptors (TLR) are integral to the recognition of extracellular or exosomal PAMPs, with TLR2 and TLR4 (Akira et al. 2006) being particularly important with respect to endometritis. Lipoteichoic acid (LTA) and peptidoglycan (PGN) are cell wall components of Gram-positive bacteria which activate TLR2 signalling, whilst LPS acts via TLR4 (Akira et al. 2006). Both TLR2 and TLR4 are expressed in granulosa cells and the CL in cattle (Gadsby, et al. 2017), with expression localised to both steroidogenic and endothelial cells (Luttgenau, et al. 2016). Lipopolysaccharide, LTA and PGN dose-dependently induced an innate immune response in granulosa cells (Price, et al. 2013). In contrast, there has been less mechanistic research into the effects of PAMPs on luteal cells *in vitro*, including luteal endothelial cells.

LPS treatment *in vivo* reduced luteal blood flow, size and progesterone secretion (Luttgenau et al. 2016), thus it is feasible that LPS adversely affects luteal function by disrupting the luteal vasculature. Indeed LPS increased apoptosis in bovine (Ali, et al. 2013; Frey and Finlay 1998) and human (Bannerman, et al. 1998; Munshi, et al. 2002) endothelial cell lines, at a range of

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treatment doses and durations. Conversely, LPS induced endothelial cell sprouting and angiogenesis in human cell lines (Chen, et al. 2009; Shin, et al. 2015; Xia, et al. 2018). Thus, it is difficult to predict the effect of LPS on ovarian endothelial cells. There is much less known of the effects of TLR2 agonists on endothelial cells, but studies to date indicate that TLR2 agonists promote endothelial cell function (Grote, et al. 2010; Saber, et al. 2011). However, there are no reports of the effects of TLR2 agonists on ovarian-derived endothelial cells, to the best of our knowledge.

To date, most studies have investigated the negative effects of RTID and bacterial PAMPs on follicular rather than luteal function. The focus of the present study was on the function of the much neglected but equally important CL. Here, we explore the phenotypic characteristics of the CL collected from cows with and without RTID. Based on the profound reduction in luteal vascularity observed in cows with RTID in a preliminary field assessment, mechanistic studies were performed to test the hypothesis that PAMPs would suppress luteal angiogenesis and function *in vitro*. For the first time, we have revealed that luteal endothelial cells are sensitive to low concentrations of LPS but were unaffected by LTA and PGN, however progesterone production was unaffected by any PAMPs. Further experiments confirmed that LPS acted on endothelial cells by increasing apoptosis and decreasing proliferation. Moreover, the inhibition of TLR4 negated the profound effects of LPS on luteal endothelial cells, indicating that LPS exerted its action through TLR4. Collectively, these experiments are a major expansion of our knowledge of PAMPs and their effects on endothelial cells derived from the ovary.

Materials and methods

Tissue collection

To assess the impact of reproductive tract inflammatory disease (RTID) on luteal structure and function, bovine reproductive tracts ~~from healthy cattle~~ were collected at abattoir. Uteri had undergone involution post-partum and attained non-pregnant size. Mature CL (day 8-16; (Ireland, et al. 1980)) were isolated from cows with (n=3) and without (n=3) RTID, which was determined by visual inspection and subsequently confirmed by histological analysis of a uterine cross section (Fig 1). CL were divided and quarters either, 1) fixed in Bouin's solution for 6h prior to processing to paraffin blocks, 2) stored at -80°C for protein extraction, or 3) homogenised for tissue progesterone content.

To determine the impact of PAMPs on luteal cells in culture, bovine ovaries were collected at abattoir and early CL (days 1-4; (Ireland et al. 1980)) were selected and dissected.

Cell culture

Luteal cells (including steroidogenic, endothelial, pericytes and fibroblasts) from early CL (n=3-6 per experiment, see figure legends for details) were enzymatically dispersed with 2mg/ml collagenase I type 1A (Sigma-Aldrich Company Ltd., Dorset, UK) and 25µg/ml DNase I (Sigma-Aldrich) in DMEM/Ham's F12 media for 2 × 45 min at 37°C, as previously described (Robinson, et al. 2008; Woad, et al. 2012). Dispersed cells were filtered (70µm) and washed thrice in supplemented EBM-2 (Lonza, Basel, Switzerland; (Robinson et al. 2008; Woad et al. 2012)). Cells were plated onto fibronectin-coated coverslips at 2×10^5 viable cells / well in a 12-well plate, and incubated at 39°C with 5% CO_2.

Spent culture medium was collected and replaced with fresh supplemented EBM-2 after 24h and thereafter every 2 days. From culture day 1, cells were treated with or without ultrapure *E.*

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coli lipopolysaccharide (LPS, TLR4 agonist), *S. aureus* lipoteichoic acid (LTA, TLR2 agonist) or *B. subtillis* peptidoglycan (PGN, TLR2 agonist) for 5 days (InvivoGen, Toulouse, France; concentrations as detailed in figure legends). Cells were further treated with or without 1μM TAK242 (TLR4 signalling inhibitor; InvivoGen). All cultures were performed in the presence of FGF2 and VEGFA. When the cells were fully confluent they were either, 1) fixed in acetone:methanol (1:1) at 4°C for 10 min prior to immunostaining or, 2) collected for protein extraction.

Progesterone analysis

To quantify progesterone content of luteal tissue, CL were homogenised in ice-cold PBS and progesterone extracted with petroleum ether (Tsang, et al. 1990). Samples were diluted 1:800 in PBS prior to analysis by progesterone ELISA (Ridgeway Science; Gloucestershire, UK: RRID:AB_2801662), with an intra-assay coefficient of variation (CV) of 5.6%.

To quantify progesterone concentration in spent culture media, samples were diluted with PBS (1:50-1:500) as appropriate and measured by the same ELISA. The lower limit of sensitivity was 0.2ng/ml, with an intra- and inter-assay CV of 6.9% and 10.5%, respectively.

Protein extraction

Luteal tissue (100mg) was homogenised on ice in 2ml of ice-cold lysis buffer [0.1M Tris pH7.4, 1mg/ml dithiothreitol, 1mg/ml SDS, 0.18mg/ml sodium orthovanadate with protease inhibitor cocktail (Sigma-Aldrich)]. Cultured luteal cells were lysed in 50μl of ice-cold cell extraction buffer (FNN0011, Thermo Scientific, Waltham, MA, USA) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and rapidly freeze-thawed five times. Both tissue and cell lysates were then sonicated and centrifuged at 13000×g for 15 min. Protein

concentrations were determined by Bradford Protein assay (Bio-Rad Laboratories Ltd., Watford, Hertfordshire, UK).

Western blotting

Relative protein expression in luteal tissue or luteal cells following LPS treatment was determined by Western blot analysis. The primary antibodies used were rabbit polyclonal anti-human CYP11A (Abcam, Cambridge, UK; RRID:AB_1310110 (Fadhillah, et al. 2014)), HSD3B (gift of Dr Richard Parker, University of Birmingham, AL, USA (Robinson, et al. 2006)), STAR (Abcam; RRID:AB_10678397 (Fadhillah et al. 2014)), and mouse monoclonal anti-human ACTA2 (Sigma-Aldrich; RRID:AB_476701 (Robinson et al. 2006)). Blots were divided to enable multiple probing with different antibodies. Protein bands were quantified by densitometry (ImageJ; U.S. National Institutes of Health, Bethesda, MD, USA; (Schneider, et al. 2012)) and normalised to Histone H3 loading control (Abcam; RRID:AB_302613). Preliminary experiments determined the presence of a single band of the appropriate size for all proteins of interest, except CYP11A. In this case a second non-specific band was observed at ~20kD, however this was neither contained in the final excised region nor quantified.

Histological analysis and immunostaining

Five-micrometre sections of uterus and CL were dewaxed with histoclear and rehydrated through graded alcohols. Uterine sections were stained with haematoxylin and eosin for histological confirmation of immune cell infiltration into the endometrium and some disruption to the luminal epithelium (Bondurant 1999).

