Abstract. Ovulation is an inflammation-like process, and cyclooxygenase-2 (COX-2)-dependent production of prostaglandin E2 (PGE2) 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 progstaglandin E2 (PGE2) 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 PGE2 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 PGE2 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 E2 (PGE2) and prostaglandin F2α (PGF2α) concentrations increase massively prior to ovulation [8, 11]. Although the concentration of several prostaglandins increases in parallel during ovulation, PGE2 is considered the key mediator [8, 11–13]. PGE2 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, PGE2 and PGF2α are involved in the differentiation of granulosa cells into lutein cells, triggering progesterone production, and corpus luteum maintenance [13, 17–19]. Progesterone and PGE2 increase simultaneously in preovulatory follicles, while the decrease in 17β-estradiol in preovulatory follicles is not correlated with PGE2 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 (lipoydroperoxides, 8-iso-PGF2α) 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 PGE2, 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 PGF2α 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 injection, 17.5 ± 2.9 mm at injection) and by clearly visible circular perfusion of the follicle wall upon ultrasonographic examination in Doppler mode.

Preovulatory follicles were identified by growth exceeding one mm 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 (Natriumchlorid; Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and further diluting with physiological saline solution (Sodiumchlorid; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) to obtain the desired concentrations. The 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 x 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

| 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 oxicam. Meloxicam was injected into preovulatory follicles at three different concentrations (172.5, 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] methanesulphonamide 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 following the slaughter of the animals in the institute's own abattoir. 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. 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 × 10^3 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% CO2.

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 trial). The supernatant was collected after 4 or 24 h of cultivation and cell-free stored at −20°C.

Hormone analysis

Prostaglandin E2 (PGE2), 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.

PGE2 concentrations were estimated using the 96-well PGE2 High Sensitivity ELISA Kit (Enzo Life Sciences, Lörbach, 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 PGE2, and the intra- and inter-assay coefficients of variation were tested and established in our group for cell experiments [27].

Fig. 1. Concentrations of prostaglandin E2 (PGE2) in the follicular fluid of untreated preovulatory control follicles 16 and 21 h after administration of GnrH (Con 16 and Con 21, respectively) and PGE2 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.
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
coefficient 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. As the test of normality failed for estrogen and PGE2 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 PGE2, 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. PGE2 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 PGE2 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 PGE2). 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 PGE2, estradiol, and progesterone production after cultivation for 4 or 24 h. PGE2 concentration in the control was 1866 ± 224 pg/ml/106 cells after 4 h of cultivation (Fig. 2A). The PGE2 level was tenfold higher (19516 ± 8446 pg/ml/106 cells) after 24 h of cultivation (Fig. 2B). Compared to the controls, all COX inhibitor-treated groups showed significantly lower PGE2 concentrations, with an overall mean of 525 ± 18 pg/ml/106 cells and 605 ± 76 pg/ml/106 cells after 4 and 24 h of cultivation, respectively (all P < 0.05). No significant increase in PGE2 concentration was observed between 4 and 24 h of culture. Treatment with 10 µM meloxicam resulted in slightly but significantly higher PGE2 concentrations after 24 h of cultivation compared to the other treatment groups (1111 ± 363 pg/ml/106 cells; Fig. 2B). The control and treatment groups together exhibited average estradiol concentrations of 633 ± 52 ng/ml/106 cells in the supernatants after 4 h of cultivation (Fig. 2C), with a significant increase to 973 ± 81 ng/ml/106 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/106 cells after 4 h of cultivation, whereas all treatment groups taken together showed an average of 99 ± 7 ng/ml/106 cells (Fig. 2E). After 24 h of incubation, the progesterone concentration increased significantly three- to fourfold up to 294 ± 22 ng/ml/106 cells in the control (P = 0.003) and up to 331 ± 23 ng/ml/106 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 PGE2 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 PGE2 levels to a basal concentration. This is comparable to other studies that documented a massive decrease in PGE2 levels in the FF after administration of COX inhibitor at different doses [36–38]. Furthermore, the results confirmed the known increase in PGE2 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 PGE2 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 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 PGE2, 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 PGE2 production in the cell culture. The effect on PGE2 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 PGE2 reduction (by COX inhibitor treatment) and estradiol production in this study. Similarly, Wang et al. (2012) reported no correlation between PGE2 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, PGE2 increases the mRNA levels of CYP19A1 (a key gene of estrogen biosynthesis) and aromatase levels, whereas, in luteinized granulosa cells, PGE2 does not affect steroidogenesis [45]. Progesterone production was not altered in our in vitro experiments. This is contrary to the generally accepted hypothesis that PGE2 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 PGE2 levels were decreased in preovulatory follicles, ovulation was not inhibited by trilostane. Thus, altered P4 production in connection with decreased PGE2 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 PGE2 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 PGE2α [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 lipooxygenase 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 5-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.

