THE SURVIVAL AND DEVELOPMENT OF SHEEP EGGS FOLLOWING COMPLETE OR PARTIAL REMOVAL OF THE ZONA PELLUCIDA

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Summary. The effect of ‘Protease’ on the zona pellucida of fertilized and unfertilized sheep eggs was examined, and the effects of the treatment and of mechanical removal of part of the zona on the subsequent development of fertilized eggs was assessed in vitro and in vivo.

The zonae of follicular eggs were digested by ‘Protease’ within 25 min of exposure. In ovulated eggs, the resistance of the zona to digestion decreased with age. Zonae of eggs collected 2 days after oestrus (Day 2) were extremely resistant whereas nearly all the Day-5 eggs lost their zonae within 10 min. Fertilization had little effect upon the susceptibility of zonae to digestion by ‘Protease’.

Neither age of egg nor method of treating the zona had any effect on subsequent development of fertilized Day-2 and Day-6 eggs in culture, but there were effects of both factors on the proportion of eggs which developed to normal Day-25 embryos. ‘Protease’ treatment of Day-2 and Day-3 eggs failed to digest the zona completely and a high proportion of such eggs developed to normal embryos whereas only a few eggs of similar age, in which part of the zona was removed mechanically, developed normally. The zonae of Day-4 to Day-6 eggs were invariably removed by ‘Protease’ and the method of treatment had no effect on the proportion of eggs which developed to normal embryos. Irrespective of method of treatment, more Day-6 than Day-4 eggs developed to normal embryos. Culture for 2 days following treatment did not increase the survival of Day-6 eggs when they were transferred to recipients.

Ovulation and ageing of eggs appear to be associated with changes in the zona pellucida which influence its susceptibility to digestion by proteolytic enzymes. In early cleavage stage eggs, the major rôle of the zona may be protection of the inner cell mass from the uterine environment.

INTRODUCTION

In many mammalian species the zona pellucida prevents polyspermic fertilization (Braden, Austin & David, 1954) and may also be responsible for the normal development of fertilized eggs during early cleavage stages by maintaining the
intact integrity of the inner cell mass. In the mouse, rat, rabbit and pig, a relatively intact zona is essential for the continued development in vivo of recently fertilized eggs (Nicholas & Hall, 1942; Seidel, 1952, 1956, 1960; Tarkowski, 1959; Moore, Adams & Rowson, 1968; Moore, Polge & Rowson, 1969). Blastocysts and eight-cell eggs of the rat and mouse, from which the zonae have been completely removed, will show continued development in vitro, but on transfer to recipients only blastocysts show continued development (Bronson & McLaren, 1970; Modlinski, 1970; Brun & Psychoyos, 1972). In the sheep, Moor & Cragle (1971) found that eight-cell eggs, from which the zonae were removed enzymatically, failed to show continued development both in vitro, and after transfer to recipient ewes.

The zona appears to be necessary during early cleavage stages, but in most species it is shed during expansion of the blastocyst, exposing denuded cells to the uterine environment. At about the time the zona is lost, critical changes must occur in the characteristics of the egg or the surrounding environment.

The aims of the present study were to examine the effects of a proteolytic enzyme on the zona pellucida of sheep eggs and to determine the potential for further development of sheep eggs of known ages following damage to, or removal of, the zona pellucida.

**MATERIALS AND METHODS**

Eggs which had been collected from Merino ewes in which multiple ovulation had been induced with an equine anterior pituitary extract (HAP) were used in two experiments. The eggs were aspirated from ovarian follicles or were recovered by flushing the oviducts and uterine horns in vivo with Dulbecco phosphate buffer (C.S.L. Melbourne: composition in g/l: NaCl, 8·0; KCl, 0·2; Na₂HPO₄ (anhydrous), 1·15; KH₂PO₄, 0·2; CaCl₂, 0·1; MgCl₂, 0·1) enriched with 10% heterologous sheep serum (DB+10% S).

