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Original Article

Inhibition of lipopolysaccharide-induced suppression of luteal function in isolated perfused bovine ovaries

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Abstract. Recently, we observed that lipopolysaccharide (LPS) suppresses corpus luteum (CL) function in isolated perfused ovaries. It remained unclear if this suppression was due to increased luteal $PGF_{2\alpha}$ secretion or LPS-induced apoptosis. Therefore, possible impacts of $PGF_{2\alpha}$ and LPS were inhibited by a non-steroidal anti-inflammatory drug (flunixin) and an endotoxin-binding agent (polymyxin B), respectively. Bovine ovaries with a mid-cycle CL were collected immediately after slaughter and perfused for 240 min. After 50 min of equilibration, either flunixin or polymyxin B (5 µg/ml of each) were added to the perfusion medium of six ovaries, respectively. All ovaries (n = 12) were treated with *E. coli* LPS (0.5 µg/ml) 60 min after the onset of perfusion, and received 500 I.U. of hCG after 210 min of perfusion. Progesterone and $PGF_{2\alpha}$ were measured in the effluent perfusate every 10 and 30 min, respectively. Biopsies of the CL were collected every 60 min to determine the mRNA expression of the cytokine *TNFA* and factors of apoptosis (*CASP3*, *-8*). Flunixin-treatment inhibited the increase of $PGF_{2\alpha}$ after LPS-challenge that was observed in the polymyxin B-treated (PX-LPS) ovaries. After hCG-stimulation, progesterone secretion increased ($P < 0.05$) in group PX-LPS but not in the flunixin-treated (F-LPS) ovaries. Compared to initial values before LPS-challenge, luteal mRNA expression of *TNFA* and *CASP3* was increased ($P < 0.05$) in group F-LPS at 120 and 180 min, respectively, and those of *CASP8* was decreased ($P < 0.05$) in PX-LPS at 60 and 120 min after LPS-treatment. In conclusion, although flunixin managed to inhibit $PGF_{2\alpha}$, it did not suffice to successfully prevent LPS-induced apoptosis. However, endotoxin-binding polymyxin B resulted in luteal responsiveness to hCG after LPS-challenge.
Key words: Corpus luteum, Endotoxin, Flunixin, Polymyxin B, Prostaglandin $F_{2\alpha}$

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Inflammatory diseases, such as endometritis and mastitis, play a pivotal role in dairy cows and reduce their reproductive performance [1, 2]. To investigate the effects of inflammation on cows' fertility, treatment with lipopolysaccharide (LPS), the endotoxin from the outer membrane of gram-negative bacteria, was used as a model [3–5]. In this model, different routes of administration of LPS were investigated, namely intravenous [6], intrauterine [7], and intramammary [8]. During inflammation, LPS induces the production of prostaglandins (PGs) by macrophages, monocytes and endothelial cells [9]. Furthermore, LPS activates the nuclear factor kappa B (NF-κB), which leads to the expression of multiple proinflammatory cytokines [10]. These cytokines are released from the activated macrophages and stimulate in turn the neutrophils to produce reactive oxygen species [11]. Moreover, it is noteworthy that some cytokines, such as tumor necrosis factor α (TNFA) and interferon (IFN) γ, may directly reduce fertility due to their cytotoxic effect on luteal cells [12].

When given intravenously, *Escherichia coli* (*E. coli*) LPS transiently reduced size and blood flow of the bovine corpus luteum (CL), as well as blood progesterone (P_4) concentrations [6]. Since $PGF_{2\alpha}$ metabolite concentrations were also increased after LPS treatment [6], it was speculated that an enhanced uterine release of $PGF_{2\alpha}$ induced premature luteolysis. However, LPS induced apoptosis in luteal monolayer cultures without the influence of endometrium-derived $PGF_{2\alpha}$ [13]. Therefore, a recent study used the *in vitro* model of a perfused ovary to investigate whether the LPS-induced effects on the bovine CL were mediated *via* LPS-induced release of $PGF_{2\alpha}$ or directly by LPS [14]. In that study, the reduced P_4 secretion seemed to be caused predominantly by an increase in LPS-induced apoptosis, but an impact of luteal $PGF_{2\alpha}$ could not be excluded.

To further investigate the impact of luteal $PGF_{2\alpha}$ and LPS on the suppression of luteal function, an attempt was made in the present study to inhibit their different modes of action by a non-steroidal anti-inflammatory drug (NSAID) and an endotoxin-binding agent, respectively.

The NSAID flunixin inhibits the enzyme cyclooxygenase and therefore blocks the synthesis of the eicosanoid inflammatory mediators, such as PGs [15, 16]. Furthermore, flunixin has antioxidative properties [17], and inhibits the activation of NF-κB [18] and the increase in cytokine levels [19, 20]. In LPS-treated mice, for instance, flunixin inhibited the increase in TNFA, interleukin 1β, and interleukin 10 [19]. However, flunixin is unable to directly bind the LPS molecule [21]. The use of flunixin for the treatment of endotoxemia relies on its modulatory function on acute hemodynamic changes [20], i.e. changes in systemic blood pressure, cardiac output, and organ perfusion. Following the recommended intravenous dosing of 2 mg flunixin per kg body weight in cattle, maximum blood concentrations of 5 µg/ml of flunixin were reached within 3.5 h [22].

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Polymyxin B is an antibiotic with endotoxin-binding properties due to its high affinity to the lipid A of LPS [23–25]. In human medicine, polymyxin B was successfully used to reduce blood endotoxin levels in patients with sepsis [26–28]. In this respect, 1 mg of *E. coli* LPS is bound by 55 µg of polymyxin B [24]. However, polymyxin B not only binds LPS but also inhibits the binding activity of NF-κB, and therefore inhibits the new expression of TNFA [10]. The minimal inhibitory concentration of polymyxin B for *E. coli* was reported 1.43 µg/ml [29] and 5 µg/ml [30]. Therefore, therapeutical serum concentrations of 3 to 5 µg/ml were recommended for polymyxin treatment [22].

