Follicular growth and regression during the 8 days after hypophysectomy in sheep

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Summary. Ewes were hypophysectomized on Day 0 and ovariectomized 1, 2, 4 or 8 days later. There was no effect of hypophysectomy on the overall population of follicles > 0.8 mm in diameter during the time studied. However, the growth of healthy follicles > 2 mm in diameter was prevented by Day 2. Turnover of follicles was very active in the ovaries of hypophysectomized ewes as shown by peaks in the proportion of follicles in early atresia at Day 4 and in advanced atresia at Days 1 and 8. By Day 8, most of the measures of the population of follicles 0.8 to 2 mm in diameter were back to the values of Day 0 ewes. The mitotic index of the granulosa cells of the healthy follicles exhibited a similar pattern with a nadir at Day 2 followed by a return at Days 4 and 8 to values similar to Day 0 ewes.

Ink-marked preovulatory follicles underwent a steady decrease in their histological size after hypophysectomy and this was associated with time-related changes in the health status of these follicles. By Day 1, 4 out of 7 follicles were still healthy while at Days 2, 4 and 8, all follicles were in advanced, late and collapsing atresia respectively. There was no evidence of an ability of PMSG (1000 i.u.) to rescue large follicles in advanced atresia (48 h after hypophysectomy). Furthermore, at 24 h after hypophysectomy, only 2 out of 5 follicles were maintained. However, PMSG partly overcame the depressing effects of hypophysectomy on the population of follicles 0.8 to 2 mm in diameter.

It is concluded that (1) active follicular turnover can occur in the absence of gonadotrophins; (2) it takes about 8 days for a large follicle to disappear in the ovarian stroma; and (3) it is unlikely that rescue of atretic follicles massively contributes to the ovulatory response after PMSG.

Introduction

Atresia is undoubtedly a major feature of folliculogenesis. In sheep, whatever the breed or stage of the oestrous cycle, 50–70% of the population of large antral follicles is atretic (Brand & de Jong, 1973; Turnbull *et al.*, 1977; Cahill *et al.*, 1979). While the morphological and functional changes induced by atresia are well documented (Tsafirri & Braw, 1984), only limited information is available on the time needed for a follicle to disappear by atresia in large domestic animals. This has been due not only to the difficulty in following individual follicles for extended periods of time but also to the need for a precise definition of the start of the atretic process. These difficulties can be alleviated by monitoring the kinetics of individual follicles with ink labelling after hypophysectomy. Repeated ink labelling of individual follicles can be used to follow the time-related regression of these follicles (Driancourt & Cahill, 1984). Hypophysectomy has been shown to induce atresia in most of the large follicles in rodents (hamster: Taya & Greenwald, 1980; rat: Braw *et al.*, 1981) and in sheep (Dufour *et al.*, 1979). Therefore the first aim of this study was to follow the regression of large preovulatory follicles after induction of atresia by hypophysectomy.

The histological features of sheep ovaries have only been studied at 4 and 70 days after hypophysectomy (Dufour *et al.*, 1979). The second aim of this study was therefore to investigate the short-term changes induced by hypophysectomy in the proportion of healthy follicles and granulosa cell proliferation.

Rescue of atretic follicles by PMSG has been demonstrated in rodents (mice: Peters, 1979; Byskov, 1979) but no clear evidence of a similar process has been obtained in large animals (Dott *et al.*, 1979). As the progress of atresia was synchronized by hypophysectomy, the last aim of this work was to observe whether follicles at known stages of atresia could be rescued by PMSG.

Materials and Methods

Experiment I: dynamics of atresia after hypophysectomy. During the breeding season, oestrus in 20 crossbred ewes (Border Leicester × Merino) was synchronized by the insertion of intravaginal progestagen-impregnated sponges (Repromap: Upjohn, Kalamazoo, MI, U.S.A.). At 30–36 h after sponge removal and before oestrus was detected, ewes were anaesthetized by an intravenous injection of thiopentone sodium (Intraval: May and Baker, Dagenham, U.K.) followed by maintenance with a halothane–oxygen mixture. During a mid-ventral laparotomy, the large pre-ovulatory follicle(s) were measured and marked with dots of ink in the ovarian stroma surrounding them (Driancourt & Cahill, 1984). Immediately after the laparotomy, the ewes were hypophysectomized using the transnasal, trans-sphenoidal approach (Clarke *et al.*, 1983). On each of Days 1, 2, 4 and 8 after hypophysectomy, 4 ewes were ovariectomized following careful measurement of the size of the marked follicles. The 4 control ewes were ovariectomized when the ovaries contained follicles at the preovulatory stage and are referred as Day 0 ewes.