For luteal tissue sections and luteal cells grown on coverslips, endothelial cells were localized with von Willebrand Factor (VWF), while perivascular pericytes were localized with smooth muscle actin (ACTA2) immunostaining (Robinson et al. 2006). Primary antibodies were rabbit

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polyclonal anti-human VWF (20µg/ml; Dako / Agilent, CA, USA; RRID:AB_2315602) or mouse monoclonal anti-human ACTA2 (5µg/ml; Sigma-Aldrich; RRID:AB_476701). For negative control slides, primary antibodies were replaced with equivalent concentrations of rabbit or mouse IgG.

For luteal tissue sections, antigen retrieval was additionally performed by incubating with either 0.4mg/ml trypsin, 0.25mg/ml $CaCl_2$ in PBS at room temperature (RT) for 15 min [for VWF], or boiling in 10mM sodium citrate buffer (pH 6) for 10 min [for ACTA2]. Endogenous peroxidase activity was quenched with 0.3% (v/v) hydrogen peroxide in methanol for 5 min at RT. After 2 washes in PBS, 20% normal goat serum [for VWF] or 4% normal horse serum [for ACTA2] was applied for 30 min at RT. Primary antibodies were then incubated overnight at 4°C.

Sections / coverslips were washed in PBS and incubated with biotinylated anti-rabbit IgG (Vector Laboratories Ltd., Peterborough, UK) or biotinylated horse anti-mouse IgG (Vector Laboratories) for 30 min at RT. This was followed by PBS washes and then incubation with ABC (Vector Laboratories) for 30 min at RT. After washing, diaminobenzidine (Vector Laboratories) was applied for 1-5 minutes, then washed and counterstained with haematoxylin before dehydration through graded alcohols. Tissue sections were mounted using Permount (Fisher Scientific, Loughborough, UK) whilst the cell culture coverslips were removed from each culture well and further dehydrated in xylene before mounting onto a glass slide with DPX mountant (Sigma-Aldrich).

Dual label immunofluorescence

To determine endothelial cell proliferation, coverslips were simultaneously incubated with mouse anti-human Ki67 (diluted 1:100, Vector Laboratories; RRID:AB_2314697 (Robinson et al. 2006)) and 20µg/ml biotinylated *Griffonia (Bandeiraea) simplicifolia* isolectin B4

(Vector Laboratories; RRID:AB_2314661 (Plendl, et al. 1996)) in a humidified chamber at 4°C overnight. Targets were localised following incubation with Texas-Red conjugated anti-mouse IgG (20µg/ml; Vector Laboratories) and fluorescein-labelled avidin DCS (20µg/ml; Vector Laboratories) for 1h at RT.

To determine endothelial cell apoptosis, coverslips were simultaneously incubated with rabbit anti-human cleaved caspase-3 (diluted 1:300, Cell Signaling Technology, Danvers, MA, USA; RRID:AB_10897512 (Zielniok, et al. 2014)) and 20µg/ml biotinylated *Griffonia (Bandeiraea) simplicifolia* isolectin B4 (Vector Laboratories; RRID:AB_2314661) in a humidified chamber at 4°C overnight. Targets were localised following incubation with Alexa Fluor 594 conjugated anti-rabbit IgG (10µg/ml; Thermo Fisher) and fluorescein-labelled avidin DCS (20µg/ml; Vector Laboratories). All coverslips were washed twice with PBS and mounted in Vectashield with DAPI (Vector Laboratories).

Quantification of immunostaining

All image analysis was performed using Image Pro-Plus 6.0 (Media Cybernetics, Wokingham, UK). The quantification of VWF or ACTA2 immunostaining of luteal tissue was as previously described (Robinson et al. 2006), with two sections selected for each CL sample. For each section, 10 fields of view were randomly captured under 20x objective using Leica DM4000B microscope (Leica Microsystem (UK), Ltd, Milton Keynes, UK), equipped with a QImaging micropublisher 5.0 RTV colour camera (QImaging (UK) Ltd, St Helens, UK) and the area of positive staining was determined. The formation of endothelial cell (EC) networks in culture was analysed (Robinson et al. 2008; Woad et al. 2012) by capturing 12 images of VWF immunostaining per coverslip under a 5× objective. The total area of EC networks, number of branch points and the extent of branching was determined. For each coverslip, the data was

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expressed as either % relative to control for that particular culture or the actual value recorded as indicated.

To estimate apoptotic and proliferation indices by dual labelling, five non-overlapping images of each coverslip were captured under the 40× objective lens using a Leica DM5000B fluorescence microscope, equipped with a Leica DFC350fx camera (Leica Microsystem (UK), Ltd). Only endothelial cells (isolectin positive) with nuclear caspase-3 (green) or Ki67 (red) staining were counted. The total number of DAPI stained endothelial cell nuclei was then counted and indices calculated.

Statistical analyses

Statistical analyses were performed in Genstat 19.0 (VSN International, Hemel Hempstead, UK). All data were checked for normality using residual plots and homogeneity of variance by Bartlett's test. Data were log transformed where appropriate. Differences were considered significant at $P<0.05$ and results are presented as mean and associated SEM. The effect of RTID on luteal weight, % area of staining (VWF, ACTA2), progesterone content and relative protein expression were analysed by t-test.

The effects of PAMPs on luteal cells in culture were determined by randomised block ANOVA, with treatment as factor and blocked by culture, followed by Tukey's multiple comparisons test. The number of independent pools of luteal cells per experiment is indicated in the figure legends.

Results

Reproductive tract inflammatory disease is associated with reduced luteal progesterone content and steroidogenic capacity

In the control cows, all uterine layers appeared histologically normal, with an intact luminal epithelium, extensive gland formation and minimal infiltration of immune cells (Fig 1A, B). In contrast, in cows with RTID, there was some disruption to the luminal epithelium, extensive infiltration of immune cells into the sub-epithelial layer and dilatation of the blood vessels (Fig 1C, D). All control and RTID cows had an apparently active CL present at the time of tissue collection, without signs of regression.

Corpora lutea from cows with confirmed RTID were 1.2-fold smaller than those from control cows ($P<0.05$; Table 1). Similarly, luteal progesterone content (1.2-fold; $P<0.05$) and total luteal progesterone content (1.5-fold; $P<0.05$) were reduced in cows with RTID compared to control cows (Table 1).

Reproductive tract inflammatory disease was associated with a significant reduction in luteal steroidogenic protein expression (Fig 2). Luteal CYP11A protein expression was reduced by 1.5-fold ($P<0.05$), HSD3B by 4.6-fold ($P<0.01$), and STAR by 1.6-fold ($P<0.01$) in the presence of RTID versus controls.

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Reproductive tract inflammatory disease is associated with reduced luteal vascularity

An extensive microvasculature was observed in CL of control cows in the mid-luteal phase, such that the majority of steroidogenic cells were in close proximity to endothelial cells (Fig 3A). In contrast, the vascularity appeared markedly reduced in cows with RTID, with a limited localisation of endothelial cells (Fig 3B) and large areas of parenchyma devoid of endothelial cells. Quantification of VWF staining revealed a 3.7-fold reduction in the degree of luteal vascularisation in cows with RTID versus controls ($P<0.05$, Fig 3C).

In the CL of control cows, there was considerable localisation of pericytes (ACTA2-positive cells) throughout the luteal parenchyma. The expression pattern across the luteal section was similar to that for VWF and ACTA2-staining was particularly evident in larger capillaries (Fig 3D). In cows with RTID, there was a 3.5-fold reduction in luteal pericyte coverage ($P<0.001$; Fig 3E, F). Western blot analysis of normalised luteal ACTA2 expression revealed an 8-fold reduction in ACTA2 expression in cows with RTID (1.67±0.02 vs. 0.21±0.05, $P<0.001$) (data not shown).

Luteal endothelial cell network formation is dramatically reduced by treatment with lipopolysaccharide, but not lipoteichoic acid or peptidoglycan

Cells from early corpora lutea were challenged with a range of PAMPs, to determine their impact on endothelial cell (EC) network formation and progesterone production. Luteal cells were cultured in the presence and absence of ultrapure LTA, PGN or LPS for 5 days, at two different concentrations as recommended by the manufacturer (InvivoGen, Toulouse, France) and previously reported (Bromfield and Sheldon 2011).

