Ram-specific effects on in-vitro fertilization and cleavage of sheep oocytes matured in vitro*

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Summary. The present experiments were designed to identify possible male-specific effects on early embryonic development in vitro. Sheep oocytes were matured in vitro for 24–26 h and then fertilized in vitro using equal numbers of viable spermatozoa from 1 of 6 Clun Forest rams. At 15–18 h after insemination, oocytes were either fixed and examined for fertilization and polyspermy or further cultured in modified M 199 medium for 3 days in an oviduct epithelial co-culture system. There were significant differences in 5 separate trials between the rams with respect to the rate of fertilization, degree of polyspermy and cleavage rate after monospermic fertilization. The mean rate of fertilization varied from 89% in Ram B to 43% in Ram C while the percentage of polyspermic eggs varied from 5 to 34%. Both the absolute number of embryos cleaving to the 16-cell stage and the calculated percentage of monospermic eggs reaching the 16-cell stage differed markedly between groups of eggs fertilized by different rams. The results indicate that the development of sheep eggs in vitro is differentially affected by the ram from which the spermatozoa are collected.

Keywords: ram effect; in-vitro fertilization; embryo development

Introduction

Efficient methods of in-vitro maturation and fertilization are of considerable importance for the future development of the techniques of nuclear transplantation and genetic manipulation in farm animals. The technique developed by Cheng (1985) provides a method for obtaining consistently high rates of fertilization of sheep oocytes matured in vitro. Moreover, it has been shown by transfer that the embryos produced in this manner are viable; 10 lambs were born from 7 out of 16 recipient ewes which received oocytes at 16 h after in-vitro insemination (Cheng et al., 1986). There is evidence that the spermatozoa from individual bulls and boars differ in their ability to fertilize eggs in vitro (Hanada, 1985a; Iritani et al., 1986; E. J. C. Polge, personal communication). The present study was carried out to evaluate whether similar variations exist in the semen of rams. Because of the marked effect of seasonality on fertility in rams, all the present experiments were carried out from November 1986 until March 1987.

Materials and Methods

Ovaries were obtained from sheep killed at a local abattoir, transported to the laboratory within 1 h of slaughter and the cumulus-oocyte complexes were collected from follicles of 2–5 mm in diameter by the method of Moor & Truenson (1977). The technique used for maturation was the same as that previously shown to give a high percentage of

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viable metaphase II oocytes (Staigmiller & Moor, 1984). Oocytes, together with additional granulosa cells, were cultured for 24–26 h at 39°C in a system that provided gentle agitation to the medium.

Freshly ejaculated semen, collected separately from 6 randomly selected Clun Forest rams (21–22 months old), was capacitated by a modification of the method described by Cheng (1985). Briefly, each semen sample was maintained undiluted at 20°C for 4 h and then washed three times (1000 g, 4 min) in PBS. Subsequently it was resuspended in 2 ml TCM 199 with Earle’s salts (Gibco Europe Ltd, Paisley, U.K.), pH 7.8, supplemented with 20% (v/v) heat-inactivated sheep serum collected on the day after oestrus (TCM 199-SS) (Cheng, 1985). The sperm concentration in the suspension was determined and the sample was adjusted to 2 ml containing $2 \times 10^8$ spermatozoa/ml and kept at 20°C for 40 min before it was further diluted with TCM 199-SS at pH 7.4 (Cheng, 1985) to a final concentration of $1 \times 10^6$ cells/ml. Hypotaurine and isoproterenol (Sigma Ltd, Poole, U.K.) were added immediately before in-vitro insemination at a final concentration of 10 and 16 μM respectively.

After maturation groups of oocytes coming from different ovaries were denuded of their cumulus cells by pipetting, washed in PBS (Cheng, 1985) and incubated in the prepared sperm suspension for 15–18 h at 39°C under controlled humidity (95%) and atmospheric conditions (5% CO₂ in air). At the end of the incubation period part of the oocytes from each group were fixed (acetic acid and alcohol, 1:3 v/v) and stained with 1% aceto-lacmoid for evidence of fertilization. The others were cultured for a further 3 days in Medium 199 supplemented with 10% fetal calf serum (Sera-Lab Ltd, Crawley Down, U.K.) in the presence of a monolayer of oviduct cells. These were collected from ewes on the 2nd day after oestrus and then cultured for 3 days before embryos were put on them (Gandolfi & Moor, 1987). Oocytes were considered as being fertilized when penetrated swollen sperm head(s) with residual sperm tail(s) or the female and male pronuclei with residual sperm tail(s) were visible. For evaluating cleavage, observations were made twice daily using an inverted microscope; after the 3-day culture period the oocytes were fixed and stained and the numbers of nuclei were recorded.

