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Escherichia coli lipopolysaccharide administration transiently suppresses luteal structure and function in diestrous cows

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Abstract

The objective was to characterize the effects of *Escherichia coli* lipopolysaccharide (LPS) endotoxin (given i.v.) on luteal structure and function. Seven nonlactating German Holstein cows, 5.1±0.8 years old (mean±s.e.m.), were given 10 ml saline on day 10 (ovulation=day 1) of a control estrous cycle. On day 10 of a subsequent cycle, they were given 0.5 μg/kg LPS. Luteal size decreased (from 5.2 to 3.8 cm^2, $P\leq0.05$) within 24 h after LPS treatment and remained smaller throughout the remainder of the cycle. Luteal blood flow decreased by 34% ($P\leq0.05$) within 3 h after LPS and remained lower for 72 h. Plasma progesterone (P_4) concentrations increased ($P\leq0.05$) within the first 3 h after LPS but subsequently declined. Following LPS treatment, plasma prostaglandin (PG) F metabolites concentrations were approximately tenfold higher in LPS-treated compared with control cows (9.2 vs 0.8 ng/ml, $P\leq0.05$) within 30 min, whereas plasma PGE concentrations were nearly double ($P\leq0.05$) at 1 h after LPS. At 12 h after treatment, levels of mRNA encoding *Caspase-3* in biopsies of the corpus luteum (CL) were increased ($P\leq0.05$), whereas those encoding *StAR* were decreased ($P\leq0.05$) in cattle given LPS vs saline. The CASP3 protein was localized in the cytoplasm and/or nuclei of luteal cells, whereas StAR was detected in the cytosol of luteal cells. In the estrous cycle following treatment with either saline or LPS, there were no significant differences between groups on luteal size, plasma P_4 concentrations, or gene expression. In conclusion, LPS treatment of diestrus cows transiently suppressed both the structure and function of the CL.

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Introduction

Inflammation in cattle is associated with low fertility, attributed to endotoxin-induced production of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and regression of the corpus luteum (CL; Moore *et al*. 1991, Hockett *et al*. 2000, Sheldon *et al*. 2009). For example, cows with severe bacterial metritis had smaller CL, which produced less progesterone (P_4; Williams *et al*. 2007). Following i.v. administration of *Escherichia coli* lipopolysaccharides (LPS) in pro-estrus cows, ovulation was delayed 4 days, and the interval from ovulation to CL formation and to increased peripheral P_4 concentrations was both prolonged (Suzuki *et al*. 2001, Lavon *et al*. 2008). I.v. administration of endotoxin on days 7–9 of the estrous cycle in cows caused rapid increases in serum concentrations of P_4 and PGF metabolites (13,14-dihydro-15-keto-$PGF_{2\alpha}$; PGFM), followed by a transient decrease in P_4 concentrations, although the duration of the estrous cycle was not significantly altered (Gilbert *et al*. 1990). In that study, a high dose of LPS (5 μg/kg) was used; in some cows, it caused severe cyanosis, fever, ptyalism, diarrhea for 24 h, and recumbency for up to 5 days. However, in another study (Lavon *et al*. 2008), 0.5 μg/kg LPS given i.v. affected ovarian function and caused systemic responses that were not life threatening. It was noteworthy that LPS was given i.v. in this study, as the objective was to determine the systemic effects of LPS, not the effects of LPS derived from a specific location (e.g. uterus or mammary gland).

P_4 is primarily synthesized in the CL, but small amounts are also synthesized in the adrenal cortex.

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Cholesterol, the precursor of P_4, must be transported to the inner mitochondrial membrane; the StAR protein (*StAR*) is involved in this rate-limiting step of steroidogenesis (Stocco & Clark 1996). The CL also produces PG, particularly during the early stages of the estrous cycle and during luteolysis. The rate-limiting step in PG production is conversion of arachidonic acid to PGG2 by cyclooxygenase-2 (*PTGS2* (*COX2*)), the inducible, highly regulated form of cyclooxygenase (Wiltbank & Ottobre 2003, Slough *et al*. 2011). During luteolysis, activation of *Caspase-3* has a pivotal role in selective destruction of key structural and functional cellular proteins (Casciola-Rosen *et al*. 1996, Thornberry & Lazebnik 1998). Furthermore, PGE might contribute to maintenance of luteal function until 21 days post-estrus, perhaps even causing a prolonged luteal phase in *postpartum* cows (Gimenez & Henricks 1983, Opsomer *et al*. 2000, Sheldon *et al*. 2009).

There are apparently no reports characterizing, at the subcellular level, the relationship between endotoxin exposure and luteal function in cows. However, an ultrasound-guided biopsy of the CL would be a noninvasive means of investigating the effects of endotoxin exposure on luteal tissue. Therefore, the objectives of this study were to characterize the *in vivo* effects of endotoxin on luteal function and structure (order consistent with title) and in particular mRNA levels for *Caspase-3*, *StAR*, and *PTGS2*, and their respective proteins, in the CL of cyclic, nonlactating cows.

Results

Clinical response and interovulatory interval

None of the cows had any systemic reactions after administration of saline solution. In contrast, following LPS treatment, all cows had tachycardia and tachypnoea, combined with an expiratory grunt (starting ~15 min after treatment), followed by muscle tremors. Furthermore, all cows had ptyalism and cyanosis, starting 1 h after treatment and persisting for 8–12 h. Three cows had an immediate febrile response, with peak temperatures (39.5–40.4 °C) between 3 and 12 h after treatment. Three cows developed diarrhea within the first 12 h after administration (it persisted for 8 h). In some cows, feed intake did not resume until 6 h after treatment, whereas water intake was suspended until ~8 h after administration. However, it was noteworthy that all but one cow appeared clinically normal by 24 h after LPS treatment. In the remaining cow, clinical signs had abated by 48 h after treatment.

