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Inhibition der Effekte von Lipopolysaccharid auf das Corpus luteum am isoliert perfundierten Ovar des Rindes

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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 an increased luteal $PGF_{2\alpha}$-secretion or an 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), respectively. Bovine ovaries with a mid-cycle CL were collected after slaughter and perfused for 240min. After 50min of equilibration, flunixin and polymyxin B (5µg/mL of each) were added to the perfusion medium of six ovaries respectively. All ovaries were treated with *E.coli* LPS (0.5µg/mL) 60min after the onset of perfusion, and received 500I.U. of human chorionic gonadotropin (hCG) after 120min of perfusion. Progesterone and $PGF_{2\alpha}$ were measured in the effluent perfusate every 10 and 30min, respectively. Biopsies of the CL were collected every 60min 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-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 the initial values before LPS-challenge, luteal mRNA expression of *TNFA* and *CASP3* was increased ($P<0.05$) in group F-LPS at 120 and 180min, respectively, and those of *CASP8* was decreased ($P<0.05$) in group PX-LPS at 60 and 120min after LPS-treatment. In conclusion, complete inhibition of any impact of $PGF_{2\alpha}$ by flunixin did not prevent LPS-induced apoptosis. However, polymyxin resulted in luteal responsiveness to hCG after LPS-challenge.

Zusammenfassung

Lipopolysaccharid (LPS) hemmt die Funktion des Corpus luteum in isoliert perfundierten Ovarien. Es ist unklar, ob diese Hemmung auf einer erhöhten lutealen $PGF_{2\alpha}$-Sekretion oder LPS-induzierten Apoptose beruht. Daher wurden $PGF_{2\alpha}$ und LPS durch ein NSAID (Flunixin) bzw. einen Endotoxinfänger (Polymyxin) inhibiert. Bovine Ovarien mit Blütegelbkörper wurden am Schlachthof entnommen und während 240min perfundiert. Nach 50min Äquilibrierung wurden Flunixin bzw. Polymyxin B (je 5µg/ml) zum Perfusionsmedium von je sechs Ovarien hinzugefügt. Alle Ovarien wurden 60min nach Perfusionsbeginn mit *E.coli* LPS (0,5µg/ml) behandelt und erhielten nach 120min Perfusion 500I.E. hCG. Progesteron (P_4) und $PGF_{2\alpha}$ wurden alle 10 bzw. 30min im abfliessenden Perfusat gemessen. Gelbkörperbioptate zur Bestimmung der mRNA Expression des Zytokins *TNFA* und der Apoptosemarker (*CASP3, -8*) wurden alle 60min entnommen. Die Flunixin-Behandlung hemmte den Anstieg von $PGF_{2\alpha}$ nach LPS-Stimulation, der bei mit Polymyxin behandelten Ovarien (PX-LPS) beobachtet wurde. Nach hCG-Stimulation stieg die P_4-Sekretion in Gruppe PX-LPS an, aber nicht bei den mit Flunixin behandelten Ovarien (F-LPS). Die luteale mRNA Expression von *TNFA* und *CASP3* nahm in Gruppe F-LPS zu, während die Expression von *CASP8* in Gruppe PX-LPS abnahm. Zusammengefasst verhindert eine Inhibition von $PGF_{2\alpha}$ durch Flunixin die LPS-induzierte Apoptose nicht, während Polymyxin die luteale Ansprechbarkeit gegenüber hCG nach LPS-Stimulation aufrechterhält.

Inhibition of lipopolysaccharide-induced luteolysis in isolated perfused bovine ovaries

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Short title: Inhibition of LPS-effects on bovine CL *in vitro*

1. Introduction

Inflammatory diseases, such as endometritis and mastitis, play a pivotal role among diseases in dairy cows and reduce their reproductive performance (Barker *et al.* 1998, LeBlanc *et al.* 2002). 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 (Suzuki *et al.* 2001, Lavon *et al.* 2008, Williams *et al.* 2008, Herzog *et al.* 2012, Lüttgenau *et al.* 2016a, Lüttgenau *et al.* 2016b). During inflammation, LPS induces the production of prostaglandins (PGs) by macrophages, monocytes and endothelial cells (Andreasen *et al.* 2008). Furthermore, LPS activates the nuclear factor kappa B (NF-κB), which leads to the expression of many proinflammatory cytokines (Tsuzuki *et al.* 2001). These cytokines are released from the activated macrophages and stimulate in turn the neutrophils to produce reactive oxygen species (Er *et al.* 2009). 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 (Petroff *et al.* 2001).

