A study of the population of primordial and small follicles in the sheep*

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Summary. The population of primordial and small follicles in adult ewes of high (3·1) and low (1·6) ovulation rate was studied by histological methods. The small follicle population which has a skewed frequency distribution in relation to either follicle or oocyte size can be divided according to morphological features into dormant, transitory and growth phases.

It is suggested that initiation of follicle growth involves firstly the passage of follicles from the dormant to the transitory category and (if it occurs) is independent of gonadotrophins; and secondly, the passage from the transitory category to the growth phase. The second step is dependent on gonadotrophins and the rate of such passage is higher in ewes with a high ovulation rate.

The overall mean follicle size of small follicles is larger in ewes with a low ovulation rate, suggesting that either they have structurally different small follicles or their reserve of small follicles is less depleted than in ewes with a high ovulation rate.

Introduction

The total follicle population consists of a large reserve of primordial and small follicles (hereafter called small follicles) and a much smaller number of larger follicles in the growth phase. Passage of follicles from this reserve pool of small follicles to the growth phase, i.e. follicle growth initiation, remains poorly understood although Krarup, Pedersen & Faber (1969) have shown it to be influenced by the number of small follicles whilst Peters, Byskov, Lintern-Moore, Faber & Anderson (1973) have shown it to be independent of gonadotrophins. Land (1970) and Cahill, Mariana & Mauléon (1979) considered the small follicle population to be a single homogeneous population in which the breed of ewe with the lower ovulation rate had significantly more small follicles. However, a direct relationship has been found between the number of follicles in the growth phase and ovulation rate (Cahill *et al.*, 1979). Therefore around the time follicles enter the growth phase there is a change-over in the relative numbers of small and growing follicles between ewes of high and low ovulation rate.

The present study was undertaken to determine whether the small follicle (< 0·07 mm diam.) population was a homogeneous population and whether these follicles differ morphologically between two breeds of sheep.

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Materials and Methods

Experiment 1

During December (mid-breeding season) 6 Romanov and 6 Ile-de-France ewes (mean ovulation rates 3.1 and 1.6 respectively) were ovariectomized. All ewes were 30 months of age. Following ovariectomy both ovaries from each ewe were fixed in Bouin–Hollande's solution and later one ovary per ewe was randomly selected, serially sectioned at a thickness of 7 μm , mounted, stained with Feulgen's stain and inspected microscopically.

Only follicles less than 0.07 mm in diameter (about 3–4 layers of granulosa cells) were considered. For each of the 12 ovaries, 5 follicles from each of the following classes, 0–35.5 (area of largest cross-section 0–1000 μm^2), 35.6–50.2 (1000–2000 μm^2), 50.3–61.5 (2000–3000 μm^2) and 61.6–71.3 μm (3000–4000 μm^2) in diameter, were measured in order to cover the size range of the small follicle population. For each follicle the following variables were measured on the section where the nucleolus of the oocyte nucleus was seen; area of follicle, area of oocyte, number of granulosa cells/section, diameter of the oocyte nucleus, whether 50% or more of the granulosa cells were epithelioid or cuboid in shape and whether the follicles were arranged in a 'nest formation' (i.e. walls of 2 adjacent follicles touch) or separated from one another. The areas were measured according to the method previously described (Cahill *et al.*, 1979) and to conform with the normal convention of expressing follicle size by diameter, the area was assumed to be circular and the diameter calculated.

Experiment 2

Also during December, 5 Romanov and 5 Ile-de-France ewes, aged 20 months, were ovariectomized. After ovariectomy the exterior portion of the left ovary of each animal was cut into small cubes about 1 mm³, fixed in 3% glutaraldehyde and sectioned at a thickness of 1 μm . The sections were stained with toluidine blue and inspected microscopically. For each ovary the first 50 follicles found with 2 or less layers of granulosa cells in the section where the nucleolus was present were examined and the same variables were recorded as in Exp. 1 with the exception of the diameter of the oocyte nucleus.

