Original Article

Effects of different cyclooxygenase inhibitors on prostaglandin E_2 production, steroidogenesis and ovulation of bovine preovulatory follicles

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Abstract. Ovulation is an inflammation-like process, and cyclooxygenase-2 (COX-2)-dependent production of prostaglandin E_2 (PGE_2) is its key mediator. Balanced regulation of inflammatory processes in high-yielding dairy cows may be essential for physiological ovulation and fertility. This study aimed to elucidate the mechanisms underlying ovulation failure and cyst development after disturbing intrafollicular inflammatory cascades. Therefore, nonselective (indomethacin and flunixin-meglumine), COX-2 selective (meloxicam), and highly COX-2 selective (NS-398) inhibitors were injected into preovulatory follicles 16 h after administration of GnRH, and ovulation was monitored via ultrasound examination. Additionally, follicular fluid was collected after injection of indomethacin, meloxicam, and NS-398. Moreover, primary granulosa cell cultures from preovulatory follicles were prepared and treated with indomethacin, meloxicam, and NS-398. The concentrations of 17β-estradiol, progesterone, and prostaglandin E_2 (PGE_2) in the follicular fluid and cell supernatant were estimated. Indomethacin and flunixin-meglumine blocked ovulation, even at low doses, and led to ovarian cyst development. The selective and highly selective COX-2 inhibitors meloxicam and NS-398 were not effective in blocking ovulation. However, indomethacin, meloxicam, and NS-398 significantly and comparably reduced PGE_2 concentration *in vivo* and *in vitro* ($P < 0.05$) but had no effect on estradiol or progesterone production. This may contradict the generally accepted hypothesis that PGE_2 is a key mediator of ovulation and progesterone production. Our results suggest a connection between ovarian disorders and inflammatory actions in early postpartum cows.
Key words: Cyclooxygenase (COX), NSAID, Ovarian cyst, Ovulation, Steroids

The postpartum period of high-yielding dairy cows is characterized by a high metabolic rate and negative energy balance. Elevated metabolism increases oxidative stress and is accompanied by catabolism, which creates a state comparable to chronic inflammation [1]. In response, anti-inflammatory signals will also increase [2, 3]. During this period, fertility is reduced for various reasons, including ovulation failure and ovarian cyst development [4].

Ovulation is also comparable to an inflammatory process [5, 6]. The preovulatory LH peak induces the expression of cyclooxygenase 2 (COX-2) via LH receptors, whereas the expression of COX-1 remains basal [7–9]. The upregulation of COX-2 is the rate-limiting step in prostaglandin synthesis in preovulatory follicles [10]. This upregulation leads to increased levels of different pro-inflammatory prostaglandins in the preovulatory follicle. In cattle, intrafollicular prostaglandin synthesis increases approximately 18 h after HCG administration [8], followed by ovulation ten hours later [9].

Prostaglandins play an important role in ovulation. In particular, prostaglandin E_2 (PGE_2) and prostaglandin $F_{2α}$ ($PGF_{2α}$) concentrations increase massively prior to ovulation [8, 11]. Although the concentration of several prostaglandins increases in parallel during ovulation, PGE_2 is considered the key mediator [8, 11–13]. PGE_2 orchestrates ovulation processes, including cumulus expansion, oocyte release,

follicle rupture, and angiogenesis, via its four receptor types, which are all present in the ovary [12, 14, 15]. Several studies in different species have demonstrated that blocking the COX-2 pathway in preovulatory follicles or knocking out the *Ptgs2* gene in mice reduces intrafollicular prostaglandin synthesis and prevents ovulation [9, 16]. Moreover, PGE_2 and $PGF_{2α}$ are involved in the differentiation of granulosa cells into lutein cells, triggering progesterone production, and corpus luteum maintenance [13, 17–19]. Progesterone and PGE_2 increase simultaneously in preovulatory follicles, while the decrease in 17β-estradiol in preovulatory follicles is not correlated with PGE_2 levels [8, 18]. In addition, during ovulation, other inflammatory mechanisms are present, including the lipoxygenase (LOX) pathway and leukotrienes [20, 21], activation of proteolytic enzymes [22, 23] and angiogenic factors [24], and innate immune responses [25].

Therefore, balanced regulation of inflammatory and anti-inflammatory factors is essential for ovulation. The overlap of inflammatory-like processes during the postpartum period and ovulation likely enables crosstalk between signaling pathways, leading to ovulatory disorders.

Previous studies demonstrated relationships between biomarkers of oxidative stress (lipohydroperoxides, 8-iso-$PGF_{2α}$) and ovarian function in dairy cows and their direct interference with granulosa cell function [26, 27]. Recently, we demonstrated that intrafollicular administration of indomethacin, a nonselective COX-1 and -2 inhibitor, can prevent ovulation in a bovine model [28]. Moreover, the unruptured follicle further developed into a cystic ovarian follicle. These artificially induced cysts showed characteristics comparable to those of naturally occurring ovarian cysts and originated from a short and local disturbance of the inflammatory-like processes in the preovulatory follicle. However, the effects of different COX inhibitor classes on ovulation and cyst formation in cattle have not

been studied intensively.