In summary, flunixin inhibits the LPS-induced $PGF_{2\alpha}$ secretion by the bovine CL, whereas polymyxin B can directly bind and inactivate the LPS molecule. Furthermore, both drugs inhibit cytokine production. Using separate treatments with flunixin and polymyxin B in the isolated perfused ovary model, the present study investigates if the suppression of luteal function due to endotoxemia (as simulated by Lüttgenau *et al*. [14]) can be avoided by either the inhibition of luteal $PGF_{2\alpha}$ or the inactivation of LPS. Our hypothesis is that the LPS-induced release of $PGF_{2\alpha}$ is not mandatory for the suppression of luteal function by LPS.

Materials and Methods

Harvesting and preparation of ovaries

Twelve ovaries with *mesovarium*, an intact *tunica albuginea*, and a CL with an estimated diameter of > 20 mm that was subsequently confirmed as a mid-cycle (days 8–15) CL [31], were harvested in accordance with ethical demands from the carcasses of clinically healthy cows (*Bos taurus*; including Brown Swiss, Holstein Friesian, Red Holstein and Swiss Fleckvieh; breeds were equally distributed between the two groups) at the abattoir. The preparation and catheterization of the ovarian arteries, the flushing of the ovaries and their transport to the laboratory, and the measurements of ovarian weight and luteal size were recently described in detail [14].

Isolated perfusion of ovaries

Ovaries were perfused isolated under highly standardized microclimatic conditions. A schematic illustration of the perfusion system and the detailed composition of the perfusion medium were recently provided [14]. The perfusion medium was standardized with the use of a membrane oxygenator (Radnoti Membrane Oxygenating Chamber; Radnoti Limited, Dublin, Ireland) and water jacketed reservoirs (Water-Jacketed Reservoir 3 l and 5 l; Radnoti Limited) that were heated by a circulating bath (Immersion Thermostats, Baths and Circulators, Optima T100; Grant Instruments Ltd., Cambridgeshire, UK). Water jacketed glassware and tubings (Water-Jacketed f/tzbe ass 24", Water-Jacketed Oxygenator, Water-Jacketed Bubble Trap; Radnoti Limited) kept the temperature of the perfusion medium constant until it reached the ovary. Measurements that were performed to ensure adequate oxygenation and temperature of the perfusion medium were recently described [14]. Furthermore, an advanced volume- and pressure-controlled peristaltic pump (Minipuls 3 Peristaltic Pump; ADInstruments, Oxford, UK) was used. The pump was connected to a pump speed controlling hardware device (STH Pump Controller), a data acquisition hardware (PowerLab), and a bridge amplifier (Bridge Amp) that allowed to connect the PowerLab to the pressure transducer (Physiological Pressure Transducer; all from ADInstruments). The PowerLab was connected *via* USB to a laptop, where data acquisition and analysis software (LabChart 8; ADInstruments) were installed. The pressure transducer was inserted in the perfusion system close to accessing the ovarian artery and was connected to the PowerLab, Bridge Amp and STH Pump Controller, which in turn were connected to the LabChart software. The pressure was calibrated with the help of a transducer simulator and tester (Delta-Cal; Utah Medical Products Inc., Athlone, Ireland) and the data was inserted in LabChart. Both, pressure and flow, were continuously measured throughout the experiment. The perfusion pressure was measured in 11 out of 12 ovaries; in one ovary from group F-LPS measurement was not possible due to a technical problem. The perfusion medium was not recycled as already rationalized [14].

Study design

In consistence with the recent study by Lüttgenau *et al*. [14], all ovaries were perfused for 240 min (Fig. 1). During the first 50 min (equilibration), no agents were added to the perfusion medium. Ovaries were randomly allocated to two groups of six ovaries. In group F-LPS 5 µg/ml flunixin (Flunixine Biokema ad us. vet.; Biokema SA, Crissier, Switzerland) and in group PX-LPS 5 µg/ml polymyxin B (Polymyxin-B-sulfat KA 10 Mio IE/100 ml ad us. vet.; Kantonsapotheke Zürich, Zurich, Switzerland) were added to the medium, starting at 50 min after the start of perfusion. In both groups,

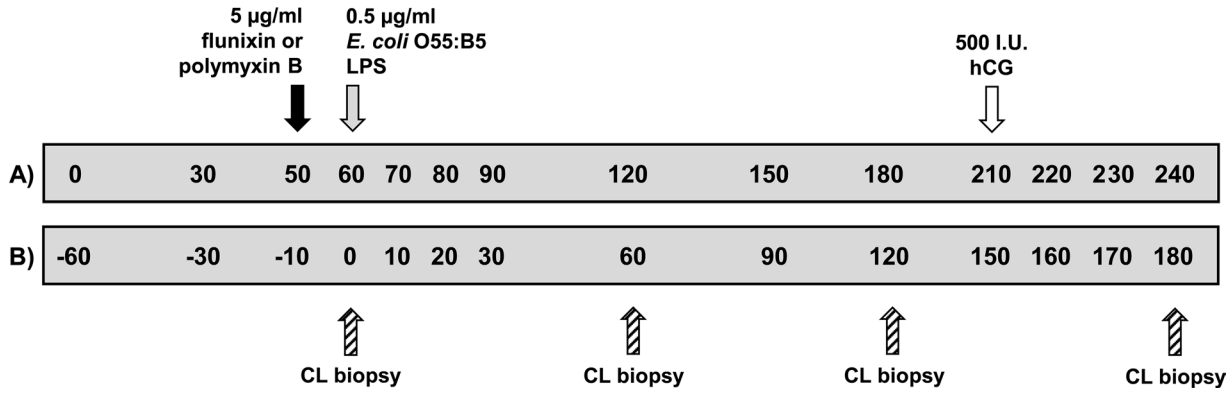

Fig. 1. Treatment schedule of isolated perfused bovine ovaries with the timeline depicted as A) minutes after the start of perfusion, and B) minutes before / after the start of lipopolysaccharide (LPS) treatment. At 50 min after the start of perfusion, ovaries were assigned randomly to receive either 5 µg/ml flunixin (n = 6) or polymyxin B (n = 6). In both groups, 0.5 µg/ml *E. coli* O55:B5 LPS and 500 I.U. human chorionic gonadotropin (hCG) were added to the perfusion medium at 60 min and 210 min after the start of perfusion, respectively. Samples of the effluent perfusion medium were collected on all times shown, and biopsies of the corpus luteum (CL) were performed every 60 min after the start of perfusion.