Statistical analysis

Due to the small number of follicles considered per ovary, animal differences were not considered and were pooled for breed comparisons. Mean follicle and oocyte diameters and frequencies of follicles according to size of the follicle were compared using analysis of variance. Three distinct categories of follicles were evident and the demarcations of these categories were arbitrarily determined. Differences in the slopes of the linear regressions describing various development features and the diameter of the oocyte nucleus were tested by covariance analysis between breeds within each category and between categories.

Results

Experiment 1

It was possible to distinguish 3 categories in the small follicle population according to the diameter of the oocyte nucleus (Text-fig. 1). The parameters of follicle diameter, number of granulosa cells per section and oocyte diameter did not change per increase in the oocyte nuclear diameter in Category I, but increased significantly in Category II whilst in Category III they increased only slightly per subsequent increase in the oocyte nuclear diameter. The slopes of the linear regressions (oocyte nuclear diameter versus follicle diameter, versus number of granulosa

cells per section, versus oocyte diameter) did not differ between breeds in any of the follicle categories but differed significantly ($P < 0.05$) between categories both within breeds and after pooling of data for both breeds. There was a significant linear regression ($P < 0.01$) between the number of granulosa cells per section and follicle size and this regression was not significantly different ($P > 0.05$) between breeds. There were significant linear relationships ($P < 0.05$) between oocyte diameter and follicle diameter and number of granulosa cells per section and these relationships did not differ between breeds, probably due to the relatively small number of follicles spread over a wide range of follicle sizes (see below, Exp. 2).

There was no significant difference between breeds in the proportion of follicles with $>50\%$ of its granulosa cells cuboid in shape. The pooled means showed that there were 31, 94 and 100% of follicles having $>50\%$ cuboid cells in Categories I, II and III respectively. Follicles in 'nest formation' were found only in Category I follicles in similar proportions for each breed (Ile-de-France, 6.6%; Romanov, 10.0%).

Text-fig. 1. The relationships between the oocyte nuclear diameter and (a) follicle size, (b) number of granulosa cells and (c) oocyte size for small follicles from 6 Romanov (■) and 6 Ile-de-France (▲) ewes (Exp. 1). Each point represents the mean from 6 ovaries for a given size of the oocyte nucleus.

Experiment 2

The frequency of small follicles when considered according to either follicle or oocyte diameter was a skewed distribution that did not differ between breeds although there was a trend that Ile-de-France ewes had slightly more larger follicles. The small follicles were significantly larger ($P < 0.05$) in diameter in the Ile-de-France (0.33 ± 0.0006 mm) than in the Romanov (0.030 ± 0.0010 mm) ewes. There were also more granulosa cells per section in the Ile-de-France follicles (8.38 ± 0.29 versus 6.27 ± 0.22 ; $P < 0.01$). However, there was no significant difference between breeds in the mean oocyte diameter (0.024 ± 0.0005 and 0.023 ± 0.005 mm).

The linear regressions between size of follicle and oocyte size were significantly different between breeds ($P < 0.01$) such that for a given size of oocyte the mean follicle size and mean number of cells were larger in the Ile-de-France than the Romanov ewes. An analysis of discrimination showed that any given follicle could be successfully classified ($P < 0.05$) according to the breed in 81% of cases simply by considering only the follicle and oocyte sizes. The regression between the number of granulosa cells present in one section and follicle diameter was significant ($P < 0.01$) and did not differ between breeds.

There was no significant difference between breeds in the proportion of follicles observed in 'nest-formation' (Ile-de-France, 11.2%; Romanov, 9.6%).

There was little difference ($P < 0.1$) between breeds in the proportion of follicles having more than 50% of granulosa cells cuboid in shape (Ile-de-France, $33.2 \pm 4.3\%$; Romanov, $16.1 \pm 6.3\%$).

Discussion

Our results show that the small follicle population of the sheep is morphologically heterogeneous, supporting the findings of Mariana (1978) and Lintern-Moore & Moore (1979) that the small follicles in the rat and mouse respectively are heterogeneous according to histochemical and morphological features.