In the control wells, the cells reached confluence with extensive formation of EC into highly-organised networks. These EC networks had multiple complex branch points and were present

across the whole coverslip. Treatment with LTA or PGN at either dose, did not affect any endothelial cell growth parameters analysed ($P>0.05$; Fig 4). In contrast, the presence of LPS visibly reduced the number and extent of EC networks (Fig 4) whilst appearing not to affect the overall degree of confluency. Image analysis (Fig 4) confirmed that LPS treatment markedly reduced the total area of EC networks at both low ($P<0.05$) and high ($P<0.001$) concentrations. Indeed, at the highest dose of LPS, the total area of EC networks was reduced by nearly 20-fold. Similarly, the total number of branch points was reduced by low ($P<0.05$) and high ($P<0.001$) concentrations of LPS. Conversely, the degree of branching within a network was only affected by the high dose of LPS ($P<0.05$).

In the control wells, luteal cell progesterone production increased with time in culture, with progesterone concentrations increasing nearly 3-fold between day 3 and 5 of culture ($P<0.001$). The progesterone production by luteal cells was, however, unaffected by incubation with LTA, PGN or LPS at any concentration, on either day 3 or 5 of culture ($P>0.05$, Fig 5).

The lipopolysaccharide-induced reduction in luteal endothelial cell network formation is reversed by inhibition of toll like receptor 4 signalling

Endothelial cell network formation was dramatically and dose-dependently reduced by LPS at concentrations from 1-1000ng/ml (Fig 6). Indeed, the total EC network area was reduced by 25% at 1ng/ml LPS ($P<0.05$). In the presence of LPS at 10ng/ml and above, only very sparse growth of endothelial cells was observed ($P<0.01$). In the control cells, TAK242 treatment reduced the total EC network area by 25% ($P<0.01$) but did not affect the number of branch points ($P>0.05$) and degree of branching ($P>0.05$). The addition of TAK242 treatment to LPS-treated luteal cells restored all EC network formation parameters to control levels ($P<0.001$ vs LPS treatment).

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Lipopolysaccharide reduces proliferation and increases apoptosis of endothelial cells

To determine how LPS might act to adversely affect EC numbers, proliferation and apoptosis indices were calculated following LPS exposure. In this experiment, the cells were fixed on day 5 prior to confluency, to better enable identification of ongoing cell dynamics. In agreement with our previous study (Woad et al. 2012), in the control wells the EC were observed in large clusters of tightly packed cells with less apparent branching (Fig 7A). Treatment with LPS reduced total EC area ($P<0.05$) and number of branch points ($P<0.05$) in a dose-dependent manner (Fig 7E, F).

Minimal apoptosis was observed in the absence of LPS (Fig 7C), however, LPS treatment dose-dependently increased luteal EC apoptosis ($P<0.001$), with a maximal apoptotic index of 45% (Fig 7G). Luteal cells (including endothelial cells) had proliferated in culture in the absence of LPS (Fig 7D), with an observed EC-specific proliferation index of 24%. LPS exposure at 1000 ng/ml significantly reduced EC proliferation by 1.4-fold ($P<0.05$) with intermediate proliferation observed at 10ng/ml LPS (Fig 7H).

Lipopolysaccharide does not alter progesterone production or steroidogenic protein expression *in vitro*

In the control wells, luteal cell progesterone production increased with time in culture, with progesterone concentrations increasing nearly 2.6-fold between day 3 and 5 of culture. In agreement with the previous experiment, progesterone production by luteal cells was unaffected by incubation with LPS at both concentrations, on either day 3 or 5 of culture ($P>0.05$, Fig 8A, B). Similarly, the expression of CYP11A, HSD3B and STAR protein on day 5 of culture was unaffected by LPS treatment ($P>0.05$, Fig 8C-E).

Discussion

This study demonstrates that RTID is associated with adverse alterations in both luteal structure and function. Total luteal P4 content was reduced 1.5-fold in cows with RTID versus control cows, reflecting diminished luteal CYP11A, STAR and HSD3B protein expression. Indeed, these observations occurred during a period when luteal structure and function are stable (days 8-16) with respect to weight, steroidogenic enzyme expression and activity (Couet, et al. 1990; Mann 2009; Pescador, et al. 1996). In addition, luteal vascularity was dramatically reduced, as judged by both endothelial and perivascular cell coverage and would be expected to further contribute to a critical functional insufficiency and decreased likelihood of achieving a pregnancy.

This extends previous findings, whereby repeated intrauterine LPS infusions resulted in decreased plasma P4 concentrations (Luttgenau et al. 2016; Williams, et al. 2008) and steroidogenic factor mRNA expression was reduced following *in vivo* LPS challenge (Herzog, et al. 2012; Luttgenau et al. 2016). Similarly, high levels of uterine pathogens were associated with reduced plasma progesterone concentrations (Williams et al. 2007). Notably, the presence of RTID has not prevented final follicular growth or ovulation, but has attenuated the resulting CL, both structurally and functionally.

Luteal blood flow was transiently suppressed following LPS challenge in cows (Herzog et al. 2012). This lowered luteal perfusion was predicted to be the result of systemic alterations and increased vascular permeability (Herzog et al. 2012). The present study suggests for the first time that LPS associated with RTID also induces deleterious luteal microvascular changes.

Using a physiologically relevant mixed luteal cell culture (Robinson et al. 2008; Woad et al. 2012), we have demonstrated that LPS adversely affects endothelial cell network formation, with a dramatic 95% loss of EC cells observed in response to the highest concentration

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(1µg/ml), which is similar to the upper range of LPS detected in the follicular fluid of cows with clinical endometritis (mean 176ng/ml, range 4.3–875.2ng/ml; (Herath et al. 2007)). Furthermore, total endothelial network area was sensitive to even low concentrations of LPS (1ng/ml). In contrast, alternative pathogen associated molecules LTA and PGN had no impact on angiogenesis versus controls at any concentration.

LPS has been shown to promote angiogenesis in a range of settings; stimulating endothelial cell proliferation, migration and microvessel sprouting (Chen et al. 2009; Shin et al. 2015; Xia et al. 2018) resulting in potential pro-angiogenic clinical applications (Ma, et al. 2015). In contrast, deleterious effects of LPS on cultured endothelial cells have also been observed, resulting in endothelial dysfunction and cell death (Ali et al. 2013; Bannerman et al. 1998; Frey and Finlay 1998; Munshi et al. 2002). On the other hand, TLR2-ligands have been shown to promote angiogenesis, significantly increasing endothelial cell proliferation, tube formation and migration both *in vivo* and *in vitro* (Grote et al. 2010; Saber et al. 2011).

Notably, the LPS-induced inhibition of angiogenesis occurred despite the presence of the predominant stimulators of angiogenesis, FGF2 and VEGFA (Robinson et al. 2008; Woad et al. 2012). Both FGF2 and VEGFA were exogenously supplemented in culture, in addition to any potential endogenous production. Indeed, significant *in vitro* VEGFA production was previously observed in a related luteinising follicular angiogenesis system (Nwachukwu 2019). VEGFA is a known endothelial survival factor, with potent anti-apoptotic activity (Alon, et al. 1995; Ferrara, et al. 2003). Furthermore, pre-treatment of human umbilical vein endothelial cells with VEGFA was protective against LPS-induced apoptosis (Munshi et al. 2002), although the degree of protection conferred by VEGFA decreased at higher concentrations of LPS (1µg/ml).

Toll-like receptors are critical for microbial detection and host defence (Akira et al. 2006), and whilst TLRs are predominantly expressed by immune cells, they have been localised to the reproductive tract, including normal and malignant human ovaries and ovarian tumour cells (Zhou, et al. 2009; Zuo, et al. 2017). Cumulus oocyte complexes of ovulating follicles express a range of immune-related genes including TLR4 (Hernandez-Gonzalez, et al. 2006; Shimada, et al. 2006). Bovine granulosa cells of developing follicles express mRNA encoding *TLR4* and its co-receptors *CD14* and *MD-2* (Herath et al. 2007). Furthermore, bovine corpora lutea express *TLR2* and *TLR4* mRNA (Gadsby et al. 2017; Nicholls, et al. 2016) and TLR2 and 4 immunoreactivity was localised to luteal endothelial and steroidogenic cells (Luttgenau et al. 2016). The presence of critical TLR signalling components is also supported by the observation that bovine granulosa cells initiate an inflammatory response to pathogen associated molecular patterns including LPS, whilst oocyte maturation is perturbed in response to LPS (Bromfield and Sheldon 2011; Price et al. 2013).

