Characterization of calf follicular fluid and its ability to support cytoplasmic maturation of cow and calf oocytes

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The aims of the present study were to characterize the follicular fluid from prepubertal calf follicles of known size and quality and to study the ability of follicular fluid to support cytoplasmatic maturation of calf and cow oocytes. Follicular fluid was obtained from 67 calf follicles classified according to size (S: small < 6 mm, M: medium 6–8 mm and L: large > 8 mm in diameter) and quality (HY: healthy, EA: early atretic and A: atretic). Quality was first determined by mitosis:pycnosis ratios in granulosa cell smears and confirmed by insulin-like growth factor binding protein (IGFBP) patterns. There was approximately 90% agreement between the two methods of follicle classification and on this basis the calf follicular fluid was pooled into nine groups. The accuracy of this pooling was confirmed by evaluation of oestradiol concentrations in the nine pools of follicular fluid using radioimmunoassay. Increases in follicle size were characterized by a decreased intensity of bands for IGFBP-2, IGFBP-5 and IGFBP-4, an increase in the proportion of healthy follicles and a decrease in the proportion of follicles in the early stages of atresia. This finding is in agreement with previously published results in cows. All classes of calf follicular fluid contained lower concentrations of oestradiol than previously reported for corresponding classes of cow follicular fluid. Cow oocytes were matured in M199 alone, or supplemented with 10% fetal calf serum (FCS), or 10% calf follicular fluid from one of three pools (LHY, LEA, LA), fertilized, and cultured for 8 days in synthetic oviduct fluid. Addition of FCS or calf follicular fluid to cow oocytes during in vitro maturation increased the yield of blastocysts on day 8 over the control (23%, 21/91), FCS (39%, 37/96, P < 0.05), LA (41% 21/52, P < 0.05), LEA (32%, 28/88), LHY (36%, 32/88), although not significantly in all cases. The rate of hatching of blastocysts was also improved: control (38%, 8/21), FCS (54%, 20/37), LA (62%, 13/21), LEA (75%, 21/28, P < 0.02), LHY (59% 19/32). In contrast, the addition of either FCS, calf follicular fluid or cow follicular fluid did not improve development of calf oocytes compared with the unsupplemented control. In conclusion, it is probable that serum and follicular fluid contain factors that stimulate the acquisition by oocytes, during maturation, of developmental competence and to which prepubertal oocytes are unable to respond. Specific receptors for these factors may develop only around puberty.

Introduction

The ability to produce embryos from oocytes recovered from fetal (Betteridge et al., 1989) or prepubertal calves (Armstrong et al., 1991; Georges and Massey, 1991) offers the potential for markedly reducing the generation interval in cattle, thereby substantially accelerating the rate of genetic gain that can be achieved through embryo transfer. In addition, calf ovaries contain many more follicles visible on the surface than do those of adult cows; this is due to senescence during the lifetime of the animals (Erickson 1966; Revel et al., 1995), thus offering the potential of recovering many more oocytes from a given number of ovaries. However, oocytes from calf ovaries are less competent to develop than those from cow ovaries (Seidel et al., 1971; Lèvesque and Sirard, 1994; Revel et al., 1995; Damiani et al., 1995, 1996; Khatir et al., 1996). Although fertilization and cleavage rates do not differ greatly between calf and cow oocytes, blastocyst yields are significantly reduced with calf oocytes (Revel et al., 1995; Khatir et al., 1996). It is likely that the deficiency in developmental capacity of calf oocytes is due to abnormal cytoplasmic maturation of these oocytes. Indeed, calf oocytes have been shown to exhibit a delay in organelle migration following in vitro maturation (IVM) as well as abnormal chromatid and microtubule configurations (Damiani et al., 1996).

Lack of developmental competence in oocytes of prepubertal animals is not restricted to cows; it has been reported in mice (Eppig and Schroeder, 1989), goats (Izquierdo et al., 1995;...
Mogas et al., 1995) pigs (Pinkert et al., 1989) and sheep (Boland and Gordon, 1977; O’Brien et al., 1996). Thus, pubertal oocytes represent a good negative model for the study of the mechanisms involved in acquisition of developmental competence.