It is evident that the frequency of follicles according to follicle diameter is a skewed distribution which can be divided into three categories (Text-fig. 2). We should like to suggest the first is a category of dormant follicles since follicles in this category are very small, have no relationship between oocyte parameters (oocyte size and nuclear diameter) and follicle parameters (number of cells or size), have a low proportion of cuboid cells and often are arranged in 'nest formation'. The two experiments are comparable as the age difference of the two groups of ewes is unlikely to affect the small follicle population and, if we assume that shrinkage following the two fixation techniques was similar (as the regressions of number of cells with follicle size were not significantly different ($P > 0.05$) between the two methods), we can extrapolate that approximately 81% and 72% of small follicles in the Romanov and Ile-de-France ewes respectively are in this category. Extrapolation from the estimates of Cahill *et al.* (1979) for the total numbers of small follicles per ovary show that there are 24 700 and 40 500 follicles per Romanov and Ile-de-France ovary respectively in this dormant category.

We suggest that the second category be termed a transitory category because the follicular parameters are very sensitive to any change in oocyte parameters and it is probably from this category that follicles enter the growth phase. The transitory category commences when a follicle has a mean oocyte nuclear diameter of 0.013 mm, which coincides with a follicle diameter of 0.034 mm, and contains 14 cells on the largest cross-section. This cut-off point very closely resembles the situation found in the mouse for which 10 cells on the largest cross-section is the indicator for the start of follicle growth and RNA polymerase activity increases (Lintern-Moore & Moore, 1979). Similar extrapolations show that there are 19% and 28% or 5500 and 15 700 small follicles in this category in Romanov and Ile-de-France ewes respectively.

Text-fig. 2. A schematic representation of the small follicle population of ewes with high (●, Romanov) and low (▲, Ile-de-France) ovulation rates.

Approximately three follicles enter the rapid growth phase per day per ewe (Turnbull, Braden & Mattner, 1977) and, if no further atresia of small follicles occurs, then the number of follicles in the transitory categories represents 10 and 28 years' supply of oocytes for Romanov and Ile-de-France ewes respectively; this is generally sufficient for a ewe's normal fertile reproductive life-span.

At an oocyte nuclear diameter of 0.02 mm (Category III) follicles can be considered with certainty to have entered the growth phase, when there are 3–4 layers of granulosa cells and a follicular diameter of 0.06 mm. Cahill *et al.* (1979) found that follicles 0.06 mm or larger in diameter are more numerous in ewes with a higher ovulation rate. There is therefore a change-over in the relative number of follicles between the transitory category and growth phase; i.e. the Romanov ewes, which have the higher ovulation rate, have fewer follicles in the dormant and transitory phases but more follicles in the growth phase hence the number of follicles recruited from the transitory to the growth phase must be higher in ewes with a higher ovulation rate.

Dufour, Cahill & Mauléon (1979) found that after long-term hypophysectomy there is a decrease in the number of follicles with more than 3 layers of granulosa cells and so recruitment from the transitory to the growth phase in the sheep is under the control of the gonadotrophins. It is probable that follicles in the transitory category acquire FSH receptors (Ryle, 1972) and then selectively enter the growth phase under gonadotrophic control. Recruitment of follicles from the dormant to the transitory category remains unresolved. Krarup *et al.* (1969) and Peters *et al.* (1973) suggest that such recruitment may operate by an intra-ovarian mechanism independent of gonadotrophins.

The overall mean follicle size and the mean number of granulosa cells per section were higher in the Ile-de-France than the Romanov ewes in Exp. 2. This difference persisted even when follicles were considered in relation to a given oocyte size. Such a breed difference was not found

in Exp. 1, probably due to the smaller number of follicles considered per breed in this size range and, with 7 μm thick sections, the measurements of oocyte and follicular areas is less precise. This difference in the size of the follicle relative to the oocyte size between breeds has two possible explanations: (1) the process of oogenesis may differ between the two breeds, resulting in a basic structural difference of the small follicles between the breeds, or (2) since the largest follicles of the transitory category probably enter the growth phase (Mariana & Meyer, 1979), then what we are seeing in the adult animal is a partly depleted stock of small follicles because as the larger follicles move into the growth phase only the smaller follicles remain. The influence (if any) of this observed structural difference of the small follicles between breeds, on the recruitment of follicles to the growth phase remains unknown.

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