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 (Herzog *et al.* 2012). Since $PGF_{2\alpha}$ metabolite concentrations were also increased after LPS treatment (Herzog *et al.* 2012), it was speculated that an enhanced uterine release of $PGF_{2\alpha}$ induced premature luteolysis. However, LPS induced apoptosis in luteal monolayer cultures (Mishra & Dhali 2007), although an influence of endometrium-derived $PGF_{2\alpha}$ was excluded. Therefore, a recent study (Lüttgenau *et al.* 2016c) 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. 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 luteolytic effects, 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 (Vane 1971, Cheng *et al.* 1998). Furthermore, flunixin has antioxidative properties (Konyalioglu *et al.* 2007), and inhibits the activation of NF-κB (Bryant *et al.* 2003) and the increase in cytokine levels (Yazar *et al.* 2007, Chalmeh *et al.* 2013). In LPS-treated mice, for instance, flunixin inhibited the increase in TNFA, interleukin 1β, and interleukin 10 (Yazar *et al.* 2007). However, flunixin does not have the ability to bind directly the LPS molecule (Parviainen *et al.* 2001). The use of flunixin for the treatment of endotoxemia relies on its modulatory function on acute hemodynamic changes (Chalmeh *et al.* 2013).

Polymyxin B is an antibiotic with endotoxin-binding properties due to its high affinity to the lipid A of LPS (Morrison & Jacobs 1976, Cavaillon & Haeffner-Cavaillon 1986, Bucklin *et al.* 1995). In human medicine, polymyxin B was successfully used to reduce blood endotoxin levels in patients with sepsis (Uriu *et al.* 2002, Cruz *et al.* 2007, Cruz *et al.* 2009). In this respect, 1 mg of *E. coli* LPS is bound by 55 µg of polymyxin B (Morrison & Jacobs 1976). However, polymyxin B not only binds LPS but also inhibits the binding activity of NF-κB, and therefore inhibits the new expression of TNFA (Tsuzuki *et al.* 2001).

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 luteolysis due to endotoxemia is mediated predominantly by luteal $PGF_{2\alpha}$ or by LPS-induced apoptosis.

2. Materials and Methods

2.1. Ovaries

Ovaries with *mesovarium* were harvested from the carcasses of clinically healthy cows (*Bos taurus*; including Brown Swiss, Holstein Friesian, Red Holstein and Swiss Fleckvieh) that were slaughtered at a commercial abattoir. Twelve ovaries containing a CL with an estimated diameter of >20 mm (subsequently confirmed as a mid-cycle CL) and intact *tunica albuginea* as well as *mesovarium* with ovarian vessels were used.

2.2. Preparation of ovaries

Immediately after the ovary was recovered, the *ramus uterinus* and all branches of the ovarian artery with a similar diameter were ligated (Polysorb 0®; Corvidien, Dublin, Ireland), and a 16-gauge venous catheter (Vygonüle T; Vygon, Écouen, France) was fixed within the ovarian artery with three circular ligatures (Polysorb 0®). The preparation and catheterization of the ovarian artery, the flushing of the ovary, and its transport to the laboratory was recently described in detail (Lüttgenau *et al.* 2016c).

In the laboratory, the ovary with vascular pedicle was weighed using a precision balance (Mettler PM 400®; Mettler-Toledo, Greifensee, Switzerland), and the average diameter of the CL was determined with ultrasonography (Aquila Esaote Pie Medical; Esaote Biomedica, Cologne, Germany). Only ovaries with a luteal diameter >20 mm were used.

2.3. Isolated perfusion of ovaries

Ovaries were fixed on the bipod as described recently (Lüttgenau *et al.* 2016c). An incubator for newborn humans (Atom Infant Incubator V-850; Atom Medical Corporation, Tokyo, Japan) and an advanced (compared to Lüttgenau *et al.* 2016c) perfusion system were used to ensure a controlled perfusion under standardized microclimatic conditions (temperature, 37.5 to 38.5 °C; relative humidity, 75 to 85 %). A schematic illustration of the complete set-up is shown (Fig. 1).

As medium for the isolated perfusion of the ovary, a modified Tyrode's solution was used (for detailed composition see Lüttgenau *et al.* 2016c). Tyrode's solution was oxygenated with carbogen (Oxycarbon medizinal; Carbagas, Domdidier, Switzerland) using a membrane oxygenator (Radnoti Membrane Oxygenating Chamber; Radnoti Limited, Dublin, Ireland). To ensure adequate oxygenation, the pH and partial pressures of oxygen and carbon dioxide in the perfusion medium were measured before and after its passage through the ovary. Measurements were performed at the start of perfusion and then every 60 min until the end of perfusion using a blood-gas analysis system (Rapidlab 248 TM; Siemens, Munich, Germany). According to the results of blood-gas analysis, the supply of carbogen and the flow rate were adjusted to sustain a physiologic pH (target value, 7.40). The perfusion medium was heated in water jacketed reservoirs (Water-Jacketed Reservoir 3 L and 5 L; Radnoti Limited) by a heated circulating bath (Immersion Thermostats, Baths and Circulators, Optima T100; Grant Instruments Ltd, Cambridgeshire, UK). Water jacketed glasswares 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. Every 10 min, the temperature of the perfusion medium was measured directly before entering the ovarian artery, by inserting a thermometer in the perfusion system. According to these measurements, temperature of the water circulation was adjusted to maintain the medium's temperature between 37 and 38 °C.