0.5 µg/ml *E. coli* O55:B5 LPS (Sigma-Aldrich, St. Louis, MO, USA) were given to the medium at 60 min after the start of perfusion. As well-established studies regarding blood concentrations of LPS in cows with inflammatory diseases were lacking, LPS concentration in the ovarian follicular fluid of cows with uterine inflammation was used based on bibliographical data. The used concentration of 0.5 µg LPS per ml is within the range of LPS concentrations recommended in previous studies [5, 32] and equal to the LPS concentration used in the precursive study of Lüttgenau *et al*. [14]. Flunixin, polymyxin B, and LPS treatments were continued until the end of the perfusion time. For all ovaries, 500 I.U. human chorionic gonadotropin (hCG; Chorulon 1500®; MSD Animal Health GmbH, Luzern, Switzerland) were added to the perfusion medium at 210 min after the start of perfusion. The used dosage of 500 I.U. was the minimum effective dose of hCG to induce ovulation and to increase endogenous P_4 production in cattle [33], and reliably and reproducibly increased luteal P_4 synthesis in isolated perfused bovine ovaries [14].

To assess the effect of polymyxin B on luteal release of PGs, a preliminary experiment was performed on three ovaries treated with polymyxin B alone during the treatment period of perfusion (Supplementary Table 1). In this experiment, concentrations of PGE_2 and $PGF_{2\alpha}$ were measured in the effluent perfusate at 10, 40, 70, 100, 130, 160, and 190 min after start of polymyxin B treatment (equivalent to 0, 30, 60, 90, 120, 150, and 180 min after start of LPS treatment in groups F-LPS and PX-LPS).

Lactate, creatine kinase, P_4, PGE_2, and $PGF_{2\alpha}$

Lactate concentration and the activity of creatine kinase (CK) were used as markers of hypoxia and cell death [34, 35] to ensure that the ovary remained in a functional state during the perfusion.

In the effluent perfusion medium, concentrations of lactate, P_4, PGE_2, and $PGF_{2\alpha}$ and the activity of CK were measured every 30 min throughout the perfusion period. Additionally, P_4, PGE_2, and $PGF_{2\alpha}$ levels were determined at 50 min after the start of perfusion, and P_4 levels were also measured every 10 min within 30 min after the treatments with LPS and hCG. The methods for determination of lactate, CK, P_4, and $PGF_{2\alpha}$ were recently described [14]; for analysis of PGE_2, a high-sensitivity ELISA kit (Enzo Life Sciences AG, Lausen, Switzerland) was used. To assure the comparability between different test kit lots, PG concentrations of samples at 120 min after the start of perfusion were analyzed with each ELISA kit. Concentrations of P_4 and PGE_2 that exceeded the standard concentrations of the respective tests were determined after dilution (1:10).

Analyses of lactate, CK, PGE_2, and $PGF_{2\alpha}$ had lower detection limits of 0.04 mmol/l, 5 U/l, 72 pg/ml, and 2.0 pg/ml, respectively. For measurements below these limits, 0.04 mmol/l, 5 U/l, 72 pg/ml, and 2.0 pg/ml were used as respective arbitrary values to facilitate statistical analysis. Additionally, for PGE_2 measurements above the upper detection limit of 10,000 pg/ml, the arbitrary value of 10,000 pg/ml was used.

The range of standard concentrations for the P_4 test was 0.03 to 53 ng/ml, intra- and inter-assay coefficients of variation were ≤ 8.5% and ≤ 8.7%, respectively, and 50% of relative binding (ED50) occurred at 1.56 to 1.77 ng/ml. For the PGE_2 test, the range of standard concentrations was 7.81 to 1,000 pg/ml, intra- and inter-assay coefficients of variation were ≤ 9.8 and ≤ 12.6%, and ED50 occurred at 127.8 pg/ml. For the $PGF_{2\alpha}$ test, the range of standard concentrations was 1.95 to 2,000 pg/ml, intra- and inter-assay coefficients of variation were ≤ 7.2 and ≤ 11.0%, and ED50 was 81 pg/ml.

Corpus luteum biopsy and expression analysis

Corpus luteum biopsy was performed after 60, 120, 180, and 240 min of perfusion. A detailed description of the collection and storing of luteal samples was provided recently [14]. The use of a biopsy needle allowed repeated biopsy sampling from a single CL without impairing its subsequent function [36].

Luteal mRNA expression was determined for the proinflammatory cytokine *TNFA*, the apoptotic enzymes *CASP3* and *-8*, and the prostaglandin synthases *PGES* (*PTGES*) and *PGFS* (*AKR1B1*).

Total RNA from luteal tissue samples was extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). Homogenization of the tissues was achieved with the Qiagen TissueLyser II and 2.8 mm ceramic beads (2 ml Reinforced Tubes w/ 2.8 mm Ceramic Beads 50 Pack; LabForce, Muttenz, Switzerland). RNA concentration and integrity were quantified using the NanoDrop 2000 (peqLab) and the Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany), respectively. RNA integrity numbers ranged from 9.1 to 10.0 (average 9.9). Five hundred nanograms of RNA were reverse transcribed using the M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega, Madison, WI, USA) as recently described [37].

