Induction of cystic ovarian follicles (COFs) in cattle by using an intrafollicular injection of indomethacin

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Abstract. The aim of this study was to establish a model to induce cystic ovarian follicles (COFs) in cattle using the cyclooxygenase inhibitor, indomethacin. Eighteen Holstein-Frisian cattle were synchronized with prostaglandin F2alpha (PGF2α) and gonadotropin-releasing hormone (GnRH). Ultrasound-guided transvaginal intrafollicular injections were performed in 23 preovulatory follicles with different concentrations of indomethacin 16 h after GnRH administration. An injection of 0.2 ml 35 µM indomethacin solution (resulting in a final concentration of 8 µg/ml in the follicular fluid) was the minimal dosage leading to COF formation. The induced COFs reached a maximum mean diameter of 36.9 ± 4.5 mm eleven days after injection. The estrous cycle was extended to 25–39 days. Luteinization was first observed 4 days after injection, accompanied by a slight increase in plasma progesterone concentration. The bioactivity of indomethacin was demonstrated by the decrease of prostaglandin E2 in the follicular fluid of three animals. The method presented here is minimally invasive and allows for the generation of defined COFs for further investigations.

Key words: Bovine, Cyclooxygenase, Follicle injection, Ovarian cysts, Ovulation

R eproductive disorders, especially cystic ovarian follicles (COFs), are a major problem during the post-partum period of lactating dairy cows [1]. COFs lead to prolonged calving intervals and result in increased labor costs, veterinary treatments, and a higher loss due to premature culling [2]. This contradicts the aim of sustainable agriculture and animal welfare. The incidence of COF formation is about 6–30% [1, 3]. Multiparous, lactating dairy cows are more often affected by a COF than heifers or beef cows [4]. COFs are commonly defined as anovulatory follicles, larger than 25 mm in diameter, which occur in the absence of any luteal tissue and persist longer than 10 days on one or both ovaries [5]. However, other definitions state that the cysts are between 17–20 mm in diameter and persist for a shorter duration of time [6, 7]. In most studies COFs are macroscopically and hormonally divided into follicle-theca- or luteal cysts. Roughly, follicular cysts have a thin wall and usually produce estradiol. Luteal cysts have a wall which is thicker than 3 mm and they produce substantial amounts of progesterone [7]. COFs are dynamic structures which can regress spontaneously, transform from follicular to luteal cysts, and can be replaced by new cysts or persist [4, 5, 8]. Approximately 60% of COFs developed during early post-partum period regress spontaneously [7]. Investigations into the underlying mechanisms of cyst formation in naturally occurring cysts are difficult, because the developmental stage of a COF cannot be precisely determined, especially in the early stages due to the unknown time point of presumed failed ovulation. A generally accepted hypothesis for the pathogenesis of COF formation is that dysfunctions in the estradiol-mediated positive-feedback mechanism of the hypothalamic-pituitary-ovarian axis lead to an aberrant, insufficient or low gonadotropin releasing hormone/luteinizing hormone (GnRH/LH) surge, accompanied by suprabasal progesterone levels [7, 9, 10]. However, studies have shown that experimentally produced suprabasal progesterone levels, successfully suppress LH secretion in cows, but only persistent follicles developed, which did not show typical cyst growth dynamics [11]. Furthermore, it is well known, that cows with COF react adequately to a GnRH signal [12]. Moreover, data have shown that high LH levels can lead to COF formation [13]. The results of further studies even suggest that the pulsatile LH signals may be necessary for cystic growth [9, 10], and that no primary dysfunction of the pituitary must exist before COF formation [14]. Apart from the systemic hormonal reasons for COF formation, there are some additional local intraovarian factors which are thought to lead to or stimulate the required conditions for COF development of anovulatory follicles [15].