For perfusion, a volume- and pressure-controlled peristaltic pump (Minipuls 3 Peristaltic Pump; ADInstruments, Oxford, UK) was used. The peristaltic 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) was installed. The flow was manually calibrated with a measuring cylinder and a chronograph prior to the start of each experiment, and the data was inserted in the LabChart software that allowed to continuously measure and possibly modify the flow of the perfusion medium. The pressure was measured with the transducer that was inserted in the perfusion system close to accessing the ovarian artery. The pressure transducer 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. The pressure was continuously measured throughout the experiment. The flow was adjusted to achieve a value of approximately 2 mL/min per gram ovarian tissue, as proposed (Stähler & Huch 1971). To avoid artifacts caused by accumulation of metabolites or hormones, the perfusion medium was not recycled.

2.4. Study design

All ovaries were perfused for 240 min. During the first 50 min (equilibration), no agents were added to the perfusion medium. Ovaries were randomly allocated to two different 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, 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. Flunixin, polymyxin B, and LPS treatments were continued until the end of the perfusion time. For all ovaries, human chorionic gonadotropin (hCG, 500 I.U.; Chorulon 1500®; MSD Animal Health GmbH, Luzern, Switzerland) was added to the perfusion medium at 210 min after the start of perfusion.

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

Effluent perfusion medium was sampled every 10 min throughout the entire perfusion period. At each time of sampling, three native aliquots were collected and stored at -20 °C.

To ensure that the ovary remained in a physiological state, lactate concentration and activity of creatine kinase (CK) were used as markers of hypoxia and cell death (Richter *et al.* 2000; Lüttgenau *et al.* 2016c). For these measurements, which were performed every 30 min during the perfusion period, the Cobas Mira Plus analyzer (Roche, Basel, Switzerland) was used as described recently (Lüttgenau *et al.* 2016c).

Concentrations of P_4 were measured in the effluent perfusion medium every 10 min throughout the perfusion period, using a radioimmunoassay (Kit RIA immunotech IM1188 lot 150817C; Beckman Coulter GmbH, Krefeld, Germany). The range of standard concentrations for this 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.

Starting at 60 min of perfusion, concentrations of PGE_2 and $PGF_{2\alpha}$ were measured every 30 min in the effluent perfusion medium. Therefore, high-sensitivity ELISA kits (Enzo Life Sciences AG, Lausen, Switzerland) were used. For PGE_2 and $PGF_{2\alpha}$ tests, the range of standard concentrations was 7.81 to 1000 pg/mL and 1.95 to 2000 pg/mL, respectively. Intra-

and inter-assay coefficients of variation were ≤9.8 and ≤12.6 % for PGE_2 and ≤7.2 and ≤11.0 % for $PGF_{2\alpha}$, respectively, and ED50 for PGE_2 and $PGF_{2\alpha}$ occurred at 127.8 pg/mL and 81 pg/mL, respectively.

2.6. Corpus luteum biopsy and expression analysis

One biopsy sample (approximately 15 x 1 x 1 mm) was obtained from the maximum diameter (including cells from the periphery and the center) of the CL after 60, 120, 180, and 240 min of perfusion. A detailed description of the collection and storing of luteal samples was provided recently (Lüttgenau *et al.* 2016c).

Luteal mRNA expression was determined for the proinflammatory cytokine *TNFA*, the apoptotic enzymes caspase (*CASP*) 3 and -8, and the PGE- (*PGES/PTGES*) and PGF (*PGFS/AKR1B1*) synthases.

Total RNA from luteal tissue samples was extracted using the miRNeasy Mini Kit (Qiagen). 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 (Pistek *et al.* 2013).

Luteal mRNA expression was determined in a two-step quantitative real-time PCR (qPCR) 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 qPCR was performed in a reaction volume of 10 µL, consisting of 5 µL Kapa SYBR Fast Universal

qPCR Kit, 0.4 µL of each primer (10 µM), 0.07 µL VisiBlue (TATAA Biocenter, Göteborg, Sweden), 3.13 µL water, and 1 µL cDNA. The primers used to amplify specific fragments referring to selected regulated genes 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 against the geometrical mean of the five reference genes *YWHAZ*, *H3F3A*, *CNOT11*, *SUZ12* and *TBP* (ΔC_q). 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 (Livak and Schmittgen, 2001).