Khatir et al. (1996) showed previously that the lower developmental competence of calf oocytes cannot be explained by differences in the protein profiles of the oocyte itself. Thus, it would seem that factors other than the oocyte may be instrumental in determining its competence. Several authors have shown that the developmental capacity of the bovine oocyte may be affected by the size (Pavlok et al., 1992; Lonergan et al., 1994a) and quality (Hazeleger et al., 1994; Blondin and Sirard, 1994a, b; Carolan et al., 1996) of the follicle from which it is obtained. These findings suggest that the follicular fluid that constitutes the constantly changing microenvironment in which the oocyte develops plays a major modulating role in determining its fate.

Many studies have attempted to characterize the composition and to examine the effect of the presence of follicular fluid during oocyte maturation on subsequent development (Andersen et al., 1976; Ayoub and Hunter, 1993; Lonergan et al., 1994a, Romero and Seidel, 1994; Sun et al., 1994; Sirard et al., 1995; Carolan et al., 1996). The ability of follicular fluid to support oocytes during IVM appears to be influenced by its origin. Carolan et al. (1996) showed that the action of follicular fluid on cytoplasmic maturation of cow oocytes varies with follicle quality but not with size. Furthermore, it has been shown that there are differences in the protein profiles of follicular fluid from calf and cow ovaries, suggesting that these differences in the protein content of follicular fluid may in part explain the lower developmental competence of calf compared with cow oocytes (Khatir et al., 1996). Among the factors in follicular fluid that stimulate development, the presence of epidermal growth factor (EGF) has been identified in different species. We have previously shown that the presence of EGF during maturation of cow oocytes significantly increases developmental competence (Lonergan et al., 1996).

In the present study, we aimed to characterize more precisely the follicular fluid from calf follicles of known size and quality and to evaluate the ability of this follicular fluid, pooled according to size and quality of the follicles it was obtained from, to support cytoplasmic maturation of calf and cow oocytes. In addition, the effect of EGF during the maturation of cow and calf oocytes was examined.

Materials and Methods

Collection and classification of follicular fluid

Ovaries from calves, approximately 3–4 months old, were collected at a local abattoir and transported on ice to the laboratory. A total of 67 individual follicles were dissected free of ovarian tissue. These follicles were measured and grouped into three categories (< 0 mm; 6–8 mm; > 8 mm). Follicular fluid from each follicle was aspirated with a 26 G needle, centrifuged at 300 g for 7 min, and the supernatant stored at –20°C. Follicle quality was determined as described by Monniaux (1987). For each follicle, a smear of granulosa cells was prepared on histological slides, fixed in methanolic-formaldehyde-acetic acid (80:15:5), and stained with Feulgen. On the basis of the Feulgen coloration, follicles were classified as normal (frequent mitosis, rare or no pycnotic granulosa cells), early atretic (infrequent mitosis, intermittent pycnotic granulosa cells), or atretic (no mitosis in granulosa cells and numerous pycnotic bodies). Cow follicular fluid from large follicles (> 8 mm) recovered under similar conditions (Carolan et al., 1996) was classified as above.

Biochemical analysis of follicular fluid

A sample of follicular fluid from each of the 67 follicles was assessed for insulin-like growth factor-binding protein (IGFBP) patterns by western ligand blotting using the method previously described by Hossenlopp et al. (1986). Briefly, 1.5 μl of follicular fluid from each pool was submitted to 12% SDS-polyacrylamide gel electrophoresis under nonreducing conditions. The separated proteins were electroblotted onto nitrocellulose filters (0.45 μm pore size) which were treated with PBS (0.01 mol l⁻¹, pH 7.4) containing 0.1% (v/v) Nonidet P-40, 0.5% (w/v) gelatin, and 0.1% (v/v) Tween-20, and then incubated overnight at 4°C with 1 x 10⁶ c.p.m. ¹²⁵I-labelled IGF-II in a buffer containing 0.03 mol NaH₂PO₄ 1 x 10⁻³, 500 μl Tween-20 1 x 10⁻¹, 200 mg EDTA 1 x 10⁻³, pH 7.4. After incubation, membranes were washed in PBS containing 0.1% Tween-20, air dried, and submitted to autoradiography by exposure to Amersham Hyperfilm MP with intensifying screens for one night at –70°C. Binding specificity was demonstrated using membranes with an excess of unlabelled IGF-II.