Due to the major obstacle that cyst formation can only be investigated retrospectively, different in vivo models have been established to simulate the process of COF formation in cattle, which are more or less coherent to the hypothesized pathogenesis of COF. Most of these studies have used systemic hormonal treatments to interfere with the hypothalamic-pituitary-axis. Such models included, for instance, a prolonged supplementation of progesterone [11] or the use of systemic estradiol administration [16]. Both models successfully block ovulation and lead to the formation of anovulatory follicles,
which were described as becoming cystic. Another method to create anovulatory follicles up to 23 mm in diameter, is a repeated systemic injection of adrenocorticotropic hormone (ACTH) [17]. Previous studies have focused more on a local intraovarian intervention. In this and other studies, ovulation was inhibited by the administration of different cyclooxygenase (COX) inhibitors [18–20]. The upregulation of COX enzymes, especially COX-2, and the resulting increase of prostaglandins in the preovulatory follicle is an essential factor for ovulation [21]. COX-2 upregulation in the follicle is induced by an LH surge. COX-2 increases about 18 h after human chorionic gonadotropin (hCG) or GnRH administration in cattle. The resulting ovulation occurs approximately 10 h after the COX upregulation [21]. COX inhibitors suppress the increase in prostaglandins in the preovulatory follicle and successfully block ovulation [18–20, 22], but the subsequent development of these anovulatory follicles is unclear. In these studies, the COX pathway was downregulated by specific- (NS-398) [18] or non-specific COX inhibitors (flunixin and indomethacin) [19, 20, 22] in cattle. The inhibitors were either given as a systemic treatment over several days or were administered directly into the follicle. Additionally, in humans the systemic use of non-steroidal anti-inflammatory drugs (meloxioca or rofecoxib) varied between 19–39 days. A difference in the development of COFs depending on the use of heifers or lactating cows was not observed.

Plasma progesterone and estradiol concentrations were measured in 5 animals which developed a COF after intrafollicular injection of indomethacin (Fig. 3). As expected, the concentrations of progesterone decreased significantly after PGF2α injection to 0.17 ± 0.01 ng/ml on the day of GnRH injection (P < 0.05). Thereafter, the plasma progesterone concentrations slowly increased after the disturbed ovulation (Fig. 3). On day 7 after the injection of indomethacin, the mean progesterone concentration was 0.9 ± 0.19 ng/ml and the highest concentration of 1.08 ± 0.22 ng/ml was measured 11 days after the injection. Intrafollicular injections are advantageous because they allow for local administration of inhibitors or antibodies in a certain follicle, and they allow us to study their effects on follicles without taking systemic effects into account.

The before mentioned models have presented an opportunity to manipulate and study the ovulatory process. However, to our knowledge, there have been no long-term follow up studies that characterize the subsequent development of the generated anovulatory follicles, their influence on the ovarian function, and their regression. Therefore, the aim of the study was to establish the intrafollicular injection of a COX-inhibitor as a model for COF formation in cattle. Herein, the subsequent development of the anovulatory follicles into COFs after intrafollicular injection, were characterized by morphological and endocrinological parameters.

Briefly, preovulatory follicles were produced by a synchronization protocol with an injection of prostaglandin F2alpha (PGF2α) followed by an injection of GnRH 54 h later. The ovulation is expected about 28 h after the GnRH administration [21]. In a first part of the study transvaginal ultrasound guided intrafollicular injections with 0.2 ml of a 279 µM indomethacin solution were performed into the preovulatory follicles 16 h after the GnRH treatment to prevent ovulation and induce COF formation. Follow up ultrasound examinations were performed and blood samples were collected to monitor the morphological and functional development of COFs until the next occurring ovulation. In the second part of the study, decreasing concentrations of indomethacin were intrafollicularly injected to determine the lowest effective concentration which leads to COF formation. The third aspect of this study was to determine the biochemical activity of the minimal effective dose of indomethacin as measured by the PGE2 concentration in the follicular fluid. Therefore, PGE2 concentrations were measured in injected and subsequently aspirated follicles as well as in control follicles.