This study aimed to investigate the effects of different COX inhibitors on prostaglandin production, steroidogenesis, and ovulation in bovine preovulatory follicles. The effects of nonselective COX-1 and -2 inhibitors and selective and highly selective COX-2 inhibitors on PGE_2, estradiol, and progesterone production were compared *in vivo* and *in vitro*.

Material and Methods

In vivo experiments

In vivo experiments included the generation of preovulatory follicles, intrafollicular injections, and subsequent ovulation monitoring or follicular fluid aspiration. Animal experiments were approved by the federal state of Mecklenburg Western-Pomerania, Germany (LALLF M-V TSD 7221.3-1-038/12; TSD/7221.3-1-010/16).

In total, 28 German Holstein heifers and 15 cows in their first lactation were used. The animals were housed in the experimental facility for cattle at the Research Institute for Farm Animal Biology (FBN) in Dummerstorf, Germany. Preovulatory follicle generation and intrafollicular injections were performed following a previously described protocol by Lapp *et al*. [28]. Briefly, for the generation of preovulatory follicles, cattle in diestrus received an injection of a $PGF_{2\alpha}$ analog (2 ml PGF Veyx® forte, 0.25 mg/ml Cloprostenol; Veyx-Pharma GmbH, Schwarzenborn, Germany) to induce luteolysis. If regression of the corpus luteum (decreased size and vascularization) and growth of a dominant follicle was observed by ultrasound examination 54 h after $PGF_{2\alpha}$ analog, intramuscular injection of a GnRH analog (2 ml Gonavet Veyx®, 50 µg/ml gonadorelin acetate [6-D-Phe]; Veyx-Pharma GmbH) was administered to induce ovulation. The preovulatory follicle was identified by growth exceeding one millimeter per day after prostaglandin administration (mean diameter 17.5 ± 2.9 mm at injection) and by clearly visible circular perfusion of the follicle wall upon ultrasonographic examination in Doppler mode.

Intrafollicular injections of different COX inhibitors were performed 16 h after GnRH administration. Follicle injections were performed transvaginally with ultrasound guidance. Prior to the injection procedure, epidural anesthesia with procainhydrochlorid (5 ml Procamidor 20 mg/ml; WDT, Garbsen, Germany) and sedation with xylazine (0.75 ml Xylariem, 23.3 mg/ml xylazinhydrochlorid; Ecuphar, Oostkamp, Belgium) were performed. Follicle injections were performed using a custom-made ovum pick-up device mounted

with a 6.5 MHz sector finger-tip-probe (EUP-F331; Hitachi Medical, Tokyo, Japan) and equipped with a changeable 25-gauge needle (Sterican; 0.5 × 40 mm; B. Braun, Melsungen, Germany). The volume of the injected substance was constant at 0.2 ml. The substances used, their concentrations, and the number of injections are presented in Table 1. Approximately 15 min after the injection procedure, follicle intactness was determined by ultrasound examination. The effects on ovulation were monitored by ultrasonographic examination beginning the day after the injection and lasting up to four days after injection. In general, the animals were used only once per treatment (Table. 1). However, in the case of treatment with 35 µM indomethacin (n = 4), 172 µM meloxicam (n = 5), NS-398 (n = 7), and NaCl (n = 6), one animal was used again.

To obtain follicular fluid (FF) for hormone analysis, further injected follicles and untreated control follicles were aspirated (n = 3 per group) using 21-gauge needles (Sterican; 0.8 × 40 mm; B. Braun). Aspiration of FF was performed five hours after follicle injection of COX inhibitor (21 h after GnRH administration) or for untreated control follicles 16 h and 21 h after GnRH administration. The animals were usually only used once per injection treatment followed by aspiration. However, in the case of treatment with indomethacin, one animal was used once again. The injected-aspirated follicles had a mean diameter of 15.8 ± 3.2. No significant blood admixtures or clots were observed in aspirates. FF was cooled immediately on ice, centrifuged at $500 \times g$ for 10 min at 4°C to remove blood or cell debris, and stored at –20°C until analysis. The results from follicle aspirations were already partly used in a technology report [28].

COX inhibitors and control solutions for follicle injections

Indomethacin is a nonselective inhibitor of COX-1 and -2 derived from indole acetic acid derivatives. Preovulatory follicles were injected with 0.2 ml of a solution of 70 µM, 35 µM, or 5 µM indomethacin. This solution was prepared by dissolving indomethacin (Indomethacin 99%, Merck KGaA, Darmstadt, Germany) in ethanol (ROTIPURAN® ≥ 99.8%, p.a., Ethyl alcohol; Carl Roth GmbH+Co. KG, Karlsruhe, Germany) and further diluting with physiological saline solution (Natriumchlorid; Carl Roth GmbH + Co. KG) to obtain the desired concentrations. The *in vivo* experiments with indomethacin were performed in a parallel subproject, and the data were published in a previous technology report [28]. Ten follicles injected with indomethacin in the subproject were used in this study to test the ability of indomethacin to inhibit ovulation (Table 1). Three additional