Luteal mRNA expression was determined in a two-step quantitative real-time PCR (qPCR) as described recently [14], using the CFX384 Real-Time PCR Detection System (Bio-Rad, Munich, Germany) and the Kapa SYBR Fast Universal qPCR Kit (KK4618; Kapa Biosystems, London, UK). The coefficient of variation of the qPCR was below 1%. The primers used to amplify specific fragments referring to selected regulated genes were identical with those in the study of Lüttgenau *et al*. [14] and are shown in Table 1. The primer-specific annealing temperatures are outlined. The cycle number (C_q) required to achieve a definite SYBR Green fluorescence signal was calculated by the regression method (Bio-Rad CFX Manager 3.1). The C_q was inversely correlated with the logarithm of the initial template concentration. The C_q determined for the target genes were normalized (ΔC_q) against the geometrical mean of the five reference genes tyrosine 3-monooxygenase/tryptophan 5-mono-oxygenase activation protein-zeta (*YWHAZ*), histone (*H3F3A*), CCR4-NOT transcription complex – subunit 11 (*CNOT11*), suppressor of zeste 12 homolog - Drosophila (*SUZ12*), and TATA box binding protein (*TBP*). To avoid negative digits, while allowing the estimation of a comparison between two genes, data were presented as means ± SEM added to the arbitrary value 10 (ΔC_q). Thus, a high ΔC_q proportionally resembled high transcript abundance [38].

Postprocessing of ovaries

All ovaries (except one from group F-LPS) were perfused with stained (Patent blue; Sigma-Aldrich) perfusion medium and dissected to test for smaller leakages of the ovarian vessels and homogenous perfusion of the luteal tissue as described recently [14]. In one ovary (group F-LPS), the dissection of the CL was unintentionally performed before staining. However, delayed staining revealed no leakage from the ovarian artery and homogenous perfusion of the remaining half of the CL. Therefore, due to the established criteria, all ovaries were included in the study.

Statistical analyses

Statistical analyses were conducted using the Statistical Analysis System V9.3 (SAS Institute Inc., Cary, NC, USA). The distribution of the data was tested for normality by means of the Shapiro-Wilk-test (PROC UNIVARIATE). Repeated measures ANOVA (PROC GLM) was performed to assess the influence of treatment, time, and treatment-by-time interaction. To control the type I error rate, Tukey's HSD test was applied. Significant results were further evaluated

Table 1. Sequences and accession numbers of PCR primers for assayed genes from bovine corpus luteum cells, and length and annealing temperature (AT) of PCR products

Gene	Gene symbol	Reference [acc. no.]	Forward primer [5'-…-3']	Reverse primer [5'-…-3']	PCR product [bp]	AT [°C]
Tumor necrosis factor α	*TNFA*	NM_173966.3	CCACGTTGTAGCCGACATC	ACCACCAGCTGGTTGTCTTC	108	60
Caspase 3	*CASP3*	NM_001077840.1	AACCTCCGTGGATTCAAAATC	TTCAGGRTAATCCATTTTGTAAC[1]	114	60
Caspase 8	*CASP8*	NM_001045970.2	TGTCACAATCGCTTCCAGAG	GAAGTTCAGGCACCTGCTTC	183	60
Prostaglandin E synthase	*PGES* (*PTGES*)	NM_174443.2	TCCTGGTCTTCTTCCTGGG	CCCAGACAATCTGCAGGG	132	60
Prostaglandin F synthase	*PGFS* (*AKR1B1*)	NM_001012519.1	ATACAAGCCGGCGGTTAAC	TGTCTGCAATCGCTTTGATC	188	60
Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta	*YWHAZ*	NM_174814.2	AGGCTGAGCGATATGATGAC	GACCCTCCAAGATGACCTAC	141	60
Histone	*H3F3A*	NM_001014389.2	ACTGGCTACAAAAGCCGCTC	ACTTGCCTCCTGCAAAGCAC	233	60
CCR4-NOT transcription complex, subunit 11	*CNOT11*	XM_582695.6	TCAGTGGACCAAAGCCACCTA	CTCCACACCGGTGCTGTTCT	170	60
Suppressor of zeste 12 homolog (Drosophila)	*SUZ12*	NM_001205587.1	CATCCAAAAGGTGCTAGGATAGATG	TGGGCCTGCACACAAGAATG	160	60
TATA box binding protein	*TBP*	NM_001075742.1	CAGAGAGCTCCGGGATCGT	CACCATCTTCCCAGAACTGAATAT	194	60

[1] degenerate multispecies primer, R = A or G.

using a Student's *t*-test (PROC MEANS) for dependent pairwise comparisons and a single-factor ANOVA (PROC GLM) for independent pairwise comparisons. In case of non-normal data (applicable to the flow of the perfusion medium), Wilcoxon's signed rank test (PROC UNIVARIATE) for dependent pairwise comparisons and Kruskal-Wallis-test (PROC NPAR1WAY) for independent pairwise comparisons were used. Data were presented as mean ± SEM or median ± mean absolute deviation (MAD), depending on the distribution of the data, and differences were considered significant at $P \leq 0.05$.

Results

The median (± MAD) interval between death of the cow and begin of the perfusion was 59.0 ± 2.2 min (range, 47–72 min) and did not differ ($P > 0.05$) between groups F-LPS and PX-LPS. The mean (± SEM) diameter of the CL was 28.0 ± 1.2 mm (range, 26.9–29.5 mm) and did not differ ($P > 0.05$) between the groups. The mean (± SEM) pressure was 121.6 ± 3.6 mmHg and there was neither a treatment effect, a time effect, nor a treatment-by-time interaction ($P > 0.05$ for each). The temperature and the flow of the perfusion medium did not differ between the groups ($P > 0.05$) and the mean (± SEM) temperature and median (± MAD) flow were 37.19 ± 0.05°C and 34 ± 3.3 ml/min, respectively. All ovaries showed contractions of the vascular pedicle that were not quantified. A subjective intensification of the contractions over time was observed in both groups. During perfusion, ovaries with vascular pedicle increased in weight due to edema in the *mesovarium*. The mean (± SEM) increase was 56.7 ± 7.8 g and did not differ ($P > 0.05$) between groups.

