Ovarian features contributing to the variability of PMSG-induced ovulation rate in sheep

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Summary. In Exp. 1, ovulation rate was measured in three groups of Romanov ewes given two injections of 600 i.u. PMSG 3 weeks apart with the ewes intact (Group I, N = 8), a similar treatment with the ewes intact at the first injection and unilaterally ovarioctomized at the second (Group II, N = 8), or unstimulated ewes which were hemispayed at the same time as Group II ewes (Group III, N = 6).

In Exp. 2, the follicular population of one ovary was correlated with the number of ovulations induced by 600 i.u. PMSG in the contralateral ovary (10 Romanov ewes).

From 8.4 ± 1.8 (Group I) and 8.2 ± 3.3 (Group II) CL at the first injection, PMSG-induced ovulation rate at the second injection decreased to 3.9 ± 1.8 and 3.7 ± 1.2 in Groups I and II respectively, a value similar for ewes with 1 or 2 ovaries. Furthermore, despite no major changes in the number of antral follicles after the first injection, there was no correlation (r = -0.09) between the response to the two successive injections in intact ewes.

Comparison of the ovarian status of the ovary removed before the PMSG injection (Group II ewes of Exp. 1, ewes of Exp. 2) to the number of CL found in the remaining ovary demonstrated that PMSG-induced ovulation rate was (i) not correlated with the overall antral follicle population (r = 0.62 in Exp. 1, r = 0.49 in Exp. 2), (ii) significantly correlated (r = 0.74, P < 0.05, in Exp. 1; r = 0.85, P < 0.01, in Exp. 2) with the number of healthy follicles 0.8-2.0 mm in diameter, and (iii) negatively correlated with the number of healthy follicles >2 mm in diameter (r = -0.29 in Exp. 1; r = -0.61, P < 0.05 in Exp. 2).

Introduction

Attempts to get accurate control of the numbers of eggs shed after treatment with exogenous gonadotrophins have always been unsuccessful. While some factors of variation have been identified (breed: Saumande et al., 1978; FSH/LH ratio of the gonadotrophin used: Chupin et al., 1984; Murphy et al., 1984; day of the cycle when the stimulation is performed: Phillippo & Rowson, 1975; Sreenan et al., 1978) and while the mechanisms of PMSG action have been clarified (Dott et al., 1979), our understanding of the factors responsible for the great variability in gonadotrophin-induced ovulation rate is still very limited. In cattle, this variability was linked to the number of follicles in the growth phase (Monniaux et al., 1983). It was, however, surprising that no improvement of the correlation value linking follicle numbers and ovulation rate was noted when medium to large healthy follicles (i.e. the most likely to be involved in the response) were considered. The present work was undertaken to examine whether (i) the number of corpora lutea after administration of exogenous gonadotrophins was closely related to quantitative features (total number of follicles) of the ovarian follicular population, and (ii) qualitative characteristics of the follicular population (follicular size distribution, extent of atresia, presence of large healthy/atretic follicles) could modulate the ovarian response.
Animals and experimental design

Exp. 1: effects of repeated stimulations and of unilateral ovariectomy on PMSG-induced ovulation rate. During the late breeding season (January–February), at the INRA experimental farm of Grignon, two successive treatments for control of oestrus were performed with fluorogestone-impregnated sponges (Intervet, Angers, France) inserted for 14 days to 22 mature (3–5 years old) Romanov ewes. On the day before sponge removal, ewes were randomly allocated to three groups. Group I ewes (N = 8) underwent a first intramuscular PMSG injection (600 i.u. Chronogest: Intervet) on the day before sponge removal and a further similar injection (600 i.u.) at the same time of the second synchronization treatment. The interval between the two injections was 3 weeks. Group II ewes (N = 8) had the same treatment as Group I ewes for their first treatment cycle, but on the day preceding sponge removal of the second synchronized cycle, they were unilaterally ovarioectomized under general anaesthesia (Pentothal: Abbott, Paris, France), and then given PMSG (600 i.u.). Group III ewes (N = 6) acted as controls and were synchronized, but not given PMSG, and unilaterally ovarioectomized at their second synchronized cycle in the same way as Group II ewes.

The dosage of 600 i.u. PMSG was selected because of the high natural ovulation rate of the Romanov breed (about 3) and of the positive relationship between natural and PMSG-induced ovulation rate (Bindon et al., 1986). It proved to be clearly superovulatory (see 'Results'), confirming that high natural ovulation rate may be associated with high sensitivity to gonadotrophins (Bindon et al., 1986). The number of corpora lutea was counted at laparoscopy 6 days after sponge removal.