Table 1. COX inhibitors used for intrafollicular injections, their concentrations in the 0.2 ml injection volume, and their probability of inhibiting ovulation

Group	Substance	Injected concentrations	Injected/ovulated follicles	Inhibition of ovulation (%)
Nonselective COX inhibitors	Indomethacin	70 µM	4/0	100
	Indomethacin	35 µM	4/1	75
	Indomethacin	5 µM	2/2	0
	Flunixin	338 µM	7/0	100
	Flunixin	56 µM	3/3	0
Selective COX-2 inhibitor	Meloxicam	1725 µM	4/1	75
	Meloxicam	172 µM	5/5	0
	Meloxicam	57 µM	3/3	0
Highly selective COX-2 inhibitor	NS-398	60 µM	7/7	0
Control solutions	Ethanol	0.5%	3/3	0
	NaCl	0.9%	6/6	0
	DMSO	0.4%	3/3	0

follicles were injected with 0.2 ml of 35 μM indomethacin and aspirated five hours later to analyze follicular fluid (Fig. 2). The indomethacin experiments in the subproject [28] were temporarily carried out in parallel with the other COX inhibitor experiments using the same materials, methods, and study designs.

Meloxicam is a selective COX-2 inhibitor belonging to the group of oxicames. Meloxicam was injected into preovulatory follicles at three different concentrations (1725, 172, and 57 μM), which were prepared by diluting Melovem® (20 mg/ml meloxicam; Dopharma Research B.V., Raamsdonksveer, Netherlands) with physiological saline solution (B. Braun Vet Care, Melsungen, Germany) at a respective ratio of 1:33, 1:333, and 1:1000. Twelve preovulatory follicles were injected with 0.2 ml of meloxicam solution to test for ovulation inhibition (Table 1). Three additional follicles were injected with 0.2 ml of 172 μM meloxicam solution for follicle aspiration.

NS-398 is an N-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulfonamide and is a highly selective COX-2 inhibitor. A 79.6 mM stock solution with 25 mg NS-398 (NS-398 > 98%; Merck KGaA) per milliliter dimethylsulfoxide (DMSO > 99.7%; Merck KGaA) was prepared. The stock solution was diluted in two steps with physiological saline solution (Isotonische Natriumchlorid-Solution ad us. vet.; B. Braun Vet Care) to obtain a final concentration of 60 μM. Seven follicles were injected with 60 μM NS-398 to test for ovulation inhibition (Table 1) and three for subsequent follicle aspiration (Fig. 1).

Flunixin-meglumine is a nonselective inhibitor of COX-1 and -2 from the fenamic acid group. It was used at 338 and 56 μM. To prepare these solutions, Niglumine® (50 mg/ml flunixin, corresponding to 82.9 mg/ml flunixin-meglumine; Alfavet, Neumünster, Germany) was diluted 1:3000 and 1:1000, respectively, with physiological saline solution (Isotonische Natriumchlorid-Solution ad us. vet.; B. Braun Vet Care). Ten preovulatory follicles were injected with 0.2 ml of flunixin solution to test for ovulation inhibition (Table 1).

Follicle injections with 0.2 ml of physiological saline solution (Isotonische Natriumchlorid-solution ad us. vet.; B. Braun Vet Care; n = 6) were used as vehicle control for flunixin and meloxicam. For NS-398, control injections with 0.4% DMSO in physiological saline (n = 3) were performed. Additionally, injections of 0.2 ml of a 0.5% ethanol solution (ROTIPURAN®; ≥ 99.8% ethyl alcohol) diluted in physiological saline to three follicles served as vehicle controls for indomethacin injections (Table 1).

Culture of mural granulosa cells (MGCs)

Granulosa cells for cell culture were obtained from four different preovulatory follicles (n = 4) with a mean diameter of 18.5 ± 1.17 mm. Preovulatory follicles were produced and identified as described above. Follicles were obtained 16 h after GnRH administration following the slaughter of the animals in the institute's own abattoir. In one case, the follicle was obtained by transvaginal ovariectomy according to the method described by Reisinger and modified by Richter [29] and established in our group for cell experiments [27]. Ovaries were immediately shipped on ice to the laboratory.

MGCs were prepared and cultured as previously described [27]. After obtaining the ovary, the basal lamina from the preovulatory follicle was manually prepared, and the inner follicular wall was dissected from the ovary. MGCs were separated using a cell strainer (MAC smart strainers, 100 μm, Miltenyi Biotec, Bergisch Gladbach, Germany) and flushed with a 1% bovine serum albumin (BSA) solution (BSA ≥ 96%; Sigma Aldrich, Merck KGaA). After filtration, the cells were centrifuged at 300 × g for 10 min at 4°C. The cell sediment was resuspended in Aqua Dest. for erythrocyte lysis.