The mean cycle length of the control cycle was 21.0 ±0.9 days (mean ± S.E.M.), whereas it was 25.0 ±2.5 days ($P=0.138$) for the cycle following LPS treatment The minimum and maximum lengths of the cycles were 18 and 24 days in control cows and were 19 and 38 days in LPS-treated cows, with two cows in the latter group having a prolonged cycle (28 and 38 days respectively).

Luteal area and luteal blood flow

All cows had single ovulations. Following treatment with saline or LPS, five of seven cows had a CL with a cavity, whereas no cavity was present in the CL of the other two cows.

For luteal area, there was a group by time interaction ($P\leq0.001$), but no effect of group or time ($P=0.115$ and 0.083 respectively). Luteal area decreased in LPS-treated cows (from 5.2 to 3.8 cm^2, $P\leq0.05$) within 24 h after treatment and remained at that size until 216 h after treatment (Fig. 1). In control cows, luteal area decreased from 4.6 cm^2 at the start of treatment to 3.4 cm^2 at 216 h after treatment. Between 24 and 72 h after LPS administration, luteal area was smaller in LPS-treated cows compared with control cows ($P\leq0.05$). However, starting 5 days after treatment, luteal area was not significantly different between groups. Similarly, luteal area was not significantly different between groups in the following estrous cycle after treatment with either saline or LPS (Fig. 2A).

For luteal blood flow (LBF), there were effects of group, time, and a group by time interaction ($P=0.002$, 0.019, and ≤0.0001 respectively; Fig. 3A). There was no significant difference in LBF between groups −1.0 h before treatment. However, at 3 h after treatment, LBF had decreased by 34% in LPS-treated cows ($P\leq0.05$). Between 24 and 72 h after LPS treatment, LBF rebounded and reached nearly baseline values again ($P<0.05$). In control cows, LBF remained nearly

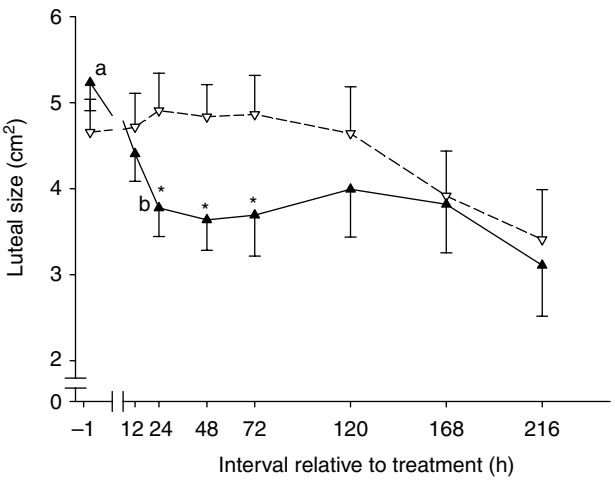

Figure 1 Mean ± S.E.M. luteal size in cows given saline (open triangle) or *E. coli* lipopolysaccharide (LPS; filled triangle) on day 10 of the estrous cycle. *Values differed between saline- and LPS-treated cows ($P\leq0.05$). [a,b]Within LPS-treated cows, values without a common superscript differed ($P\leq0.05$).

constant during the investigation period ($P>0.05$). Until 48 h, LBF was lower in LPS-treated vs control cows ($P\leq0.05$).

Plasma P_4 concentrations, PGFM, and PGE concentrations

For plasma P_4 concentrations, there were effects of time and group by time ($P\leq0.0001$ and ≤0.0001 respectively; Fig. 3B), but no effect of group ($P=0.5083$). Plasma P_4 concentrations were stimulated by endotoxin; within the first 30 min after administration, they increased dramatically (from 4.8 to 8.6 ng/ml, $P\leq0.05$). Thereafter, plasma P_4 concentrations decreased until 9–24 h when the concentrations reached their nadir (~1.9 ng/ml, $P\leq0.05$). Furthermore, 5 days (120 h) after LPS treatment, plasma P_4 concentrations had recovered (3.8 ng/ml). Plasma P_4 concentrations were higher ($P\leq0.05$) in LPS-treated than in control cows within the first 3 h after treatment. Plasma P_4 concentrations did not differ ($P>0.05$) between groups from 4 to 6 h after LPS treatment, whereas between 9 and 72 h after treatment, plasma P_4 concentrations were lower in LPS-treated compared with control cows ($P\leq0.05$). However, at 5 days (120 h) after treatment, there were no differences in plasma P_4 concentrations between groups anymore ($P>0.05$), nor were there differences in plasma P_4 concentrations between groups in the estrous cycle after treatment with either saline or LPS ($P>0.05$; Fig. 2B).

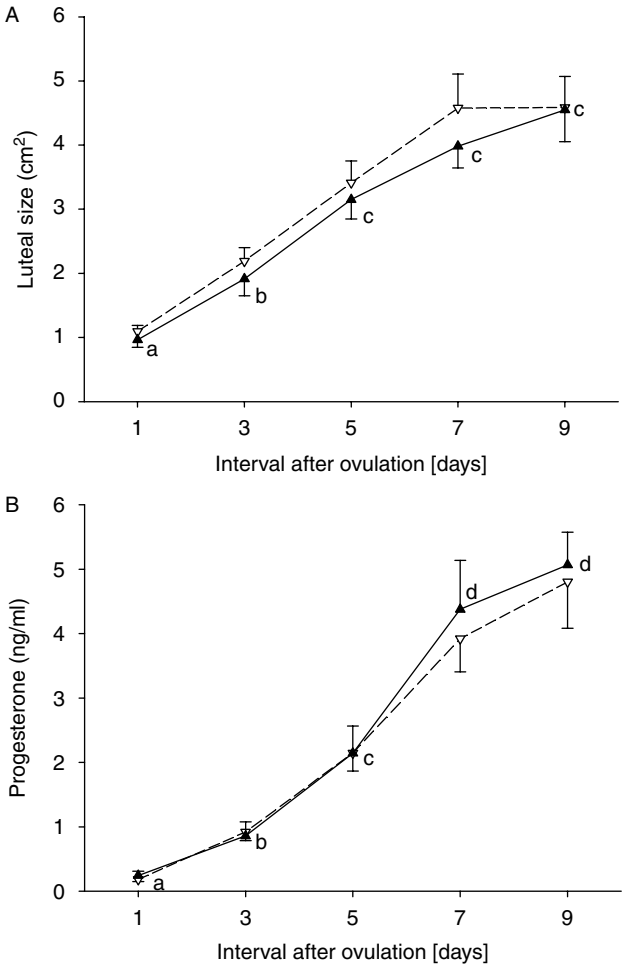

Figure 2 Mean ± S.E.M. luteal size (panel A) and plasma progesterone concentration (panel B) in cows in the estrous cycle after treatment with saline (open triangle) or *E. coli* lipopolysaccharide (LPS; filled triangle). [a,b,c,d]Within LPS-treated cows, values without a common superscript differed ($P\leq0.05$).