There was approximately 90% agreement between these two methods of classification. However, where there was a discrepancy, the classification was based on the histological results. Thus, on the basis of these two classifications, the calf follicular fluid was pooled into nine groups: small healthy (SHY; n = 13); small early atretic (SEA; n = 13); small atretic (SA; n = 8); medium healthy (MHY; n = 10); medium early atretic (MEA; n = 4); medium atretic (MA; n = 7); large healthy (LH; n = 6); large early atretic (LEA; n = 2); large atretic (LA; n = 4) and aliquoted for subsequent addition to the maturation medium.

Analysis of the IGFBP patterns and oestradiol concentrations was carried out in the nine pooled samples to confirm our initial classification. The oestradiol concentration was determined by radioimmunoassay without extraction (Terqui et al., 1988).

Protein analysis of follicular fluid

Pools of follicular fluid from cow (LHY, LEA, LA) and calf (SHY, SEA, SA, MHY, MEA, MA, LHY, LEA, LA) follicles were used to study the protein profiles. A volume of 2 μl of each pool was diluted at 1:25 in sample buffer (Laemmli, 1970), heated at 100°C for 3 min and stored at –20°C until electrophoresis. Thawed samples were centrifuged at 13 000 g for 5 min. Three replicates were carried out. The total protein content of the follicular fluid was measured as previously described by Bradford (1976). A volume of 10 μl of sample, corresponding to 40 μg of protein was resolved. Gels were
silver-stained, using a protocol modified from Morrissey (1981) to observe the constitutive proteins. Briefly, gels were prefixed in a solution of 40% (v/v) methanol and 10% (v/v) acetic acid in 50% water for 1 night and fixed for 30 min in 10% (v/v) glutaraldehyde. They were then washed five times (10 min) in water and immersed in 5 µg dithiothreitol ml⁻¹ for 30 min. They were subsequently treated with 0.1% (w/v) silver nitrate for 30 min, rinsed rapidly with water and developed (solution of 0.3 mol sodium carbonate 1⁻¹ containing 0.0135% formaldehyde). Staining was stopped by washing in 10% (v/v) acetic acid. Finally, the gels were photographed and analysed visually.

LH and oestradiol concentration in follicular fluid aspirates

In a separate experiment, the LH and oestradiol concentrations in pools of follicular fluid from cow and calf follicles, collected after routine aspiration of follicles of 2–6 mm diameter were assessed by radioimmunoassay (Terqui et al., 1988).

In vitro maturation (IVM) fertilization (IVF) and culture (IVC)

Maturation and fertilization methods were adapted from those previously described by Mermillod et al. (1993). Cumulus–oocyte complexes (COCs) were obtained by aspiration of follicles, 2–6 mm in diameter, from the ovaries of slaughtered, prepubertal calves (3–4 months old) and adult cows. All follicles completely surrounded by unexpanded cumulus cells were used. They were washed four times in modified Dulbecco’s PBS (supplemented with 36 mg pyruvate l⁻¹, 50 µg gentamycin ml⁻¹ and 0.5 mg BSA ml⁻¹ (Sigma fraction V, Sigma Co. Ltd, St Louis, MO). Groups of up to 50 oocytes were transferred to four-well plates (Nunc, Roskilde) containing 500 µl of medium for 24 h maturation at 37°C in an atmosphere of 5% CO₂ in air with maximum humidity.

Two controls were used: Tissue Culture Medium 199 (M199, Sigma Co. Ltd), as a lower defined control to which the follicular fluid under test was added, and M199 plus 10% heat-treated fetal calf serum (FCS, Sigma Co. Ltd) as an upper control (Loneran et al., 1994b).