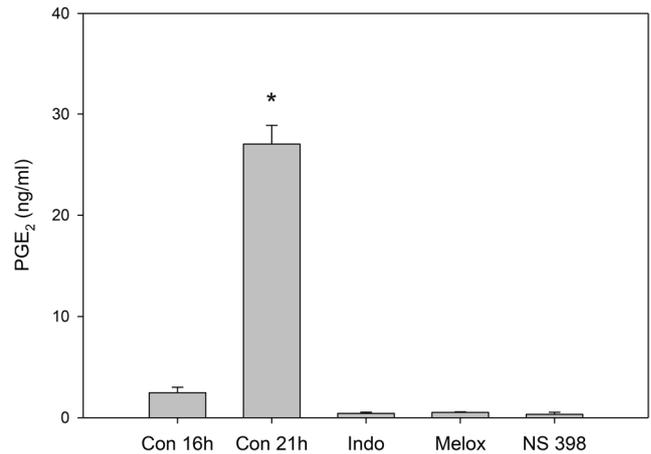**Fig. 1.** Concentrations of prostaglandin E_2 (PGE_2) in the follicular fluid of untreated preovulatory control follicles 16 and 21 h after administration of GnRH (Con 16 and Con 21, respectively) and PGE_2 concentrations in preovulatory follicles 21 h after GnRH administration, after injection of 0.2 ml of a 35 μM indomethacin (Indo), a 172 μM meloxicam (Melox), or a 60 μM NS 398 solution 5 h earlier (i. e. 16 h after GnRH administration). n = 3 in each group; * $P < 0.05$, Student-Newman–Keuls Method.

Thereafter, 2 × phosphate buffered saline (PBS), diluted from 10 × PBS (Sigma Aldrich), was added. Cells were centrifuged at 300 × g for 10 min at 4°C and resuspended in 500 ml of culture medium (DMEM/Ham's F-12 liquid medium without L-glutamine; Biochrom, Berlin, Germany) containing 10% penicillin/streptomycin (10.000 U/ml, 10.000 μg/ml; Biochrom) and 5% FBS superior standardized serum (Biochrom). Cells were counted via conduction using a Coulter counter and tested for viability by propidium iodide staining and flow cytometry quantification. Aliquots (100 μl) of cell suspension were plated in 96-well culture plates in 100 μl of medium. Thus, 140–220 × 10³ cells were seeded in each well. The results of the hormone analysis were corrected according to 10^6 cells (pg/ml/10^6 cells; ng/ml/10^6 cells). Cultivation was performed at 37°C in humidified chambers containing 5% CO_2.

Based on the *in vivo* experiment, the effects of the three different COX inhibitor classes on cultivated MGCs were tested. The nonselective COX-1 and -2 inhibitor indomethacin, selective COX-2 inhibitor meloxicam, and highly selective COX-2 inhibitor NS-398 were applied to the cell culture. Based on the *in vivo* experiments, indomethacin was used at final concentrations of 50 and 100 μM, meloxicam at 10 and 100 μM, and NS-398 at 10 and 50 μM. All treatments were performed in parallel in two wells per trial, with four biological replicates (cells from one preovulatory follicle per trail). The supernatant was collected after 4 or 24 h of cultivation and cell-free stored at –20°C.

Hormone analysis

Prostaglandin E_2 (PGE_2), 17-beta-estradiol (E2), and progesterone (P4) concentrations of the follicular fluids of aspirated follicles and follicles collected for cell culture and the supernatants of primary granulosa cell cultures were estimated.

