Collection of oocytes and production of blastocysts in vitro from individual, slaughtered cows

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Summary. On four occasions ovaries from a total of 35 cows were collected separately at the abattoir where they had been killed. The age of 20 of these cows was recorded. Oocytes from these ovaries were collected separately and were submitted to in vitro maturation, in vitro fertilization and in vitro culture procedures. Ovaries of 34 randomly chosen cows were pooled and treated as the control. Ova from individual cows were cultured in 10μl droplets and those from pooled ovaries were cultured in groups of 50 in 50μl droplets of oviductal cell-conditioned medium. The 35 cows treated individually supplied 493 oocytes (mean 14·1 oocytes per cow) with high individual variation (sd = 10·0; range = 0–38) and 47 expanded blastocysts (9·5% of oocytes; mean 1·3 blastocysts per cow; range = 0–6). Among these cows, 16 produced one or more blastocysts. Considerable variation in average development rates was detected over the four replicate experiments (11·3, 4·0, 9·0 and 13·5%). The 34 cows treated as the control supplied 397 oocytes (mean 11·7 oocytes per cow) and 44 expanded blastocysts (11·1% of oocytes; mean 1·3 blastocysts per cow) with high variations between replicates (11·1, 4·0 and 18·1%). No difference was observed between individual and pooled ovaries regarding either the number of oocytes, the rate of blastocyst formation, or the number of blastocysts per cow. No effect of age was detected.

The conclusion was that culture of zygotes in small groups does not impair bovine embryo development in vitro but that high individual variation in oocyte number and the rate of embryonic development may explain the variable results observed in this study between replicate experiments and in general in bovine IVF. These variations will impair the prediction of blastocyst production from individual cows of high genetic value.

Keywords: in vitro fertilization; cow; embryo; blastocyst; breeding; oviduct; conditioned medium

Introduction

In vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC) of bovine oocytes can supply the large numbers of embryos required for preimplantation studies and for use in the new biotechnologies such as embryo cloning or production of transgenic offsprings. In addition, IVF could find some application in the breeding of cattle of high genetic value. For example, IVF could produce the last calves from a cow of high genetic value after it had been killed or allow the rapid reconstitution of a herd killed for health reasons. Another application of IVF may be the mass production at low cost of beef cattle embryos that would improve the commercial value of calves produced by dairy breeds (Gordon, 1991). Furthermore, recent development of the ultrasound guided technique for oocyte collection in live cows (Kruip et al., 1991; van der Schans et al., 1991) confirmed that IVF could be an alternative method to the current embryo production procedures involving superovulation.
Breeding applications of IVF in cows implies the identification of the mother and, as a consequence, the separate handling of small numbers of oocytes and embryos from individual cows. Some recent studies in mice have shown that the regulation of preimplantation embryo development in *vitro* may depend on paracrine factors originating in the reproductive tract (Ménézo *et al.*, 1989; Ouhibi *et al.*, 1990) but could also be influenced by some autocrine factors secreted by embryos themselves (Rappolee *et al.*, 1988; Paria & Dey, 1990).

In cows, the well described 8–16-cell stage block (Thibault, 1966) can be overcome *in vitro* by using either co-culture of embryos with oviductal epithelial cells (Eyestone & First, 1989; Marquant-Le Guienne *et al.*, 1989) or media conditioned by these cells (Eyestone *et al.*, 1990, 1991; Boccart *et al.*, 1991). However, the embryos are generally cultured in large groups in small volumes of medium, favouring the action of a possible autocrine factor. Embryo production from the small numbers of oocytes produced by single cows could be impaired by the dilution of autocrine factors during embryo culture.

In this study, we investigated the oocyte yield from individual cows killed at the abattoir and the ability of these oocytes to develop to the blastocyst stage when cultured in small groups after undergoing IVM, IVF and IVC.

**Materials and Methods**

**Culture of oviductal cells and conditioning medium**

All reagents were obtained from Sigma (St Louis, USA) unless specified otherwise. Cultures of oviductal cells were initiated as described by Eyestone & First (1989). Cow oviducts were obtained from the abattoir without consideration of the stage of the oestrous cycle of the donor. After dissection, the oviducts were briefly immersed in 70% ethanol and the epithelium was gently scraped using a microscope slide. Mucosal tissue was then transferred to a conical tube containing 10 ml of tissue culture medium 199 (TCM 199; Gibco, Scotland) supplemented with 10% heat treated fetal calf serum. The tissue was then washed twice in the same medium and finally resuspended in 50 volumes of medium for seeding into 25 ml culture flasks (6 ml per flask). After 4 days, the medium was renewed and unattached cells discarded. Confluence was reached 6 days after seeding. For preparation of the conditioned medium, 6 ml of culture medium was added to a confluent culture of bovine oviductal epithelial cell monolayer in a 25 cm² flask. The conditioned medium was collected every two days, centrifuged at 500 g for 10 min and stored at 4°C. After harvesting three times on the same monolayer, the collected conditioned medium was pooled and frozen (−20°C) in 1 ml aliquots.