For the first part of the study, 5 preovulatory follicles were injected with 0.2 ml of 279 µM indomethacin. The mean follicle diameter on the day of injection was 18.3 ± 0.9 mm. All follicles failed to ovulate. The mean diameter was 18.3 ± 0.9 mm 24 h after the injection. The anovulatory follicles showed a significant increase in size during the next 11 days (Fig. 1). On day 4 after injection, the mean diameter was 30.8 ± 2.8 mm. Moreover, a slight thickening of the follicular wall and an increase in vascularization (Color-Doppler mode) were observed from day 4 onwards (Fig. 2). The largest mean-diameter (36.9 ± 4.5 mm) was measured on day 11 post-injection. After day 11, the COFs decreased in size and the follicular wall grew thicker. On day 18, the mean diameter was 26.7 ± 4.5 mm and for the first time, was smaller than previous examinations (Fig. 1). As time went on, the COFs became compact and completely regressed. One COF ruptured during an ultrasonography examination on day 7. Thereafter, the COF developed into a Corpus luteum-like structure. The length between intrafollicular injection and regression of the COF, followed by a new ovulation and physiological Corpus luteum formation varied between 19–39 days. A difference in the development of COFs depending on the use of heifers or lactating cows was not observed.
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The plasma estradiol concentrations showed the opposite course compared to the progesterone profile (Fig. 3). The mean estradiol concentration on the time point of injection was $10.8 \pm 1.5$ pg/ml (day of induced estrus), but peaked on day 4 after injection with a mean concentration of $15.8 \pm 2.1$ pg/ml. However, estradiol concentrations varied considerably between animals (Fig. 3). In fact, in one animal, an elevation of the estradiol level in the plasma occurred not until eleven days after injection. Due to the variability, no significances were observed.

As a vehicle control, 0.2 ml of a 0.5% ethanol solution was injected into 5 preovulatory follicles with a mean diameter of $16.2 \pm 1.2$ mm. All vehicle-treated follicles ovulated within 24 h after injection and developed a Corpus luteum (Fig. 2). The Corpus luteum reached a size of $25.1 \pm 1.1$ mm at day 11 after injection.

For the second part of the study, 0.2 ml of decreasing concentrations of indomethacin were injected into preovulatory follicles to detect the minimal effective dose for ovulation prevention (Table 1). COFs developed even after an injection of 35 µM indomethacin solution (n = 4 injections), whereas injections of 5 µM indomethacin solution (n = 2 injections) resulted in ovulations. Overall, the anovulatory follicles (later COFs) from this point, gained a mean diameter of $28.7 \pm 3.5$ mm 4 days after injection. The COFs that developed after the injection of 35 µM indomethacin, gained a mean diameter of $30.8 \pm 3.5$ mm 4 days after injection. The ovulated follicles developed a Corpus luteum with a mean diameter of $17.6 \pm 2.3$ mm on day 4.

The third aspect of this study was concerned with investigating the effect of the lowest inhibitory dose of indomethacin on the synthesis of PGE$_2$. Therefore, 3 follicles were injected 16 h after GnRH administration with the minimal effective dose of indomethacin (0.2 ml at 35 µM). The follicles were aspirated 5 h later (21 hours after GnRH administration). As controls, untreated preovulatory follicles were aspirated 16 h (n = 3) and 21 h (n = 5) after the GnRH injection.
PGE₂ concentration could not be statistically secured. A mean concentration of 0.4 ± 0.1 ng/ml 21 h after the GnRH treatment (Fig. 4). Despite the administration. A mean concentration of 2.5 ± 0.5 ng/ml (21 h after GnRH, Fig. 4). Follicles receiving an injection of 35 µM indomethacin solution 5 h prior to the aspiration showed the lowest concentrations of PGE₂. Their mean PGE₂ concentration was 0.4 ± 0.1 ng/ml 21 h after the GnRH treatment (Fig. 4). The later time point in this protocol was chosen because of the expected upregulation of COX (18 h after GnRH treatment).

In this study the time point of intrafollicular injection was 14 to 16 h after the gonadotropin-releasing hormone (GnRH) administration. However, the variation in the time point of injection was important to note. The initiation of luteinization might be explained by a maintained estradiol production during the first days. Estrogen is the key-mediator in growing follicles. It inhibits apoptosis, increases proliferation in dominant follicles, and provides positive feedback for LH secretion.

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### Table 1. Administered solutions of indomethacin, and the resulting final concentrations in the follicular fluid of injected follicles, the numbers of injected and the subsequent ovulated follicles

| Concentration | Final concentration | Number of injections | Ovulated follicles |
|---------------|---------------------|----------------------|-------------------|
| 279 µM        | 64 µg/ml            | 5                    | 0                 |
| 140 µM        | 32 µg/ml            | 3                    | 0                 |
| 70 µM         | 16 µg/ml            | 4                    | 0                 |
| 35 µM         | 8 µg/ml             | 4                    | 1                 |
| 5 µM          | 1.14 µg/ml          | 2                    | 2                 |

