Micromorphology of antral follicles in cattle after prostaglandin-induced luteolysis, with particular reference to atypical granulosa cells

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Dutch Friesian heifers ($n = 13$) and cows ($n = 13$) were used to obtain information about the number, size and micromorphology of antral follicles (≥ 3 mm in diameter) in cattle after induction of luteolysis with the $\text{PGF}_{2\alpha}$ analogue luprostiol. Special attention was paid to the presence of atypical granulosa cells in these follicles to obtain additional data to help evaluate the hypothesis that these cells are markers of follicular atresia. Animals were injected i.m. with 15 mg of the synthetic prostaglandin on day 10 or day 11 of the oestrous cycle. The ovaries were collected on day 12, that is 48 and 24 h after injection of luprostiol, respectively. After prostaglandin-induced luteolysis, the mean number of medium-sized and large nonatretic follicles and of medium-sized atretic follicles had not changed in heifers and in cows, compared with those of untreated animals. However, in heifers, contrary to cows, the development of a preovulatory-sized follicle was initially accompanied by an increase in the number of large definitely atretic follicles. Atypical granulosa cells can be considered as markers for a lower quality follicle, on the basis of their absence in preovulatory-sized follicles and their presence in large numbers in a high proportion of definitely atretic follicles. If it is assumed that only a nonatretic follicle without atypical granulosa cells will grow to preovulatory size, growth of this follicle within 2 days after prostaglandin treatment was almost 9 mm and over 10 mm in heifers and cows, respectively. In cows, most growth of follicles was observed at the first day after prostaglandin treatment; in heifers, this occurred one day later.

Introduction

Prostaglandins are formed in many tissues and have various functions (Walpole, 1975). In cattle, prostaglandins from the uterus evoke regression of the corpus luteum, which leads to reduced progesterone concentrations, followed by the development of a preovulatory follicle, the start of a preovulatory LH surge and eventually ovulation (Louis *et al.*, 1975; Hansel and Convey, 1983). Endogenous prostaglandins are cleared rapidly from the circulation (Walpole, 1975). More stable prostaglandin analogues, such as cloprostenol (Estrumate: ICI, Macclesfield) and luprostiol (Prosolvlin: Intervet, Boxmeer), are commonly used for their luteolytic action or for synchronization of oestrus and ovulation (Wright and Malmo, 1992). Although the luteolytic effect of these exogenous prostaglandins has been well documented (Hansel and Convey, 1983), little information is available on the micromorphology of the population of bovine antral follicles after luteolysis induced by prostaglandin analogues (Ireland and Roche, 1982).

Prostaglandins are often given in combination with pregnant mares' serum gonadotrophin (PMSG) to evoke a superovulation response in farm animals (Phillipo and Rowson, 1975; Dieleman and Bevers, 1987; Gallo *et al.*, 1992). We recently reported the effects of PMSG on the number and the micromorphology of antral follicles in heifers (van den Hurk *et al.*, 1992; van den Hurk and Dijkstra, 1992). In these studies, atypical granulosa cells could be morphologically distinguished from normal granulosa cells. On the basis of ultrastructural findings, we hypothesized that the presence of atypical granulosa cells in follicles hitherto called nonatretic was associated with the onset of follicular atresia. Information on the possible involvement of atypical granulosa cells in follicular atresia can be extended by more detailed studies on the occurrence of these cells in atretic and nonatretic follicles, including the preovulatory-sized ones that develop after prostaglandin-induced luteolysis.

The present study on bovine antral follicles was carried out to obtain more information about micromorphology, number and size of these follicles after luteolysis induced by the $\text{PGF}_{2\alpha}$ analogue luprostiol. In this respect, special attention was paid

to the presence of atypical granulosa cells in antral follicles to obtain more information to assess the hypothesis that these cells are markers of follicular atresia.