Lipopolysaccharide infusion in the ewe disrupted the pre-ovulatory rise in oestradiol, however this was not always associated with inhibited LH pulsatility, suggesting that the effects of LPS are not solely neuroendocrine in nature (Battaglia, et al. 2000). In support of direct ovarian effects, LPS has been shown to inhibit follicular steroidogenesis *in vitro*. LPS supressed bovine granulosa cell production of oestradiol (Shimizu, et al. 2012) whilst progesterone production did not change in response to LPS. In contrast, LH-stimulated or luteinising bovine theca cells responded to LPS challenge with reduced progesterone production (Magata, et al. 2014; Shimizu, et al. 2016), suggesting a greater sensitivity of theca cells to LPS during luteinisation. Conversely, LPS stimulated progesterone production by bovine luteal cells *in vitro*, although there was no additive effect of LH and LPS (Grant, et al. 2007). Collectively, this would indicate that luteal steroidogenesis is not adversely sensitive to LPS. In the current study, there is an apparent discrepancy between the lack of effect of LPS on progesterone production *in*

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vitro and reduced luteal progesterone content in RTID cows *ex vivo*. However, a possible explanation is that the reduced luteal vasculature resulted in decreased gonadotrophin or nutrient availability such that progesterone synthesis capabilities were reduced. Alternatively, PAMPs from the infected uterus could have suppressed hypothalamus-pituitary gland function thus decreasing luteal support. Furthermore, adverse alterations to follicle growth in response to LPS may also impact subsequent luteal function.

In the present study, TLR2 ligands failed to induce changes in steroidogenesis or angiogenesis. In contrast, challenge with TLR2 ligands, LTA or Pam3CSK4, led to an inflammatory response in bovine granulosa cells of both developing and dominant follicles (Bromfield and Sheldon 2011; Price et al. 2013) and the accumulation of inflammatory mediators, IL6 and IL8. Oestradiol production was reduced concurrent with this inflammatory response, although progesterone was unaffected (Price et al. 2013). PGN also decreased oestradiol production in granulosa cells from large but not small bovine follicles (Shimizu et al. 2012). It remains to be established whether the LH surge and luteinisation alters the sensitivity to some PAMPs or whether TLR2 signalling only targets steroidogenesis downstream of progesterone.

The specificity of endothelial response to LPS and not LTA or PGN suggests that TLR4 signalling is a more critical regulator of luteal angiogenesis than TLR2 signalling. Alternatively, LPS signalling may be acting via downstream pathways exclusively induced by TLR4, rather than those common to all TLRs, namely those mediated by the downstream effector MyD88. This could include the activation of the interferon response factor 3 by LPS in a MyD88-independent manner which upregulates type 1 interferons (Dauphinee and Karsan 2006), which have been shown to inhibit angiogenesis (Yildirim, et al. 2015). The lack of response to TLR2 is not thought to be the result of the absence of heterodimerisation partners

TLR1 or 6, since mRNA expression of both TLRs was detected in bovine luteal tissue (Gadsby et al. 2017; Nicholls et al. 2016).

LPS failed to evoke a response in the presence of TAK242, which is a selective small molecule inhibitor of TLR4. TAK242 potently inhibits the intracellular domain interactions of TLR4 without any effect on other TLRs (Kawamoto, et al. 2008) and inhibits both MyD88-dependent and independent pathways (Takashima, et al. 2009) irrespective of LPS concentration. This suggests that the luteal actions of LPS are mediated via TLR4, rather than other cellular pathways (Hagar, et al. 2013; Kayagaki, et al. 2013). Interestingly, treatment with TAK242 reduced total EC area in the absence of LPS. The exact mechanism is unclear, however, it suggests the presence of underlying endogenous TLR4 activity and this may be LPS-independent. Indeed, alternative ligands including saturated and polyunsaturated fatty acids (e.g. palmitic acid, linoleic acid) can activate TLR4 and the subsequent effects are variable and complex (Rogero and Calder 2018).

Angiogenesis is inhibited in response to LPS, however it remains unclear whether this is as a result of LPS sensing directly by endothelial cells or indirectly via luteal steroidogenic cells. TLR4 has been immunolocalised to both EC and steroidogenic cells in bovine CL (Luttgenau et al. 2016), and both endothelial cells and granulosa cells and cell lines (Herath et al. 2007; Munshi et al. 2002; Zuo et al. 2017) express TLR4, supporting both possible cellular responses.

LPS has been shown to injure endothelium from a range of tissues (Fujita, et al. 1998; Koide, et al. 1996) and to lead to pathological cell damage, including during sepsis (Hotchkiss, et al. 1999). Furthermore, LPS induced endothelial apoptosis via the activation of caspase-mediated cell death pathways and up-regulation of pro-apoptotic signalling *in vitro* (Munshi et al. 2002). In the present study, LPS-induced endothelial cell loss resulted from a combination of increased apoptosis and reduced proliferation. LPS has been shown to inhibit neural progenitor

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cell proliferation (Ekdahl, et al. 2003) and to suppress critical mediators of cell cycle progression (Cohn, et al. 2010). The number of viable bovine luteal cells was reduced in response to short-term LPS treatment (Grant et al. 2007), whilst in contrast, LPS did not alter the survival of developing or luteinising bovine theca or granulosa cells over 96 hours of culture (Magata et al. 2014; Shimizu et al. 2016; Shimizu et al. 2012).

In conclusion, the present study will be instrumental in developing new thinking into the mechanisms by which the TLR4 agonist LPS adversely affects ovarian function. Specifically, RTID profoundly reduced luteal vascularity in animals that had previously ovulated, while LPS dramatically decreased the ability of primary endothelial cells to form networks, even in the presence of exogenously-added pro-angiogenic factors. At the same time, LPS had no detectable effect on progesterone production or steroidogenic protein expression. This strongly indicates that ovarian endothelial cells are targeted by LPS and that they are particularly sensitive to even low concentrations of LPS. Equally, it is entirely feasible that LPS would adversely affect follicular endothelial cells, thereby reducing follicular competence. The evidence provided in this novel study on the direct impact of LPS on the functional development of the previously neglected CL is a major expansion of our understanding of the mechanisms by which bacterial infections have detrimental effects on ovarian function and fertility.

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Figure Legends

Figure 1: **Reproductive tract inflammatory disease is associated with immune cell infiltration into the endometrium and disruption to the luminal epithelium**

Histological analysis of the uterus from control cows (CONT; A, B) and cows with reproductive tract inflammatory disease (RTID; C, D). In cows with RTID, there was some disruption to the luminal epithelium (arrows), while the luminal epithelium was intact in the control cows. Importantly, there was extensive infiltration of immune cells (arrowheads) in the sub-epithelial layer. Images in B and D are under higher magnification (40x) and the scale bars are 50-200µm.

Figure 2: Reproductive tract inflammatory disease is associated with reduced luteal steroidogenic protein expression.

Protein expression of CYP11A (A), HSD3B (B) and STAR (C) was quantified and normalised to HISTH3. Significant reductions in luteal CYP11A, HSD3B and STAR expression were observed in cows with reproductive tract inflammatory disease (RTID; + ; n=3) *vs*. control (CONT; - ; n=3); *, $P<0.05$; **, $P<0.01$. Data are mean±SEM.

Figure 3: Reproductive tract inflammatory disease is associated with reduced luteal vascularity.

Immunohistochemical localisation of von Willebrand factor (VWF; A, B), and smooth muscle actin (ACTA2; D, E) in bovine corpora lutea. The VWF and ACTA2 staining (black arrows) was more extensive in control cows (CONT; A, D) compared to those with reproductive tract inflammatory disease (RTID; B, E). Significant reductions in % area of VWF (C) and ACTA2 (F) staining were observed in cows with RTID *vs*. control (*, $P<0.05$; ***, $P<0.001$). Data are mean±SEM from 3 control and 3 RTID cows. Scale bars represent 100µm. Negative controls

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(inset A and D) following replacement of each primary antibody with equivalent concentrations of IgG.