Cumulus expansion was visually assessed under a stereo-microscope after maturation. Oocytes were then washed four times in PBS and once in fertilization medium before being transferred in groups of up to 50 to four-well plates containing 250 µl of fertilization medium (TALP), containing 10 µg heparin-sodium salt ml⁻¹ (167 U mg⁻¹, Calbiochem, San Diego, CA) per well. Motile spermatozoa were obtained by centrifugation of frozen-thawed spermatozoa on a Percoll (Pharmacia, Uppsala) discontinuous density gradient (2 ml at 45%, over 2 ml at 90%) for 20–30 min at 700 g at room temperature. Spermatozoa from the same ejaculate of one bull was used throughout all experiments. Viable spermatozoa, collected at the bottom of the 90% fraction were washed in TALP and pelleted by centrifugation at 100 g for 10 min at room temperature. Spermatozoa were counted in a haemocytometer and diluted in the appropriate volume of TALP to give a concentration of 4 × 10⁶ spermatozoa ml⁻¹; 250 µl of this suspension was added to each fertilization well to obtain a final concentration of 2 × 10⁶ spermatozoa ml⁻¹. Plates were then incubated for 18–21 h in 5% CO₂ in humidified air at 37°C.

Culture took place in modified synthetic oviduct fluid droplets (25 µl) (SOF; Carolan et al., 1995) under paraffin oil in a humidified atmosphere of 5% CO₂, 5% O₂, 90% N₂ at 37°C. Cumulus cells were removed from presumptive zygotes by gentle vortexing in PBS. The zygotes were subsequently washed twice in PBS and twice in SOF before being transferred in groups of 20–30 to the culture droplets (1 zygote µl⁻¹ medium). No serum was added during culture (Khatir et al., 1996). Cleavage was assessed 48 h after placement in culture (non-cleaved, 2–4 cells, 5–8 cells). The number of embryos developing to the expanded blastocyst stage was assessed on days 6–8 of culture (that is, 72 h after insemination). Hatching was recorded on day 8 of culture and expressed as a percentage of day 8 blastocysts.

Experiment 1: effect of the presence of calf or cow follicular fluid during in vitro maturation on subsequent embryo development.

The aim of this experiment was to determine whether calf follicular fluid contained an active factor. This was determined by adding it to the maturation medium of cow oocytes, since previous studies have shown that such oocytes are responsive to follicular fluid from adult ovaries (Carolan et al., 1996). A total of 415 immature cow COCs were used for this experiment. After recovery, the COCs were randomly allocated to one of five maturation media: M199 alone, M199 supplemented with 10% FCS, or M199 supplemented with 10% calf follicular fluid from one of three pools (LIY, LEA, LA). This experiment was repeated three times. After IVM, oocytes were inseminated and the presumptive zygotes were cultured as described above.

To confirm the results of the experiment described above and to determine the effect of cow follicular fluid on calf oocytes, a total of 624 immature calf COCs were allocated to one of the following three maturation treatments: M199 alone or M199 supplemented with 10% calf follicular fluid or 10% cow follicular fluid. Three replicates were carried out. After IVM, oocytes were inseminated and the presumptive zygotes cultured as described above.

Experiment 2: effect of maturation conditions during IVM of cow and calf oocytes on subsequent development.

Immature COCs from cow and calf oocytes were cultured in parallel in one of four maturation media: M199 alone or M199 supplemented with 10 ng EGF ml⁻¹, 10% FCS or 10% follicular fluid from large healthy follicles (calf follicular fluid for calf oocytes; cow follicular fluid for cow oocytes). Three replicates were carried out with a minimum of 30 COCs per replicate per group.

Statistical analyses

Raw data were analysed by chi-squared analysis or Fischer’s exact test where appropriate. P < 0.05 was considered significant.