**In vitro maturation (IVM) and in vitro fertilization (IVF)**

The methods used were those described by Sirard *et al.* (1988) for maturation and by Parrish *et al.* (1986) for fertilization. All operations were performed at 39°C under 5% CO₂ in humidified air.

**Oocyte collection and maturation.** Ovaries from a total of 35 cows of different breeds were collected separately at an abattoir on four separate occasions (10, 10, 10 and 5 cows, respectively). Ovaries were collected in saline at 20°C and treated within 3 h of death. On three of these occasions, ovaries from 34 additional cows were pooled and treated in a similar way. Oocytes were collected by aspiration of 2–5 mm follicles. Only oocytes completely surrounded by compact cumulus cells (Leibfried & First, 1979) were selected and washed in modified Tyrode's medium (low bicarbonate Tyrode supplemented with albumin, lactate and pyruvate: TALP; Ball *et al.*, 1983; Parrish *et al.*, 1986). Groups of up to 20 were transferred in 100 µl droplets of maturation medium (TCM 199 containing 10% heat treated fetal calf serum, 1 µg oestradiol ml⁻¹, 5 µg porcine luteinizing hormone ml⁻¹ and 0.5 µg porcine follicle-stimulating hormone ml⁻¹) under mineral oil into 60 mm Petri dishes for 24 h. Oocytes from each donor cow were treated separately during maturation and fertilization. During maturation, the number of oocytes per 100 µl droplet ranged from one to 20 for the individual cows.

**Fertilization.** Mature oocytes were washed in TALP and groups of 10 were transferred to 50 µl droplets of fertilization medium (TALP containing 10 µg heparin ml⁻¹; Calliochem, San Diego, USA) under oil. Spermatozoa were selected by centrifugation of thawed semen on a Percoll (Pharmacia, Uppsala) discontinuous density gradient (45/90%) for 30 min at 700 g. Semen from the same ejaculate was used throughout the experiment. Live spermatozoa (at the bottom of the 90% Percoll fraction) were washed in TALP and pelleted by centrifugation at 100 g for 10 min. Spermatozoa were counted in a haemocytometer and diluted in the appropriate volume of TALP. Two microliters of the suspension was added to each fertilization droplet to give a final concentration of 2 × 10⁵ spermatozoa ml⁻¹. Dishes were then incubated for 18 h. For the individual cows, the number of oocytes per 50 µl droplet ranged from one to 10.
Embryonic culture

Fertilized ova were washed in culture medium (TCM 199 supplemented with 10% heat-treated fetal calf serum). Cumulus cells were removed by repeated pipetting and the embryos were cultured in droplets of conditioned medium under mineral oil. Ova from individual cows were cultured in 10 μl droplets, regardless of their number (two to 38 ova per droplet), whereas ova from pooled ovaries were cultured by groups of about 50 in 50 μl droplets. The number of expanded blastocysts was determined on day seven of culture. Results were analysed by χ² and by Student's t test for mean values and correlation coefficients.

Results

Number of oocytes

The number of oocytes harvested from each cow ranged from 0 to 38 with a mean value of 14.1 and a standard deviation (SD) of 10.0. Considerable variation among individuals was detected on each of the four occasions on which oocytes were collected (SD = 12.2, 11.1, 5.7 and 7.6). No significant difference was detected between the average number of oocytes collected from individual cows on the four different occasions (15.1, 14.2, 13.4 and 13.3 respectively, Table 1). The average number of oocytes per cow (Table 1) did not differ significantly when ovaries were treated separately (14.1) or as a pool (11.7). The number of oocytes produced by younger animals (17.3 per cow, Table 2) was not statistically different from that produced by cows more than 3 years old (12.5 per cow). Unfortunately, due to poor abattoir facilities, the age of only 20 individual cows was known (collections one and two). There were seven cows under 3 yrs old in collection one and two in collection two and three cows of more than 3 yrs old in collection one and eight in collection two. When the 35 cows treated individually were classified according to their oocyte yields (Table 3), the different classes were well distributed over the four collections.