The corresponding final concentrations in the follicular fluid were calculated for the estimated average follicle diameter of 18.1 mm.

administration. A mean concentration of 2.5 ± 0.5 ng/ml PGE₂ was measured in follicles 16 h after GnRH injection, whereas in the later aspirated control follicles the concentration were 13.7 ± 6.8 ng/ml (21 h after GnRH, Fig. 4). Follicles receiving an injection of 35 µM indomethacin solution 5 h prior to the aspiration showed the lowest concentrations of PGE₂. Their mean PGE₂ concentration was 0.4 ± 0.1 ng/ml 21 h after the GnRH treatment (Fig. 4). Despite the obvious numerical differences between groups, the changes in the PGE₂ concentration could not be statistically secured.

Our results show that an intrafollicular injection of indomethacin successfully blocks the ovulation of preovulatory follicles and results in COF formation. Although an aberrant LH signal due to a disruption in the hypothalamic-pituitary-ovarian-axis is the most common hypothesis for COF formation, the exact pathophysiology remains unknown. In contrast to other models and the common hypothesis, the model presented here includes a GnRH induced LH surge. We show that a disruption of local intraovarian factors alone, in a preovulatory follicle is sufficient to create conditions resulting in COF formation. The anovulatory follicles retained their growth, became cystic and formed later on the situation of suprabasal P₄ concentration as observed in cystic cows [7]. For several models in which disturbance of the hypothalamic-pituitary-ovarian-axis occurs, the development of only persistent follicles was described and a regression was seen after the abolishment of the administered substances [11, 17]. Comparable to the presented results, a study of Gümen et al. (2005) showed that COF formation after an LH surge is possible [14]. This leads to the assumption that the LH signal could be important for further growth of the anovulatory follicles. The increasing growth rates of the follicle to a cyst seen in this model will provide insight into the processes and triggering signals that cause that an unruptured follicle will develop into a cyst and not undergo atresia or persistence.

Indomethacin, a COX-1 and COX-2 inhibitor, is known to produce anovulatory follicles [18, 20, 22] and this could be confirmed in the present study. The mentioned studies performed intrafollicular injections at the time of GnRH or 2 h after GnRH administration. In this study the time point of intrafollicular injection was 14 to 16 h later. The later time point in this protocol was chosen because of the expected upregulation of COX (18 h after GnRH treatment) [21] and the plasma half-life of indomethacin (5.3 h) [27]. After successful ovulation inhibition, the anovulatory follicles of the current study developed into a COF within a few days. The maximum COF diameter of 36.9 ± 4.5 mm in this study is comparable to a study, which observed COF formation after long-term systemic COX inhibitor treatment (flunixin) in cows. They reported maximum diameters of 36.2 ± 2.9 mm [19]. Anovulatory follicles, which were formed with different approaches (e.g. by ACTH or prolonged progesterone treatment), remained smaller in size (20–25 mm) [11, 17]. Depending on the cyst definition, the diameter of the COFs produced in this study were comparable to naturally occurring cysts [7].

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### Fig. 4. Prostaglandin E₂ (PGE₂) concentrations in follicular fluids of untreated follicles 16 h and 21 h after gonadotropin-releasing hormone (GnRH) administration and in follicles 21 h after GnRH, but injected with a solution of 0.2 ml of 35 µM indomethacin 5 h prior.

The corresponding final concentrations in the follicular fluid were calculated for the estimated average follicle diameter of 18.1 mm.

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stagnation and the growth of the induced COFs remained until day 11 after injection which is in contrast to other studies. While anovulatory follicles induced by NS-398 or systemic flunixin administration, began to luteinize within the first 44–48 h after failed ovulation, the COFs induced using our protocol showed a prolonged period of 4 days to the beginning of luteinization. Therefore, NS-398 induced anovulatory follicles were described further as LUF [18] due to the relatively early luteinization but not as COFs. This observation is similar to studies in humans, which reported LUF development in women after the oral consumption of meloxicam over several days [24].

In this study, delayed luteinization was accompanied by a delayed increase in plasma progesterone concentrations. The progesterone concentration remained at suprabasal levels, which is the same for naturally occurring cysts [1]. Progesterone concentrations that are characteristic for a physiological Corpus luteum were not reached. Follicles treated with the specific COX-2 inhibitor NS-398, which were later described as LUF, reached serum progesterone levels of ≥ 2.7 ng/ml on day 8 after injection [18].