Results

Classification of follicular fluid

The distribution of follicles according to size and quality is shown (Table 1). Of the follicles obtained, 51% were less than
Table 1. Distribution of follicles (n = 67) dissected from calf ovaries classified according to size and quality based on histology (no statistical differences)

<table>
<thead>
<tr>
<th>Size (mm)</th>
<th>n</th>
<th>Healthy (%)</th>
<th>Early atretic (%)</th>
<th>Atretic (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 6</td>
<td>34</td>
<td>38</td>
<td>38</td>
<td>24</td>
</tr>
<tr>
<td>6–8</td>
<td>21</td>
<td>48</td>
<td>19</td>
<td>33</td>
</tr>
<tr>
<td>&gt; 8</td>
<td>12</td>
<td>50</td>
<td>17</td>
<td>33</td>
</tr>
</tbody>
</table>

Fig. 1. Autoradiograph of western ligand blotting of insulin-like growth factor binding proteins (IGFBPs) in calf follicular fluid from follicles classified according to size and quality. Molecular masses are expressed in kDa. SHY: small healthy; SEA: small early atretic; SA: small atretic; MH: medium healthy; MEA: medium early atretic; MA: medium atretic; LHY: large healthy; LEA: large early atretic; LA: large atretic.

6 mm, 31% were between 6 and 8 mm and 18% were greater than 8 mm in diameter. When the diameter of the follicle was greater than 6 mm, there was an increase in the proportion of healthy follicles and a corresponding decrease in the proportion of follicles in the early stages of atresia.

IGFBP patterns in follicular fluid

A representative autoradiograph of western ligand blotting of the follicular fluid pools is shown (Fig. 1). All samples displayed the IGFBP-3 doublet at 44–42 kDa. Follicular fluid from small follicles displayed intense bands at 35 and 24 kDa in addition to poorly defined bands at 30 kDa, corresponding to IGFBP-2, IGFBP-4 and IGFBP-5, respectively (Monget et al., 1993). The intensity of the IGFBP-3 doublet did not change with the size or quality of the follicle. However, larger follicle size was characterized by a decrease in the intensity of IGFBP2, IGFBP5, and IGFBP4, while atresia was characterized by increased intensity of these bands.

Oestradiol concentration in follicular fluid pools

Concentrations of oestradiol (ng ml⁻¹) in the calf follicular fluid pools were as follows: SHY: 2.3 ± 0.003, SEA: 0.6 ± 0.068, SA: 0.3 ± 0.044, MHY: 22.6 ± 0.9, MEA: 0.4 ± 0.02, MA: 0.6 ± 0.016; LHY: 99.1 ± 5.5, LEA: 1.1 ± 0.05, LA: 0.6 ± 0.034. An increase in follicular size in healthy or early atretic follicles was characterized by a progressive increase in oestradiol concentrations, while onset of atresia was characterized by a sharp decrease in oestradiol concentrations.

LH and oestradiol concentration in follicular fluid aspirates

A comparison of the concentrations of oestradiol and LH in follicular fluid pools routinely aspirated from cow and calf follicles, 2–6 mm in diameter, demonstrated that the concentration of oestradiol in follicular fluid from cow ovaries was twice that from calf ovaries (12.7 ± 5.5 ng ml⁻¹ versus 6.3 ± 2.1 ng ml⁻¹, respectively). Similarly, the concentration of LH in follicular fluid aspirates from cow ovaries was more than twice that from calf ovaries (4.0 ± 0.3 ng ml⁻¹ versus 2.0 ± 0.2 ng ml⁻¹, respectively), based on three replicates.

Protein patterns in calf follicular fluid

The protein content in follicular fluid did not differ among the nine grades of calf follicle (SHY, SEA, SA, MHY, MEA, MA, LHY, LEA, LA) (Fig. 2), except for minor variations, such as bands of 37 and 44 kDa which characterized the three classes of small follicle (not indicated in gel). However, bands of 22, 25, 34 and 45 kDa were less intense in calf than in cow follicular fluid. In addition, the bands of 22 and 25 kDa and a series of bands between 25 and 34 kDa were more intense in cow non-atretic follicular fluid than in cow atretic follicular fluid and all classes of calf follicular fluid. It is of interest that these differences between cow and calf follicular fluid were also observed in the follicular fluid pools confirming these results (see Fig. 2).