For PGFM, there were effects of group and time, and a group by time interaction ($P=0.0042$, ≤0.0001, and ≤0.0001 respectively; Fig. 4A). At 30 min after LPS administration, plasma PGFM concentrations were approximately tenfold higher in LPS-treated compared with saline-treated cows (9.2 vs 0.8 ng/ml, $P\leq0.05$). These concentrations remained elevated in LPS-treated cows at 4 h after exposure (2.8 vs 0.6 ng/ml, $P\leq0.05$), but there was no significant difference between groups from 6 h to the end of the investigations.

Plasma PGE concentrations were nearly twice as high in LPS-treated vs control cows at 1 h after treatment (3.61 vs 1.98 ng/ml respectively, $P\leq0.05$; Fig. 4B). At 4 h after treatment, there was no significant difference between groups ($P>0.05$), whereas 24 h after treatment, PGE concentrations were lower in saline-treated than in LPS-treated cows ($P\leq0.05$). There was no significant difference between groups 24 h before the next ovulation ($P>0.05$). However, it was noteworthy that the two LPS-treated cows with prolonged cycles had the highest PGE concentrations 24 h before the next ovulation (3.3 and 8.6 ng/ml respectively).

Gene expression and immunohistochemistry

The mRNA encoding *Caspase-3* was more than twice as high in LPS-treated cows vs control cows at 12 h after treatment ($P\leq0.05$; Fig. 5), with no significant difference in the following cycle. Luteal mRNA expression of *StAR* was lower in LPS- than in saline-treated cows 12 h after treatment ($P\leq0.05$; Fig. 5), with no significant difference between groups on day 10 of the estrous cycle after treatment with either saline or LPS ($P>0.05$; Fig. 5). There was no difference in mRNA encoding *PTGS2* in LPS- or saline-treated cows 12 h after challenge ($P>0.05$; Fig. 5).

CASP3 was predominantly present in the cytoplasm of luteal cells in control cows (Fig. 6). In LPS-treated cows, there was a strong nuclear reaction of CASP3 after LPS exposure (12 h). Endothelial cells were stained infrequently, whereas PTGS2 immunoreaction was detected in connective tissue cells (also endothelial cells) and to a much lower extent in luteal cells after LPS treatment (Fig. 6). Luteal cells were strongly positive for StAR protein (Fig. 6). Unfortunately, the small size and number

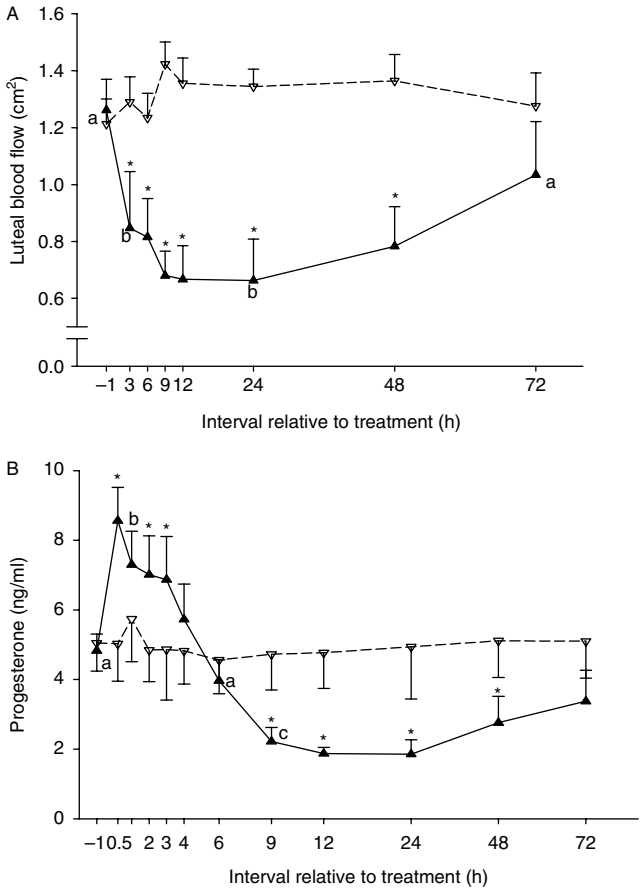

Figure 3 Mean±S.E.M. luteal blood flow (panel A) and plasma progesterone concentration (panel B) in cows given saline (open triangle) or *E. coli* lipopolysaccharide (LPS; filled triangle) on day 10 of the estrous cycle. *Values differed between saline- and LPS-treated cows ($P \leq 0.05$). [a,b]Within LPS-treated cows, values without a common superscript differed ($P \leq 0.05$).

of biopsies per cow precluded quantification of the immunoreaction and thus formal statistical comparisons between groups.

Discussion

To our knowledge, this is the first detailed assessment regarding the effects of LPS on luteal function and structure in cows. There was temporary suppression of CL function and structure after i.v. administration of LPS endotoxin in diestrus, nonlactating cows. Luteal size, LBF, and P_4 all decreased within 24 h after LPS treatment. Similarly, in a previous study, when LPS was given 42 h after PGF (which was intended to induce luteal regression and ovulation), CL formation was delayed compared to control cows (Suzuki *et al.* 2001). Furthermore, as CL diameter was smaller during the first *postpartum* cycle in cows with many vs fewer uterine pathogens, it was concluded that metritis had deleterious effects on ovarian function and that it contributed to infertility (Williams *et al.* 2007).