Materials and Methods

Animals

Experiments were carried out from October to December 1991 with Dutch Friesian heifers ($n = 13$; 450–500 kg) and cows ($n = 13$; 600–750 kg). Housing and detection of oestrus were as described by Dieleman *et al.* (1983). The time when animals allowed mounting on at least two consecutive occasions was regarded as the onset of oestrus and the day on which this occurred was defined as day 0. Luteal function was assessed by determination of the progesterone concentration in the peripheral blood according to the method described by Dieleman and Bevers (1987). Animals used in these studies had 8–10 nmol progesterone l^{-1} in the peripheral blood on day 10 (that is, during the midluteal stage) of the oestrous cycle. At day 10, three heifers (14–16 months of age) and three cows (4–6 years of age) were injected with 15 mg of the prostaglandin analogue, luproliol (Prosolvin; Intervet), whereas another five heifers and five cows were injected at day 11 with the same compound. All ovaries were collected by ovariectomy at day 12, i.e. 48 h or 24 h after prostaglandin treatment, respectively. Ovaries obtained from five untreated heifers and five untreated cows on day 12 of the oestrous cycle served as controls. Previous studies (P. L. A. M. Vos, unpublished) showed that oestrus occurs 3 days after prostaglandin injection at the midluteal phase of cows.

Histology

Ovaries from heifers were fixed in Bouin's fluid, dehydrated in a graded series of ethanol and embedded in paraffin wax. These ovaries were sectioned on a microtome. From cow ovaries, all follicles ≥ 3 mm were excised, measured and then processed for routine histological examination as described above. Serial sections, 5 μm thick, were stained with periodic acid–Schiff–haematoxylin. All antral follicles ≥ 3 mm were counted and micromorphologically evaluated, following criteria described by van den Hurk *et al.* (1992). In addition, the number of atypical granulosa cells was established in at least three cross-sections through each follicle, including the largest cross-section; the number of atypical granulosa cells per cross-section in nonatretic and atretic follicles was compared. Antral follicles were allocated to two size categories according to diameter (3–5.9 mm and ≥ 6 mm). The diameter of each follicle was calculated according to Choudary *et al.* (1968). Values were corrected for 10% shrinkage according to Krup and Dieleman (1982). In each size category, mean numbers of a specific type of follicle were calculated; the data for treated and untreated animals were then compared.

Statistical analysis

The χ^2 test for k independent samples (Siegel, 1959) was applied to compare the numbers of atypical granulosa cells in nonatretic, lightly atretic and definitely atretic follicles

≥ 3 mm. The nonparametric Kruskal–Wallis one-way ANOVA followed by the Mann–Whitney U test (Siegel, 1959) were used to compare data on the number of nonatretic and atretic follicles in corresponding size categories. The level of statistical significance was set at $P < 0.05$.

Results

Atypical granulosa cells, characterized by their elongated shape and oblong nucleus were found in nonatretic, lightly atretic and definitely atretic follicles (Fig. 1a–c) both in untreated and prostaglandin-treated animals. In nonatretic and lightly atretic follicles atypical granulosa cells were found in the basal area of the granulosa. In definitely atretic follicles, they were also found in more apical regions. In heavily atretic follicles, the granulosa had reduced to a single layer or few layers of cells in which atypical granulosa cells were absent (Fig. 1d). The numbers and percentages of nonatretic, lightly atretic and definitely atretic follicles ≥ 3 mm without (0), with small (1–5), with moderate (6–50) and with large (> 50) numbers of atypical granulosa cells per cross-section through a particular follicle are given (Table 1). Data from untreated and treated heifers and cows are combined. The results show that the proportion of follicles with large numbers of atypical granulosa cells is higher in the category of definitely atretic follicles than in nonatretic follicles ($P < 0.01$), and follicles in the lightly atretic category were intermediate.