PGE_2 concentrations were estimated using the 96-well PGE_2 High Sensitivity ELISA Kit (Enzo Life Sciences, Lörrach, Germany) according to the manufacturer's instructions. Samples and standards were tested in duplicates. The sensitivity for the assay was 13.4 pg/ml of PGE_2, and the intra- and inter-assay coefficients of variation
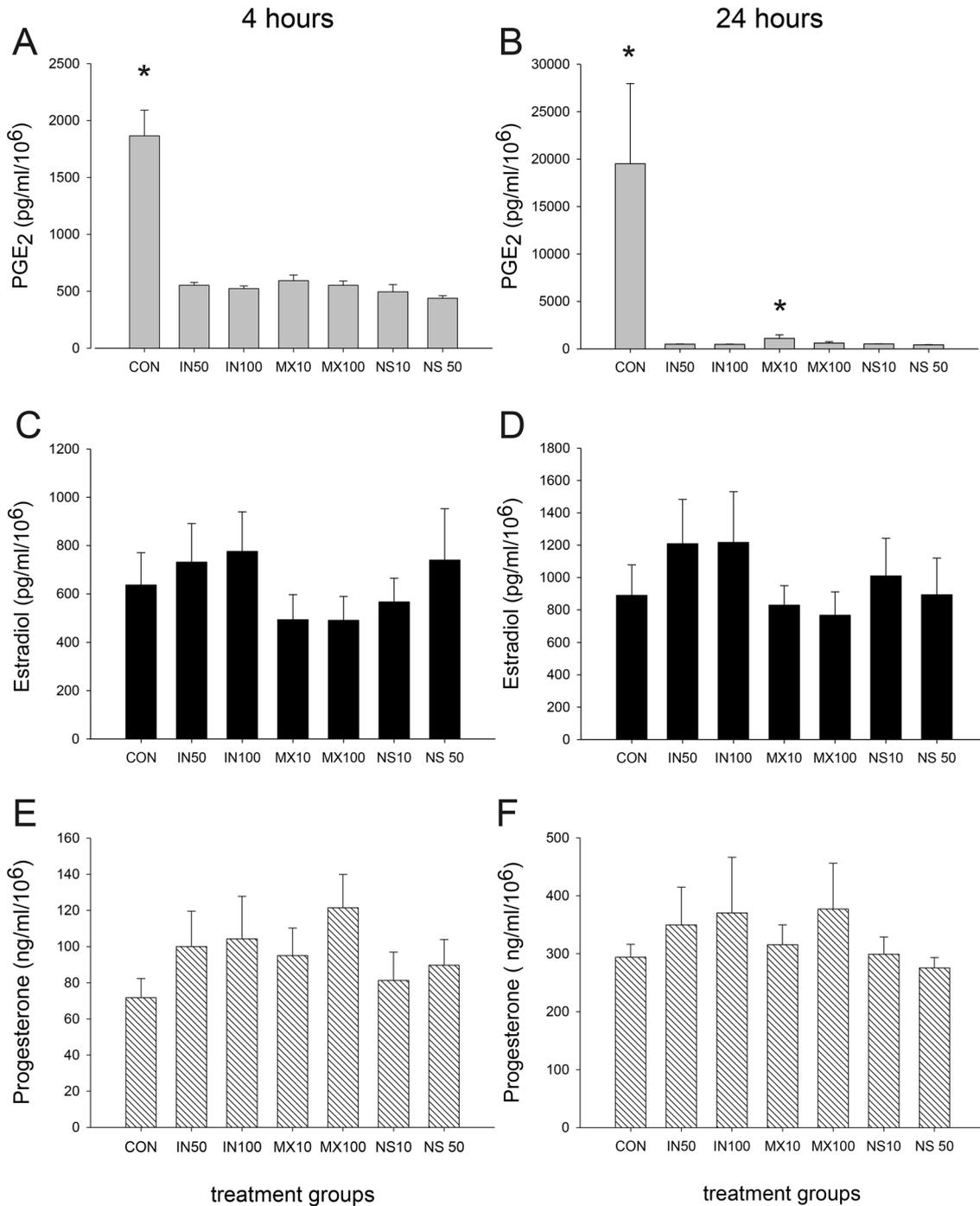
Fig. 2. Concentrations of prostaglandin E_2 (PGE$_2$), 17β-estradiol (E2), and progesterone (P4) in the supernatant of mural granulosa cell cultures after 4 and 24 h of cultivation. Granulosa cells for primary cell cultures were obtained from preovulatory follicles (16 h post-GnRH administration) and cultured with 50 or 100 μM indomethacin (IN50/IN100), 10 or 100 μM meloxicam (MX10/MX100), and 10 or 50 μM NS 398 (NS10/NS50). * $P < 0.05$, Student-Newman–Keuls Method; CON, control.

were 8.9 and 3%, respectively, according to the manufacturer's specifications.

Progesterone and 17β-estradiol concentrations in the cell supernatants were analyzed using a direct 3H-RIA in-house assay, as previously described [30]. Briefly, a 1,2,6,7-H(N) progesterone tracer (Hartmann Analytik, Braunschweig, Germany) was used for P4 quantification using a direct 3H-radioimmunoassay (RIA). Antibodies were obtained from rabbits immunized with the 11-OH-progesterone

conjugate. The intra- and inter-assay coefficients of variation were 7.6 and 9.8%, respectively. 17β-Estradiol levels in FF were measured using 3H-RIA after extraction with ethyl ether. The sensitivity of the assay was 3 pg/ml. The intra- and inter-assay coefficients of variation were 6.9 and 9.9%, respectively. 17β-Estradiol levels in the cell supernatant were estimated using an ELISA kit (No 501890: Cayman Chemical, Ann Arbor, MI, USA). According to the manufacturer, the assay's sensitivity was 20 pg/ml, and the intra- and inter-assay

coefficients of variation were 12.1 and 8.4%, respectively.

Statistical analyses

SigmaPlot Version 11.0 statistical software (Systat Software Inc., San Jose, CA, USA) was used for data processing. Data are reported as mean ± standard error. Hormone levels in the FF were first analyzed with one-way ANOVA. For progesterone concentrations, a subsequent pairwise multiple comparison procedure was performed using the Holm–Sidak method. As the test of normality failed for estrogen and PGE_2 in the FF, a Kruskal–Wallis test was performed. A subsequent pairwise multiple comparison test was performed using the Student-Newman–Keuls method. Hormone levels in the supernatants between treatments within one collection time point were analyzed using one-way ANOVA. In the case of PGE_2, the test of normality failed. Hence, the Kruskal–Wallis test and a pairwise multiple comparison test using the Student-Newman–Keuls method were performed. Differences between 4 and 24 h of cell culture within the treatment groups were analyzed using a paired t-test. If the normality test failed, a Wilcoxon signed-rank test was performed. The methods used are described in the text and legends.