**Experiment 1.** Fertilized and unfertilized eggs which had been collected from the oviducts and uteri of donor ewes 2 to 5 days after they had been mated to entire or vasectomized rams were incubated with a ‘Protease’ from *Streptococcus griseus* (Sigma). Follicular eggs which had been collected, either 2 to 3 days after the onset of oestrus (‘unovulated eggs’), or 12 to 24 hr before the estimated time of ovulation (‘preovulatory eggs’) were similarly treated with ‘Protease’. Eggs from atretic follicles were discarded, as were those eggs which were not surrounded by at least one layer of corona radiata cells. ‘Protease’ was prepared as a 0·5% solution (w/v) in DB and the solution was sterilized by passage through cellulose filters (0·45-μm pore diameter, ‘Millipore’). The eggs were added to 2 ml ‘Protease’ solution, incubated at 30 to 35°C, and the times taken for the zonae to be digested were recorded.

**Experiment 2.** Fertilized eggs which had been collected from the oviducts and uteri of donor ewes 2 to 6 days after the onset of oestrus were either incubated in 0·5% ‘Protease’ as in Exp. 1, or a portion of the zona was mechanically torn off to expose the blastomeres. Following treatment, the eggs were transferred to recipient ewes or cultured in vitro for 3 days. A further group of Day-6 eggs
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which had been treated enzymatically or mechanically were cultured in vitro for 2 days and then transferred to recipients.

Mechanical removal of a portion of the zona was achieved by micromanipulation using sterile glass needles and crooks (Pl. 1, Fig. 1) attached to micromanipulators ('Leitz Wetzlar'). Manipulations were carried out at 30 to 35°C with the eggs held in DB+20% S in small concave glass dishes. In all cases, enough of the zona was removed to ensure that the inner cell mass was directly exposed to the environment.

Culture procedures

Culture was carried out at 37.5°C under normal atmospheric conditions in sterile pyrex glass tubes containing 2 ml medium under a layer (3 to 4 mm) of light-weight paraffin oil. The medium used was DB+20% S enriched with 10% fetal calf serum.

In this laboratory, this medium regularly supports growth of two-cell eggs to eight cells, eight-cell eggs to about twenty cells and morulae to expanded and hatched blastocysts.

Eggs which had been exposed to 'Protease' were washed three times in 6 to 8 ml DB+20% S before culture or transfer. All eggs, apart from those destined for transfer, were examined after culture both as fresh preparations and after staining with 1% orcein.

Transfer procedures

Following removal of the zonae, one or two eggs were transferred to each recipient ewe which had been run with a harnessed vasectomized ram and inspected for oestrus twice daily. Day-2 eggs (day of oestrus = Day 0) were transferred to the oviducts, Day-4 and Day-6 eggs were transferred to the uterine horns, and equal numbers of Day-3 eggs were transferred to the oviducts and uterine horns. All transfers were made to the oviducts or uterine horns ipsilateral to the ovary which contained the corpus luteum. When eggs were transferred immediately after the removal of their zonae, the transfers were made to synchronous recipients which had been first observed in oestrus at the same time as their respective donors. Day-6 eggs were cultured for 48 hr and then transferred to recipients which were first served 12 hr after their donors. This was done in an effort to compensate for any retardation in development that might have occurred during culture.

Recipients which did not return to oestrus following transfer were killed to recover embryos on Day 25. The embryos were classified as normal, retarded or resorbing according to the criteria of normal development described by Bryden, Evans & Binns (1972).

RESULTS

Experiment 1. Effect of 'Protease' on the zona pellucida

There was a marked effect of age of egg and a minor effect of fertilization on the susceptibility of the zona to 'Protease' (Table 1). Follicular eggs, irrespective of whether they were collected 12 to 24 hr before any follicles had ruptured
(preovulatory eggs) or 2 to 3 days after oestrus when most follicles had ruptured (unovulated eggs), were extremely susceptible to 'Protease' digestion and the zonae of all fifty-four follicular eggs were completely removed within 25 min of exposure.