Figure 4: Treatment with lipopolysaccharide, but not lipoteichoic acid or peptidoglycan dose dependently decreased the formation of luteal endothelial cell networks *in vitro*.

Luteal cells were cultured for 5 days, then fixed and endothelial cell (EC) networks identified by von Willebrand Factor immunocytochemistry. Extensive EC networks (brown, arrowheads) were formed in control (CONT) wells (A, E). Representative photomicrographs show the effects of treatment with lipoteichoic acid (LTA) at 10ng/ml (B) or 1000ng/ml (F), peptidoglycan (PGN) at 100ng/ml (C) or 10000ng/ml (G), or lipopolysaccharide (LPS) at 10ng/ml (D) or 1000ng/ml (H), on EC network formation. Scale bars represent 500μm. Total EC network area (i), number of branch points (ii) and branch points per network (iii) were quantified. LPS in a dose-dependent manner reduced the total EC network area, number of branch points and branch points per network (Treatment *vs*. control *, $P<0.05$; ***, $P<0.001$). The formation of EC networks was unaffected by LTA and PGN at either concentration. Data are mean+SEM from 3 independent cultures.

Figure 5: Luteal progesterone production *in vitro* was unaffected by treatment with microbial components.

Luteal cells were cultured for 5 days in the absence or presence of lipoteichoic acid (LTA; A), peptidoglycan (PGN; B) or lipopolysaccharide (LPS; C) and the progesterone concentration of spent media was measured by ELISA on days 3 and 5. Progesterone production increased with time in culture ($P<0.001$) but was unaffected by treatment. Data are mean+SEM from 3 independent cultures.

Figure 6: Toll like receptor 4 signalling blockade reversed the lipopolysaccharide-induced decrease in endothelial cell network formation *in vitro*.

Luteal cells were cultured for 7 days in the absence (A, F) or presence (B-E, G-J) of lipopolysaccharide (LPS), without (A-E) or with (F-J) 1μM TAK242 co-incubation to inhibit toll-like receptor (TLR)4 signalling. Endothelial cell (EC) networks (brown, arrowheads) were identified by von Willebrand Factor immunocytochemistry. The branch points are indicated by arrows. Scale bars represent 200μm. Total EC network area (i), number of branch points (ii) and branch points per network (iii) were quantified. LPS significantly reduced EC network parameters and this was reversed in the presence of TAK242. Data are mean+SEM from 4-6 independent cultures. Significance (P<0.05) between groups is indicated by different letters a>b>c.

Figure 7: Lipopolysaccharide reduced endothelial cell area, acting via increased apoptosis and reduced proliferation

Luteal cells were cultured for 5 days in the absence (A, C and D) or presence (B) of 1000ng/ml lipopolysaccharide (LPS). Large clusters of endothelial cells (EC, black asterisks and white dotted lines) with signs of branching and network formation occurring (black arrows) were identified by von Willebrand Factor immunocytochemistry (brown; A, B) or isolectin staining (green; C, D). Total EC area (E) and number of branch points (F) were quantified. Overlay images of endothelial-specific expression of activated caspase-3 (pink; C) and Ki67 (pink; D) were quantified and presented as apoptotic (G) and proliferation (H) indices. Apoptotic and proliferating EC are labelled with white arrows, while white arrowheads indicate apoptotic and proliferating non-endothelial cells. Nuclear counterstain, DAPI, blue. Data are mean+SEM

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from 3 independent cultures. Significance (P<0.05) between groups is indicated by different letters a>b>c.

Figure 8: Lipopolysaccharide does not alter progesterone production or steroidogenic protein expression *in vitro*

Luteal cells were cultured for 5 days in the absence or presence of lipopolysaccharide (LPS) and the progesterone concentration of spent media was measured by ELISA on days 3 (A) and 5 (B). Protein expression of CYP11A (C), HSD3B (D) and STAR (E) was quantified and normalised to HISTH3. Data are mean+SEM from 3 independent cultures.

Table 1: Reproductive tract inflammatory disease (RTID) is associated with reduced luteal weight and progesterone content

	Control (n=3)	**RTID** (n=3)	**P value**
CL weight (g)	4.17 ± 0.18	3.47 ± 0.15	0.04
Luteal progesterone content (ng/mg)	1827 ± 45	1493 ± 88	0.03
Total luteal progesterone (µg)	7625 ± 497	5186 ± 443	0.02