Ovarian lactate production and CK activity did neither show a treatment effect ($P > 0.05$) nor a treatment-by-time interaction ($P > 0.05$) but a time effect ($P < 0.0001$ and $P = 0.003$, respectively). Mean (± SEM) lactate concentrations decreased from 0.45 ± 0.06 mmol/l during the equilibration time to 0.18 ± 0.01 mmol/l during the treatment period. Similarly, CK activity decreased from 65.9 ± 13.8 U/l during the equilibration time to 9.5 ± 1.3 U/l during the treatment period. Before the start of any treatment, neither the concentrations of lactate nor the activity of CK differed ($P > 0.05$) between F-LPS and PX-LPS groups. Measurements below the indicated lower detection limits were found in group F-LPS for CK (21 out of 54 measurements), and in group PX-LPS for lactate (2 out of 54) and CK (26 out of 54).

Regarding PGE_2 and $PGF_{2\alpha}$ concentrations in the effluent perfusate, there was a treatment effect ($P = 0.0003$ and $P = 0.003$, respectively), a time effect ($P < 0.0001$ and $P = 0.0003$, respectively), and a treatment-by-time interaction ($P < 0.0001$ and $P = 0.0003$, respectively). Before the start of any treatment, the concentrations of PGE_2 and $PGF_{2\alpha}$ did not differ ($P > 0.05$) between F-LPS and PX-LPS groups, but concentrations of PGE_2 and $PGF_{2\alpha}$ were higher ($P \leq 0.03$) in group PX-LPS compared to group F-LPS during the treatment period (Fig. 2A and B). During the equilibration period (before treatment), PGE_2 and $PGF_{2\alpha}$ levels decreased in both groups ($P = 0.03$; Fig. 2A and B). After the start of treatment, PGE_2 and $PGF_{2\alpha}$ levels increased in group PX-LPS ($P \leq 0.04$; Fig. 2A and B), whereas levels did not differ in group F-LPS ($P > 0.05$; Fig. 2A and B). Prostaglandin E_2 and $PGF_{2\alpha}$ measurements below the indicated lower detection limits were found in group F-LPS (36 out of 60 and 43 out of 60 measurements, respectively), and PGE_2 measurements above the indicated upper detection limit were found in group PX-LPS (4 out of 60 measurements).

Progesterone concentrations in the effluent perfusate did neither show a treatment effect ($P > 0.05$) nor a treatment-by-time interaction ($P > 0.05$) but a time effect ($P = 0.003$). During the perfusion period, the concentration of P_4 did not differ ($P > 0.05$) between F-LPS and PX-LPS groups (Fig. 3). In group F-LPS, P_4 levels decreased ($P = 0.04$) at 120 and 150 min after LPS-challenge, whereas P_4 levels remained constant ($P > 0.05$) over time in group PX-LPS (Fig. 3). After stimulation with hCG, P_4 concentrations increased ($P = 0.03$) in group PX-LPS but did not differ ($P > 0.05$) in group F-LPS (Fig. 3). The percental increase in P_4 concentrations after hCG treatment was also significant in group PX-LPS but not in group F-LPS, and a higher ($P = 0.02$) P_4 increase in group PX-LPS compared to group F-LPS was revealed at 10 min after hCG stimulation (Fig. 4).

Luteal mRNA abundance of *TNFA* did neither show a treatment effect ($P > 0.05$) nor treatment-by-time interaction ($P > 0.05$) but a time effect ($P = 0.05$). The expression of *TNFA* mRNA did not differ ($P > 0.05$) between the groups (Fig. 5A). However, an increase ($P = 0.04$) in *TNFA* mRNA was observed between 0 and 120 min after LPS-challenge in group F-LPS (Fig. 5A), whereas no difference ($P > 0.05$) over time was found in group PX-LPS.

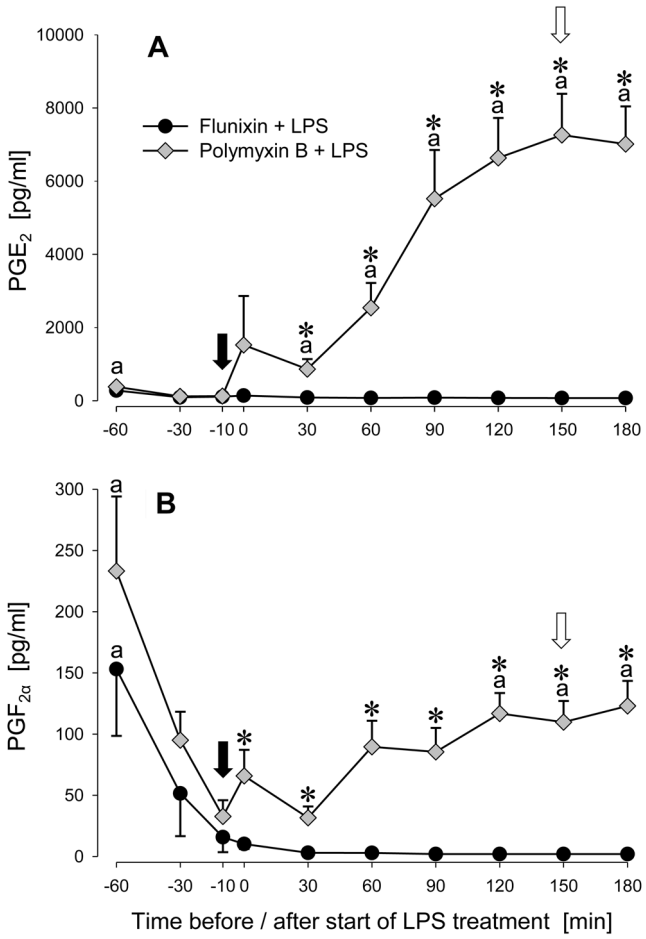

Fig. 2. Changes (means ± SEM) in PGE_2 (A) and $PGF_{2\alpha}$ (B) concentrations of the effluent perfusate from ovaries treated with flunixin and LPS (F-LPS; n = 6) and ovaries treated with polymyxin B and LPS (PX-LPS; n = 6) during a 60-min perfusion period before and 180-min period after the start of LPS-challenge; The black arrow indicates the start of flunixin / polymyxin treatment; The white arrow indicates the time of hCG treatment; The letter "a" represents a difference between times ($P \leq 0.05$) compared to 10 min before the start of LPS-challenge (i.e. the starting time of flunixin / polymyxin treatment) within groups indicated; An asterisk represents a difference ($P \leq 0.05$) between groups F-LPS and PX-LPS at times indicated.