Table 1. Yield of oocytes and blastocysts obtained from separated or pooled ovaries in four separate experiments

<table>
<thead>
<tr>
<th>Experiment and Treatment</th>
<th>Number of cows</th>
<th>Number of oocytes</th>
<th>Number of oocytes per cow</th>
<th>Number of blastocysts</th>
<th>Number of blastocysts per cow</th>
<th>Number of blastocysts per oocyte (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Separated</td>
<td>10</td>
<td>151</td>
<td>15.1</td>
<td>17</td>
<td>1.7a</td>
</tr>
<tr>
<td></td>
<td>Pooled</td>
<td>3</td>
<td>36</td>
<td>12.0</td>
<td>4</td>
<td>1.3a</td>
</tr>
<tr>
<td>2</td>
<td>Separated</td>
<td>10</td>
<td>142</td>
<td>14.2</td>
<td>6</td>
<td>0.6b</td>
</tr>
<tr>
<td></td>
<td>Pooled</td>
<td>19</td>
<td>179</td>
<td>9.4</td>
<td>7</td>
<td>0.4b</td>
</tr>
<tr>
<td>3</td>
<td>Separated</td>
<td>5</td>
<td>67</td>
<td>13.4</td>
<td>6</td>
<td>1.2ab</td>
</tr>
<tr>
<td></td>
<td>Pooled</td>
<td>13</td>
<td>182</td>
<td>14.0</td>
<td>33</td>
<td>2.5a</td>
</tr>
<tr>
<td>4</td>
<td>Separated</td>
<td>10</td>
<td>133</td>
<td>13.3</td>
<td>18</td>
<td>1.8a</td>
</tr>
<tr>
<td></td>
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<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Total</td>
<td>Separated</td>
<td>35</td>
<td>493</td>
<td>14-1</td>
<td>47</td>
<td>1.3a</td>
</tr>
<tr>
<td></td>
<td>Pooled</td>
<td>34</td>
<td>397</td>
<td>11.7</td>
<td>44</td>
<td>1.3a</td>
</tr>
</tbody>
</table>

nd: Not determined.

a,bValues with different superscripts within a column are significantly different (P < 0.05).

Blastocyst production

The oocytes from cows treated individually produced between 0 and 6 blastocysts (SD = 1.8) with a mean value of 1.3 transferable embryos per cow (Table 1). However, oocytes from 19 of
these cows did not produce any blastocysts. The percentage of oocytes forming blastocysts ranged from 0 to 100% with a mean value of 9.5%. The number of blastocysts obtained per cow and the percentage of oocytes forming blastocysts was the same for pooled ovaries as for ovaries treated separately (1.3 transferable embryos per cow, 11.1% of the oocytes).

The number of oocytes forming blastocysts and the number of blastocysts produced per cow differed significantly \((P < 0.05)\) between the different collection times for the oocyte from individual ovaries and for oocytes from pooled ovaries (Table 1). The number of blastocysts per cow ranged from 0.4 to 2.5 and the percentage of oocytes forming blastocysts from 4.0 to 18.1%. However, when the results of the four collections were taken together, the percentage of oocytes forming blastocysts and the average number of blastocysts produced did not differ significantly between the group of cows treated individually and those treated together (9.5% and 11.1%, and 1.3 and 1.3, respectively, Table 1).

### Effect of age and oocyte yield

The nine younger cows (1–3 years) produced twice as many blastocysts per cow (1.6) than older cows (0.8) (Table 2). However, due to considerable variation between individuals, this difference was not significant. Furthermore, the percentage of oocytes forming blastocysts did not differ between the two age groups (9.0 versus 6.6%). The number of blastocysts produced per cow increased with the oocyte yield (Fig. 1a, \(r^2 = 0.151\)), but no significant correlation was found between the number of oocytes per cow and the percentage forming blastocysts as shown by the comparison of groups of cows represented in Table 3 and by percentage of blastocysts formed plotted against oocyte numbers (Fig. 1b). There was no significant difference in the percentage of oocytes forming blastocysts between cows that gave only a few oocytes (11.1%) and those cows that gave more than 30 oocytes (8.8%). No correlation was detected between numbers of oocytes and the percentage developing into blastocysts (Fig. 1b, \(r^2 = 0.002\)).
In our study we produced blastocysts from ovaries of individual cows by a complete in vitro procedure. This confirms the results recently obtained by Funahashi et al. (1991). Our results also suggest that the IVM–IVF–IVC procedure may be used successfully in breeding cattle of high genetic value. However, the yield in terms of oocytes per cow was highly variable (from 0 to 38, mean = 14.1). Furthermore, the percentage of oocytes forming blastocysts was low (9.5% on average) and also variable (0 to 100%). The number of embryos obtained from each individual cow was low and variable (0 to 6 transferable embryos per cow). The embryo yield per cow may be improved in at least two ways: first by increasing the number of oocytes recovered (for example by dissection of follicles) and second, by increasing the number developing into blastocysts by optimization of culture conditions (such as the method of producing the conditioned medium, the choice of basic medium, and growth factors or other protein additions). Nevertheless, IVF may already be a useful alternative to the classic technique of superovulation in some particular cases of breeding.