Results

In the first part of the study, nonselective, selective, or highly selective targeting COX-2 inhibitors were injected at different doses in preovulatory follicles to investigate their ability to inhibit ovulation (Table 1). Injection of a standardized volume (0.2 ml) and dose (70 μM) of indomethacin in the follicle reliably blocked ovulation, and an injection of indomethacin at 35 μM inhibited ovulation in three of four cases (75%, Table 1). The unruptured follicles developed into ovarian cysts. Reliable prevention of ovulation has not been achieved with meloxicam injections in the present study. Only the highest concentration of meloxicam (1725 μM) led to ovulation failure in three of four cases. Injection of 60 μM NS-398 did not block ovulation in seven trials. Intrafollicular injections of control solutions, i.e., the different solubilizers of the COX inhibitors we used (ethanol for indomethacin, NaCl for flunixin and meloxicam, DMSO for NS-398), did not hamper ovulation (Table 1). Additional follicle injections with flunixin-meglumine were performed to prove the effectiveness of nonselective COX inhibitors. Injection of 338 μM flunixin-meglumine inhibited ovulation comparable to the inhibition achieved by 70 μM indomethacin. However, the lower tested concentration of flunixin solution (56 μM) did not affect ovulation. Unruptured follicles also developed into ovarian cysts.

To test the injection method and maintenance of the bioactivity of the COX inhibitors in the follicles, we injected three different COX inhibitors (nonselective, indomethacin; selective, meloxicam; highly selective, NS-398) into three preovulatory follicles 16 h after the administration of GnRH. For this purpose, the inhibitors were used at low concentrations (indomethacin, 35 μM; meloxicam, 172 μM; NS-398, 60 μM). At these concentrations, indomethacin blocked ovulation, while the other inhibitors did not. Five hours after injection (i.e. 21 h after GnRH), treated follicles and 16 and 21 h after GnRH administration, untreated follicles were aspirated. PGE_2 concentration in the untreated preovulatory follicles was significantly higher in the follicles aspirated 21 h after GnRH treatment than that in the follicles aspirated after 16 h (Fig. 1). Interestingly, all COX inhibitors at the tested concentrations comparably and significantly reduced the PGE_2 concentration in the follicular fluid to a basal level compared to that in the control follicles 21 h after GnRH (P < 0.05; Control 21 h: 28.5 ± 2.8; mean of all COX inhibitors: 0.4 ± 0.07

ng/ml PGE_2). Follicular fluids obtained 16 h or 21 h after GnRH administration (untreated control follicles) showed progesterone concentrations of 137 ± 34 and 176 ± 17 ng/ml, respectively. The progesterone concentration in follicles treated with indomethacin, meloxicam, and NS-398 were 82 ± 15, 125 ± 33, and 64 ± 26 ng/ml, respectively. Estradiol concentrations were 109 ± 8 and 95 ± 13 ng/ml in the FF of control follicles 16 and 21 h after GnRH treatment, respectively. The E2 concentration of follicles treated with indomethacin, meloxicam, and NS-398 was 42 ± 8, 105 ± 57, and 25 ± 11 ng/ml, respectively. The steroid concentrations are presented using descriptive statistics only. A reliable statistical evaluation appeared to be of little significance (low power) because high variations in steroid content were observed in the preovulatory follicles, and only a few follicles were investigated. Therefore, the effects of the different COX inhibitor classes on P4 and E2 production were tested in primary cell cultures from preovulatory follicles under standardized *in vitro* conditions. Figure 2 shows the effects of indomethacin, meloxicam, and NS-398 on PGE_2, estradiol, and progesterone production after cultivation for 4 or 24 h. PGE_2 concentration in the control was 1866 ± 224 pg/ml/10^6 cells after 4 h of cultivation (Fig. 2A). The PGE_2 level was tenfold higher (19516 ± 8446 pg/ml/10^6 cells) after 24 h of cultivation (Fig. 2B). Compared to the controls, all COX inhibitor-treated groups showed significantly lower PGE_2 concentrations, with an overall mean of 525 ± 18 pg/ml/10^6 cells and 605 ± 76 pg/ml/10^6 cells after 4 and 24 h of cultivation, respectively (all P < 0.05). No significant increase in PGE_2 concentration was observed between 4 and 24 h of culture. Treatment with 10 μM meloxicam resulted in slightly but significantly higher PGE_2 concentrations after 24 h of cultivation compared to the other treatment groups (1111 ± 363 pg/ml/10^6 cells; Fig. 2B). The control and treatment groups together exhibited average estradiol concentrations of 633 ± 52 ng/ml/10^6 cells in the supernatants after 4 h of cultivation (Fig. 2C), with a significant increase to 973 ± 81 ng/ml/10^6 cells after 24 h of cultivation (P < 0.001, Wilcoxon signed-rank test; Fig. 2D). However, no significant differences in estradiol production were detected among the groups after the two culture periods. The progesterone concentration in the control groups was approximately 72 ± 11 ng/ml/10^6 cells after 4 h of cultivation, whereas all treatment groups taken together showed an average of 99 ± 7 ng/ml/10^6 cells (Fig. 2E). After 24 h of incubation, the progesterone concentration increased significantly three- to fourfold up to 294 ± 22 ng/ml/10^6 cells in the control (P = 0.003) and up to 331 ± 23 ng/ml/10^6 cells in the treated groups (P < 0.001; Wilcoxon signed-rank test; Fig. 2F). No significant difference was observed in progesterone levels among the groups after 4 or 24 h of cultivation.