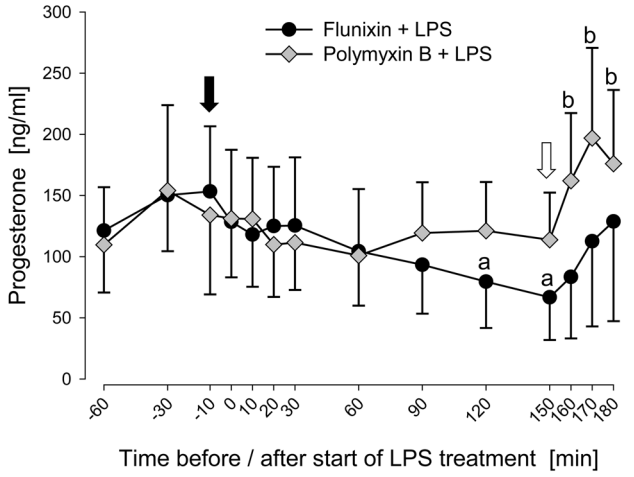

Fig. 3. Changes (means ± SEM) in P_4 concentrations of the effluent perfusate from ovaries treated with flunixin and LPS (F-LPS; n = 6) and ovaries treated with polymyxin B and LPS (PX-LPS; n = 6) during a 60-min perfusion period before and 180-min period after the start of LPS-challenge; The black arrow indicates the start of flunixin / polymyxin treatment; The white arrow indicates the time of hCG treatment; The letter "a" represents a difference between times ($P \leq 0.05$) compared to 10 min before the start of LPS-challenge (i.e. the starting time of flunixin / polymyxin treatment) within groups indicated; The letter "b" represents a difference between subsequent times ($P \leq 0.05$) compared to 150 min after the start of LPS-challenge (i.e. the time of hCG stimulation) within groups indicated.

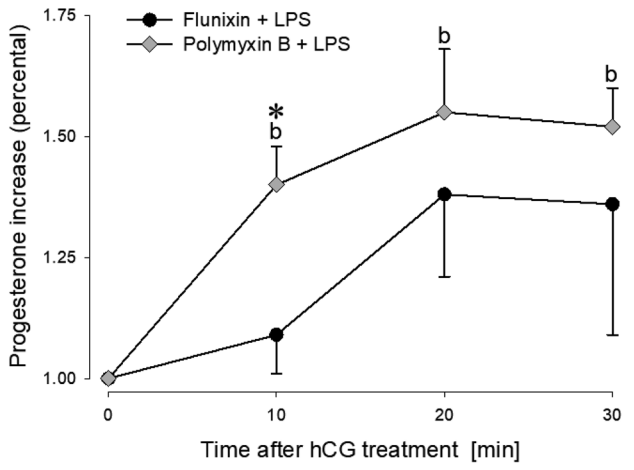

Fig. 4. Percental changes (means ± SEM) in P_4 concentrations of the effluent perfusate from ovaries treated with flunixin and LPS (F-LPS; n = 6) and ovaries treated with polymyxin B and LPS (PX-LPS; n = 6) during a 30-min perfusion period after the time of hCG stimulation (i.e. 150 min after the start of LPS-challenge); The letter "b" represents a difference between times ($P \leq 0.05$) compared to 0 min after hCG treatment within groups indicated; An asterisk represents a difference ($P \leq 0.05$) between groups F-LPS and PX-LPS at times indicated.

Luteal mRNA expression of caspase (*CASP*)*3* and *CASP8* did neither show a treatment effect ($P > 0.05$) nor a treatment-by-time interaction ($P > 0.05$) but a time effect was evident ($P = 0.004$). The mRNA expressions of *CASP3* and *CASP8* did not differ ($P > 0.05$) between the groups (Fig. 5B). Increased *CASP3* mRNA ($P = 0.03$) was observed in group F-LPS at 180 min (compared with 0 min) after the start of LPS treatment (Fig. 5B), whereas the expression of *CASP3* did not differ ($P > 0.05$) over time in group PX-LPS. The expression of *CASP8* mRNA remained stable ($P > 0.05$) over time in group F-LPS, whereas a decreased expression ($P = 0.03$) at 60 and 120 min after LPS-challenge compared to the start of the treatment period was observed in group PX-LPS (Fig. 5B).

Luteal mRNA expression of PGE- (*PGES*) and PGF (*PGFS*) synthases did neither show a treatment effect, a time effect nor a treatment-by-time interaction ($P > 0.05$ for each). The mRNA abundance of *PGES* and *PGFS* after LPS-challenge did neither differ ($P > 0.05$) between groups F-LPS and PX-LPS nor within groups at any time compared to the pre LPS treatment values (Fig. 5C).