2.7. Postprocessing of ovaries

After the 240-min study period, ovaries with *mesovarium* were weighed again to estimate perfusion-induced edema. Subsequently, 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 and homogenous staining of the CL. In the experiments, less than one-quarter of perfusion medium leaked from the ovarian artery before reaching the ovary and the CL was homogenously stained; therefore, all ovaries were included in the study. Between each perfusion of an ovary, all glassware and tubing that were in contact with the perfusion medium were cleaned and steam sterilized.

2.8. Statistical analyses

The median (± mean absolute deviation, 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 and did not differ ($P > 0.05$) between the groups. Based on their dimensions, CL were designated as mid-cycle (Days 8-16; Day 1 = ovulation) according to Herzog *et al.* (2010).

The perfusion pressure was measured in 11 out of 12 ovaries; in one ovary from group F-LPS measurement was not possible due to technical problems. The mean (± SEM) pressure was 121.6 ± 3.6 mmHg and there was no treatment or time effect ($P > 0.05$). 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.

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, 0.03 mmol/L, 4 U/L, 71 pg/mL, and 1.9 pg/mL were used as arbitrary values for lactate, CK, PGE_2, and $PGF_{2\alpha}$, respectively, to facilitate statistical analysis. Additionally, for PGE_2 measurements above the higher detection limit of 10,000 pg/mL, the arbitrary value of 10,001 pg/mL was used.

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*time interaction. Significant results were further evaluated 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, 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 ± MAD, depending on the distribution of the data, and differences were considered significant at $P \leq 0.05$.

3. Results

Ovarian lactate production and CK activity did not show a treatment ($P > 0.05$) but a time effect ($P < 0.0001$ and $P = 0.0012$, 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.

Regarding PGE_2 and $PGF_{2\alpha}$ concentrations in the effluent perfusate, there was a treatment ($P = 0.0004$ and $P = 0.0002$, respectively) and a time effect ($P < 0.0001$ and $P = 0.0001$, respectively). Concentrations of PGE_2 and $PGF_{2\alpha}$ were higher in group PX-LPS compared to group F-LPS (Fig. 2A and B). Over time, PGE_2 and $PGF_{2\alpha}$ levels increased in group PX-LPS ($P = 0.03$; Fig. 2A and B), whereas levels did not differ in group F-LPS ($P > 0.05$; Fig. 2A and B).

Progesterone concentrations in the effluent perfusate did not differ neither between the treatment groups nor over time (both $P > 0.05$). On a per-day basis, P_4 concentrations in group F-LPS started to decrease during LPS-challenge and reached lower levels ($P < 0.04$) at the time of hCG stimulation compared with the start of the treatment period. In group PX-LPS, an increase ($P < 0.05$) in P_4 levels was observed at 20 and 30 min after hCG stimulation (Fig. 3).

Luteal mRNA abundance of *TNFA* did not show a treatment effect ($P > 0.05$). However, an increase ($P = 0.04$) in *TNFA* mRNA was observed between 0 and 120 min after LPS-challenge in group F-LPS (Fig. 4A), whereas no difference ($P > 0.05$) over time was found in group PX-LPS.

Luteal mRNA expression of *CASP3* did not show a treatment ($P > 0.05$) but a time effect ($P = 0.08$). Increased *CASP3* mRNA ($P < 0.03$) was observed in group F-LPS at 180 min (compared with 0 min) after the start of treatment (Fig. 4B), whereas the expression of *CASP3* did not differ ($P > 0.05$) over time in group PX-LPS.

The mRNA expression of *CASP8* did not differ ($P > 0.05$) between the groups. Over time, *CASP8* mRNA remained stable ($P > 0.05$) in group F-LPS, whereas a decreased expression ($P < 0.05$) at 60 and 120 min after LPS-challenge compared to the start of the treatment period was observed in group PX-LPS (Fig. 4B).

Luteal mRNA expression of *PGES* and *PGFS* did not show a treatment effect ($P > 0.05$). Furthermore, the mRNA abundance of *PGES* and *PGFS* after LPS-challenge did not differ ($P > 0.05$) at any time from that before LPS treatment (Fig. 4C).

4. 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 (Coskun *et al.* 2009). Furthermore, high lactate concentration and CK activity at the start of perfusion, indicating hypoxia and cell death (Ahrén *et al.* 1972), 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 (Richter *et al.* 2000) and bovine ovaries (Lüttgenau *et al.* 2016c). Additionally, the advanced perfusion system in the present study, using an infant incubator, water jacketed glassware and tubing, and specific controlling hard- and software, allowed to maintain highly standardized conditions for the ovaries. Thus, in our opinion, the results obtained during the treatment period of the present study are reliable.