Semen from each ram was tested in 3–5 separate in-vitro trials for fertilization and cleavage ability. The observations for each ram, over days, were found to be very consistent and consequently comparisons between the 6 rams were carried out on the pooled data for each ram. Since the parameters of interest in this study were proportions based on relatively small integer values, inter-ram comparisons were examined using Fisher’s Exact Test as described by Bradley (1968).

**Results**

The rate of maturation to metaphase II in the present study ranged from 79 to 93% with a mean of 89.1% (Table 1). This table also shows the fertilization rates (including monospermy and polyspermy) of oocytes inseminated with spermatozoa collected from the 6 test rams. The fertilization rates were expressed as the percentage of the number of oocytes maturing to the second metaphase since no sperm penetration was found in the oocytes that had not matured to the second metaphase. There were numerous significant differences in the fertilization rates between rams which are detailed in Table 1. In particular the total number of oocytes fertilized using semen from Rams A, B and E was significantly higher than that of Rams C and D. The percentage of polyspermic oocytes was the highest for Ram B and this figure was significantly higher than those for

<table>
<thead>
<tr>
<th>Rams</th>
<th>No. of oocytes examined (replicates)</th>
<th>No. of oocytes matured* (%)</th>
<th>% of oocytes fertilized (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total†</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Monospermic†</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Polyshpermic‡</td>
</tr>
<tr>
<td>A</td>
<td>40 (4)</td>
<td>35 (88)</td>
<td>74 (26)</td>
</tr>
<tr>
<td>B</td>
<td>40 (3)</td>
<td>36 (90)</td>
<td>89 (32)</td>
</tr>
<tr>
<td>C</td>
<td>40 (5)</td>
<td>37 (93)</td>
<td>43 (16)</td>
</tr>
<tr>
<td>D</td>
<td>41 (5)</td>
<td>38 (93)</td>
<td>53 (20)</td>
</tr>
<tr>
<td>E</td>
<td>40 (5)</td>
<td>37 (93)</td>
<td>84 (31)</td>
</tr>
<tr>
<td>F</td>
<td>38 (5)</td>
<td>30 (79)</td>
<td>67 (20)</td>
</tr>
</tbody>
</table>

|$\text{D} < \text{B(0-1),E(1)}$  
|$\text{C} < \text{A(1),B(0-1),E(1)}$  
|$\text{B} > \text{C(5),E(5),F(5)}$

*Oocytes were fixed after maturation for 24–26 h and additional culture with spermatozoa for 15–18 h.
†Percentages of the number of oocytes matured to the second metaphase.
‡Percentages of the number of fertilized oocytes.
§Comparisons of rams and % level of significance (Fisher’s Exact Test).
Table 2. Cleavage in vitro of sheep follicular oocytes inseminated in vitro with ejaculated spermatozoa from different rams

<table>
<thead>
<tr>
<th>Rams</th>
<th>No of oocytes cultured* (replicates)</th>
<th>% of oocytes fertilized with monospermy†</th>
<th>% of oocytes cleaved to 2-cell</th>
<th>4-cell</th>
<th>8-cell</th>
<th>16-cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>28 (3)</td>
<td>57</td>
<td>86</td>
<td>82</td>
<td>79</td>
<td>50</td>
</tr>
<tr>
<td>B</td>
<td>27 (3)</td>
<td>58</td>
<td>74</td>
<td>67</td>
<td>56</td>
<td>56</td>
</tr>
<tr>
<td>C</td>
<td>27 (3)</td>
<td>35</td>
<td>44</td>
<td>44</td>
<td>41</td>
<td>26</td>
</tr>
<tr>
<td>D</td>
<td>29 (3)</td>
<td>50</td>
<td>48</td>
<td>48</td>
<td>41</td>
<td>28</td>
</tr>
<tr>
<td>E</td>
<td>26 (3)</td>
<td>73</td>
<td>85</td>
<td>85</td>
<td>85</td>
<td>69</td>
</tr>
<tr>
<td>F</td>
<td>28 (3)</td>
<td>60</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>68</td>
</tr>
</tbody>
</table>

§A > C(1),D(1)  #E > C(1),D(1)  F > C(5),D(5)  E > B(5),C(1),D(1)

*Oocytes were cultured for 3 days in TCM 199 + 10% FCS with sheep oviduct cells.
†Data from Table 1.
§Comparisons of rams and % level of significance (Fishers Exact Test).