Neither the mean number of medium-sized (3.0–5.9 mm) antral (nonatretic plus atretic) follicles nor that of large (≥ 6 mm) antral follicles in prostaglandin-treated heifers (Table 2) and cows (Table 3) was significantly different from that of untreated animals. In heifers, however, the mean number of medium-sized nonatretic follicles was significantly enhanced at 24 h after prostaglandin treatment, when compared with those at 48 h (Table 2). This was due to the development of at least one medium-sized nonatretic follicle without atypical granulosa cells in each heifer. These latter medium-sized follicles were absent in both heifers and cows at 48 h after prostaglandin treatment; however, all of these animals had one large nonatretic (preovulatory-sized) follicle that lacked atypical granulosa cells (Fig. 1e). The mean numbers of medium-sized atretic (lightly atretic plus definitely atretic plus heavily atretic) follicles in prostaglandin-treated heifers (Table 2) and cows (Table 3) was not significantly different from that of the corresponding control animals. When atretic follicles were subdivided, on the basis of the extent of atresia, prostaglandin-induced luteolysis had not influenced the mean number of medium-sized lightly atretic, definitely atretic or heavily atretic follicles in both heifers and cows. This was also the case for the large atretic follicles of cows. In heifers, however, the mean number of large atretic follicles had increased at 24 h after prostaglandin treatment, owing to increased numbers of definitely atretic follicles. At 48 h after prostaglandin treatment, these values had returned to control values.

The mean size of the largest antral follicle (either nonatretic or atretic), the largest atretic follicle, the largest nonatretic follicle (either with or without atypical granulosa cells) and the largest nonatretic follicle without atypical granulosa cells in the ovaries of untreated and prostaglandin-treated animals are

Fig. 1. Photomicrographs of sections of antral follicles. Periodic acid–Schiffs (PAS)–haematoxylin. Part of (a) a nonatretic follicle with an atypical granulosa cell (arrow), (b) a lightly atretic follicle with a few atypical granulosa cells (arrows) and pyknotic nuclei (arrowheads), (c) a definitely atretic follicle with numerous atypical granulosa cells (arrows) and pyknotic nuclei, (d) a heavily atretic follicle, and (e) a preovulatory-sized follicle. Note the absence of atypical granulosa cells. Bars represent 10 µm. G: granulosa; T: theca interna; L: follicular lumen; BL: basal lamina.

shown (Table 4). The mean size of the largest antral follicle did not change after prostaglandin treatment. The mean size of nonatretic follicles, on the contrary, was enhanced and was 6.5 and 8.4 mm within 2 days after prostaglandin treatment in heifers and cows, respectively. In this period, the mean size of the largest atretic follicle decreased 3.2 and 9.3 mm in heifers and cows, respectively. The mean diameter of nonatretic follicles without atypical granulosa cells was 8.7 and 10.4 mm larger in heifers and cows, respectively, at 48 h after PG

Table 1. Numbers and percentages (%) of nonatretic, lightly atretic and definitely atretic follicles ≥ 3 mm with 0, 1–5, 6–50 or > 50 atypical granulosa cells per cross-section in cattle ($n = 26$)

Number of atypical granulosa cells	Follicle category		
	Nonatretic (%)	Lightly-atretic (%)	Definitely-atretic (%)
0	24 (40.0)	5 (5.0)	0 (0.0)
1–5	18 (30.0)	15 (14.9)	8 (7.4)
6–50	18 (30.0)	60 (59.4)	52 (47.7)
> 50	0 (0.0)	21 (20.7)	49 (44.9)

Table 2. The effect of the prostaglandin analogue luproliol (PG) at 24 h and 48 h after injection on the mean number ($\pm$ SEM) of medium-sized (3.0–5.9 mm) and large (≥ 6 mm) ovarian follicles in heifers