The synthesis of PGE_2 and $PGF_{2\alpha}$ was inhibited in group F-LPS due to the treatment with the cyclooxygenase inhibitor flunixin. In contrast, there was a significant increase in PGE_2 and $PGF_{2\alpha}$ production in group PX-LPS. The CL is rich in arachidonic acids, the precursor of PGs, and luteal PGs contribute to the regulation of the CL (Arosh *et al.* 2004). Since PGE_2 is a luteotropic factor (Arosh *et al.* 2004), the inhibition of its synthesis can be judged as a detrimental effect of flunixin on the CL. In a recent study (Lüttgenau *et al.* 2016c), the impact of luteal $PGF_{2\alpha}$ on the LPS-induced apoptosis of the CL could neither be proved nor excluded. In the present study, the complete inhibition of any impact of $PGF_{2\alpha}$ in group F-LPS did not prevent LPS-induced apoptosis. Consequently, luteal $PGF_{2\alpha}$ does not seem to be responsible for luteolysis after LPS-challenge. However, it is noteworthy that $PGF_{2\alpha}$ of different origin, namely luteal and endometrial, is supposed to play a differing role in bovine luteal function (Kobayashi *et al.* 2001, Skarzynski *et al.* 2001, Shirasuna *et al.*

2004). Therefore, conclusions regarding the impact of luteal $PGF_{2\alpha}$ on the CL cannot necessarily be adapted to the effect of endometrial $PGF_{2\alpha}$.

From the start of the treatment period until hCG-challenge, P_4 concentrations in the effluent perfusate remained statistically unchanged in group PX-LPS but decreased in group F-LPS. Furthermore, after the application of hCG, P_4 levels increased significantly in group PX-LPS but not in group F-LPS. In a recent study (Lüttgenau *et al.* 2016c), 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 not able to block the LPS-induced suppression of luteal responsiveness to hCG. Several in vivo studies (Aké-López *et al.* 2005, Guzeloglu *et al.* 2007, Geary *et al.* 2010, von Krueger & Heuwieser 2010) 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 (Aké-López *et al.* 2005, Guzeloglu *et al.* 2007), whereas other studies did not find any effect on early embryonic loss or pregnancy rates (Geary *et al.* 2010, von Krueger & Heuwieser 2010). However, detrimental effects of flunixin itself on the CL have not been described yet. Therefore, we assume that the suppressed hCG responsiveness of the CL was caused by the detrimental effects of LPS that were not inhibited by the flunixin treatment.

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. Since TNFA is known to have cytotoxic effects on luteal cells (Petroff *et al.* 2001), an inhibition of LPS-induced apoptosis of the CL by flunixin is not likely. Consistently, flunixin treatment did not inhibit

the LPS-induced suppression of hCG responsiveness of the CL. 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.

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 the increasing evidence that polymyxin B treatment is more suitable than flunixin treatment to inhibit the LPS-induced apoptosis of the bovine CL.

Although flunixin inhibited the synthesis of PGE_2 and $PGF_{2\alpha}$, the luteal mRNA expression of *PGES* and *PGFS* was not reduced. It is known that luteal *PGES* shows an irregular pattern during the different phases of the luteal phase, whereas *PGFS* is expressed at a constant level throughout the CL lifespan (Arosh *et al.* 2004). However, the synthesis of PGE_2 and $PGF_{2\alpha}$ is not closely related to the expression of *PGES* and *PGFS*, respectively.

In conclusion, polymyxin B but not flunixin inhibits the LPS-induced suppression of luteal hCG responsiveness, although flunixin successfully inhibits luteal PG secretion. Therefore, the detrimental effects of LPS on the bovine CL in the isolated perfused ovary were most likely mediated by LPS-induced release of proinflammatory cytokines and apoptosis and not by luteolysis due to secretion of luteal $PGF_{2\alpha}$.

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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Figure legends

Fig. 1. Schematic view of the equipment used for isolated perfusion of bovine ovaries.

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 the treatment period of perfusion.

→ Treatment with 500 I.U. hCG.

[a] Difference ($P \leq 0.05$) compared to 0 min after the start of LPS-challenge in the group indicated.

* 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 the treatment period of perfusion.

→ Treatment with 500 I.U. hCG.

[a,b] Difference ($P \leq 0.05$) compared to ([a]) 0 min after the start of LPS-challenge ([b]) 150 min after the start of LPS-challenge (i.e. the time of hCG stimulation) in the group indicated.

Fig. 4. 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, black column; n=6) and ovaries treated with polymyxin B and LPS (PX-LPS, grey column; n=6) during the treatment period of perfusion.

[a] Difference ($P \leq 0.05$) compared to 0 min after the start of LPS-challenge in the group indicated.