Although the diestrous CL is the most highly perfused organ (per unit of tissue) in the body (Wiltbank *et al.* 1988), it was noteworthy that LBF decreased sharply within the first 3 h after LPS administration in this study. When there is a serious systemic problem (e.g. septicemia), blood flow is diverted from the periphery to the core (Evtushenko *et al.* 1985, Richardson *et al.* 1996); this may have accounted for the precipitous decline in luteal perfusion in this study. Furthermore, in these cows, blood samples taken 30 min after LPS treatment were dark blue, and cows had clinical symptoms of increased vascular permeability (i.e. injected or ruptured episcleral vessels), attributed to damage to capillary endothelia, and release of compounds that were vasoactive and initiated the coagulation cascade.

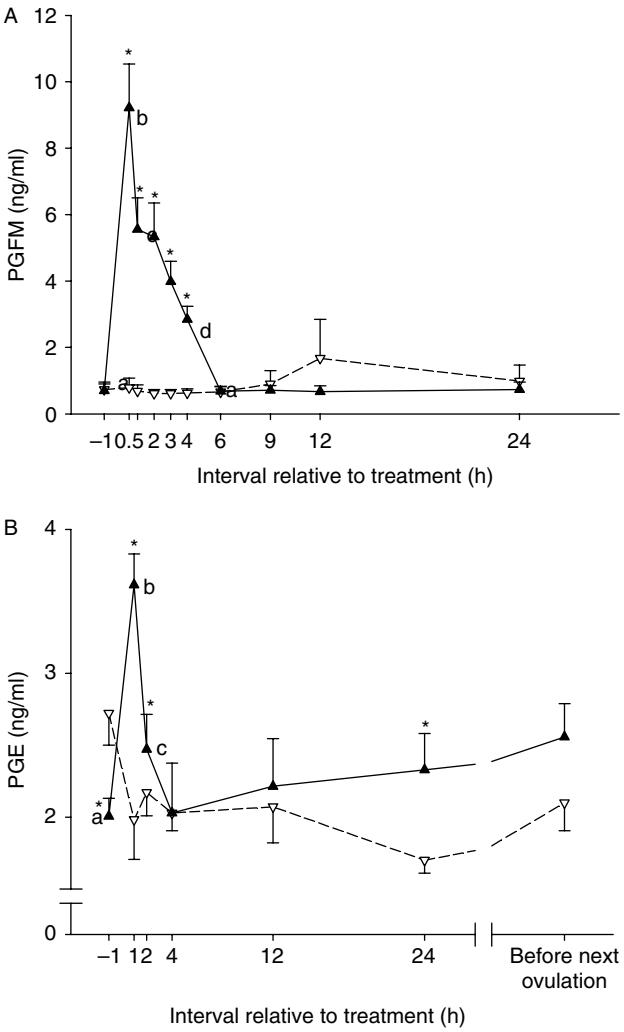

Figure 4 Mean±S.E.M. plasma concentrations of prostaglandin F metabolites (PGFM; panel A) and prostaglandin E (PGE; panel B) in cows given saline (open triangle) or *E. coli* lipopolysaccharide (LPS; filled triangle) on day 10 of the estrous cycle. *Values differed between saline- and LPS-treated cows ($P \leq 0.05$). [a,b,c,d]Within LPS-treated cows, values without a common superscript differed ($P \leq 0.05$).

Decreased LBF may have been associated with partial luteolysis. In that regard, after induction of luteolysis with PGF, LBF initially increased (30 min after PGF treatment), but it subsequently (within 4 h) decreased