Embryo development after IVM/fIVF/HIVC

Experiment 1. Addition of 10% calf follicular fluid (LHY, LEA, LA) to cow oocytes during IVM did not change the cleavage rate or the number of embryos developing to the 5–8 cell stage 72 h after insemination compared with those matured in M199 alone (Table 2). However, follicular fluid from large atretic follicles significantly increased the blastocyst yield at day 6 (29% versus 14%, P < 0.05) and day 8 of culture (41% versus 23%, P < 0.05), respectively, when compared with culture with M199 alone. Although the addition of LEA-follicular fluid or LHY-follicular fluid both increased development as we have shown with LA-follicular fluid, the difference was not significant, possibly due to the small numbers involved. Neither serum nor two of the three follicular fluid classes significantly increased the hatching rate compared with the control. Only LEA-follicular fluid had a significant effect (75% versus 38%; P < 0.05). Addition of cow follicular fluid to M199 had no effect on the subsequent development of calf oocytes (blastocyst yields for day 8 were 18% (38/213) for M199 and 13% (25/199) for M199 plus cow follicular fluid) (Fig. 3). However, the addition of calf follicular fluid significantly decreased the blastocyst yield (10%, 21/212; P < 0.02).

Experiment 2. The addition of either 10% FCS, 10 ng EGF ml⁻¹ or 10% LHY-follicular fluid from cow ovaries to cow oocytes during IVM significantly increased the blastocyst yield
on day 8 (Fig. 4). In contrast, addition of FCS or 10% LHY calf follicular fluid to calf oocytes during IVM had no effect on subsequent blastocyst yield. However, EGF did significantly increase yields on day 8, in agreement with our observations in cows.

**Discussion**

In previous studies from our laboratory, we have shown that oocytes from prepubertal calves are less competent at development than those from adult cows (Revel et al., 1993) and that there are differences in the protein profiles of follicular fluid from calf and cow follicles suggesting that the follicular fluid contains a factor(s) that may be involved in the acquisition of developmental competence (Khatir et al., 1996). In the present study, calf follicular fluid was initially classified using histological criteria. This initial classification was confirmed by western ligand blotting of IGFBPs. There was approximately 90% agreement between these two methods of classification. An increase in follicle size was characterized by a decreased intensity of bands for IGFBP-2, IGFBP-5 and IGFBP-4, while atresia was characterized by increased intensity of these bands.

![Fig. 2. Profiles of protein content of calf and cow follicular fluid (FF) according to follicular size and quality. Each lane represents 40 μg of protein. Arrows on the left indicate the principal bands expressed in kDa. Std: molecular mass standards; SHY: small healthy; SEA: small early atretic; SA: small atretic; MH: medium healthy; MEA: medium early atretic; MA: medium atretic; LHY: large healthy; LEA: large early atretic; LA: large atretic; 1: pooled calf follicular fluid; 2: pooled cow follicular fluid.](image-url)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cleaved</th>
<th>5–8 Cell</th>
<th>Blastocyst yield</th>
<th>Hatching rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>Day 6 (n (%)</td>
<td>Day 8 (n (%)</td>
</tr>
<tr>
<td>M199</td>
<td>91 (70)</td>
<td>43 (48)</td>
<td>13 (14)*</td>
<td>21 (23)*</td>
</tr>
<tr>
<td>M199 + FCS</td>
<td>96 (81)</td>
<td>50 (52)</td>
<td>28 (29)*</td>
<td>37 (39)*</td>
</tr>
<tr>
<td>M199 + FF LA</td>
<td>51 (82)</td>
<td>22 (43)</td>
<td>15 (29)*</td>
<td>21 (41)*</td>
</tr>
<tr>
<td>M199 + FF LEA</td>
<td>88 (73)</td>
<td>43 (49)</td>
<td>22 (25)*</td>
<td>28 (32)*</td>
</tr>
<tr>
<td>M199 + FF LHY</td>
<td>89 (70)</td>
<td>43 (48)</td>
<td>19 (21)*</td>
<td>32 (36)*</td>
</tr>
</tbody>
</table>

Value, are the mean of three replicates.