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

Figure 1

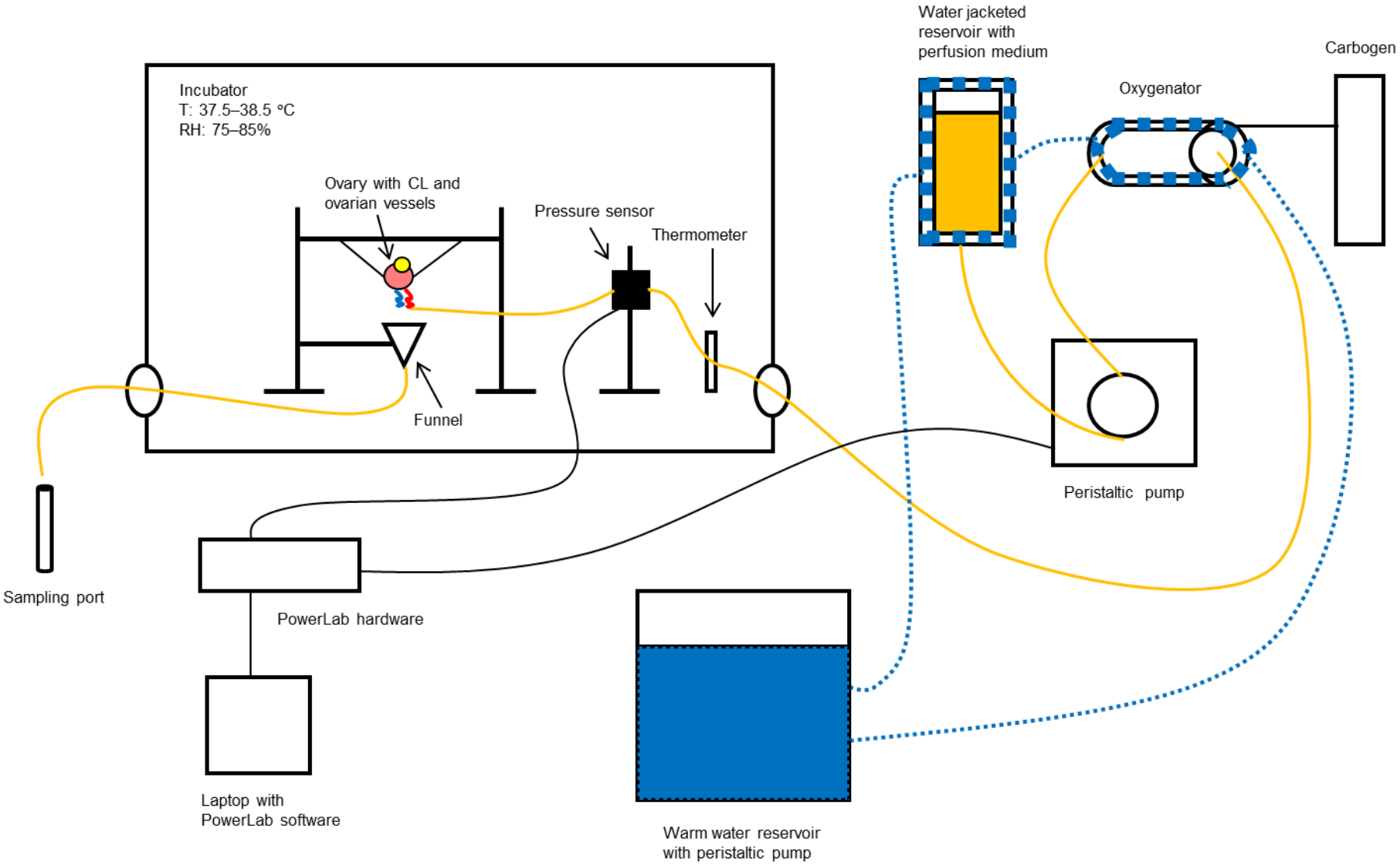

Water jacketed
reservoir with
perfusion medium
Carbogen
Oxygenator
Incubator
T: 37.5–38.5 °C
RH: 75–85%
Ovary with CL and
ovarian vessels
Pressure sensor
Thermometer
Funnel
Peristaltic pump
Sampling port
PowerLab hardware
Laptop with
PowerLab software
Warm water reservoir
with peristaltic pump