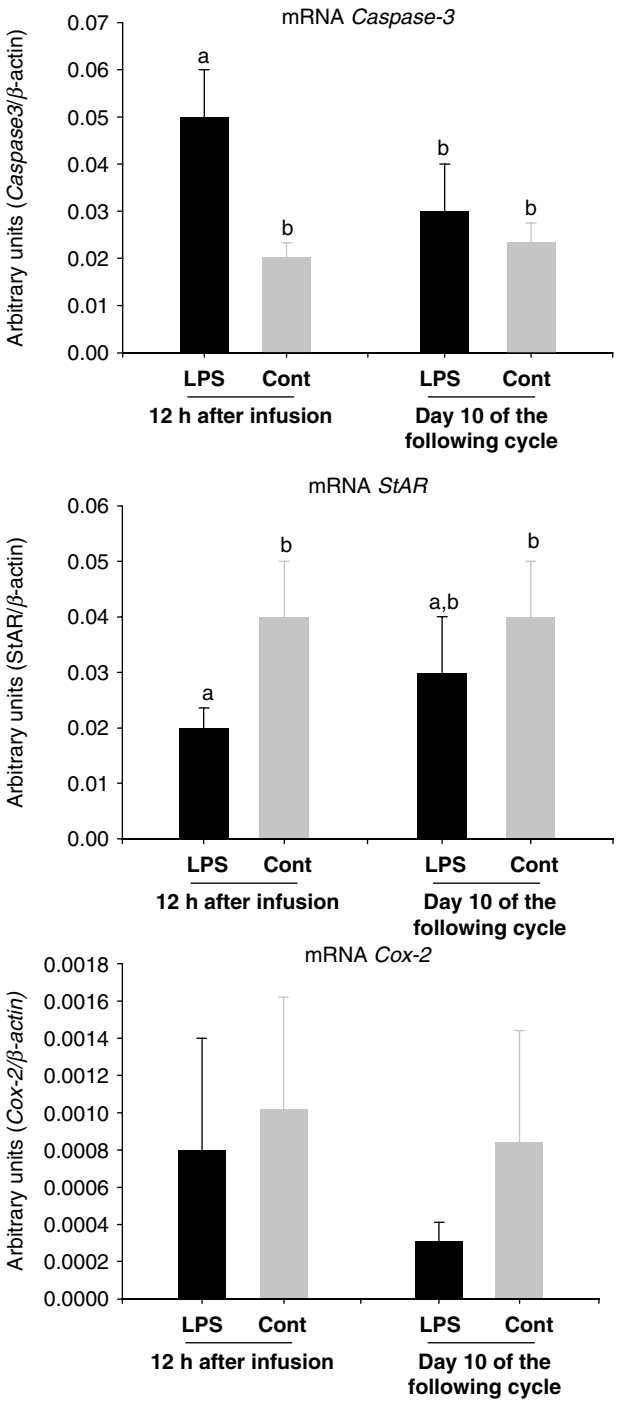

Figure 5 Mean ± S.E.M. levels of mRNA for specific enzymes at 12 h after infusion and on day 10 of the following estrous cycle in cows given saline (Cont; gray bars) or *E. coli* lipopolysaccharide (LPS; black bars). [a, b]Within a sampling period (i.e. 12 h or day 10), values differed between saline- and LPS-treated cows ($P \leq 0.05$).

(Acosta & Miyamoto 2004). For animal welfare reasons (i.e. systemic effects of LPS on the cows), transrectal sonographic examinations were not conducted immediately after LPS administration.

Decreased plasma P_4 concentrations occurred concurrent with reduced LBF; in that regard, plasma P_4 concentrations decreased to minimal values between 9 and 24 h after LPS treatment, consistent with the close correlation between LBF and P_4 (Herzog *et al.* 2010). However, there was an initial, transient increase in plasma P_4 concentrations (within the first 30 min after LPS treatment). Similarly, in a previous study (Gilbert *et al.* 1990), plasma P_4 concentrations were markedly increased within 4 h after LPS infusion; the prolonged increase in P_4 concentrations was attributed to the high dose of LPS (tenfold that used in this study). It is well established that P_4 concentrations increase following LPS treatment (Peter *et al.* 1989, Battaglia *et al.* 1997, Takeuchi *et al.* 1997). This increase was attributed to adrenal origin, as similar rises were induced by endotoxin in ovariectomized heifers (Kujjo *et al.* 1995) and ewes (Battaglia *et al.* 1997). Suzuki *et al.* (2001) suggested that LPS caused suppression of the reproductive axis at the level of the hypothalamus, anterior pituitary, or both, associated with central activation of the neuroendocrine stress axis, resulting in temporary increases in both P_4 and cortisol. In this study, plasma P_4 was lower in LPS-treated cows compared with control cows at 9 h after treatment. Similarly, systemic P_4 concentrations were decreased over several days in cows given an intrauterine infusion of LPS (Williams *et al.* 2008). In this study, 72 h after LPS treatment, there was some recovery in plasma P_4 concentrations, whereas 5 days after treatment, plasma P_4 concentrations were not significantly different from those in saline-treated cows Furthermore, average cycle length was not significantly altered after LPS treatment in the current study, in agreement with a previous report (Gilbert *et al.* 1990). The profound increase in PFGM concentrations within 30 min after LPS administration obviously did not induce complete luteal regression, presumably due to an insufficient release of $PGF_{2\alpha}$. In addition, we inferred that PGE contributed to luteal cell recovery, as it regulates luteal lifespan and generally has effects opposite to those of $PGF_{2\alpha}$. In that regard, intrauterine infusion of PGE delayed the decline in P_4 concentrations and prolonged the estrous cycle in cattle (Akinlosotu *et al.* 1986), and it counteracted the effects of $PGF_{2\alpha}$ in gilts treated with indomethacin (Akinlosotu *et al.* 1988). Interestingly, in the two cows with a prolonged estrous cycle, PGE concentrations were maximal at the next ovulation, consistent with the well-known luteotrophic effect of PGE. In previous studies, chronic intrauterine infusion of PGE in the uterine horn ipsilateral to the CL-containing ovary prevented spontaneous luteolysis (Magness *et al.* 1981, Weems *et al.* 1992, 2006) or premature luteolysis induced by $PGF_{2\alpha}$

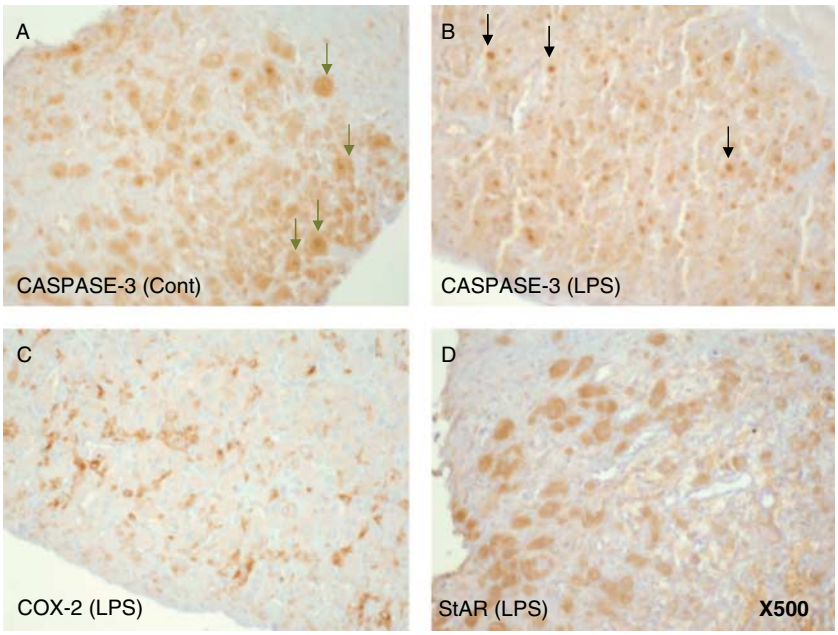

Figure 6 Immunolocalization of CASP3 (CASPASE-3), PTGS2, and StAR in biopsies of corpora lutea of cows given saline or *E. coli* lipopolysaccharide (original magnification: ×500). (A) CASP3 (CASPASE-3) immunostaining was present in cytoplasm in cows given saline- (Cont; green arrows). (B) LPS-treated cows had predominantly nuclear staining 12 h after *E. coli* lipopolysaccharide administration (LPS; black arrows). (C) PTGS2 was localized in stroma cells between luteal cells in LPS-treated cows. (D) StAR antibody strongly stained the cytoplasm of luteal cells in LPS-treated cows.