Follicles	Heifers		
	Untreated	PG 24 h	PG 48 h
Antral (3–5.9 mm)	9.4 (3.3)	10.0 (1.5)	8.7 (3.9)
Nonatretic	1.8 (1.0)	2.6 (1.5) ^b	0.0 (0.0)
NA – AGCs	0.2 (0.2)	1.2 (0.2) ^b	0.0 (0.0)
NA + AGCs	1.4 (0.9)	1.4 (0.5)	0.0 (0.0)
Atretic	7.6 (3.6)	7.4 (1.4)	8.7 (3.9)
LA	3.2 (2.7)	2.4 (0.9)	2.3 (1.8)
DA	1.2 (0.7)	2.8 (0.8)	4.0 (1.5)
HA	3.2 (1.2)	2.2 (0.7)	2.3 (0.7)
Antral (≥ 6 mm)	3.0 (1.0)	8.0 (1.8)	2.0 (0.6)
Nonatretic	0.4 (0.2)	0.6 (0.4)	1.0 (0.0)
NA – AGCs	0.0 (0.0)	0.2 (0.2)	1.0 (0.0) ^a
NA + AGCs	0.4 (0.2)	0.4 (0.4)	0.0 (0.0)
Atretic	2.6 (1.1)	7.4 (1.6) ^{a,b}	1.0 (0.6)
LA	2.0 (1.3)	2.0 (0.8)	0.3 (0.3)
DA	0.6 (0.4)	4.6 (1.5) ^{a,b}	0.0 (0.0)
HA	0.0 (0.0)	0.6 (0.4)	0.7 (0.3)

NA – AGCs: nonatretic follicles without atypical granulosa cells; NA + AGCs: nonatretic follicles with atypical granulosa cells; LA: lightly atretic follicles; DA: definitely atretic follicles; HA: heavily atretic follicles.

^{a,b}Values significantly ($P < 0.05$) different from untreated heifers, and heifers at 48 h after luproliol treatment, respectively.

treatment compared with that from untreated animals. The largest increase in mean diameter of nonatretic follicles without atypical granulosa cells was observed on the first day after prostaglandin treatment in cows, and at the second day in heifers.

Discussion

In ovaries of heifers and cows at 48 h after PG treatment, that is, from 1 day before oestrus, the largest follicle is nonatretic, lacks atypical granulosa cells and is the only nonatretic follicle present in the class of follicles larger than 6 mm. Quirck *et al.* (1986) demonstrated that, in prostaglandin-treated heifers, the

Table 3. The effect of the prostaglandin analogue luprostitol (PG) at 24 h and 48 h after injection on the mean number ($\pm$ SEM) of medium-sized (3.0–5.9 mm) and large (≥ 6 mm) ovarian follicles in cows

Follicles	Cows		
	Untreated	PG 24 h	PG 48 h
Antral (3–5.9 mm)	10.2 (1.9)	11.4 (2.9)	6.0 (1.7)
Nonatretic	1.6 (0.5)	1.6 (0.7)	0.0 (0.0)
NA – AGCs	0.6 (0.2)	0.6 (0.4)	0.0 (0.0)
NA + AGCs	1.0 (0.4)	1.0 (0.4)	0.0 (0.0)
Atretic	8.6 (1.8)	9.8 (2.5)	6.0 (1.7)
LA	2.0 (0.6)	4.8 (1.0)	2.3 (1.3)
DA	3.6 (0.7)	2.4 (1.2)	2.7 (0.9)
HA	3.0 (1.0)	2.6 (1.6)	1.0 (0.6)
Antral (≥ 6 mm)	2.0 (0.5)	3.8 (1.1)	1.7 (0.8)
Nonatretic	0.4 (0.2)	1.0 (0.3)	1.0 (0.0)
NA – AGCs	0.2 (0.2)	0.8 (0.2)	1.0 (0.0) ^a
NA + AGCs	0.2 (0.2)	0.2 (0.2)	0.0 (0.0)
Atretic	1.6 (0.4)	2.8 (0.9)	0.7 (0.6)
LA	0.2 (0.2)	0.6 (0.6)	0.3 (0.3)
DA	1.2 (0.2)	1.2 (0.6)	0.3 (0.3)
HA	0.2 (0.2)	1.0 (0.5)	0.0 (0.0)

NA – AGCs: nonatretic follicles without atypical granulosa cells; NA + AGCs: nonatretic follicles with atypical granulosa cells; LA: lightly atretic follicles; DA: definitely atretic follicles; HA: heavily atretic follicles.