Conflict of interests: The authors declare no conflicts of interest.

Acknowledgments

The authors thank Veronika Tesch and Christian Plinski for their excellent support in the barn and the laboratory.

References

1. Bradford BJ, Yuan K, Farney JK, Mamedova I, Sordillo LM, Bradford BJ. TNF-a altered inflammatory responses, impaired health and productivity, but did not affect glucose or lipid metabolism in early-lactation dairy cows. J Vet Med Sci 2006; 171: 206–228. [Medline] [CrossRef]
2. Störis J, Richards JS. Purification and characterization of a novel, distinct isoform of prostaglandin endoperoxide synthase induced by human chorionic gonadotropin in granulosa cells of rat preovulatory follicles. J Biol Chem 1992; 267: 6382–6388. [Medline] [CrossRef]
3. Pereira de Morais F, Amaral D’Avila C, Cuenato de Oliveira F, Ávila de Castro N, Díaz Vieira A, Schneider A, Machado Pfeifer LF, Cantarell Poguaro LM, Ferreira R, Germano Ferst J, Tomazelle Rosani M, Nunes Cerra M, Dias Gonçalves PB, Lucia T Jr, Garziera Gasperin B. Prostaglandin F2α regulation and function during ovulation and luteinization in cows. Theriogenology 2021; 171: 30–37. [Medline] [CrossRef]
4. Kim SO, Duffy DM. Mapping PTGERs to the ovulatory follicle: regional responses to the ovulatory PGE$_2$ signal. Reprod Biol Endocrinol 2018; 16: 43. [Medline] [CrossRef]
5. Weems CW, Weems YS, Randel RD. Prostaglandins and reproduction in female farm animals. Front Vet Sci 2018; 5: 99–105. [Medline] [CrossRef]
6. Gaytan F, Bellido C, Gaytán M, Morales C, Sánchez-Criado JE. Differential effects of RU486 and indomethacin on follicle rupture during the ovulatory process in the rat. Biol Reprod 2003; 69: 99–105. [Medline] [CrossRef]
7. Störis JLL, Boerboom D, Antanya M. Prostaglandins and ovulation: from indomethacin to PGHS-2 knockout. In: ey A (ed.) Ovulation: Evolving Scientific and Clinical Concepts. New York: Springer 2000: 208–220.
8. Elvin JA, Yan C, Matzuk MM. Growth differentiation factor-9 stimulates progesterone synthesis in granulosa cells via a prostaglandin E$_2$/EP2 receptor pathway. Proc Natl Acad Sci USA 2008; 97: 10288–10293. [Medline] [CrossRef]
9. Wang J, Shen X, Huang XH, Zhu ZM. Follicular fluid levels of prostaglandin E$_2$ and the effect of prostaglandin E$_2$ on steroidogenesis in granulosa-lutein cells in women with moderate and severe endometriosis undergoing in vitro fertilization and embryo transfer. Chin Med J (Engl) 2012; 125: 3985–3990. [Medline]
10. Nösveder GD, Juangel JL, Silva PJ, Rijlyson MK, Melanthus EW. Mechanisms controlling the function and life span of the corpus luteum. Physiol Rev 2000; 80: 1–29. [Medline] [CrossRef]
11. Krurus S, Jinno M, Ebara H, Yonezawa T, Kawamimani M. Inhibition of ovulation by a lipoxigenase inhibitor involves reduced cyclooxygenase-2 expression and prostaglandin E$_2$ production in gonadotropin-treated immature rats. Reproduction 2009; 137: 59–66. [Medline] [CrossRef]
12. Silva JRV, Lima FE, Souza ALP, Silva ABW. Interleukin-1β and TNF-a systems in ovulatory follicles and their roles during follicular development, oocyte maturation and ovulation. Zoote 2020; 28: 270–277. [Medline] [CrossRef]
13. Bakke LJ, Dow MP, Cassar CA, Peters JW, PARLEY JR, Smith CW. Effect of the proinflammatory cytokines on matrix metalloproteinase (MMPs)-14, MMP-2, and tissue inhibitor of metalloproteinases-2 expression within bovine periovulatory follicle and luteal tissue. Biol Reprod 2002; 66: 1627–1634. [Medline] [CrossRef]
14. Curry TE Jr, Osteen KG. The matrix metalloproteinase systems: changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. Endo Rev 2003; 24: 428–465. [Medline] [CrossRef]
15. Trau HA, Davis JS, Duffy DM. Angiogenesis in the primate ovulatory follicle is stimulated by luteinizing hormone via prostaglandin E$_2$. Biol Reprod 2015; 92: 15. [Medline] [CrossRef]
16. Richards JS, Liu Z, Shimada M. Immune-like mechanisms in ovulation. Trends Endocr Metab 2008; 19: 191–196. [Medline] [CrossRef]
17. Veronight A, Viergutz T, Plinski C, Heidtmann JM. Postpartum levels of luteinizing hormone in cattle and milk phospholipid fractions as biomarker of oxidative stress in first-lactating dairy cows. Prostaglandins Other Lipid Mediat 2014; 112: 34–38. [Medline] [CrossRef]
18. Heidtmann JM, Veronight A, Krüger B, Plinski C, Viergutz T. LOX-1 regulates endothelium via intracellular calcium release from bovine granulosa cells. Cytometry A 2014; 85: 88–93. [Medline] [CrossRef]
19. Lapp R, Rütten V, Viergutz T, Heidtmann JM, Veronight A. Induction of cystic ovarian follicles (COFs) in cattle by using an intrafollicular injection of heparin. J Reprod Dev 2020; 66: 181–188. [Medline] [CrossRef]
20. Dirksen G, Baumgartner W. Immunemed. Zeitschrift für medizinische und chirurgische. Stuttgart. Parv 2006; XLI: 1325 S.
21. Schneider B, Bellmann A, Becker F, Bambang Poernomo S, Rehfeldt C, Nürburg M, Kanzit W. Granulosa inovulation in periovulatory heifers after GnRH analogs measured by immunocytochemistry. Angiogenesis in the primate ovulatory follicle is stimulated by luteinizing hormone via prostaglandin E$_2$. Biol Reprod 2015; 92: 15. [Medline] [CrossRef]
22. De Silva M, Reeves JS. Indomethacin inhibition of ovulation in the cow. J Reprod Fertil 1985; 75: 547–549. [Medline] [CrossRef]
23. Löschet W, Ungemach FR, Röker R, Pharmacoktherapie bei Haus- und Nutztieren. In: Löschet W, Ungemach FR, Röker R, Ungemach FR (eds.). Berlin: Parey; 2002: XIX, 531.
24. Vane JRS, Botting JM. Selective COX inhibitors: pharmacology, clinical effects and therapeutic potential, proceedings of a conference held on March 26-21, 1997, in Canues, France. In: Vane JRS, Botting JH (eds). Dordrecht [u.a.]: Kluwer [u.a.]; 1998: IX, 150 S.
preovulatory rise in intrafollicular progesterone may not be required for ovulation in human by the selective COX-2 inhibitor rofecoxib: a randomized double-blind study. Hum Reprod 2001; 16: 1323–1328. [Medline] [CrossRef]