(Reynolds *et al.* 1981, Weems *et al.* 1985). Furthermore, PGE (given i.m.) increased P_4 concentrations for 72 h in cows (Kimball & Lauderdale 1975).

The mRNA encoding *Caspase-3* increased considerably 12 h after LPS administration. *Caspase-3* has been described primarily in CL of other species in relation to induced luteolysis (Rueda *et al.* 1999, Carambula *et al.* 2002) but has apparently not been described in physiological luteolysis. In this study, there was an increase in *Caspase-3*, but LPS only caused a temporary suppression of CL function and morphology, without complete luteolysis. Interestingly, localization of CASP3 in either the cytoplasm or nucleus, in the absence of apparent nuclear degeneration, has been associated with several non-apoptotic functions in other tissues (Wagner *et al.* 2011). Functions proposed are participation in cellular proliferation and cell cycle regulation, as well as differentiation of numerous cell types (Schwerk & Schulze-Osthoff 2003). Therefore, we inferred that the shift of CASP3 after LPS treatment to the nuclei of the cells was not only an expression of luteolysis but also of luteal cell regeneration. The mRNA encoding *StAR* decreased 12 h after LPS administration, concurrent with reduced plasma P_4 concentrations in these cows. Although the effects of LPS treatment on *StAR* have apparently not been reported, values for mRNA encoding *StAR* were positively correlated with serum P_4 concentrations on days 5 and 14 in mares (Slough *et al.* 2011). As *StAR* activity is the rate-limiting role in steroidogenesis (Stocco & Clark 1996), we inferred that the decreased *StAR* activity was the cause rather than the consequence of reduced plasma P_4 concentrations. In contrast to its effect on *StAR*, LPS had no significant effect on the mRNA encoding *PTGS2*. In previous studies in cattle, luteal *PTGS2* mRNA expression was highest in the early luteal phase (Kobayashi *et al.* 2002), with increasing values in mature bovine CL after induction of luteolysis by PG (Tsai & Wiltbank 1997, 1998, Diaz *et al.* 2000, Narayansingh *et al.* 2002, Shirasuna *et al.* 2004).

In this study, a single bolus of LPS did not significantly affect luteal functions in the following estrous cycle, suggesting that this LPS treatment had no effect on ovarian follicular development. Cattle with metritis, particularly cases associated with *E. coli* infection, have reduced ovarian follicle growth and function and are less likely to ovulate (Peter *et al.* 1989, Sheldon *et al.* 2002, Williams *et al.* 2007). Perhaps follicle growth and development is differentially influenced by acute vs chronic LPS exposure.

The dosage of 0.5 μg/kg LPS was based on the previous reports (Nugent *et al.* 2002, Lavon *et al.* 2008). The amount used was sufficient to provoke inflammatory symptoms without risking the long-term health or even life of animals. Furthermore, by taking CL biopsies during diestrus of the LPS cycle as well as during the following estrous cycle on the corresponding day, effects of LPS treatment, and no residual effects of treatment on the subsequent CL, were demonstrated.

In conclusion, giving 0.5 μg/kg LPS to clinically healthy, cyclic cows provoked a temporary depression in luteal function and morphology but had no effect on CL development in the following cycle. Whether this extent of luteal depression has any influence on fertility or even an existing early pregnancy is the topic of current studies.

Materials and Methods

Cows

This study was conducted (between March and September 2010) in the Clinic for Cattle, University of Veterinary Medicine, Hannover, Germany. The experimental protocol was reviewed and approved and the research was conducted in accordance with German legislation on animal welfare (Lower Saxony Federal State Office for Consumer Protection and Food Safety, 33.9-42502 – 04-09/1782). Seven nonlactating German Holstein cows, clinically healthy and with no apparent reproductive abnormalities, were used. These cows were 5.1±0.8 years old (mean±S.E.M.; range, 2.4–8.2), primiparous or pluriparous, and were at least 4 months *postpartum*. They were tethered in stalls and given *ad libitum* access to hay and water.

Study design

Ovulation was synchronized with an Ovsynch protocol (Pursley *et al.* 1995). A GNRH analog (10 μg buserelin, Receptal, Intervet, Unterschleißheim, Germany) was given s.c. Seven days later, a $PGF_{2\alpha}$ analog (657.5 μg cloprostenol-natriumsalz, Estrumate, Essex, Munich, Germany) was given i.m., followed by 10 μg of the GNRH analog given s.c. 48 h later. Transrectal ultrasonographic examinations were performed 12, 24, and 36 h after the last GNRH treatment to detect ovulation (defined as day 1 of the estrous cycle). On day 9, a polyethylene catheter was inserted into a jugular vein. Each cow first underwent a control treatment: 10 ml of 0.9% saline was administered (over a 1-min interval) via the catheter on day 10 of the estrous cycle. In the following cycle, transrectal ultrasonography (to examine the ovaries) was done every other day until day 10. Then, the Ovsynch protocol was repeated and the next ovulation confirmed. On day 10 of that cycle, the LPS trial was conducted. An LPS solution (0.5 μg/kg body weight *E. coli*, O55:B5; L2880, Sigma–Aldrich) in 10 ml sterile water (B Braun, Melsungen) was given i.v. (over an interval of ∼1 min). After administration of either saline or LPS solutions, the catheter was flushed with 25 ml of 0.9% saline solution. In the cycle following the LPS trial, cows were investigated, as described previously for the saline trial, until day 10.