Discussion

In this experiment, all ovaries had an ischemic time period of less than 72 min before re-perfusion. The critical ischemic time was previously determined to be 120 min in rat ovaries [39]. Furthermore, high lactate concentration and CK activity at the start of perfusion,

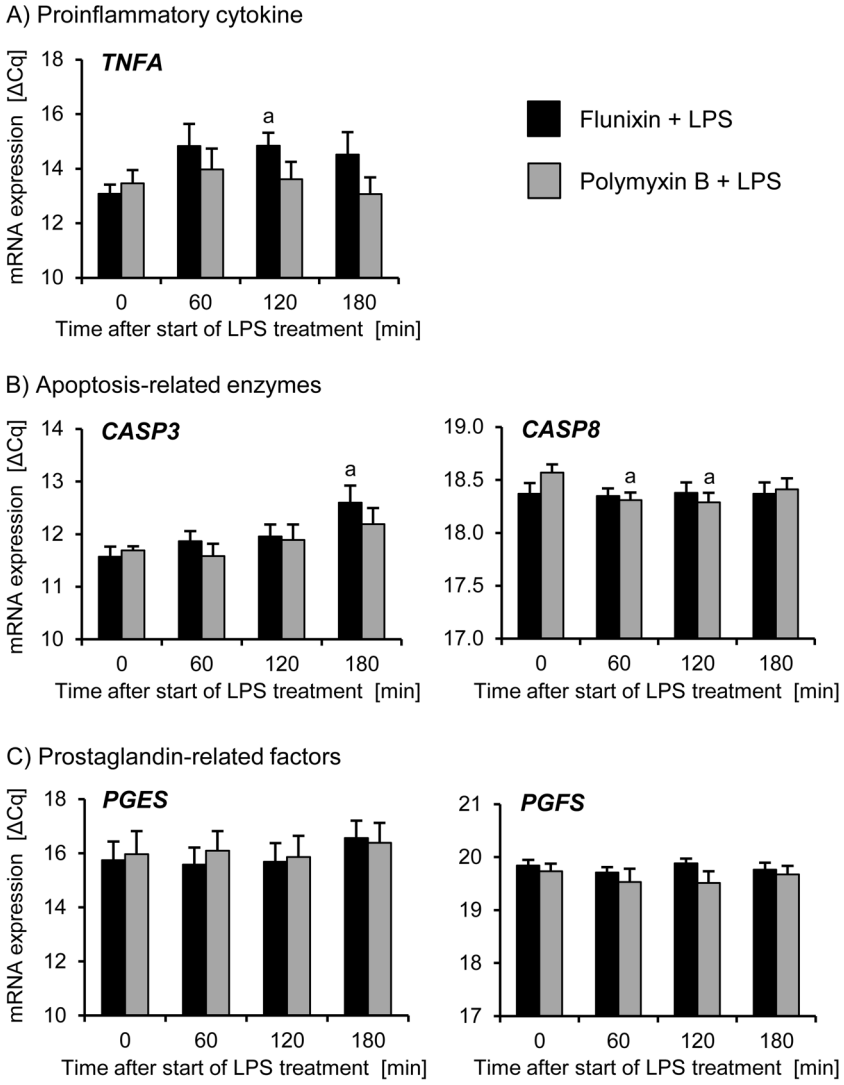

Fig. 5. Changes (means ± SEM) in luteal mRNA expression of tumor necrosis factor α (*TNFA*), caspase (*CASP*) *3*, *CASP8*, prostaglandin E synthase (*PGES*), and *PGFS*, of ovaries treated with flunixin and LPS (F-LPS; n = 6) and ovaries treated with polymyxin B and LPS (PX-LPS; n = 6) during the treatment period of perfusion; Note the log-scale of gene expression data; The letter "a" represent a difference between times ($P \leq 0.05$) compared to 0 min after the start of LPS-challenge within groups indicated.

indicating hypoxia and cell death [34], decreased rapidly during the equilibration time and remained stable at low levels during the treatment period. The same observation was made in previous studies after successful re-perfusion and oxygenation of human uteri [35] and bovine ovaries [14], indicating the maintenance of highly standardized conditions for the ovaries in the present study. Diameters of the CL ranged from 26.9 to 29.5 mm, equal to a cross-sectional area of luteal tissue ranging from 5.7 to 6.8 cm^2. In previous studies, corpora lutea of this size were only found during the mid-luteal phase [40, 41].

Concentration of PGE_2 and $PGF_{2\alpha}$ in the effluent perfusate were moderately and considerably increased at the start of re-perfusion, respectively. This result was to be expected since several prostaglandins, including PGE_2 and $PGF_{2\alpha}$, were released in response to oxidative stress, inflammation, and cell damage [42–44]. However, concentrations of PGE_2 and $PGF_{2\alpha}$ decreased rapidly during the 50 min of equilibration and reached basal levels before the start of treatments, indicating successful re-perfusion. The treatment with the cyclooxygenase inhibitor flunixin in group F-LPS inhibited the synthesis of PGE_2 and $PGF_{2\alpha}$ and kept it on basal concentrations. In contrast, there was a significant increase in PGE_2 and $PGF_{2\alpha}$ production in group PX-LPS, starting at 40 min and 130 min after treatment with LPS-binding polymyxin B, respectively. This increase was comparable to that observed after LPS-challenge in the study of Lüttgenau *et al*. [14], indicating that polymyxin B is not suitable to inhibit the production of PGs after LPS-challenge. It is probable that polymyxin B alone (without LPS) also induces PG production, since higher $PGF_{2\alpha}$ concentrations in group F-LPS compared to PX-LPS were already observed immediately before the LPS-challenge. Similar to the interaction of polymyxin B with the bacterial outer membrane, it mitigatedly permeabilizes eukaryotic membranes leading to cell swelling and histamine release [45, 46], both being associated with the release of PGs. Taken together, the present study enables to investigate the effect of LPS on the bovine CL in the absence and presence of luteal PGs.