All ewes received daily glucocorticoid replacement therapy (Dexadrenon: Intervet, Cambridge, U.K.) until slaughter. To assess the completeness of hypophysectomy, ewes were given a challenge of GnRH after ovariectomy, bled every 15 min for 2 h starting immediately before the challenge and gonadotrophin concentrations were measured (Lee *et al.*, 1976).

After ovariectomy, the marked follicles were carefully dissected and the isolated follicles and the ovaries were fixed in Bouin's solution, serially sectioned at a 10 µm thickness and one section out of 10 was mounted and stained with Feulgen's stain (Cahill *et al.*, 1979). Follicles >0.8 mm in diameter, i.e. those mostly affected by hypophysectomy according to Dufour *et al.* (1979), were counted and checked for atresia. Normal follicles were defined as having fewer than 5 pycnotic bodies along or amongst the granulosa layer. Five stages of atresia were defined. Early atretic stage 1 follicles had 5–100 pycnotic bodies mostly amongst the granulosa cells with still some granulosa cells undergoing mitosis. Early atretic stage 2 follicles had 100–200 pycnotic bodies, mostly along the antrum, and mitotic activity of the granulosa cells was markedly decreased. Advanced atretic stage 1 follicles had numerous pycnotic bodies in a distinct granulosa layer. Advanced atretic stage 2 follicles had numerous pycnotic bodies in a vanishing granulosa layer. Late atretic follicles had no granulosa cells apart from around the oocyte.

The mitotic index of the granulosa cells of healthy follicles was calculated as described by Cahill *et al.* (1985).

Experiment II: rescue of atretic follicles by PMSG. During the late breeding season, and 3 weeks after Exp. I, the oestrous cycles of 8 crossbred (Border Leicester × Merino) ewes were synchronized by progestagen sponges (Repromap: Upjohn). As in Exp. I, 30–36 h after sponge removal, ewes had their preovulatory follicle(s) measured and marked, they were then hypophysectomized. At 24 or 48 h after hypophysectomy all ewes (4/group) received an injection of 1000 i.u. PMSG (Folligon: Intervet) and after a further 48 h they were ovariectomized.

Post-operative care of the hypophysectomized ewes and histological techniques were similar to those for Exp. I.

Statistical methods. Because of the limited number of ewes per group precluding assessment of normality of distributions, non-parametric testing (Siegel, 1956), mainly the Kruskal–Wallis one-way analysis of variance and the Mann–Whitney U test, was used.

Results

The lack of an LH/FSH increase after the GnRH challenge demonstrated that hypophysectomy was complete in all ewes.

Experiment I

Short-term changes in folliculogenesis after hypophysectomy. There was no effect of treatment on the overall total (healthy + atretic) population of follicles >0.8 mm in diameter (Table 1). In contrast, there was a clear difference in their size distribution since the total number of follicles >2 mm in diameter underwent a steady decrease from Day 0 to Day 8 (Day 0 vs Day 8, $P < 0.02$) (Table 1).

Table 1. Time-related changes in the number, health status and mitotic index of the follicles between Day 0 and 8 after hypophysectomy

	Days after hypophysectomy				
	0	1	2	4	8
Total no. of follicles > 2 mm diam.	2.7 ± 1.6	2.3 ± 1.5	1.6 ± 0.5	2.0 ± 0.8	0
Proportion of healthy follicles > 2 mm (%)	54.5 ± 23	43.7 ± 51.5	0	0	—
Total no. of follicles > 0.8 mm diam.	13.4 ± 7.0	9.7 ± 3.0	9.5 ± 1.3	14.2 ± 4.3	8.6 ± 2.1
Proportion of healthy follicles > 0.8 mm (%)	26.5 ± 9.5	23.6 ± 9.2	17.7 ± 9.4	35.7 ± 16.1	35.0 ± 6.2
Mitotic index of healthy follicles > 0.8 mm (%)	-1.08 ± -0.29	-1.01 ± -0.22	-0.75 ± 0.07	-0.99 ± -0.37	-1.37 ± 0.62