*Yield as a percentage of oocytes cleaved.

**Values in the same column with different superscripts differ significantly (P < 0.05).

FF: follicular fluid; LA: large atretic; LEA: large early atretic; LHY: large healthy.

Table 2. Effect of follicular fluid from prepubertal calf ovaries on in vitro maturation of cow oocytes

![Downloaded from Bioscientifica.com at 12/21/2018 01:41:01PM via free access](image-url)
The intensity of the IGFBP-3 band did not change with follicle quality or size. These results are consistent with data obtained in adult sheep (Monget et al., 1993) and cattle (Carolan et al., 1996). On the basis of these two parameters, the samples of calf follicular fluid were pooled into nine groups. IGFBP profiles and oestradiol concentration were analysed for the nine groups to confirm this classification. There was a progressive increase in oestradiol concentration with increasing follicle size for healthy or early atretic follicles. In contrast, oestradiol concentration did not increase in large atretic follicles. This finding is in agreement with previously published studies in cows (Krup and Dieleman, 1982, 1985; Dieleman et al., 1983; Carolan et al., 1996).

Fraser et al. (1989) demonstrated that, during the prepubertal period, gonadotrophin production is much lower and concentrations in serum decrease even though GnRH synthesis is normal. In the present study, the amount of LH in cow follicular fluid aspirates was approximately twice that found in calf follicular fluid (4.0 ± 0.3 ng ml⁻¹ versus 2.0 ± 0.2 ng ml⁻¹, respectively). Dodson et al. (1988) reported that mean plasma LH concentrations in prepubertal heifers decreased from 0.96 to 0.76 ng ml⁻¹ between 3 and 15 weeks of age and had increased to 1.21 ng ml⁻¹ by 35 weeks of age. In addition, oestradiol concentrations changed with follicle quality in a manner similar to that previously observed for cows (Carolan et al., 1996). However, cow follicular fluid contains approximately twice the amount of oestradiol as calf follicular fluid (12.7 ± 5.5 ng ml⁻¹ versus 6.3 ± 2.1 ng ml⁻¹, respectively). These results may be explained by the fact that the lower concentration of LH decreases the production of androgens by theca cells, thereby inducing a reduction in the secretion of oestradiol.

A comparison of the protein content of the nine classes of calf follicular fluid with cow follicular fluid (LHY, LEA, LA) showed that the bands of 22, 25, 34 and 45 kDa were less intense in calf follicles than in cow follicles, irrespective of the follicle size and status. This is in agreement with our previous study with pooled follicular fluid (Khatir et al., 1996). However, we did not observe any significant variations in protein content among the nine classes of calf follicular fluid apart from minor variations in the intensity of bands such as 37 and 44 kDa in the three classes of calf follicular fluid from small follicles. However, it should be noted that the bands of 22 and 25 kDa were less intense in all classes of calf follicular fluid, as well as in cow atretic follicular fluid, compared with non-atretic cow follicular fluid. Whether these proteins play a role in the acquisition of developmental competence is at present unclear.

Reports in the literature on the effect of follicular fluid on oocyte maturation and embryo development in vitro are controversial. Some authors have shown follicular fluid to be inhibitory (Tsafirri et al., 1977; Chari et al., 1983; Sato and Koide, 1986; Ayoub and Hunter, 1993), while others have shown it to be stimulatory (Loneragan et al., 1994a; Sirard et al., 1995; Carolan et al., 1996). In the study reported here, the addition of calf follicular fluid to the maturation medium of cow oocytes tended to increase blastocyst yield when compared with M199 alone (29% versus 14% on day 6 and 41% versus 23% on day 8, respectively), this effect being significant for LA-follicular fluid. This result indicates that calf follicular fluid contains at least some stimulatory activity as does adult follicular fluid (Carolan et al., 1996). In a preliminary experiment, it was observed that the addition of serum or calf follicular fluid from any of the nine classes during IVF of calf oocytes failed to stimulate development (results not shown). In addition, in the experiments reported here, neither cow nor calf follicular fluid improved the developmental capacity of calf oocytes. This discrepancy may be linked to the inability of calf oocytes to respond to these supplements which may be due to the lack of certain receptors for some factors (gonadotrophins, growth factors) present in follicular fluid and serum or to the inability of calf oocytes to activate these factors.