For ovulated eggs, the zonae of all Day-2 unfertilized, and the majority of Day-2 fertilized eggs were resistant to 'Protease' and even after incubation for 12 hr the zonae, although somewhat thinned, were still intact. The zonae of Day-3 eggs were almost equally distributed between rapid digestion and resistance to 'Protease', and by Day 5 the susceptibility to 'Protease' had markedly increased and the zonae of most eggs (61 of 64: 95%) were completely digested within 10 min.

Table 1. The effect of age of sheep eggs on the time taken to remove the zona pellucida with 'Protease'

<table>
<thead>
<tr>
<th>Age of egg (days after oestrus)</th>
<th>No. of eggs</th>
<th>Total no. of eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfertilized eggs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preovulatory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unovulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>43</td>
<td>44</td>
</tr>
<tr>
<td>5</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>Total</td>
<td>159</td>
<td>231</td>
</tr>
<tr>
<td>Fertilized eggs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>37</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>44</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td>Total</td>
<td>70</td>
<td>150</td>
</tr>
</tbody>
</table>

* Follicular eggs—preovulatory, collected 12 to 24 hr before ovulation; unovulated, collected 2 to 3 days after oestrus.

All other eggs had been ovulated.

Table of \( \chi^2 \): proportion of Day-2 to Day-5 eggs denuded by enzyme treatment

<table>
<thead>
<tr>
<th>Source of deviation</th>
<th>d.f.</th>
<th>( \chi^2 )</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of egg</td>
<td>3</td>
<td>143.71</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fertilization</td>
<td>1</td>
<td>1.42</td>
<td>N.S.</td>
</tr>
<tr>
<td>Interaction</td>
<td>3</td>
<td>41.66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>186.79</td>
<td></td>
</tr>
</tbody>
</table>
Within fertilized eggs, the division into rapid digestion and resistance to 'Protease' was not as clear cut as in unfertilized eggs. In 18% of fertilized Day-2 eggs, the zonae were rapidly digested while the zonae of all unfertilized Day-2 eggs were resistant to digestion. The zonae of all unfertilized Day-4 eggs were rapidly digested while in 10 of 44 fertilized Day-4 eggs the zonae were still present even after incubation for 12 hr.

Experiment 2. Development of fertilized eggs

In preliminary studies, it had been found that exposure of fertilized eggs to 'Protease' for more than 10 min resulted in separation of blastomeres with subsequent failure to show further cleavage in culture. Hence, incubation of eggs in 'Protease' in this experiment was restricted to a maximum of 10 min irrespective of whether or not the zonae were completely removed. Incubation of Day-2 and Day-3 eggs for 10 min thinned the zonae by some 25 to 40%, but in all eggs the zonae remained intact. Zonae of Day-4 and Day-6 eggs were invariably digested within 10 min and the eggs were removed from 'Protease' immediately their zonae were lost.

At the time of collection, Day-2 eggs consisted of two to four cells; Day-3 eggs of eight cells; Day-4 eggs of eight to sixteen cells and Day-6 eggs were morulae of more than twenty cells. Eggs which developed in culture from four (or less) cells to eight cells; from eight cells to about twenty cells (Pl. 1, Fig. 2), and from twenty or more cells to blastocysts (Pl. 1, Fig. 3), were considered to have shown a significant degree of development in culture. In Day-3 and older eggs, these rates of cleavage were similar to those which occurred in vivo. In Day-2 eggs, however, development to eight cells during culture for 48 hr was indicative of a retarded rate of cleavage. Examination of the unstained orcein-stained preparations did not reveal any gross abnormality in the cultured eggs. Neither the age of an egg nor the method of treatment (enzymatic or mechanical) had any effect upon the subsequent development of eggs in culture (Table 2).