Ultrasonography and determination of LBF

Transrectal B-mode ultrasonographic examinations were done at −1, 12, 24, 48, 72, and 216 h relative to treatment (saline or LPS); following ovulation, similar examinations were done every second day until day 9 of the following estrous cycle. All these examinations were conducted by the same operator, using a Logiq Book XP ultrasound scanner (General Electrics Medical Systems, Jiangsu, People's Republic of China), equipped with a 10.0 MHz linear-array transducer (General Electrics Yokogawa Medical Systems, Tokyo, Japan). Three cross-sectional images with maximal areas of the respective CL were recorded (using B-mode sonography). Luteal areas were measured offline (PixelFlux, version 1.0, Chameleon Software, Leipzig, Germany). The cross-sectional area of the CL was determined from each image. If the CL had a cavity, the cross-sectional area of the cavity was measured and subtracted from the total area (Kastelic *et al.* 1990). The mean of the cross-sectional areas of the three images was calculated and used for statistical analyses.

Power-flow Doppler was used for color blood flow mapping of the CL in various transverse sections. Investigations were done at −1, 3, 6, 9, 12, 24, 48, and 72 h in relation to administration of saline or LPS. Fixed, preinstalled Doppler system controls were used to preclude variations in recording. Three power-flow images were recorded at the maximum blood flow cross section; care was taken to locate the entire CL within the Doppler sample box to avoid flash artifacts and to evaluate maximal blood flow within the CL. PixelFlux was used to quantify (off-line) the area of color pixels within the CL, which was considered a semiquantitative measure of LBF. For further processing, the mean of the three single images was calculated. Image acquisition and processing were done as described (Jordan *et al.* 2009, Herzog *et al.* 2010).

Blood samples and determination of plasma P_4, PG metabolites, and PGE concentrations

At 1.0 and 0.5 h before administration of saline or LPS, blood samples were collected to characterize concentrations of P_4, PGFM, and PGE; the mean of these two samples was used as a pretreatment base concentration for that day. In addition, blood samples were collected (via the catheter) at 0.5, 1, 2, 3, 4, 6, 9, 12, 24, 48, 72, and 120 h after treatment, as well as every other day until day 9 of the following estrous cycle. For all these samples, plasma was separated by centrifugation (3000 ***g***, 15 min at 4 °C) within 30 min after collection, and samples were stored at −20 °C pending analysis.

Plasma P_4 concentrations were determined with a commercial chemiluminescence immunoassay (Immulite, Siemens Healthcare Diagnostics, Deerfield, IL, USA). The lower detection limit was 0.5 ng/ml, and the intra- and interassay coefficients of variation (CVs) was <10%. Plasma PGFM concentrations were determined with a competitive enzyme immunoassay (Mishra *et al.* 2003). The PGFM–HRP conjugate and antiserum were supplied by Prof. Meyer (Physiology Weihenstephan, Technische Universitaet Muenchen, Freising, Germany), whereas the PGFM used for the standard curve was purchased from Sigma. The antiserum had minimal cross-reactions with any of the related PGs, PGE_2, PGEM, PGA_2, PGAM, and $PGF_{2\alpha}$ (<0.01%, Guven & Ozsar 1993). The lowest detection limit for PGFM was 25 pg/ml. The intra- and interassay CVs were 3.5 and 11.4% respectively.

Plasma PGE concentrations were determined using a commercial PGE_2 enzyme immunoassay (Prostaglandin E2 EIA Kit, Biotrend Chemikalien GmbH, Köln, Germany). Before analysis, plasma samples were subjected to solid-phase extraction (C18 Sep-Pak Light Columns, Waters GmbH, Eschborn, Germany). In brief, C18 Sep-Pak Light Columns were pretreated with methanol (Sigma–Aldrich) and distilled water; thereafter, 1 ml plasma (diluted in 0.2 ml methanol) was added to the column. The elution medium was methyl formate (2 ml). The eluate was dried in an Eppendorf concentrator (Model 5301) and resuspended in extraction buffer (Prostaglandin E2

enzyme immuno assay (EIA) Kit, Biotrend Chemikalien GmbH). The following EIA for PGE2 was performed according to the manufacturer's instructions. The minimal detectable concentration was 0.2 ng/ml and the intra- and interassay CVs were 9.9 and 17.4% respectively. The described cross-reactivities were 100% for PGs E_2, A_1, A_2, B_1, B_2, and E_1; 85.5% for 6-keto-PGE_1; 17.7% for PGE_3 and $F_{1\alpha}$; and 2.0% for 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (all other cross-reactivities were $<0.3\%$).

CL biopsy

CL biopsies were done on experimental day 10 of the saline cycle and the LPS cycle respectively (12 h after treatment). Additionally, after each trial, biopsy samples were collected on day 10 of the following estrous cycle. To reduce rectal contractions during the biopsy procedure, cows were given caudal epidural anesthesia (80 mg procaine hydrochloride; Procasel 2%, Selectavet, Weyarn-Holzolling, Germany). Two samples (each $\sim 15 \times 1 \times 1$ mm) were obtained from the maximum diameter of the CL, using an RNase-free (RNase-ExitusPlus; AppliChem, Darmstadt, Germany) semiautomatic high-speed biopsy needle (TEMNO Evolution; Fa. Walter, Baruth/Mark, Germany). For these biopsies, the same ultrasound scanner described earlier was used (it was equipped with a 7.5 MHz convex transducer). The biopsy needle and the ultrasound transducer were guided transvaginally using a bearing system (type Hannover) and the CL was placed immediately anterior to the vaginal fornix by transrectal manipulation. At least two tissue samples per cow were recovered. One tissue sample was immediately placed in a sterile DNase- and RNase-free cryo tube (Fa. Brand, Wertheim, Germany), frozen in liquid nitrogen, and stored at -80 °C until expression analysis was done, whereas the second sample was formalin fixed and routinely embedded in paraffin. This method allowed repeated biopsy sampling from a single CL without impairing its subsequent function, as described (Tsai *et al.* 2001).