Exp. 2: correlation between ovarian status before PMSG injection and ovulatory response. During the mid-breeding season (December) at Nouzilly, the oestrous cycles of 10 mature (2–4 years old) Romanov ewes were synchronized with sponges. These ewes were treated in the same way as Group II ewes of Exp. 1 at their second treatment cycle (i.e. unilaterally ovarioectomized and given 600 i.u. PMSG on the day before sponge removal). Histological examination of the ovary removed before stimulation provided information on the ovarian follicular population which was compared with the number of corpora lutea counted at laparoscopy 6 days after sponge removal.

Histological techniques and follicular populations

Immediately after surgery, the ovaries were fixed in Bouin–Holland’s solution, serially sectioned at a thickness of 10 μm, one section out of 6 was mounted, stained with haematoxylin and examined. Antral follicles (i.e. presenting a clear cavity of >1000 μm² among the granulosa cells) were counted and measured using the section containing the oocyte. The area of all the follicles was measured in μm² and then converted to mm, assuming that the follicle was spherical. The follicles were then ranked in five classes as previously defined (Driancourt & Mariana, 1982), each of them coinciding with one of the main events of follicular development (class 1: 0-20–0-32 mm, appearance of antrum; class 2: 0-32–0-50 mm, increase in the mitotic index of the granulosa cells; class 3: 0-50–0-80 mm, appearance of atresia; class 4: 0-80–2-0 mm, plateau at the maximum values of the mitotic index of the granulosa cells; class 5: >2-0 mm, ability of these follicles to undergo preovulatory enlargement).

All follicles were classified as normal or atretic. A follicle was said to be atretic when 5 or more pycnotic bodies were found in the section studied (Brand & de Jong, 1973; Cahill et al., 1979; Driancourt et al., 1985). Three stages of atresia were defined. Early atretic follicles present a few pycnotic bodies (<200) within the antrum or amongst the granulosa cells but the granulosa layer is intact, sometimes featuring mitotic figures. Advanced atretic follicles are extremely pycnotic with the granulosa layer starting to disintegrate into the antrum. Late atretic follicles are devoid of granulosa cells except around the oocyte. Follicles lacking an oocyte or in which the antrum was invaded by fibroblasts were not considered in this study. In all the healthy class 4 and 5 follicles, on a sample of 2000 granulosa cells per follicle, the numbers of cells undergoing mitosis were counted as previously described (Cahill et al., 1985) in the section bearing the oocyte, to obtain an estimate of the mitotic index of the granulosa cells.

Statistical methods

Because of the lack of normality in the distributions of ovulation rate, follicle numbers and mitotic indices, non-parametric testing was used (Siegel, 1956), mainly the Spearman rank correlation coefficient to estimate relationship between parameters, the Mann and Whitney U test to compare means and the Wilcoxon test to compare paired means.

Results

Effect of repeated stimulations and of unilateral ovariectomy on PMSG-induced ovulation rate (Exp. 1)

At the first treatment cycle, the ovulation rate of Group I and II ewes was 8-4 ± 1-8 (s.d.) (range 5–11) and 8-2 ± 3-3 (range 4–14) respectively, while control ewes only had 3-0 ± 0-6 (range 2–4) corpora lutea. At the second treatment cycle, the ovulation rate of the control ewes (now
unilaterally ovariecctomized) was unaffected and there were 2.8 ± 0.8 (range 2-4) corpora lutea. In contrast, three main results emerged when the second stimulation was performed and its results compared to those of the first one: (i) the total number of ovulations of Group II ewes with one ovary (3.7 ± 1.2, range 2-6) was similar to that of Group I ewes with two ovaries (3.9 ± 1.8, range 2-8), (ii) there was no correlation between the responses to the two successive stimulations applied to Group I ewes (r = -0.09), (iii) there was a significant decrease (P < 0.01 for both groups) between the response to the initial stimulation and the later one (8.2 ± 3.3 to 3.7 ± 1.2 and 8.4 ± 1.8 to 3.9 ± 1.8 for Group II and I ewes respectively). To clarify the mechanisms involved in that decrease, the ovarian follicular populations of the ovaries of Group II ewes (which had been stimulated once) were compared with those of the ovaries of Group III ewes (Fig. 1). The mean antral population tended to be reduced after one stimulation (53.8 ± 22.1 vs 85.3 ± 36.0 for Group II and III ewes respectively, P = 0.05). This was linked to a markedly smaller population of follicles of 0.20-0.32 mm in diameter in ewes already stimulated (16.3 ± 9.8 vs 32.2 ± 13.7, P = 0.01). In contrast, there was no significant difference in the total number (healthy + atretic) of follicles in the larger size classes or in the number of healthy follicles 0.8-2.0 mm or >2 mm in diameter (Fig. 1), or in the percentage of atretic follicles in the two largest size classes.