^aValue significantly ($P < 0.05$) different from untreated cows.

Table 4. Mean diameter (mm $\pm$ SEM) of the largest antral (atretic and nonatretic), the largest atretic, the largest nonatretic (those with and those without atypical granulosa cells; AGCs) and the largest nonatretic follicle without atypical granulosa cells (NA – AGCs) in untreated heifers and cows and in animals at 24 h and 48 h after prostaglandin (PG) treatment

Follicles	Antral	Atretic	Nonatretic	NA – AGCs
Heifers				
Untreated	11.8 (1.2)	11.8 (1.2)	4.8 (1.6)	2.6 (0.5)
PG 24 h	12.1 (1.5)	12.1 (1.5)	5.5 (0.8)	4.4 (0.9)
PG 48 h	11.3 (1.3)	8.6 (2.9)	11.3 (1.3)	11.3 (1.3)
Cows				
Untreated	13.7 (0.7)	13.7 (0.7)	5.6 (1.4)	3.6 (0.7)
PG 24 h	14.3 (1.6)	10.1 (1.9)	10.7 (2.6)	10.7 (2.6)
PG 48 h	14.0 (1.8)	4.4 (1.2)	14.0 (1.8)	14.0 (1.8)

largest follicle present at 1 day before oestrus is the follicle that ovulates. Consequently, in cattle, absence of atypical granulosa cells in the largest nonatretic follicle, present at 48 h after prostaglandin treatment, can be considered a characteristic for this preovulatory-sized follicle and thus for follicle quality. The presence of atypical granulosa cells as markers for a lower quality follicle is confirmed by the observation that the proportion of follicles with large numbers of atypical granulosa cells is higher in the category of definitely atretic follicles than in nonatretic follicles. This finding clearly points to an association of atypical granulosa cells with the process of follicular

atresia and corresponds to previous observations, showing the coincidence of the presence of atypical granulosa cells with the occurrence of degenerative changes in the cytoplasm of nearby granulosa cells (van den Hurk *et al.*, 1992). Because of the presence of atretic granulosa cells, we hypothesize that a great part of the population of nonatretic follicles, known as non-atretic according to current histological criteria, is of suboptimal quality. Cells that are histologically similar to atypical granulosa cells have been demonstrated in cows (Lobel and Levy, 1968; Priedkalns *et al.*, 1968; McKenzie and Kenney, 1973; Monniaux *et al.*, 1984) and in various other mammals (Clark, 1923; Ingram, 1962; Horowitz, 1967; Koering, 1969; Brand and de Jong, 1973), but only in follicles at an advanced stage of atresia.

Administration of prostaglandin during the mid-luteal stage of the oestrous cycle initially leads to a sharp increase in the number of definitely atretic follicles larger than 6 mm in heifers, but not in cows. In the present study, contrary to body weights, injected doses of luprostitol were the same for heifers and cows. The atresia promoting effect of prostaglandin may thus be due to the relatively high doses administered to heifers. An increase of follicle atresia after prostaglandin treatment of heifers was also observed by Cheremisinov *et al.* (1986), when a relatively high dose of cloprostenol was injected. In sheep, the appearance of a widespread increase in atresia in follicles > 1 mm after prostaglandin (cloprostenol) injection, is accompanied by the emergence of a preovulatory follicle (McNatty *et al.*, 1982). In all heifers at 24 h after injection of prostaglandin increased numbers of atretic follicles ≥ 6 mm are generally accompanied by the presence of one medium-sized nonatretic follicle without atypical granulosa cells. This nonatretic follicle is probably the selected preovulatory follicle, as 48 h after prostaglandin treatment, all heifers have only one large preovulatory-sized follicle, in which atypical granulosa cells are absent.