Figure 2

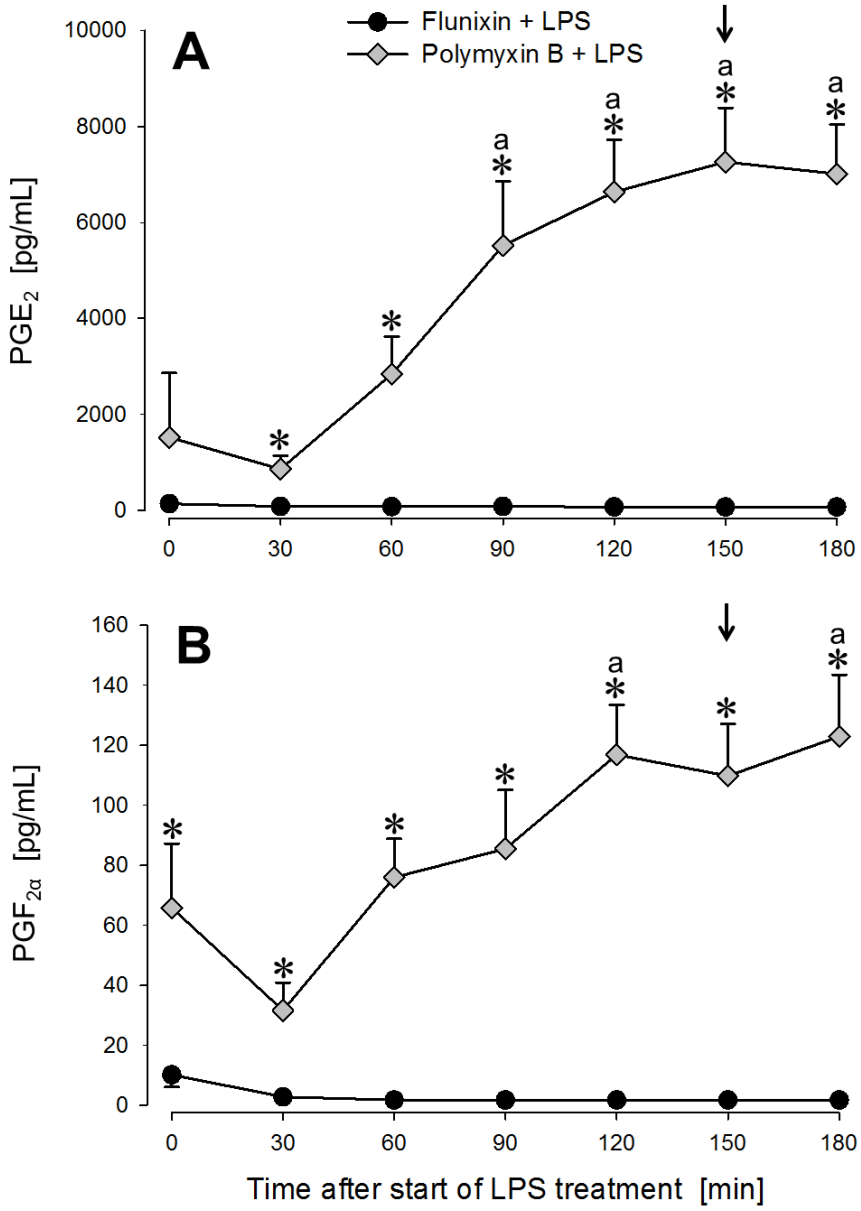

A
Flunixin + LPS
Polymyxin B + LPS
PGE2 [pg/mL]
10000
8000
6000
4000
2000
0
B
PGF2α [pg/mL]
160
140
120
100
80
60
40
20
0
0
30
60
90
120
150
180
Time after start of LPS treatment [min]