Discussion

To investigate the effects of different COX inhibitors on the ovulation of bovine preovulatory follicles, nonselective, selective, and highly selective COX-2 inhibitors were injected at different concentrations into preovulatory follicles. Indomethacin reliably blocked the ovulation of preovulatory follicles at low doses, leading to ovarian cyst development. Based on these results, a model for inducing artificial ovarian cysts in cattle has already been established [28]. Indomethacin reportedly blocks ovulation after administration directly into the ovarian tissue of cattle [31] or other species [9, 16]. Surprisingly, the selective COX-2 inhibitor meloxicam (0.2 ml of 172 μM solution) and the highly selective COX-2 inhibitor NS-398 (0.2 ml of 60 μM solution) could not block ovulation at the doses used. A very high dose (0.2 ml of 1725 μM meloxicam) did inhibit

ovulation and led to the development of ovarian cysts. This high dose of meloxicam solution resulted in a final concentration of 35 µg/ml in the follicular fluid of an average follicle with a diameter of 18 mm. This concentration is 70-fold higher than the therapeutically recommended dosage of 0.5 µg/g in cattle [32]. At these concentrations, meloxicam also partially inhibits COX-1 [33].

Selective COX-2 inhibitors have already been tested as contraceptives in women because of their potential for ovulation inhibition. Continuous administration of meloxicam in the late follicular phase, meloxicam reportedly led to delayed ovulation in women but inhibited ovulation in less than 50% of patients, and no cyst development was evident [34]. Administration of a highly selective COX-2 inhibitor (refecoxib) to women during the follicular phase also did not prevent ovulation [35]. The highly selective COX-2 inhibitor NS-398 did also not block ovulation in our study. This contradicts another report of inhibited ovulation after intrafollicular injection of NS-398 in cattle [36], where higher doses of NS-398 were used. We deliberately used minimal doses for COX inhibition to avoid any side effects and to explore the maintained bioactivity of COX at low doses.

To demonstrate the maintenance of COX activity, a representative of each inhibitor class (nonselective: indomethacin; selective: meloxicam; highly selective: NS-398) was injected at relatively low doses into preovulatory follicles. The PGE_2 concentration of the FF was estimated five hours later. This experiment revealed that the low doses of the three inhibitors had a strong and comparable effect on COX activity in the follicle and decreased PGE_2 levels to a basal concentration. This is comparable to other studies that documented a massive decrease in PGE_2 levels in the FF after administration of COX inhibitor at different doses [36–38]. Furthermore, the results confirmed the known increase in PGE_2 in preovulatory follicles starting approximately 18 h after the LH signal [8, 36, 37, 39–41]. However, we observed that ovulation did not occur in follicles injected with indomethacin, while the control and meloxicam- and NS-398-treated follicles ovulated at the concentrations used (Table 1).

Changes in PGE_2 and steroid concentrations are related to each other in preovulatory follicles. In particular, progesterone production is functionally linked to an increase in the levels of various prostaglandins [13, 17–19]. Therefore, we hypothesized that treatment of follicles with COX inhibitors hampers steroid production, which can lead to failure of ovulation [15, 17]. Some studies detected no differences in progesterone or estradiol concentrations in FF after intrafollicular injection of NS-398 or systemic flunixin administration [13, 35]. In contrast, other *in vivo* and *in vitro* studies suggest a connection between prostaglandin and steroid production in follicles [12, 15, 17, 18]. In the FF of follicles injected with the COX inhibitors, we detected the concentrations of progesterone and estradiol expected for preovulatory follicles [42, 43]. Variations in steroid levels were highly pronounced in preovulatory follicles, as previously reported [42, 43]. However, only a few follicles were investigated, so the steroid concentrations from this *in vivo* data cannot be reliably interpreted. Therefore, we examined the granulosa cells from the preovulatory follicles treated with a representative of each COX inhibitor class (nonselective: indomethacin; selective: meloxicam; highly selective: NS-398) at concentrations comparable to or even higher than the final concentrations in the FF in the *in vivo* experiments.

The untreated control cell cultures showed a 10-fold increase in PGE_2, 1.5-fold increase in E2, and 4-fold increase in P4 between 4 and 24 h of culture, and therefore a hormone pattern that is expected from viable granulosa cells from preovulatory follicles [18, 36]. All tested COX inhibitors significantly and comparably reduced PGE_2 production in the cell culture. The effect on PGE_2 concentration lasted