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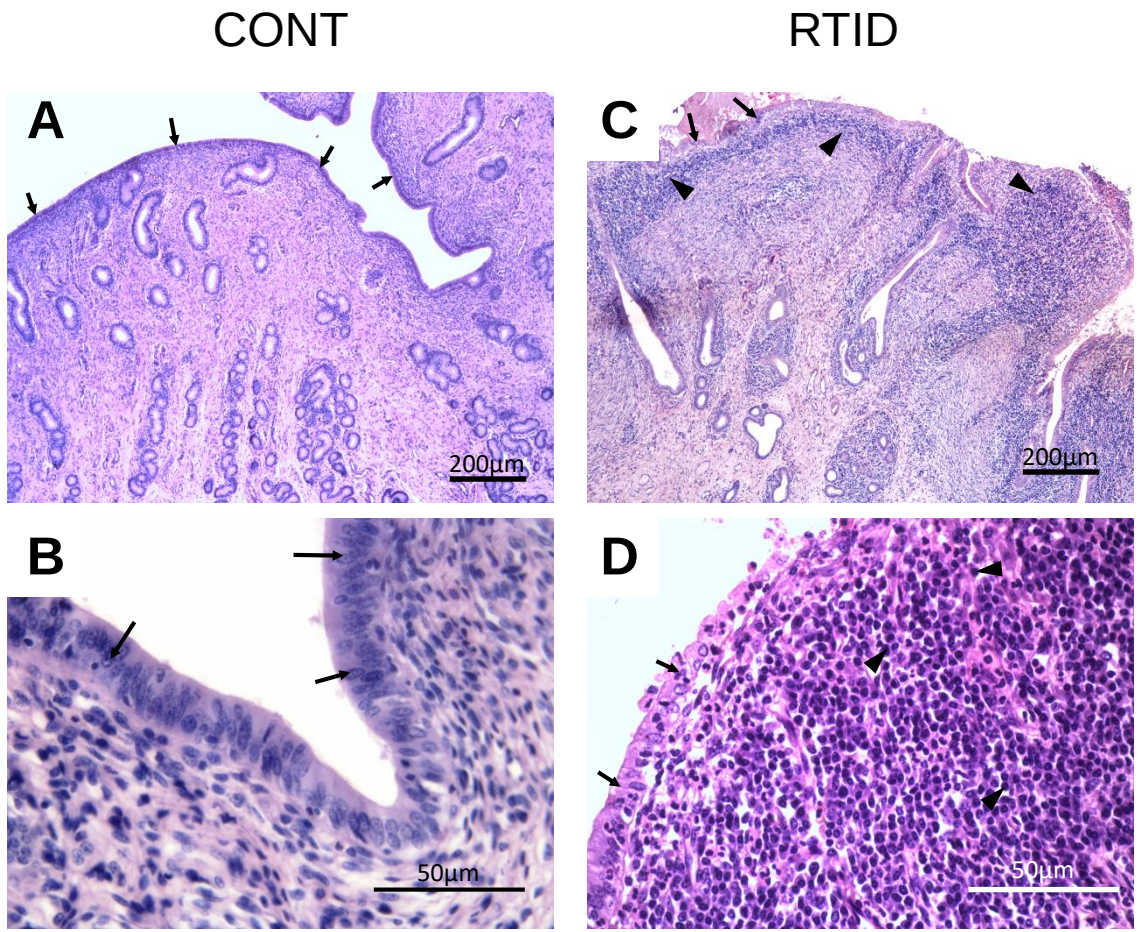
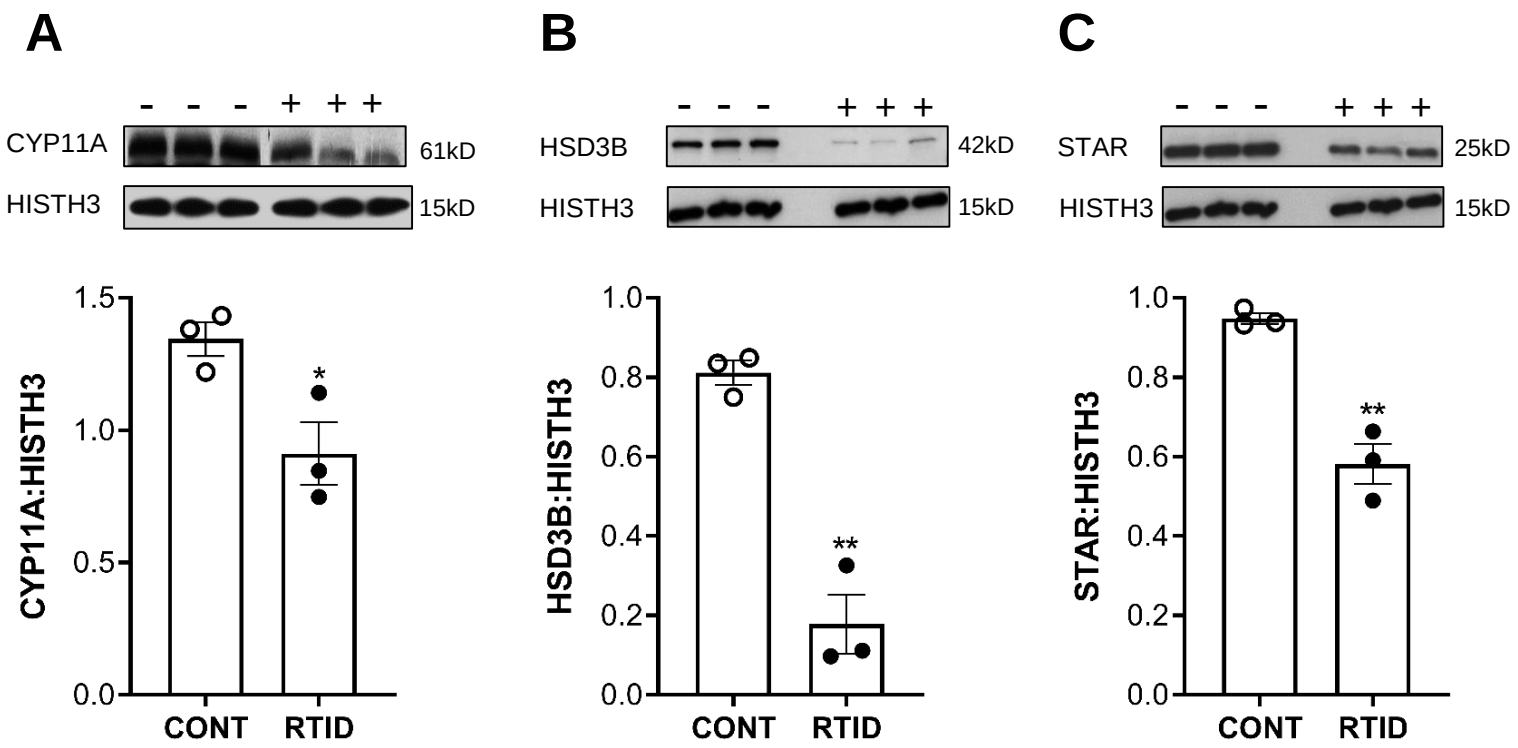
Mohammed_fig 1



Mohammed_fig 2



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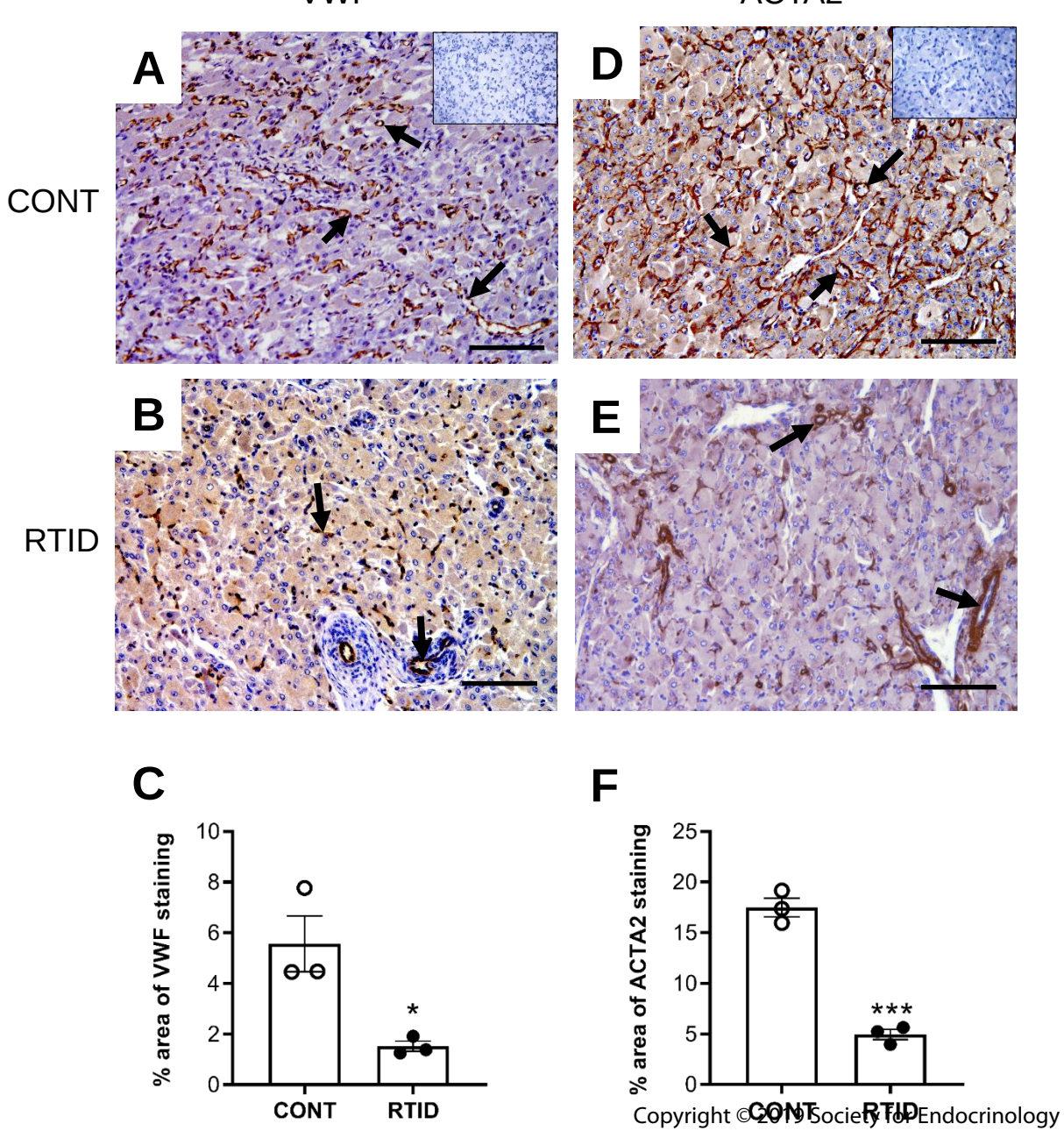
Mohammed_fig 3