Rams C, D, E and F. For the fertilization rates of monospermic oocytes the difference between Rams E and C was significant (P < 0.01) while the differences of E versus D and of F versus C and D were less marked (P < 0.06, not shown in Table 1).

Table 2 shows the cleavage ability of oocytes after in-vitro insemination using the same 6 rams. Not only fertilization rates but also the cleavage ability (2-16-cell stage) was significantly affected by a ram effect (see Table 2 for statistical details). The oocytes inseminated with spermatozoa from Rams C and D had a significantly lower ability to undergo the first cleavage than did those fertilized with the semen of Rams A, E and F. At more advanced stages, the percentage of cleaved oocytes decreased in all rams. However, 75-85% of oocytes inseminated with semen from Rams A, E and F reached the 8-cell stage, and 69 and 68% of oocytes inseminated with semen from Rams E and F reached the 16-cell stage after 3 days in culture. Indeed, the difference between Rams E and F versus Rams C and D already present at the level of monospermic fertilization was highly significant (P < 0.01) when the cleavage rate to 16-cells was considered.

The rank order of the rams for percentages of monospermic fertilization (Table 1) was the same as for percentages of oocytes cleaved to 16 cells (Table 2).

Discussion

The present study shows that the difference between rams was evident not only during in-vitro fertilization but also during cleavage. Considering only fertilized eggs, it became clear that the best cleavage rates were obtained from rams with the highest fertilization rate. Thus, Rams C and D showed poor results for fertilization and cleavage, whilst Rams A, E and F which had high fertilization rates (67-84%) also produced a high rate of 8-16-cell embryos. Although the semen from Ram B gave high fertilization rates (89%), the relatively poor percentage of 8-cell embryos (56%) was probably due to the high polyspermy (34%). Hanada (1985a) reported individual variations in the in-vitro fertilizing ability of bulls and Iritani et al. (1986) suggested that such variations could be related to the stage of the season, the age of the animals, the sperm quality and the number of ejaculations. All these possibilities have been taken into account. Firstly, to avoid seasonal effects, all our experiments were carried out in the mid-breeding season and all the comparisons in each replicate were carried out on the same day. Secondly, sperm concentrations and motility were continuously monitored and final sperm concentrations were uniform in all experiments. Thirdly, collections were made from each ram once a week and the first and second ejaculate were invariably
used for in-vitro fertilization. Other factors therefore appear to underly the male-associated differences.

We suggest that variations in either the composition of the seminal plasma or in the ratio between seminal plasma volume and sperm number may be responsible for the observed differences between fertilization rates. This suggestion is based on the knowledge that a very important part of capacitation consists in the removal or alteration of substances that are adsorbed on the sperm membrane upon its contact with the seminal plasma (Williams, 1972; Clegg, 1983; Austin, 1985). It is possible that the 4-h incubation at 20°C may have been insufficient to neutralize or remove these substances for some of the rams.

Cheng (1985) obtained a high fertilization rate (about 80%) in his series of experiments, but only 27% of zygotes underwent cleavage in a defined medium (modified BMOC-2). Hanada (1985b) reported a 20–41% fertilization and cleavage rate using Ham’s F10 + 10% sheep serum + sodium lactate. Crozet et al. (1987) obtained 63% of cleaved eggs (2–6 cells) after in-vitro fertilization of in-vitro matured oocytes in a defined medium with a high pH (7.7–7.8). In the present study, using the system of co-culture with oviduct epithelial cells developed by Gandolfi & Moor (1987), the percentage of cleaved eggs was high (2-cell, 44–86%; 8-cell, 41–85%; 16-cell, 26–69%) but depended on the ram used. Gandolfi & Moor (1987) cultured in-vivo fertilized one-cell embryos with oviducal cells, and obtained 97% early morulae and 42% expanded blastocysts after 3 and 6 days in culture, respectively. From the present results it appears that the co-culture system with the oviducal cells also provides an appropriate environment for the development of in-vitro fertilized one-cell eggs to at least the 8–16-cell stage.

In conclusion, individual rams affected not only the in-vitro fertilization rate but also the ability of zygotes to cleave after fertilization. Therefore, selection of rams with high fertilization ability is likely to be a major key for subsequent successful development in vitro.

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References


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