for 24 h, and an increase in the COX inhibitor concentration had no additional effect. Therefore, further increasing the doses for the *in vivo* experiments did not seem beneficial. It has been hypothesized that the development of an ovarian cyst could be caused by disturbed or continuing estradiol production [28, 44]. However, no relationship was observed between PGE_2 reduction (by COX inhibitor treatment) and estradiol production in this study. Similarly, Wang *et al.* (2012) reported no correlation between PGE_2 and E2 concentrations in human FF or granulosa cell cultures. Notably, our granulosa cell cultures were derived from preovulatory follicles (post-LH surge). In undifferentiated granulosa cells, PGE_2 increases the mRNA levels of CYP19A1 (a key gene of estrogen biosynthesis) and aromatase levels, whereas, in luteinized granulosa cells, PGE_2 does not affect steroidogenesis [45]. Progesterone production was not altered in our *in vitro* experiments. This is contrary to the generally accepted hypothesis that PGE_2 and progesterone production are directly functionally dependent and positively correlated [13, 17, 18, 37]. Li *et al.* (2007) found that the preovulatory rise in intrafollicular progesterone may not be required for ovulation in cattle. They injected the 3β-HSD inhibitor trilostane into preovulatory follicles to inhibit P4 synthesis. Although P4 and PGE_2 levels were decreased in preovulatory follicles, ovulation was not inhibited by trilostane. Thus, altered P4 production in connection with decreased PGE_2 levels may not explain ovulation failure in cattle.

It remained unclear whether the inhibition of ovulation is a specific property of indomethacin or due to a nonspecific COX inhibitor. Therefore, the nonspecific COX inhibitor flunixin-meglumine was injected into follicles as a proof-of-principle test for ovulation inhibition. Flunixin-meglumine belongs to the chemical group of fenamic acids, whereas indomethacin is an indole acetic acid derivative. Flunixin-meglumine also proved to be a suitable drug to reliably block ovulation in cattle, even if slightly higher doses are needed. The effective solution of flunixin-meglumine (338 µM) resulted in a final concentration of approximately 3 µg/ml in follicular fluid (calculated for an average follicle with a diameter of 18 mm). This is close to the therapeutically recommended dosage of 2 µg/g of body weight [32]. However, no data are available on the concentrations reached in the follicular fluid after systemic administration. After ovulation failure, the development of an ovarian cyst was comparable to that previously described for indomethacin [28]. The inhibition of ovulation and further cyst development by using flunixin previously required repeated systemic administrations in cattle [46].

Nothing is known about the pharmacokinetics of substances used within follicles. Therefore, we could not determine whether the inhibitory effect of the substances was comparable beyond the investigated time (21 h after GnRH administration). Even small, recurring increases in PGE_2 concentrations might be sufficient to allow the ovulation process to resume. However, in rats, ovulation inhibition by the selective COX-2 inhibitor NS-398 was not improved by the additional use of the specific COX-1 inhibitor SC 560 [47]. COX-1 and -2 are the rate-limiting enzymes at the beginning of the prostaglandin synthesis pathway (oxygenation of arachidonic acid). Therefore, COX inhibitors influence all subsequently synthesized prostaglandins and thromboxanes. A differential effect on other important prostaglandins in the ovulation process, such as $PGF_{2\alpha}$ [45, 48], cannot be excluded.

In addition to prostaglandins, the ovulation process requires more components of the inflammatory cascades [47], such as that of the lipoxygenase pathway [20]. Tanaka *et al.* (1991) suggested that the synthesis of 15-hydroxyeicosatetraenoic acid via the 15-LOX-2 pathway (15-HETE) correlates more with ovulation than the synthesis

of PGE$_2$. In their study, PGE$_2$ concentration was significantly decreased by administering low concentrations of indomethacin in rats and rabbits, but ovulation was not inhibited. When indomethacin was used at concentrations that inhibited also 15-HETE synthesis, ovulation failed. Other pro-inflammatory factors involved in ovulation are tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [21]. Both induce ovulation in rodents [49]. Flunixin-meglumine inhibited TNF-α and IL-1β production in mice [50]. In contrast, NS-398 has a pro-inflammatory effect in the IL-1-mediated inflammatory response of granulosa cells [51]. Furthermore, proteolytic enzymes, such as matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), and members of the plasminogen activator family are required for the remodeling process of the extracellular matrix during ovulation [22, 23]. In cattle, higher MMP activity has been observed in preovulatory follicles than that in ovarian cysts [52, 53]. Indomethacin injections in bovine preovulatory follicles increased the tissue inhibitor of metalloproteinase-4 and suppressed tissue plasminogen activator [37].

In conclusion, our experiments demonstrate that the nonselective COX inhibitors indomethacin and flunixin-meglumine are suitable for blocking ovulation at low doses and developing ovarian cysts. The selective COX-2 inhibitor meloxicam and the highly selective COX-2 inhibitor NS-398 did not block ovulation. All the inhibitors comparably inhibited prostaglandin E$_2$ production at the concentrations used but had no effect on estradiol or progesterone production. These findings may contradict the generally accepted hypothesis that PGE$_2$ is a key mediator of ovulation and progesterone production. Indomethacin and flunixin could have additional unknown side effects on other important pro-inflammatory factors in the ovulation process. The present results cannot appropriately substantiate these assumptions. However, our results indicate the need for further studies of the pharmacokinetics and molecular mechanisms of action of these inhibitors. This knowledge would help understand whether inflammatory pathways might link ovarian disorders and an inflammatory-like situation in postpartum cows.

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