Furthermore, amongst these follicles, the proportion of healthy follicles, while unaffected at Day 1, was dramatically reduced at Days 2, 4 and 8 after hypophysectomy (Table 1). The proportion of healthy follicles > 0.8 mm in diameter also changed with time, decreasing to reach a nadir at Day 2 after hypophysectomy (Day 0 vs Day 2, $P < 0.05$) then increasing again, although not significantly (Table 1). Time-related changes were also detected amongst the atretic follicles (Fig. 1). A significant increase in the proportion of atretic follicles in early atresia was found only at Day 4 (Day 4 vs Day 1, $P = 0.05$). In contrast to early atretic follicles, hypophysectomy induced rapid changes in the proportion of atretic follicles in advanced atresia which peaked at Day 1 (Day 1 vs Day 0, $P < 0.05$) and again, although not significantly, at Day 8. There was no significant day effect on the proportion of atretic follicles in late atresia.

Examination of the changes in the mitotic index of the granulosa cells of healthy follicles > 0.8 mm in diameter (Table 1) revealed a steady decline from Day 0 to Day 2 (Day 2 vs Day 0, $P < 0.01$) followed by a return to control values at Days 4 and 8. At Day 8 after hypophysectomy, therefore, granulosa cells were dividing as actively as in control ewes.

Dynamics of regression of the preovulatory follicles. The mean size *in vivo* of the preovulatory follicles at the first laparotomy was 7.6 ± 1.0 mm ($n = 30$). None of these preovulatory follicles ovulated following hypophysectomy and at Day 1 after hypophysectomy, their size was reduced to 5.7 ± 1.0 mm ($n = 7$) with further decreases in size, reaching 5.5 ± 1.1 mm ($n = 8$) and 3.3 ± 0.9 mm ($n = 7$) at Days 2 and 4 after hypophysectomy. By Day 8 after hypophysectomy, follicles were no longer visible on the ovarian surface. These estimates were confirmed when the follicles were measured at histological examination. From 5.0 ± 0.5 mm in Day 0 ewes, the size of the marked follicles decreased to 4.2 ± 0.9 , 4.3 ± 0.5 , 3.4 ± 0.6 and 1.1 ± 0.3 mm at Days 1, 2, 4 and 8 after hypophysectomy, respectively.

There were also time-related changes in the health status of the marked follicles. While all the follicles of the Day 0 ewes were healthy, with a mean mitotic index of $0.37 \pm 0.25\%$ at the time of sampling, by Day 1, 4 out of the 7 marked follicles were still healthy with a mean mitotic index of $0.28 \pm 0.16\%$. By Days 2 and 4, respectively, all the follicles were in advanced atresia stage 1 and in late atresia respectively. Finally, by Day 8, all the marked follicles were invaded by fibroblasts and disappearing in the ovarian stroma.

Experiment II

Rescue of atretic follicles by PMSG. One of the ewes given PMSG 24 h after hypophysectomy died. From a mean initial size of 7.3 ± 0.5 mm ($n = 10$) at the first laparotomy, the size of the

Fig. 1. Time related changes in the proportion of atretic follicles in early, advanced and late atresia during the 8 days after hypophysectomy in sheep.

marked follicles at ovariectomy was 7.5 ± 2.6 mm ($n = 5$) for the ewes given PMSG 24 h after hypophysectomy and 3.8 ± 1.8 mm ($n = 5$) for the ewes receiving PMSG 48 h after hypophysectomy, a value similar to the size of Day 4 follicles in Exp. 1. However, there were large within-group variations in the changes of size and health status of the follicles. For the ewes given PMSG 24 h after hypophysectomy, 2 follicles had decreased in size and were in advanced atresia stage 2 when sampled, 3 follicles had increased in size and were normal, with a mean mitotic index of 0.3% (2 follicles), or partly cystic and luteinized (1 follicle). Most of the follicles of the ewes given PMSG 48 h after hypophysectomy had undergone marked regressive changes and were in late atresia (4 follicles). However, one follicle of this group was still healthy although there were no mitotic divisions amongst the granulosa cells.