Among the factors in follicular fluid that stimulate development, the presence of EGF has been identified in different species. EGF concentrations in follicular fluid vary from 2 to 15 ng ml⁻¹ (humans: Franks et al., 1987; Hofmann et al., 1990; Das et al., 1992; pigs: Hsu et al., 1987). EGF, at such physiological concentrations, has been shown to have a positive effect during IVF in a variety of species (cattle: Coskun et al., 1991; Harper and Brackett, 1993; Park and Lin, 1993; Kobayashi et al., 1994; Rieger et al., 1995; Khatir et al., 1996; Loneragan et al., 1996; pigs: Sommer et al., 1992; Reed et al., 1993; Singh et al., 1993; Ding and Foxcroft 1994; rodents: Dekel and Sherizly, 1985; Downs, 1989; Das et al., 1992; humans: Das et al., 1991; Gomez et al., 1993). The effect of EGF during bovine IVF is already at a maximum at a concentration of 1 ng ml⁻¹ (Loneragan et al., 1996). Therefore, EGF in follicular fluid could in part explain the positive effect of follicular fluid during IVF of cow oocytes. In contrast, while calf oocytes respond to exogenous EGF during IVF, they do not respond to follicular fluid in the same way as adult cow oocytes do. This observation suggests that it is not EGF that is the active factor in follicular fluid, but that serum and follicular fluid contain other molecules, including EGF-like modules, such as the proteins associated with blood coagulation, fibrinolysis,
Prepubertal bovine oocyte development

Fig. 4. Effect of supplementing maturation medium (M199) with 10% FCS, 10 ng epidermal growth factor (EGF) ml⁻¹ or 10% follicular fluid from large, healthy cow or calf follicles on the developmental capacity of cow and calf oocytes. Blastocyst yields are relative to the number of oocytes undergoing IVM. Three replicates were carried out.

*Significantly different from control (P<0.05).

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neural development and cell adhesion (Campbell and Bork, 1993), that may play an important role and to which only cow oocytes are capable of responding.

In conclusion, there are differences in the protein patterns and in the concentrations of LH and oestradiol between cow and calf follicular fluid. However, calf follicular fluid, irrespective of the size and quality of the follicle from which it originates, stimulates the acquisition of developmental competence by adult oocytes during maturation, as does adult follicular fluid, but is inactive on prepubertal oocytes. Consequently, it appears that the follicular environment is not responsible for the low developmental competence of prepubertal oocytes, as these oocytes are unable to respond to the stimulatory component of follicular fluid. This failure to respond could be explained by the absence of a specific receptor expressed by COCs only after puberty. The maturation promoting activity of follicular fluid cannot be explained by the presence of EGF since calf oocytes respond to this factor. Another growth factor could be involved which remains to be identified.

H. Khatir was financed by a grant from the French government. P. Lonergan was supported by a grant from the European Commission (DG XII, BIO2-CT-94-7612) and C. Carolan received a grant from INRA (Productions Animales). The authors express their gratitude to P. Monget for his help with western ligand blots; C. Pisselet for her help in histology; D. André and C. Gauthier for help with radio-immunoassays; OGER (O. Gerard) and D. Bouchier of ‘CIA URCO’ (La Futae, Rouillon) for providing bull semen and to the staff of Tours Abattoir (M. Le Maréchal) for providing the ovaries. Thanks are also expressed to R. Duby, M. Terqui and Y. Cognie for commenting on an earlier draft of the manuscript.


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