There were major effects of both age of egg and of method of treatment on the survival and development of eggs transferred to recipients (Table 2). The effects of age and treatment were due almost entirely to a high rate of survival of Day-2 and Day-3 eggs treated with 'Protease'. In these eggs, the zonae, although thinner, remained intact after treatment, whereas in all ages of eggs treated mechanically and Day-4 and Day-6 eggs treated with 'Protease', the inner cell mass was directly exposed to the surrounding environment. In Day-4 and Day-6 eggs, there was no suggestion of any difference between methods of treatment on the proportion of eggs which developed to normal embryos (9 of 46 versus 14 of 47). When data for both treatments were pooled, and those for Day-2 and Day-3 eggs treated with 'Protease' were excluded, a significant overall increase from Day 2 to Day 6 in the proportion of eggs which developed to normal embryos was observed (1 of 24 to 16 of 46; \( P<0.01 \)). Further, there was an indication that survival rate of Day-6 eggs was greater than that of Day-4 eggs (16 of 46 versus 7 of 47; \( P<0.05 \)). For Day-3 eggs, the site of transfer had no effect upon subsequent survival. Normal embryos developed from eleven of twenty-two eggs transferred to the oviducts and nine of twenty-three eggs transferred to the uterus. Resorbing membranes with no evidence of embryo
Table 2. The effect of age of sheep eggs and of the method of removing a portion or all of the zona pellucida on development in vitro and in vivo

<table>
<thead>
<tr>
<th>Age of egg (days after oestrus)</th>
<th>No. of eggs</th>
<th>No. of recipients</th>
<th>No. of eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Developed in culture</td>
<td>Which received eggs</td>
<td>Transferred to normal embryos</td>
</tr>
<tr>
<td></td>
<td>No. of eggs</td>
<td>No. of recipients</td>
<td>No. of eggs</td>
</tr>
<tr>
<td>Mechanical removal of a portion of the zona</td>
<td>2</td>
<td>37</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>22</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>28</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>32</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>119</td>
<td>80</td>
<td>48</td>
</tr>
<tr>
<td>Enzymatic removal of zona</td>
<td>2*</td>
<td>34</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>3*</td>
<td>24</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>4†</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>6†</td>
<td>41</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>119</td>
<td>81</td>
<td>48</td>
</tr>
</tbody>
</table>

* Zona thinned but not removed.
† Zona completely removed.

Analysis of variance: development of transferred eggs to normal embryos

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>M.S.</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of egg</td>
<td>3</td>
<td>132.52</td>
<td>3.54</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Method of removal of zona</td>
<td>1</td>
<td>693.78</td>
<td>18.55</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Interaction</td>
<td>3</td>
<td>115.29</td>
<td>3.08</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Development were recovered from only three ewes, all of which had received mechanically treated eggs.

Of the sixty-five Day-6 eggs which were cultured for 2 days following enzymatic and mechanical treatment, fifty-four developed to blastocysts (Pl. 1, Fig. 3) and forty-seven of the blastocysts, selected at random, were transferred to

EXPLANATION OF PLATE 1

Photomicrographs of fresh preparations of sheep eggs treated mechanically or with 'Protease'.

Fig. 1. Glass crook and needle in the process of removing a portion of the zona of a Day-3 eight-cell egg. Note extensive tearing of the zona and the appearance of an open hole exposing the blastomeres. x 400.

Fig. 2. A Day-4 eight-cell egg developed in culture to about twenty cells following complete removal of the zona with 'Protease'. Phase contrast, x 1280.

Fig. 3. A Day-6 morula developed in culture to the blastocyst stage following removal of a portion of the zona mechanically. The blastocyst is in the process of shedding the zona by escape through the torn section of the zona. Note the appearance of normal morphology after culture and during escape from the zona. Phase contrast, x 400.
(Facing p. 102)
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thirty recipient ewes. At autopsy on Day 25, eighteen normal embryos were recovered from fifteen recipients. Three of the fifteen had one abnormal embryo (retarded or resorbing) as well as one normal embryo, and a further three recipients had one or more abnormal embryos. Development in culture to blastocysts was similar to that of Day-6 eggs in Exp. 1, which were treated in the same manner but not transferred to recipients (54 of 65 (83%) versus 57 of 73 (78%)). Culture of Day-6 eggs following treatment appeared to have little effect upon their subsequent development to normal embryos. If it is assumed that eggs which failed to develop in vitro to blastocysts were not capable of development in vivo to normal embryos, then the calculated number of normal embryos, which would have been obtained had all sixty-five Day-6 eggs been transferred to recipients after treatment and culture, was 18/47 of 54 = 21, or 32%. This is similar to the proportion of Day-6 eggs which developed to normal embryos when transferred immediately after removal of their zonae (16 of 46: 35%).