When the overall population (healthy + atretic) of follicles >0.8 mm in diameter was compared between Day 0 ewes of Exp. 1 and PMSG-treated ewes, PMSG had not increased this population at the time of ovariectomy (24 h, 9.0 ± 1.7 ; 48 h, 10.7 ± 5.8). Furthermore, PMSG treatment at 24 or 48 h after hypophysectomy only marginally increased the proportion of healthy follicles in this population (Day 0 ewes, 26.5 ± 9.5 , vs 24 h group, 31.6 ± 24.8 , vs 48 h group, 39.2 ± 28.8), mainly through a limited reduction in the extent of early atresia. Finally, PMSG treatment at 24 or at 48 h after hypophysectomy had not altered the mitotic index of the granulosa cells of healthy follicles >0.8 mm in diameter 48 h afterwards (Day 0 ewes, $1.08 \pm 0.29\%$; 24 h group, 1.03 ± 0.33 ; 48 h group, $1.22 \pm 0.74\%$).

Discussion

This study confirms and extends the results previously obtained in hypophysectomized ewes (Dufour *et al.*, 1979) by showing (1) that follicles >2 mm in diameter are acutely dependent on gonadotrophins; (2) that follicular turnover (i.e. growth and atresia) in follicles <2 mm in diameter is active in hypophysectomized ewes; and (3) that follicles in which atresia had already begun were not rescued by PMSG.

After hypophysectomy, there was a steady decline in the number of follicles larger than 2 mm so that by Day 2 after hypophysectomy there was no healthy follicle in this size range and, by Day 8, no follicles larger than 2 mm remained. That follicular growth over 2 mm is absent when gonadotrophin concentrations are not detectable confirms the results of previous studies after hypophysectomy (Dufour *et al.*, 1979) or after active immunization against GnRH (McNeilly *et al.*, 1986). Hence 2 mm, the size at which the amount of FSH receptors on the granulosa cells and of LH receptors on the thecal layer is maximum (Carson *et al.*, 1979) and at which aromatase activity starts to increase (Tsonis *et al.*, 1984a), is a key stage in folliculogenesis, separating a basal follicular growth, at least partly gonadotrophin-independent, from a tonic follicular growth acutely dependent on gonadotrophins. Extrapolation of these concepts of basal and tonic folliculogenesis to rats from the data on follicular populations in hypophysectomized animals (Paesi, 1949; Ingram, 1953) suggests that the size limit between basal and tonic follicular growth coincides with the large pre-antral stage in rats. In rats and sheep, recruitment of follicles for terminal follicular growth involves only follicles exceeding this size (Hirshfield & Midgley, 1978a,b; Driancourt & Cahill, 1984; Tsonis *et al.*, 1984b). Indeed, it has been demonstrated that recruitment is mediated by gonadotrophins (see review by Driancourt *et al.*, 1985b).

In contrast to this lack of growth of follicles over 2 mm in diameter in hypophysectomized ewes, the follicles that at the time of hypophysectomy were larger than 2 mm had some ability to cope, at least temporarily, with the lack of gonadotrophins. This is demonstrated by the fact that, at 1 day after hypophysectomy, 4 out of the 7 marked follicles were still healthy and 3 out of the 5 follicles given PMSG 24 h after hypophysectomy did not exhibit atresia 48 h afterwards. The ability of the largest follicles to remain healthy when FSH concentrations are low has previously been demonstrated for the preovulatory follicle of the mare (Palmer, 1987), the mid-cycle follicle in cattle (M. A. Driancourt & W. W. Thatcher, unpublished results) and the preovulatory follicles of the Booroola strain of Merino sheep (Driancourt *et al.*, 1985a). Another interesting feature of these follicles >2 mm in diameter is the between-follicle heterogeneity in their behaviour 1 day after hypophysectomy: 60% were healthy and 40% were in advanced atresia. This heterogeneity in the fate of the follicles after withdrawal of gonadotrophin support has also been described for barbiturate-blocked rats (Braw & Tsafiriri, 1980), since ovulatory efficiency in response to hCG decreased from 88 to 70, 52 and 31% after 1, 2, 3 and 4 days of blockade. This might be linked to the between-follicle balance in the different subtypes of granulosa cells (Erickson *et al.*, 1985; Kasson *et al.*, 1985).