Peters MW, Parsley JR, Smith GW. Inhibition of intrafollicular PGE2 synthesis and ovulation following ultrasound-mediated intrafollicular injection of the selective cyclooxygenase-2 inhibitor NS-398 in cattle. J Anim Sci 2004; 82: 1656–1662. [Medline] [CrossRef]

Li Q, Jimenez-Kressel F, Kobayashi Y, Ireland JJ, Smith GW. Effect of intrafollicular indomethacin injection on gonadotropin surge-induced expression of select extracellular matrix degrading enzymes and their inhibitors in bovine preovulatory follicles. Reproduction 2006; 133: 533–543. [Medline] [CrossRef]

Tanaka N, Espey LL, Kawano T, Okamura H. Comparison of inhibitory actions of indomethacin and prostaglandin in cathelicin in the rat. Am J Physiol 1991; 260: E170–E174. [Medline] [CrossRef]

Berisha B, Rodler D, Schams D, Sinowatz F, Pfaffl MW. Prostaglandins in superovulation induced bovine follicles during the preovulatory period and early corpus luteum. Front Endocrinol (Lausanne) 2019; 10: 407. [Medline] [CrossRef]

Hayashi KG, Matsui M, Shimizu T, Sudo N, Kida K, Miyamoto A. Involvement of pulsatile release of luteinizing hormone and growth hormone in development of codominant follicles during first follicular wave in cows. Biol Reprod 2006; 84: 113–113.