A prolonged sexual cycle was observed in 4 of 5 animals in this study. Interference of cyclicity is a known characteristic for ovarian cysts [34]. To our knowledge, no other artificial COF model has shown this unique characteristic before. Only one animal ovulated within 19 days after the indomethacin injection. However, this animal experienced a rupture of the COF due to an ultrasound examination at day 7 after injection. A rupture or a total aspiration of COF fluid is a therapeutic procedure for naturally occurring cysts allowing for a return to normal cyclicity [5, 35].

One of the aims of the development of this model was, that independent of the follicular size a fixed dose of indomethacin can be used. The initial concentration of indomethacin in this study was based on the findings of Li et al., 2006 [20]. However, to go one step further, the concentration of indomethacin was reduced in a stepwise manner to obtain the minimal effective dosage, which still disturbs the ovulation. The minimal effective dosage was 25 times lower than the doses used by Li et al., 2006. A reduction of the used COX inhibitor concentration could be helpful to reduce possible side effects of high COX inhibitor loads in the follicle, such as the diffusion and the interference to a nearby follicle. Whereas the concentration of indomethacin was reduced to a minimum, the expected effect on the COX pathway could still be observed. A clear sign for the preserved activity of low dose indomethacin injection was the massive reduction of PGE2 in the follicular fluid of injected follicles compared to untreated follicles (Fig. 4). The reduction of the PGE2 concentration due to intrafollicular COX inhibitor administration is comparable to other studies [18, 20].

In summary, the presented COF induction protocol uses a minimally invasive procedure and results in COF formation, even after physiological follicle development and LH-surge. Although the induced COFs differ in the time point, when the first signs of luteinization were observed, they meet other clinical criteria of naturally occurring ovarian cysts in cattle regarding size, morphology, persistence and interference with cyclicity. In contrast to other models with systemic hormonal treatment, the presented artificial COF model can selectively demonstrate the manipulation of ovarian functions only by local factors. Therefore, this model is useful to gain defined samples of COFs for further investigations. This will contribute to the elucidation of the underlying endocrine and molecular mechanisms of cystic ovarian disorders.

Material and Methods

Animals

In this study 23 German Holstein heifers and 2 German Holstein cows in their first lactation underwent repeated intrafollicular injections in different experimental setups of the study. The animals were between 20 and 51 months of age and housed at the Experimental Facility for Cattle at the Leibniz Institute for Farm Animal Biology in Dummerstorf, Germany. They were fed with a need based total mixed ration ad libitum. Lactating cows were milked twice a day. The experimental design was approved by the federal state of Mecklenburg Western-Pomerania, Germany (LALLF M-V TSD 7221.3-1-010/12; TSD 7221.3-1-038/12).

Follicle selection and examination

Normal cycling animals in diestrus received an intramuscular injection of 2 ml PGF Veyx® forte (0.25 mg/ml Cloprostenol, Veyx-Pharma GmbH, Schwarzenborn, Germany) to induce luteolysis. Follow up ultrasound examinations confirmed that the Corpus luteum underwent regression (decrease in size and vascularization) and growth of a dominant follicle. If the follicle had grown more than one millimeter per day and showed increased vascularization of the follicular wall, it was considered as preovulatory. Animals with a preovulatory follicle and a luteolysis of the Corpus luteum received an intramuscular injection of 2 ml Gonavet Veyx® (50 µg/ml Gonadorelin, Veyx-Pharma GmbH, Schwarzenborn, Germany) to induce an LH surge. The presence of the preovulatory follicle was controlled again via ultrasound examination 16 h after GnRH administration shortly before intrafollicular injection (Fig. 5).