![Graph showing follicle size distribution](image)

**Fig. 1.** Number of follicles per size class in ewes having received PMSG once (■) and in control ewes (□). Atretic follicles are indicated by stippled bars. Values are mean ± s.d.

**Relationships between follicle numbers before stimulation and PMSG induced ovulation rate (Group II ewes of Exp. 1, Exp. 2)**

The ovary of one ewe of Exp. 1 was damaged at surgery and one ewe of Exp. 2 did not ovulate after treatment. Both ewes were discarded from the study.

There was no significant correlation between the total (healthy + atretic) population of antral follicles and ovulation rate (Table 1). Nor was there any correlation between the number of follicles in classes 1, 2 (except in Exp. 2) and 3 and ovulation rate. In contrast, the total number (healthy + atretic) of class 4 follicles was closely related to ovulation rate (P < 0.05 in both studies). Among these class 4 follicles, 59 and 44% were healthy in Exps 1 and 2 respectively. When only healthy
class 4 follicles were considered, an increase in the correlation value was noted ($r = 0.74$, $P < 0.05$ and $r = 0.85$, $P < 0.01$). In contrast, the correlation between atretic class 4 follicles and ovulation rate was more limited and probably linked to the correlation linking healthy and atretic follicles within a class ($r = 0.77$, $P < 0.01$ between healthy and atretic class 4 follicles in Exp. 2). The total number (healthy + atretic) of class 5 follicles was not related to ovulation rate. This was the consequence of a negative correlation between the number of healthy class 5 follicles and ovulation rate together with a positive although non-significant correlation between atretic class 5 follicles and ovulation rate (Table 1).

When the features of the largest follicles were plotted against PMSG-induced ovulation rate, non-significant negative correlations between the diameter of the largest healthy follicle and ovulation rate were found. No consistent trend was found between the diameter of the largest atretic follicle and its stage of atresia and ovulation rate.

Finally, there was a general lack of correlation between the mean mitotic index of healthy class 4 and 5 follicles and ovulation rate ($r = 0.07$ and $-0.39$ respectively).

### Discussion

The aim of this study was to gain insights on the relationships between the ovarian follicular population and the variability of gonadotrophin-induced ovulation rate. This was done using two well-known features of ovarian function. (i) The similarity between ovaries of follicular populations within ewes (sheep: Cahill et al., 1979), together with the lack of short-term effect of unilateral ovariectomy on folliculogenesis (sheep: Dufour & Guilbault, 1984; cattle: Monniaux et al., 1983), was used to establish the relationship between the ovarian status before PMSG injection and the ovarian response to PMSG. (ii) The ability of the remaining ovary to maintain ovulation rate when unilateral ovariectomy is performed far enough from ovulation (Land, 1973; Findlay & Cumming, 1977) was used to observe the effect on PMSG-induced ovulation rate of halving the number of follicles.

The first important feature of this study was that the overall population of antral follicles was only a limited component of the variation of PMSG-induced ovulation rate. This is supported by three lines of evidence. Firstly, there were only non-significant correlations between the overall antral population and PMSG-induced ovulation rate ($r = 0.62$ in Exp. 1, $r = 0.49$ in Exp. 2). Secondly, there was no effect of halving the number of follicles available for stimulation through unilateral ovariectomy on PMSG-induced ovulation rate (1 ovary 3.7 ± 1.2 vs 2 ovaries 3.9 ± 1.8). Thirdly, the decrease in ovulation rate which was noted when repeated stimulations were attempted was not caused by changes in follicular populations which, with the exception of follicles forming an antrum, were unaffected by the repetition of PMSG treatment. This is in good agreement with observations in mice of strains which differ markedly in their follicular populations but exhibit very similar gonadotrophin-induced ovulation rates (Speareow, 1980).