Unfortunately, the considerable individual variation observed in our experiments will impair the predictability of the number of transferable embryos that could be obtained from one cow. This variation between individual cows was not observed by Funahashi et al. (1991), but this difference may be due to the different breeds used in these two studies. In our experiments, three out of 35 cows failed to supply suitable cumulus–oocyte complexes and in 19 no transferable embryo was produced. The variation in the number of transferable embryos per cow was probably a result of accumulated variations in the oocyte yield and in the number of oocytes developing into blastocysts and they may help to explain the experimental variable results obtained in number of bovine IVF laboratories and in our experiments (Table 1). These variations could be due to the age of cows but in our experiments, the effect of age was not significant but should be evaluated using a larger number of cows. However, the overall number of oocytes developing into blastocysts seems to be correlated with the ratio of younger to older animals in each group of cows (11.3% blastocysts in collection one with 7 younger cows out of a total of 10 and 4.0% in collection two with 2 younger cows out of a total of 10). The recently developed technique of in vivo follicle puncture (Kruip et al., 1991; van der Schans et al., 1991) could be used to investigate this hypothesis further. A continuous study of the number of oocytes supplied by a particular cow, punctured at different times could be done. Oocyte yield and number of oocytes developing into blastocysts may explain the variability of production of embryos. As previously shown (Funahashi et al., 1991), the number of blastocysts increased with oocyte yield (Fig. 1a) but in our experiments, the blastocyst rate was not correlated with number of oocytes (Fig. 1b). For example, one cow supplied only four oocytes, which all became blastocysts, whereas another one that supplied as many as 31 oocytes, did not produce any blastocysts.
Our results also indicate that the embryo:medium-volume ratio does not seem to influence the rate of development of bovine IVM–IVF embryos as the number of oocytes developing into blastocysts was the same for cows supplying less than 10 and more than 30 oocytes, although various numbers of oocytes were cultured in a fixed volume of medium (Table 3 and Fig. 1b). The number of oocytes developing into blastocysts did not seem to be influenced by the number of embryos cultured together in a fixed volume of medium (Table 3 and Fig. 1b). Further experimentation is required to confirm this observation. Previous results suggested a possible intervention of oviduct cells in overcoming the 8–16-cell block encountered by cultured bovine embryos (Eyestone & First, 1989; Gandolfi et al., 1989a; Boccart et al., 1991). Oviduct cell secretions are probably involved in this control since conditioned medium can be used as well as co-culture (Eyestone et al., 1990) even if other proteins are not added (Mermillod et al., 1992). Furthermore, some proteins secreted by the oviduct that show an expression threshold restricted to the 4–5 first days of the oestrous cycle were recently identified in cows (Boice et al., 1990; Gerena & Killian, 1990) and other species (swine: Buhi et al., 1990; sheep: Gandolfi et al., 1989b; humans: Verhage et al., 1988) and were shown to bind to the embryos (Wegner & Killian, 1991). These observations suggest a central role for oviduct and oviductal secretions in the regulation of embryo development in cattle and other species. However, autocrine factors may act on embryo development of cows in another way and influence the quality of development (for example development chronology, viability and cell number) instead of the number of oocytes developing into blastocysts. This hypothesis remains to be tested and the respective intervention of these two possible control mechanisms remains to be evaluated in different mammalian species.

The conclusions of this study are first, that culture in small groups does not impair development of bovine embryos in vitro and this observation may indicate the preponderant function of genital tract secretions in the regulation of embryo growth in cows; second, that high individual variations in oocyte number and number of oocytes developing to blastocysts may explain experimental variations in bovine IVF; third, that the possible intervention of the age of cow providing oocytes has to be confirmed by a study with large numbers of animals, and fourth, that individual slaughtered cows may be used for IVF embryo production and can produce up to six transferable embryos in our experimental conditions. However, high individual variations will impair the prediction of blastocyst production from a single cow of high genetic value since 19 of our 35 cows did not produce a blastocyst.

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