![Fig. 5. Treatment protocol: Animals in diestrus received prostaglandin F2alpha (PGF2α) followed by a gonadotropin-releasing hormone (GnRH) injection 54 h later. Intrafollicular injections of indomethacin or vehicle control were performed 16 h after GnRH administration (day 0). First ovulation detection was performed one day after follicle injection (day 1). Semiweekly ultrasonographic examinations and sample collections followed over several weeks until the next ovulation.](image-url)
**Follicular injection**

The ultrasound guided, transvaginal intrafollicular injection was conducted according to Vernunft et al., 2013 [31]. Animals received epidural anesthesia with 5 ml Procainidol (20 mg/ml Procainhydrochlorid, WDT, Garbsen, Germany) and nervous animals were additionally sedated with 0.75 ml Xylariem (20 mg/ml Xylariemhydrochlorid, Ecuphar, Ooostkamp, Belgium). The follicle injection was carried out with a custom made ovum pick-up device mounted with a 6.5 MHz sector finger-tip-probe (EUP-F331, Hitachi Medical, Tokyo, Japan) for the Picker CS 9000 ultrasound system (EUP-405, Hitachi Medical), equipped with a 25-gauge needle (Sterican needles 0.5 × 40 mm, B. Braun, Melsungen, Germany). The needle was connected to a prolongation tube and a 1 ml syringe at the outside end. The tube was filled with 0.9% saline solution (isotonische Natriumchlorid-Lösung ad us. vet., B. Braun Vet Care, Melsungen, Germany) to minimize air-filled space in the system. Shortly before the follicle injection started, the injection solution was loaded retrograde into the injection system through the needle. An air bubble remained between the test and the saline solution, which ensured separation of the test and the preloaded saline solution. After cleaning the perianal region, the ovum pick-up device was placed in the vagina and fixed with one hand. The other hand fixed the ovary with the preovulatory follicle in front of the device via transrectal manipulation. The needle was pushed through the vaginal and follicular wall until the needle is clearly visible in the antrum. The substance is injected in the follicle under ultrasound control. To decrease the risk of follicle rupture, the path of the needle should include a 3 mm bridge of ovarian tissue before entering the follicle. During the injection, the injected fluid visibly swirled around. This confirmed a successful injection (Fig. 6). After 15 min a transrectal ultrasound examination was performed to ensure that the follicle was not ruptured or leaked follicular fluid due to the previous injection.

**Part 1**

For the first part, 6 heifers and 2 lactating cows were divided randomly in two treatment groups. One group (n = 5 animals) received an intrafollicular injection of 0.2 ml of 279 µM indomethacin solution (n = 5 injections). Indomethacin (Indomethacin 99%, Merck KGaA, Darmstadt, Germany) was resuspended first in ethanol (ROTIPURAN® ≥ 99.8%, p.a., Ethylalkohol, Carl Roth GmbH+Co. KG, Karlsruhe, Germany). This solution was diluted further with physiological saline solution (Natriumchlorid, Carl Roth GmbH + Co KG, Karlsruhe, Germany) to a concentration of 279 µM indomethacin and 0.5% ethanol solution. The control group (n = 3 animals, two animals used twice) received an intrafollicular injection of 0.2 ml of a 0.5% ethanol solution as vehicle control (n = 5 injections). Ovulation was monitored by ultrasound examination one day after injection. The absence of the injected follicle and a visible development of a Corpus luteum or a vascularized structure in the same region of the preovulatory follicle was considered as ovulation. Anovulatory follicles were defined as follicles that failed to ovulate within 40 h after gonadorelin injection. Follow-up transrectal ultrasound examinations of the ovaries were performed twice a week (Monday and Thursday) for the indomethacin injected group until the onset of a new sexual cycle was detected. A new sexual cycle was defined by ultrasound examinations, the size and presence of vascularization around the growing COF or the Corpus luteum were determined.

**Blood sampling and hormonal determination**

Blood samples were taken from 5 animals with COF development after receiving an intrafollicular injection of 0.2 ml of 279 µM indomethacin. The samples were collected from the coccygeal vein in blood collection tubes for plasma preparation containing 1.6 mg EDTA-K/ml blood (S-Monovette® EDTA 9 ml, Sarstedt, Nuembrecht, Germany). Blood samples were taken twice a week from the beginning of the treatment protocol until the onset of the next sexual cycle. Plasma was separated and stored at –20°C. Progesterone and Estradiol-17β were analyzed according to Schneider et al. (2002) [36]. A quantitative 3H-RIA was used with a 1,2,6,7-H(N) progesterone tracer (Hartmann Analytik, Braunschweig, Germany) for direct progesterone measurement. The antibody was produced by immunization of rabbits versus 11-OH-progesterone conjugate. The sensitivity of the assay was 7 pg/ml. The intra- and interassay coefficient of variation were 7.6 and 9.8%, respectively. Estradiol-17β was measured by 3H-RIA after extraction by ethylther. The sensitivity of the assay was 3 pg/ml. The intra-and interassay coefficient of variation were 6.9 and 9.9%, respectively.