The progression of atresia in these large follicles was such that size was initially decreased through a reduction in the accumulation of follicular fluid since the mitotic index of the granulosa cells was unchanged 1 day after hypophysectomy. Thereafter (between Days 1 and 2) degenerative changes appeared in the granulosa cells. This was followed by a gradual reduction in the number of granulosa cells and antrum size resulting in a follicle disappearing in the ovarian stroma at Day 8 after hypophysectomy. This 8-day period for the total regression of a large follicle can be split as 1 day for the changes from healthy to advanced atretic stage 1, 2 days then being needed to reach late atresia and 4 more days for oblitative atresia. This overall estimation agrees with the observations of Smeaton & Robertson (1971) which showed that ink-injected follicles from cyclic ewes disappeared within 7 days. It might be argued that this 8 days value is an underestimate linked to the use of hypophysectomized animals since it was found that the block of the preovulatory surge of gonadotrophins with barbiturates produced a much slower progression of atresia than hypophysectomy (Braw *et al.*, 1981). However, the observation that the ewes given PMSG 48 h after hypophysectomy had a pattern of regression similar to that of untreated hypophysectomized ewes (i.e. 3.5 mm in diameter and in late atresia 4 days after hypophysectomy), despite provision of exogenous gonadotrophins between Days 2 and 4, shows that the above limitation might not be relevant in sheep. Furthermore, the mean regression rate observed in this study between Days 0 and 4 (1.1 mm/day) is similar to that found in cyclic ewes (1.5 mm/day) (Driancourt & Cahill, 1984).

A very active turnover was found between Days 0 and 8 amongst the follicles <2 mm in

diameter. After an accumulation of advanced atretic follicles at Day 1 and of early atretic follicles at Days 2 and 4, together with a reduction in the mean mitotic index of healthy follicles at Day 2, all these values were back to control values by Day 8. This fits with previous reports for sheep (Dufour *et al.*, 1979) showing that, in long-term hypophysectomized ewes, growth in terms of follicle numbers and mitotic index of the granulosa cells is at least partly maintained. Active division of granulosa cells of hypophysectomized rats has also been demonstrated (Hirshfield, 1985).

The last aim of the study was to investigate whether PMSG had some rescuing ability (Peters, 1979; Byskov, 1979) on follicles in which atresia had been synchronized by hypophysectomy. Such an activity has been suggested as contributing to the increased ovulation rate after PMSG administration, together with an increased recruitment of small follicles for preovulatory enlargement and a blockade in the initiation of atresia (Dott *et al.*, 1979). When PMSG was given 24 h after hypophysectomy, it maintained some follicles (possibly the ones which were still healthy at the time of PMSG injection) for 48 h, demonstrating a protective role from atresia. In contrast, when PMSG was injected at a time (Day 2 after hypophysectomy) when most of the follicles were already in advanced atresia stage 1, PMSG was not able to induce any improvement in the status of the marked follicles. With the timings chosen in this study, it was not possible to check whether early atretic follicles could be rescued or not. Hence, PMSG may have a limited rescuing ability, restricted, at the most, to follicles undergoing early atresia. Therefore, this effect is unlikely to play a major role in the ovarian stimulation observed when high dosages of PMSG are administered.

In conclusion, while the modulatory role of gonadotrophins on folliculogenesis is well documented, the present work establishes that most of the events of growth of follicles up to 2 mm in diameter (granulosa cell division, antrum enlargement) can proceed at least in some follicles in the absence of gonadotrophins. The heterogeneity between follicles in their need for gonadotrophin support might be one of the factors involved in the choice of the ovulatory follicle.

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