Figure 3

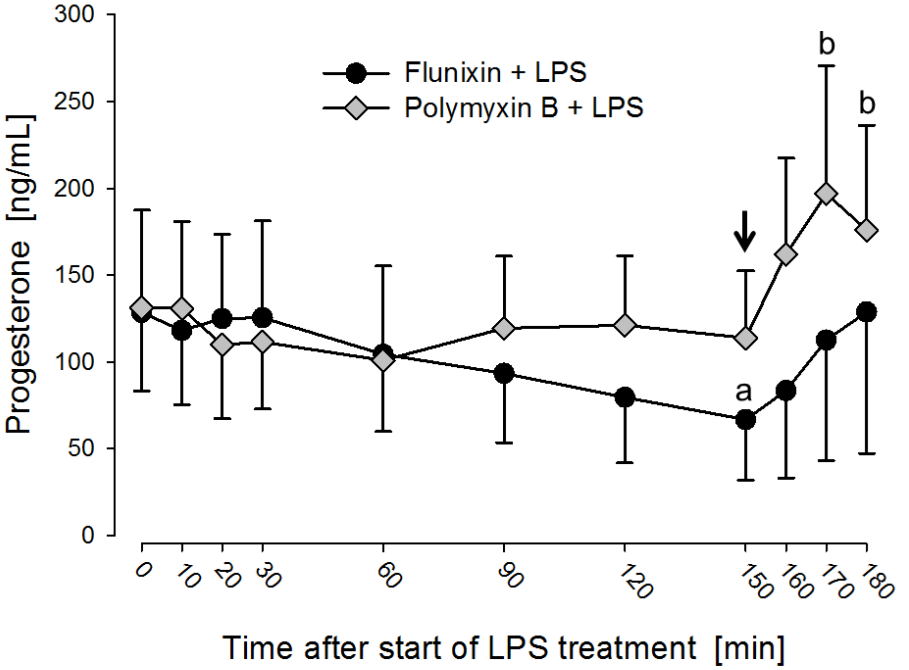
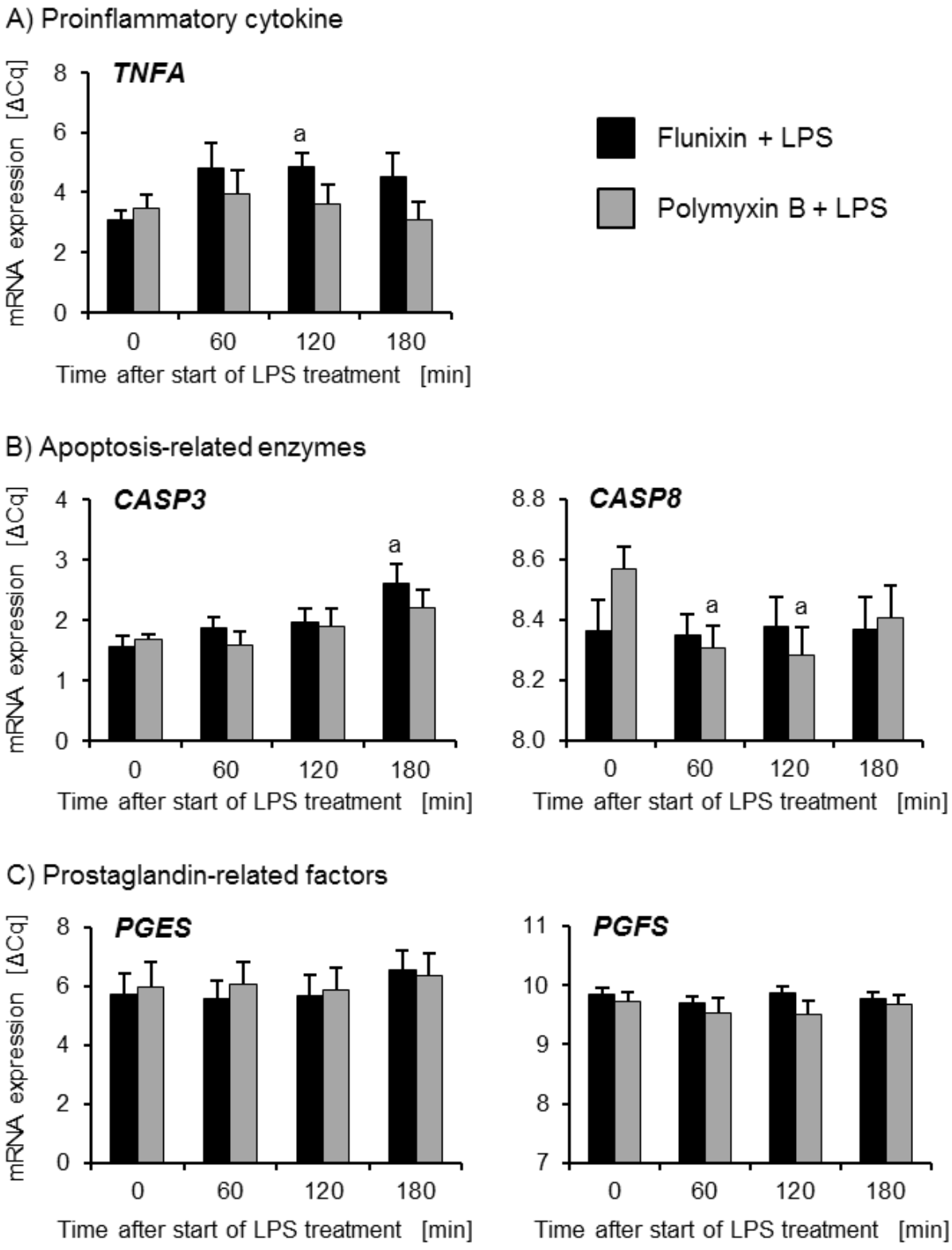

Flunixin + LPS
Polymyxin B + LPS
Progesterone [ng/mL]
300
250
200
150
100
50
0
b
b
a
0
10
20
30
60
90
120
150
160
170
180
Time after start of LPS treatment [min]

Figure 4


A) Proinflammatory cytokine
TNFA
mRNA expression [ΔCq]
8
6
4
2
0
a
0
60
120
180
Time after start of LPS treatment [min]
Flunixin + LPS
Polymyxin B + LPS
B) Apoptosis-related enzymes
CASP3
mRNA expression [ΔCq]
4
3
2
1
0
a
0
60
120
180
Time after start of LPS treatment [min]
CASP8
8.8
8.6
8.4
8.2
8.0
a
a
0
60
120
180
Time after start of LPS treatment [min]
C) Prostaglandin-related factors
PGES
mRNA expression [ΔCq]
8
6
4
2
0
0
60
120
180
Time after start of LPS treatment [min]
PGFS
11
10
9
8
7
0
60
120
180
Time after start of LPS treatment [min]

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