**Part 2**

For the second part, 4 further decreasing concentrations of indomethacin (5 µM, 35 µM, 70 µM, 140 µM) were tested for COF induction (Table 1). The corresponding final concentrations in the follicular fluid, as presented in table 1, were calculated for the

![Fig. 6. Intrafollicular injection of a preovulatory follicle. The follicle is placed via rectal manipulation in front of the vaginal ultrasound probe (a), then the needle is pushed through the vaginal and follicular wall until the needle is clearly visible in the antrum (b). The substance is injected in the follicle (c). The swirling in the follicle proved the successful injection (d).](image-url)
estimated average follicle diameter of 18.1 ± 0.5 mm and a volume of 3.2 ± 0.2 ml. The different concentrations of indomethacin were produced as described in part one. The injected volume remained at 0.2 ml. Nine heifers were randomly injected with different solutions (n = 13 injections, Table 1). After complete recovery of the physiological ovarian function three animals were used repeatedly (one animal was used two times in the group receiving a 35 µM solution of indomethacin: a second animal was used in the groups receiving a 140 µM, 70 µM and a 35 µM indomethacin solution, respectively: a third animal was used in the group receiving 140 µM as well as 70 µM indomethacin solution). Follicle selection, pre-treatment, and intrafollicular injection were performed as described in part one. On the first day after intrafollicular injection, an ultrasonographic examination was performed to monitor the occurrence of an ovulation. Anovulatory follicles were examined until day 7 after injection to ensure cyst development.

Prostaglandin E₂ (PGE₂) measurement in follicular fluid

PGE₂ was measured in aspirated follicular fluids of untreated and indomethacin injected follicles in eleven heifers. Follicular fluids were sampled via ultrasound guided transvaginal follicle aspiration. Untreated follicles were either aspirated 16 h (n = 3) or 21 h (n = 5) after GnRH treatment. Treated Follicles were injected with 0.2 ml of a 35 µM indomethacin solution 16 hours after GnRH and aspirated 5 hours later (21 h after the GnRH treatment, n = 3). The aspirated follicular fluid was stored directly on ice and centrifuged within 5 min at 500 × g for 10 min at 4°C. The cell-free supernatant was separated and stored at −20°C. PGE₂ concentration was measured in the supernatant with an ELISA (# ADI-930-001, Enzo Life Sciences, Lörrach, Germany), according to Richter et al., 2015 [37]. The sensitivity for the assay was 13.4 pg/ml PGE₂, and the intra- and inter-assay coefficients of variation were 8.9 and 3%, according to manufacturer’s specifications.

Statistical analysis

Data are presented as means with their standard errors. The statistics and graphs were carried out in R statistical software (R version 3.4.1., 2017-06-30), R Core team (2017), R: A Language and Environment for statistical computing. R Foundation for statistical computing, Vienna, Austria. URL http://www.R-project.org/. Standard errors were calculated using the package Rmisc (Ryan M. Hope (2013). Rmisc: Ryan Miscellaneous. R package version 1.5. https://CRAN.R-project.org/package=Rmisc).

The data was tested for normality with the Shapiro-Wilk test. Normal distributed data was analyzed by ANOVA (SS typ 3 for unbalanced data (PGE₂ concentration)) for repeated measurements (COF diameter, estradiol concentration). Respective multiple pairwise comparisons were calculated with Tukey-Kramer test [multicomp package: Torsten Hothorn, Frank Bretz and Peter Westfall (2008). Simultaneous inference in general parametric models. Biometrical Journal 50(3): 346–363]. Abnormally distributed data (progesterone concentration) was analyzed by the Friedman test. Multiple pairwise comparisons were calculated by Nemenyi test (PMCMCA package: Pohlert T (2014). The Pairwise Multiple Comparison of Mean Ranks Package (PMCMR). R package, URL: https://CRAN.R-project.org/package=PMCMR). P-values < 0.05 were considered to be statistically significant. Graphs were created with ggplot2 (H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2009).

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