Li Q, Jimenez-Kressel F, Bettegowda A, Ireland JJ, Smith GW. Evidence that the preovulatory rise in intrafollicular progesterone may not be required for ovulation in cattle. J Endocrinol 2007; 192: 473–480. [Medline] [CrossRef]

Boryczko Z, Bostedt H, Hoffmann B. Comparison of the hormonal and chemical composition of the fluid from bovine ovarian follicles and cysts. Reprod Domest Anim 1995; 30: 36–38. [CrossRef]

Einspanier R, Schuster H, Schams D. A comparison of hormone levels in follicle-lutein-cysts and in normal bovine ovarian follicles. Theriogenology 1993; 40: 181–188. [Medline] [CrossRef]

Todoroki J, Kaneko H. Formation of follicular cysts in cattle and therapeutic effects of controlled internal drug release. J Reprod Dev 2006; 52: 1–11. [Medline] [CrossRef]

Cai Z, Kwintkiewicz J, Young ME, Stoess C. Prostaglandin E2 increases cyp19 expression in rat granulosa cells: implication of GATA-4. Mol Cell Endocrinol 2007; 263: 181–189. [Medline] [CrossRef]

Pugliesi G, Khan FA, Hannan MA, Beg MA, Carvalho GR, Günther OJ. Inhibition of prostaglandin biosynthesis during postluteolysis and effects on CL regression, prolactin, and ovulation in heifers. Theriogenology 2012; 78: 443–454. [Medline] [CrossRef]

Gaytán M, Bellido C, Morales C, Sánchez-Criado JE, Gaytán F. Effects of selective inhibition of cyclooxygenase and lipooxygenase pathways in follicle rupture and ovulation in the rat. Reproduction 2006; 132: 571–577. [Medline] [CrossRef]

Pereira de Moraes F, Amaral D’Ávila C, Cueto A, Oliveira F, Ávila de Castro N, Díaz Vieira A, Schneider A, Machado Pfeifer LM, Ferreira R, Germano Fester J, Tomazelle Rovani M, Nunes Correia M, Dias Gonçalves PB, Lucia T Jr, Garziera Gasperin B. Prostaglandin F2α regulation and function during ovulation and luteinization in cows. Theriogenology 2021; 171: 30–37. [Medline] [CrossRef]

Machuelo Y, Emilie D. Production of ovarian cytokines and their role in ovulation in the mammalian ovary. Eur Cytokine Netw 1997; 8: 137–143. [Medline] [CrossRef]

Yazar E, Er A, Uney K, Altmann V, Elmas N. Effect of flaxseed meglineline on cytokine levels in experimental Endotoxemia in mice. Journal of Veterinary Medicine Series a-Physiology Pathology. Clin Med (Northfield Ill) 2007; 54: 352–355.

Ou HL, Sun D, Peng WC, Wu YL. Novel effects of the cyclooxygenase-2-selective inhibitor NS-398 on IL-1β-induced cyclooxygenase-2 and IL-8 expression in human ovarian granulosa cells. In vitro. Human Imm 2016; 22: 452–465. [Medline] [CrossRef]

Muthag AM, Wang X, Yang Z, Meng J, Wang X, Zhang J, Qin Z, Wang G, Li J. Study on matrix metalloproteinase 1 and 2 gene expression and NO in dairy cows with ovarian cysts. Anim Reprod Sci 2013; 152: 1–7. [Medline] [CrossRef]

Peralta MB, Baravalle ME, Belotti EM, Stassi AF, Salvetti NR, Ortega HH, Rey F, Velázquez MML. Involvement of matrix metalloproteinases and their inhibitors in bovine cystic ovarian disease. J Comp Pathol 2017; 156: 191–201. [Medline] [CrossRef]