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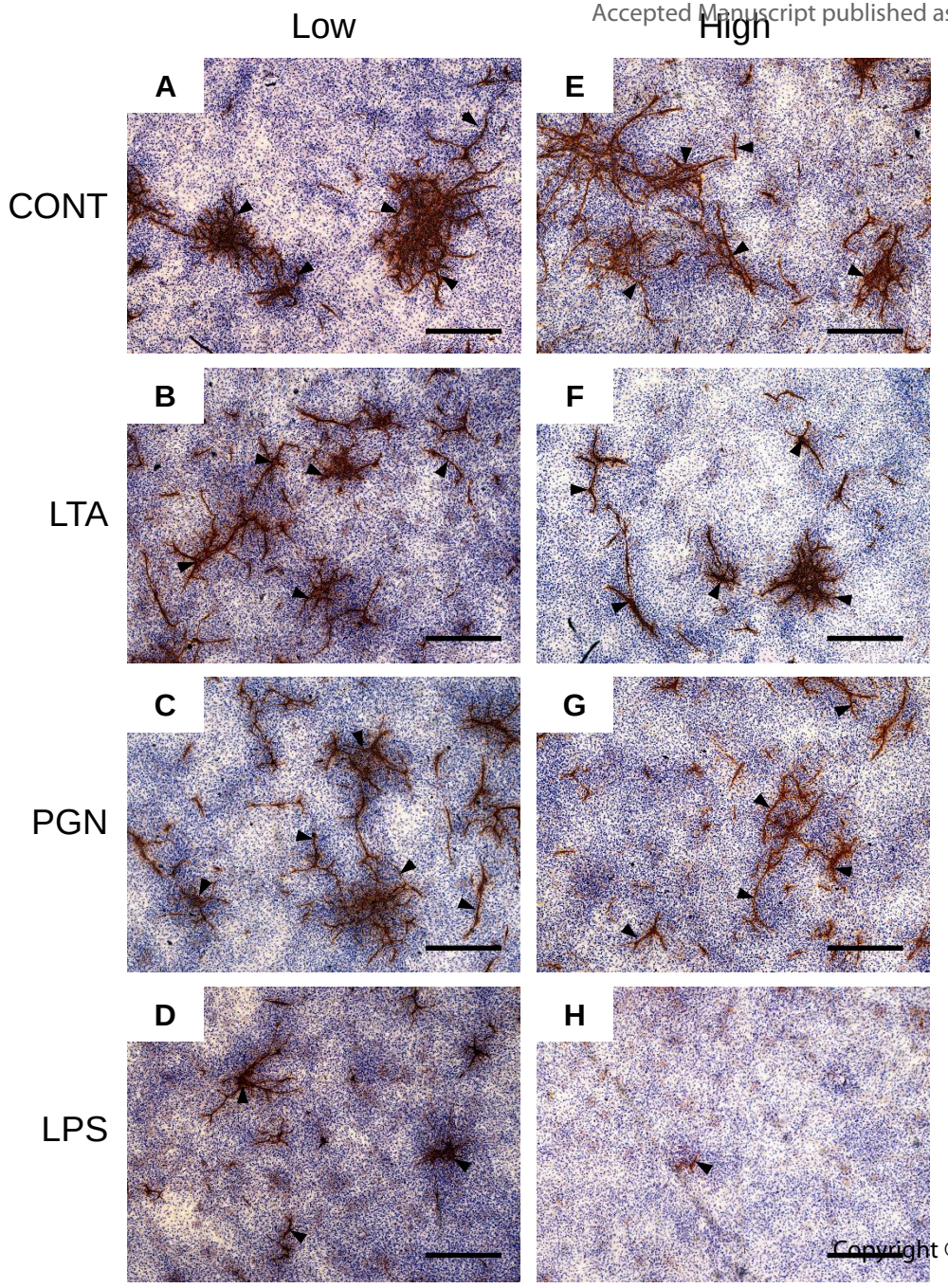
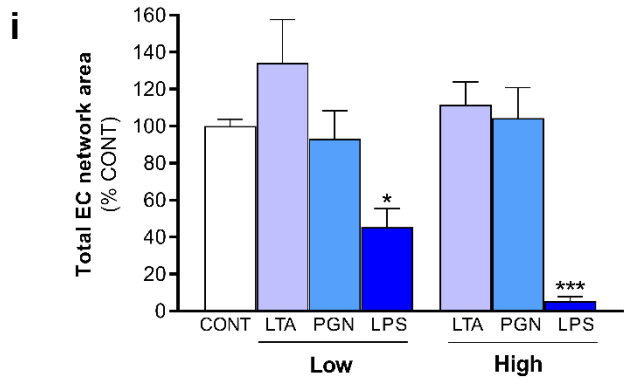
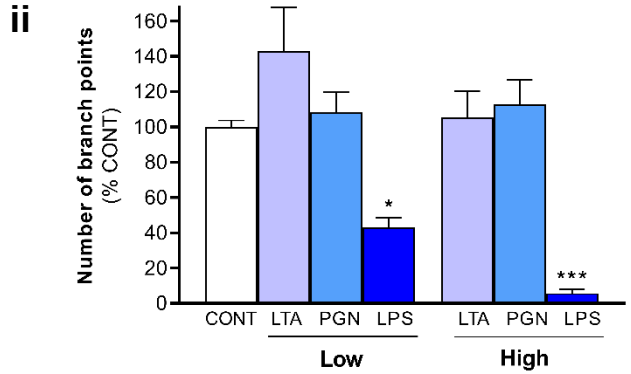
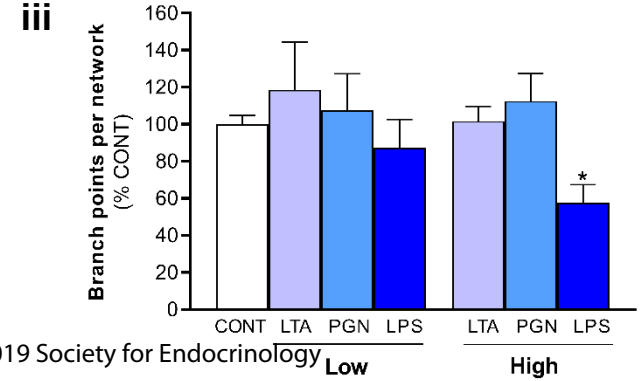
Mohammed_fig 4