The CL is rich in arachidonic acids, the precursor of PGs, and luteal PGs contribute to the regulation of the CL [47]. Since PGE_2 is a luteotropic factor [47, 48], the inhibition of its synthesis can be judged as a detrimental effect of flunixin on the CL. In the recent

study of Lüttgenau *et al*. [14], the impact of luteal $PGF_{2\alpha}$ on the LPS-induced suppression of luteal function could neither be proven nor excluded. In the present study, the complete inhibition of any impact of $PGF_{2\alpha}$ in group F-LPS did not prevent the expression of apoptotic enzymes and did not maintain luteal responsiveness to hCG after LPS-challenge. However, it is noteworthy that $PGF_{2\alpha}$ of different origin, namely luteal and endometrial, is expected to play a differing role in bovine luteal function [49–51]. Whereas endometrial $PGF_{2\alpha}$, which is released in the late luteal phase, induces functional and structural luteolysis [52], the release of luteal $PGF_{2\alpha}$ amplifies the luteolytic action of $PGF_{2\alpha}$ from the uterus within the regressing CL [51]. Therefore, conclusions regarding the impact of luteal $PGF_{2\alpha}$ on the CL in the present study using the *in vitro* model of the isolated perfused ovary cannot necessarily be adapted to the effect of endometrial $PGF_{2\alpha}$ *in vivo*.

From the start of the treatment period until hCG-challenge, P_4 concentrations in the effluent perfusate remained statistically unchanged in group PX-LPS. In contrast, P_4 concentrations in group F-LPS were decreased at 120 min and 150 min after LPS-challenge. It is noteworthy that flunixin inhibits the synthesis of all PGs, including the luteotropic eicosanoid PGE_2. Basal PGE_2 concentrations after the start of flunixin treatment might have reduced the P_4 level in group F-LPS of the present study. Due to the decreased P_4 concentrations after flunixin treatment, the P_4 level differed between F-LPS and PX-LPS groups at the time of hCG stimulation, although this difference was not significant. To exclude the influence of different starting levels, the proportion change in P_4 from the time of hCG challenge was evaluated and confirmed significantly increasing P_4 levels in group PX-LPS but not in group F-LPS. In a recent study [14], LPS abolished the hCG-induced increase in P_4 that was observed in untreated controls. The inhibition of the LPS-induced suppression of hCG-stimulated P_4 secretion in group PX-LPS indicates the maintenance of luteal viability and hCG responsiveness due to the treatment with polymyxin B. In contrast, the treatment with flunixin in group F-LPS was apparently less able to block the LPS-induced suppression of luteal responsiveness to hCG.

Several *in vivo* studies [53–56] have already investigated the effect of flunixin on luteal phase length and on maintenance of pregnancy but the results were controversial. Some studies revealed an increase of luteal phase length and a positive effect on the maintenance of early pregnancy [53, 54], whereas other studies did not find any effect on early embryonic loss or pregnancy rates [55, 56]. However, detrimental effects of flunixin itself on the CL have not been described yet. Since flunixin treatment (excluding any impact of $PGF_{2\alpha}$) could not maintain luteal responsiveness to hCG in the present study, we assume that the suppressed hCG responsiveness of the CL after LPS-challenge in the study of Lüttgenau *et al*. [14] was caused by detrimental effects of LPS other than the release of $PGF_{2\alpha}$.

Luteal mRNA expression of *TNFA* was significantly increased at 120 min compared to 0 min after the start of LPS treatment in group F-LPS. The increase in *TNFA* mRNA after pretreatment with flunixin indicates that an inhibition of PG synthesis does not avoid the LPS-induced increase in the expression of proinflammatory cytokines that was observed in the study of Lüttgenau *et al*. [14]. Since TNFA is known to have cytotoxic effects on luteal cells [12], its increased mRNA expression can be associated with luteal apoptosis. In group PX-LPS, no difference in the mRNA expression of *TNFA* was found during the treatment period, indicating that polymyxin B is more suitable to inhibit the LPS-induced expression of proinflammatory cytokines. Consistently, the NF-κB binding activity was immediately inhibited and TNFA secretion consequently suppressed after LPS neutralization with polymyxin B [10].

A significant increase in the mRNA expression of the apoptotic marker *CASP3* was observed in group F-LPS, whereas mRNA abundance of *CASP8* decreased transiently in group PX-LPS. Both results contribute to our assumption that polymyxin B treatment is more suitable than flunixin treatment to inhibit the LPS-induced apoptosis of the bovine CL as recently evidenced by increased expressions of *CASP3* and *CASP8* mRNA [14].

Although flunixin inhibited the synthesis of PGE_2 and $PGF_{2\alpha}$, the luteal mRNA expressions of *PGES* and *PGFS* were not reduced. Moreover, the expressions of *PGES* and *PGFS* mRNA remained constant over time, irrespective of the pretreatment of the CL with flunixin or polymyxin B before LPS-challenge. It is noteworthy that cyclooxygenase-2 is the rate limiting enzyme responsible for the conversion of arachidonic acid into PGH_2 (the precursor of PGE_2 and $PGF_{2\alpha}$), whereas PGES and PGFS are downstream enzymes that catalyze the conversion of PGH_2 to PGE_2 and $PGF_{2\alpha}$, respectively [57]. Luteal expression of *PGES* and *PGFS* mRNA after treatment with LPS alone was reported by Lüttgenau *et al*. [14]. It is known that luteal *PGES* shows an irregular pattern during the different phases of the luteal development, whereas there is a constant expression of *PGFS* throughout the CL lifespan [47]. However, the synthesis of PGE_2 and $PGF_{2\alpha}$ is not closely related to the expression of *PGES* and *PGFS*, respectively.

In conclusion, flunixin inhibited luteal PG secretion in isolated perfused bovine ovaries but did not suffice to successfully prevent LPS-induced apoptosis of luteal tissue. Consequently, luteal P_4 production was reduced and luteal responsiveness to hCG was suppressed after LPS-challenge. In contrast, endotoxin-binding polymyxin B did not inhibit luteal PG secretion and resulted in luteal responsiveness to hCG after LPS-challenge. Therefore, our hypothesis that the LPS-induced release of $PGF_{2\alpha}$ is not mandatory for the suppression of luteal function by LPS was corroborated. The observations in this study strongly encourage further experiments using the model of the isolated perfused ovary to directly compare the effect of treatments with LPS, flunixin, polymyxin, and consequent combinations on luteal responsiveness to hCG.

Conflict of interests: The authors have nothing to declare.

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