The second important finding of this study was the identification of the follicles which are induced to mature to ovulation by PMSG. The close correlation between the number of healthy follicles 0.8–2.0 mm in diameter and ovulation rate suggests that these follicles are those recruited

### Table 1. Correlation values linking quantitative ovarian features and ovulation rate in Exps 1 and 2

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Total no. of antral follicles</th>
<th>Total no. of follicles</th>
<th>No. of healthy follicles</th>
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<tr>
<td></td>
<td>0.20–0.32 mm</td>
<td>0.32–0.50 mm</td>
<td>0.50–0.80 mm</td>
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<td></td>
<td>0.80–2.0 mm</td>
<td>&gt;2.0 mm</td>
<td>&gt;2.0 mm</td>
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<tr>
<td>Exp. 1 (N = 7)</td>
<td>0.62</td>
<td>0.65</td>
<td>0.18</td>
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<tr>
<td></td>
<td></td>
<td>0.72*</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.74*</td>
<td>-0.29</td>
</tr>
<tr>
<td>Exp. 2 (N = 9)</td>
<td>0.49</td>
<td>-0.03</td>
<td>0.01</td>
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<tr>
<td></td>
<td></td>
<td>0.77*</td>
<td>-0.33</td>
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<tr>
<td></td>
<td></td>
<td>0.85**</td>
<td>-0.61</td>
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* $P < 0.05$; ** $P < 0.01$.
by PMSG. This result is supported by the findings that the number of follicles > 1 mm is unchanged after PMSG administration (McNatty et al., 1982) while the number of follicles > 2 mm increases (Dott et al., 1979). Follicles recruited by PMSG are much smaller than those recruited at luteolysis of an unstimulated cycle to undergo preovulatory enlargement (> 2 mm: Driancourt & Cahill, 1984; Tsonis et al., 1984). It is unclear, at present, whether such small 0.8-2.0 mm follicles, which normally heal 10-15 days to mature (Cahill & Mauléon, 1980), manage to catch up through the limited increase in the mitotic index of the granulosa cell induced by PMSG (sheep: Turnbull et al., 1977; cattle: Monniaux et al., 1983) and ovulate with an adequate number of granulosa cells. About half of these 0.8-2.0 mm follicles were atretic, an observation in good agreement with previous reports on atresia in the sheep ovary (Brand & de Jong, 1973; Turnbull et al., 1977; Cahill et al., 1979). The fact that the value of the correlation linking healthy follicles 0.8-2.0 mm in diameter and ovulation rate is actually decreased when atretic follicles are taken into account strongly suggests that they are not a component of the ovarian response to PMSG. The discrepancy between this finding and previous claims of follicular rescue from atresia by PMSG (Byskov, 1979; Peters, 1979; Monniaux et al. 1984) could be explained by the observation that there were very few follicles in early atresia (about 6%) in this class and that PMSG does not rescue follicles in advanced atresia (Driancourt et al., 1987). In contrast to healthy follicles 0.8-2.0 mm in diameter which form the reserve from which PMSG recruits follicles for ovulation, the number of follicles > 2 mm in diameter was negatively correlated with PMSG-induced ovulation rate (r = -0.29 and -0.61 in Exps 1 and 2 respectively). This has previously been reported for cattle: when response to PMSG was related to the features of the surface follicular population, ovaries devoid of large follicles and rich in small ones had the highest response (Saumande et al., 1978). Administration of PMSG, together with a luteolytic agent at different days of the luteal phase, produces a higher response after Day 8 (Phillippo & Rowson, 1975; Sreenan et al., 1978), a time when the mid-cycle follicle has started to regress in size, as detected by ultrasonic echography (M. A. Driancourt, D. Andrieu & W. W. Thatcher, unpublished results). Several compounds with direct ovarian action or indirect action through decreased gonadotrophin concentrations have been identified in follicular fluid (anti-aromatizing protein: diZerega et al., 1982; follicular growth inhibitor: Cahill et al., 1984; inhibin: Tsonis et al., 1983). Further investigations are needed to work out which of them are involved and how they act. Hypersensitivity to some of these compounds could explain the decreased response at repeated superovulations (sheep: Hulet & Foote, 1969) since follicular populations are not deeply affected by repetition of the treatments and since no PMSG antibodies have ever been found (Schams et al., 1978).

Optimization of the ovarian response to PMSG could therefore be reached by generating ovaries rich in healthy 0.8-2.0 mm follicles and devoid of healthy follicles > 2.0 mm in diameter. However, it is likely that such ovaries would not work efficiently in the absence of exogenous gonadotrophins since healthy class 5 follicles are those involved in preovulatory enlargement in naturally cyclic ewes (Driancourt & Cahill, 1984; Tsonis et al., 1984). The discrepancy in the type of follicles recruited for ovulation in unstimulated and superovulated animals could explain why there is no correlation between the responses to selection for natural and for PMSG-induced ovulation rate (Land & Falconer, 1969).

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