DISCUSSION

The changes observed in the susceptibility of the zona pellucida to digestion by 'Protease' are indicative of specific changes in the chemical properties of the zona. In the sheep, ovulation occurs 12 to 24 hr after the onset of oestrus (Killeen & Moore, 1970) so that, in relation to ovulation, Day-2 eggs were only 24 to 36 hr old, yet the properties of their zonae were quite different from those of follicular eggs. The changes in the zona associated with ovulation may result from the release of the eggs from the surrounding follicular fluids or from exposure to the oviducal environment. Day-4 eggs showed a marked change in susceptibility to digestion and it could be expected that by the 4th day after oestrus most eggs would have entered the uterus (Lang, 1969; Holst & Braden, 1972). The most simple explanation for the increased susceptibility of ovulated eggs to 'Protease' digestion lies in a change in the zona resulting either from ageing, or from exposure to the uterine environment.

Changes in the chemical properties of the zona have been reported, but only in relation to the zona reaction following penetration by spermatozoa. In rodents, the release of trypsin-like proteolytic enzymes from the cortical granules of the vitellus can be induced by contact with spermatozoa or by electrical stimulation. These enzymes appear to alter the zona and effectively block sperm penetration (Austin & Braden, 1956; Barros & Yanagimachi, 1971; Gwatkin, Williams, Hartmann & Kniazk, 1973), but the form of the change in the chemical structure of the zona has not yet been described. In the present study, fertilization had only a minor effect on the susceptibility of the zona to digestion, similar to that reported by Moor & Cragle (1971). In the sheep it would appear that penetration of the zona by spermatozoa and the resulting changes in the properties of the zona associated with the zona reaction do not markedly alter the susceptibility of the zona to digestion by 'Protease'.

Continued cleavage of eggs in culture following removal of the zona has been observed in other species (Mintz, 1962, 1964, 1965; Edwards, 1964; Tarkowski & Wroblewska, 1967; Mochow & Olds, 1968; Brackett, Killen &
Postgraduate technical were observed. The cyst. changes environment part eggs transferred present. The Committee egg blastomeres zona-free support the due high rates of development in vivo (Moore, 1970). Normal survival and development has also been obtained with eggs of more advanced cleavage stages following the transfer of eggs, cultured in a similar medium for 2 and 3 days, even after the eggs were stored for up to 2 days at 5°C (Moore & Bilton, 1973). A more likely explanation for the reduced survival of eggs with exposed blastomeres appears to lie in the rôle played by the zona in retaining the integrity of the cells, or in the ability of the zona to protect the egg from destruction by hostile factors within the uterine environment.

Bronson & McLaren (1970) suggested that failure of zona-free eight-cell eggs to survive in the mouse following transfer to the oviducts, as distinct from quite high rates of survival of zona-free blastocysts transferred to the uterus, was due to dispersion of blastomeres of the eight-cell eggs during their passage through the oviducts. The presence of intercellular connections in blastocysts of the rat and their absence in eight-cell eggs (Schlafke & Enders, 1967) was quoted to support this suggestion. Modlinski (1970) presented evidence for adherence of zona-free eight-cell mouse eggs to the oviducal epithelia. The results of the present experiment would suggest, at least in the sheep, that dispersion of blastomeres or adherence to the oviduct were not major reasons for loss of transferred eggs. No difference could be found between survival rates of Day-3 eggs transferred to the oviducts or uteri and similar low rates of survival were observed in Day-4 eggs which were all transferred to uteri. At no stage, however, were completely denuded eggs transferred to the oviducts and the presence of part of the zona could have prevented total blastomere dispersion or adherence.

If destruction of the exposed blastomeres by components of the uterine environment is responsible for loss of most of the transferred eggs, then dramatic changes must occur in either the uterine environment or the developing sheep egg at about the time when the zona is normally shed by the expanding blastocyst.

ACKNOWLEDGMENTS

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