Development of relatively large numbers of large follicles in heifers at 24 h after luprostitol treatment may be attributed to an indirect effect of the injected compound, as injection of prostaglandin evoked reduced progesterone concentrations that resulted in enhanced gonadotrophin concentrations in the circulation of farm animals, including cattle (Hansel and Convey, 1983; Jöchle *et al.*, 1987). In consequence, a number of follicles are rapidly recruited from the pool of medium-sized follicles, from which one is chosen to ovulate, and the remainder become atretic. Ko *et al.* (1991) showed that, in cows, the selected preovulatory follicle caused regression of its subordinate follicles. This dominant follicle may influence the development of other follicles, either directly by secretion of an unknown paracrine substance, or indirectly by inhibition of the FSH support to follicles, via negative feedback action of oestradiol and inhibin produced (Driancourt, 1991). Follicular regression is generally accompanied by follicular shrinkage (Rajakoski, 1960; Ingram, 1962; Marion *et al.*, 1968; Byskov, 1978), and in heifers may come to 4–7 mm per day after prostaglandin treatment, if diameters are determined by measuring dissected follicles (Quirck *et al.*, 1987). This rapid decrease in the size of nonovulatory follicles explains the strong decline in the number of large atretic follicles in heifers between 24 and 48 h after prostaglandin treatment.

In cows, the preovulatory-sized follicle appears to grow extremely quickly after injection of prostaglandin. The increase in diameter of this follicle amounts to 6.5 mm and over 8 mm for heifers and cows, respectively. If it is assumed that only a nonatretic follicle without atypical granulosa cells becomes a preovulatory-sized follicle, the mean diameters of the largest follicles of this type have to be compared. In that case, growth of the preovulatory-sized follicle within 48 h after PG treatment is almost 9 and over 10 mm for heifers and cows, respectively. These data support previous data of Dieleman *et al.* (1986), who established, by rectal palpation of a few cows, that a preovulatory follicle may grow from less than 8 mm to ovulatory size (18–20 mm) in about 2.5 days before the LH surge, thus during the period of luteal regression. The data are also in agreement with findings of Matton *et al.* (1981), who showed that destruction of all follicles larger than 5 mm at day 18 did not delay oestrus, indicating that follicles were capable of growing from <5 mm to ovulatory size within several days. On the basis of ultrasonic measurements, Quirck *et al.* (1987) observed a mean increase of 4–6 mm in diameter of preovulatory follicles in prostaglandin-treated heifers during the 3 days before oestrus. In their studies, however, diameters of follicles determined by ultrasonography were consistently 2–3 mm smaller than diameters of dissected follicles. In cows, the greatest growth of nonatretic follicles was observed at the first day after prostaglandin treatment, whereas, in heifers, the same phenomenon was observed one day later. In addition, compared with heifers, cows show a greater decrease in the size of atretic follicles at the second day after progesterone treatment. These findings suggest that luteolysis proceeds at a slower rate in heifers than in cows, and this confirms the observations of Dieleman *et al.* (1986).

In conclusion, in cows, atypical granulosa cells can be considered as markers for a lower quality follicle, based on their absence in preovulatory-sized follicles and their presence in large numbers in a high proportion of definitely atretic follicles. After prostaglandin-induced luteolysis, the mean number of medium-sized and large nonatretic follicles with atypical granulosa cells and those of atretic follicles in heifers and cows were not different from untreated animals. However, in heifers, contrary to cows, the development of a preovulatory-sized follicle is initially accompanied by an increase in the number of large definitely atretic follicles. Within 2 days after prostaglandin treatment, the mean diameter of a preovulatory-sized follicle increases almost 9 and over 10 mm in heifers and cows, respectively.

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