RNA extraction

Total RNA was extracted from CL biopsy samples using TRIzol reagent (Chomczynski & Sacchi 1987) as described by Watanabe *et al.* (2006). The yield of extracted total luteal RNA for each sample was determined by u.v. spectroscopy (optical density, 260 nm). The RNA concentration was determined with a Bio-Tech Photometer (WPA, Cambridge, UK) at 260 and 280 nm absorbance. Extracted total RNA was stored in RNA storage solution (Ambion, Austin, TX, USA) at -80 °C until used for cDNA production.

cDNA production

The RNA samples were treated with DNase using the RQ1 RNase–Free DNase kit (Promega Co.). Then, RNA (2 µl of 1 µg/µl) was incubated for 30 min at 37 °C with 1 µl RQ1 RNase–Free DNase 10× Reaction Buffer and 2 µl of 1 µg/µl RNase–Free DNase. The reaction was terminated with the addition of 1 µl RQ1 DNase Stop Solution (20 mM EGTA), and the resulting mixture was incubated again for 10 min at 65 °C. First-strand cDNA synthesis was conducted according to a commercial protocol SuperScript II Reverse Transcriptase (Invitrogen Corp.). The synthesized cDNA was stored at -30 °C.

Real-time RT-PCR

The levels of mRNA for *Caspase-3*, *StAR*, *PTGS2*, and β-actin were quantified by real-time PCR with a LightCycler (Roche Diagnostics Co.) using a commercial kit (LightCycler FastStart DNA Master SYBR Green I: Roche Diagnostics Co.). The primers were designed using Primer-3, based on bovine sequences. The amplification program consisted of 15-min activation at 95 °C followed by 40 cycles of PCR steps (15-s denaturation at 94 °C, 30-s annealing at 58 °C, and a 20-s extension at 72 °C). For quantification of target genes, a series of standards were constructed by amplifying a fragment of DNA (~150–250 bp) that contained the target sequence for real-time PCR. The primers used for real-time PCR were *Caspase-3* (NM001077840) forward, 5′-AAGCCATGGTGAA-GAAGGAA-3′ and reverse, 5′-CCTCAGCACCACTGTCTGTC-3′; *StAR* (accession no. MN174189) forward, 5′-GTGG-ATTTTGCCAATCACCT-3′ and reverse, 5′-TTATTGAAAACGT-GCCACCA-3′; *PTGS2* (AF031698) forward, 5′-TCCTG-AAACCCACTCCCAACA-3′ and reverse, 5′-TGGGCAGTCAT-CAGGCACAG-3′; β-actin (K00622) forward, 5′-CCAAGGC-CAACCGTGAGAAAAT-3′ and reverse, 5′-CCACATTCC-GTGAGGATCTTCA-3′.

The PCR products were subjected to electrophoresis, and the target band cut out and purified using a DNA purification kit (SUPRECTM-01; Takara Bio, Inc., Otsu, Japan). Three to five stepwise-diluted DNA standards were included in every PCR run. The quantification of mRNA expression was done using Light Cycler Software (version 3.5; Roche). Primer sets were tested in luteal tissue samples to confirm amplification of single bands. Before use of primers for analyzing samples, amplified products were cloned and sequenced to confirm their identity. Values were normalized using β-actin as an internal standard.

Immunohistochemistry

Sections of the luteal biopsies (4 µm thick) were mounted on saline-treated glass slides (Histobond Superior; Paul Marienfeld Laboratory Glassware, Laud-Königshofen, Germany) and dried at 37 °C for 24 h. After deparaffinization in xylene and rehydration in a series of graded ethanols, endogenous peroxidase activity was blocked by incubation in 80% ethanol (containing 2% hydrogen peroxide) for 30 min followed by three rinsing steps (3×5 min) in PBS (pH 7.2). Pretreatment with EDTA buffer (pH 9.0, 96 °C, 10 min) or citrate buffer (pH 6.0, 96 °C, 15 min) was performed for cox-2 or caspase-3 respectively to recover antibody-binding sites. This was followed by incubation in 20% normal horse (StAR) or goat (caspase-3 and cox-2) serum (in PBS) for 20 min at room temperature to avoid nonspecific protein binding. Then, sections were incubated with primary antibodies (in PBS plus 1.5% BSA) against caspase-3 (1:50; ab4051, Abcam, Cambridge, UK), cox-2 (1:20; Clone SP21, Thermo Fisher Scientific, Schwerte, Germany), and StAR (1:50; N-16, Santa

Cruz Biotechnology, Heidelberg, Germany) at 4 °C for 20 h in a moist chamber. Caspase-3 and cox-2 were detected with the DAKO Envision+ System/rabbit, HRP (DAKO, Hamburg, Germany) according to the manufacturer's protocol, whereas StAR was visualized with the SuperVision2 two-step polymer system (DCS, Hamburg, Germany) according to the manufacturer's instructions. Diaminobenzidine served as chromogen. Then, sections were washed in running tap water for 10 min and counterstained with Delafield's hematoxylin. Finally, they were dehydrated in a series of graded ethanol solutions, cleared in xylene, and mounted with Eukitt (Sigma–Aldrich).

Negative controls were produced by incubation without primary antibodies or their replacement by an isotype control (rabbit or goat IgG from serum; both were from Sigma–Aldrich) in lieu of primary antibodies. Either type of processing failed to produce a signal, thereby excluding antigen-independent staining. Positive controls were done with sections of bovine placenta or endometrium. All slides from each cow were stained and evaluated qualitatively, as the size and number of sections did not enable quantification.

Statistical analyses

A paired Student's *t*-test was used to determine the length of the estrous cycle in control vs LPS-treated cycles. Furthermore, data for LBF, luteal area, and plasma concentrations of P_4, PGFM, and PGF, were subjected to a Shapiro–Wilk test. None of these end points differed significantly from a normal distribution. Therefore, for each end point, Mixed Models ANOVA was used to determine the effects of group (LPS vs control), time, and the group by time interaction. An LSD test was used to locate differences among groups. All analyses were conducted with the Statistical Analysis System (SAS Institute, Cary, NC, USA) and $P<0.05$ was considered significant. Continuous data were presented as mean ± S.E.M.

Declaration of interest

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

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