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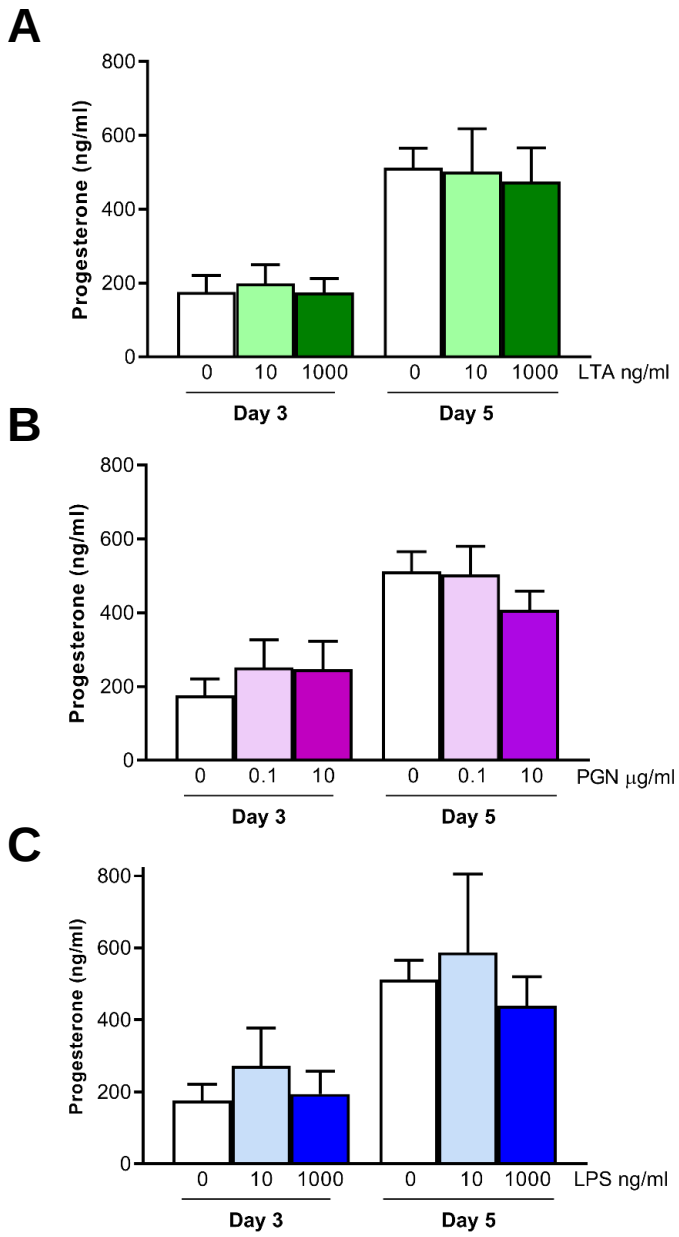
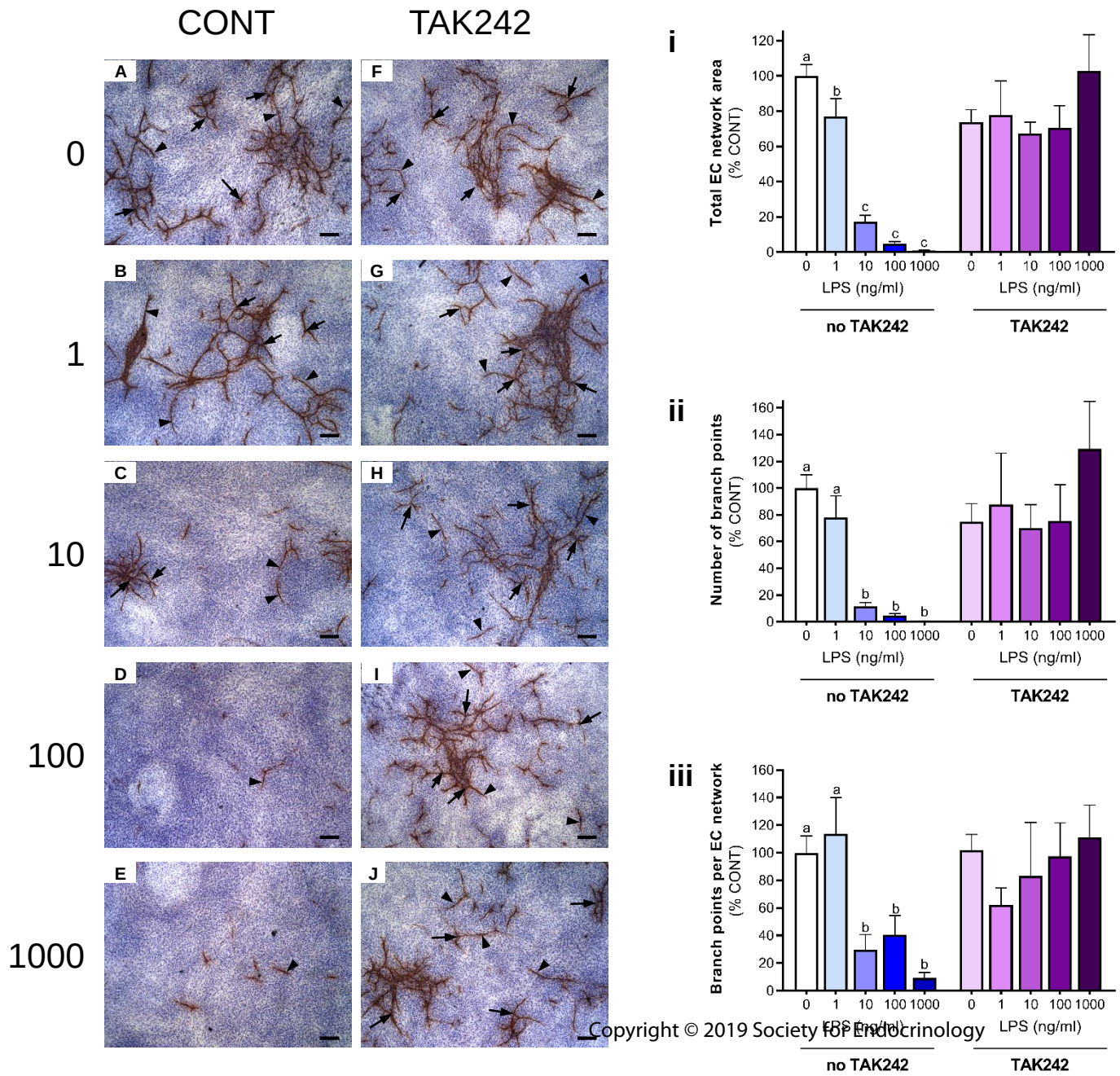
Mohammed_fig 5



Mohammed_fig 6



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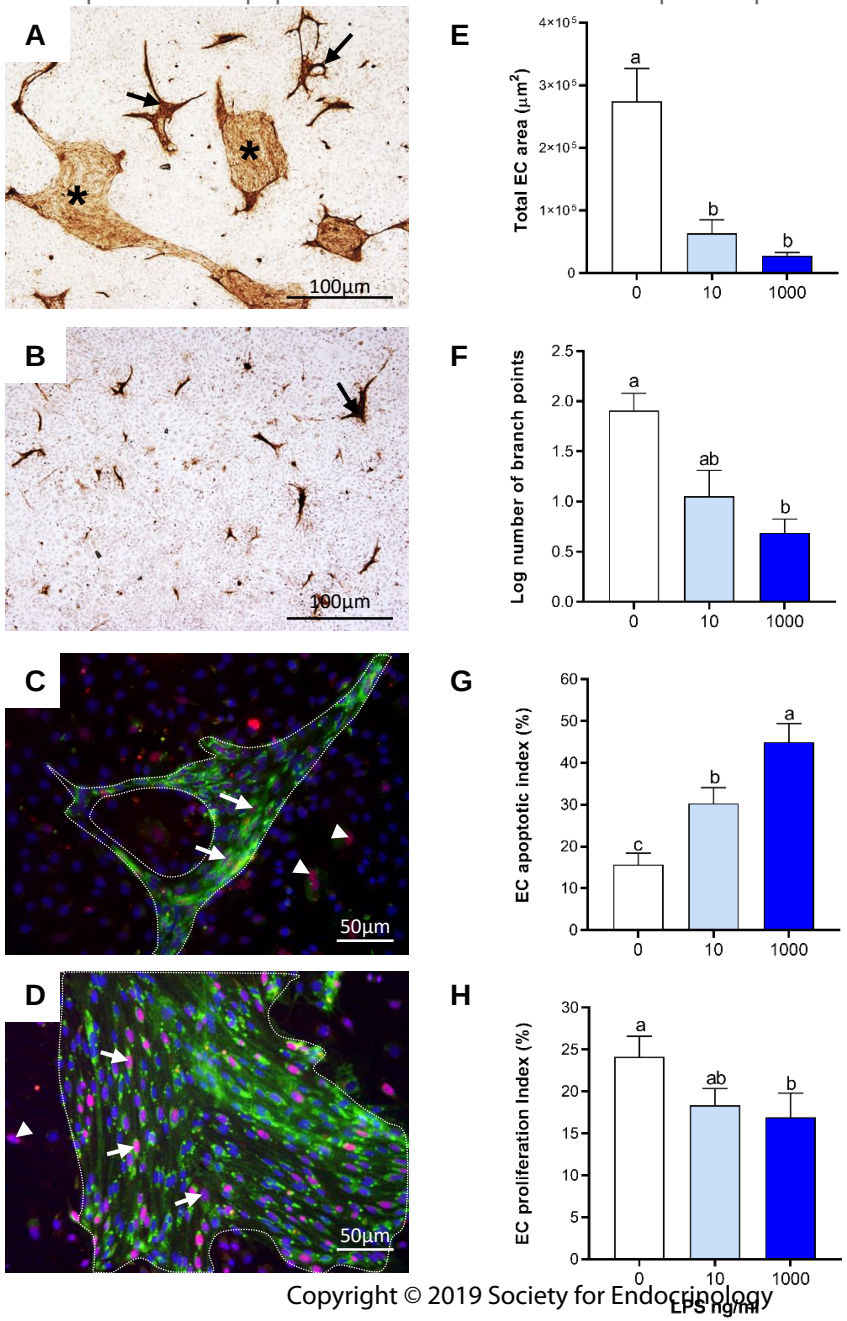
Mohammed_fig 7



Mohammed_fig 8