VARIATION IN PORCINE ZONA PELLUCIDA MORPHOLOGY FOLLOWING PRONASE TREATMENT1,2

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Summary

The effects of cell stage and day of embryo collection on the pronase-induced zona pellucida morphology in pig embryos were investigated. Three hundred and seventy-two two- to eight-cell embryos were treated for 3.0 min in 5.0% pronase in Whitten's Medium. Responses in zona pellucida morphology observed after pronase treatment were: (1) absent, (2) stretched and (3) intact. Each pig was coded as to the type of zona pellucida morphology its embryos possessed after enzyme treatment according to the following scale: (1) only intact, (2) intact and stretched, (3) only stretched, (4) stretched and absent and (5) only absent. Also, the number of embryos of a particular cell stage was expressed as a fraction of the total number of embryos collected from each pig. The incidence of intact zonae pellucidae was greatest (P<.05) among two- to three-cell embryos and the incidence of absent zonae pellucidae was greatest (P<.05) among six-to eight-cell embryos. A significant correlation (r = .79) was observed between day of embryo collection and coded zona pellucida morphology. The results suggest that the pronase-induced zona pellucida morphology observed is dependent on cell stage and day of embryo collection.

(Key Words: Pigs, Embryos, Zona Pellucida Morphology, Pronase.)

Introduction

Since Mintz's (1962) use of pronase to remove the murine zona pellucida in the production of chimeras, numerous investigators associated with the farm species have adopted the enzyme treatment. In early attempts to remove the zona pellucida of 3- to 5-d sheep embryos with .05, .2, .5 and 1.0% pronase, complete dissolution occurred only after prolonged treatment in 1.0% pronase (Pighills et al., 1968). Moor and Cragle (1971) observed variable zona pellucida solubilities of sheep embryos treated with .5% pronase and attributed the differences to variation between animals. Trounson and Moore (1974) also treated sheep embryos with .5% pronase; however, these investigators reported a significant effect in zona pellucida solubility due to age of the embryo.

During attempts in our laboratory to dissolve the zona pellucida of two-, four- and eight-cell pig embryos (Menino and Wright, 1979a,b), variable responses in zona pellucida morphology were observed following pronase treatment. Therefore, to determine if embryo age affected the pronase-induced zona pellucida morphology observed in the pig, we examined the effects of cell stage and day of embryo collection.

Materials and Methods

Embryo Collection. Forty cycling gilts, six sows and two synchronized and superovulated prepuberal gilts were used. Cycling gilts and sows were checked daily for estrus and hand-mated with boars of proven fertility. Prepuberal gilts were synchronized and superovulated with pregnant mare’s serum gonadotropin (PMSG)5 and human chorionic gonadotropin (CG)5, and bred artificially 24 h after CG with approximately 70 ml of a freshly collected raw ejaculate (Baker and Coggins, 1968). Prepuberal gilts...
displaying estrus after hormone treatment were also hand-mated.

Gilts and sows were slaughtered 24 to 120 h after breeding, and the excised reproductive tracts were maintained at 37°C during transport to the laboratory. Oviducts and uteri were flushed with Whitten's Medium (WM) lacking a bovine serum albumin (BSA) supplement (Whitten and Biggers, 1968). Flushings were recovered in embryo collection dishes and examined with a dissecting microscope for the presence of embryos (magnification ×10). Embryos were recovered from the flushings by aspiration through siliconized glass pipettes and transferred into 50 µl microdrops (Brinster, 1963) of WM with 1.5% BSA (A grade)\(^6\), under paraffin oil (Saybolt Viscosity 125/135)\(^6\). Embryos were washed by serial transfer through three microdrops before evaluation of cell stage and overall morphology with an inverted stage contrast microscope (magnification ×200).

**Pronase Preparation.** A 5.0% solution was prepared by dissolving .25 g of pronase (Protease, B grade)\(^5\) in 5.0 ml of WM. The enzyme preparation was allowed to stand overnight at 4°C before centrifugation at 3,000 x g for 10 min to remove any insoluble cellular debris. The pronase solution was adjusted to pH 7.2 to 7.6 and sterilized by filtration (.22 µm). Sterile 5.0% pronase in WM was stored in disposable 16 × 125 mm Falcon plastic culture tubes at 4°C. Before use, approximately .5 ml of pronase solution was aseptically pipetted into a disposable 35 × 10 mm Falcon plastic tissue culture dish and incubated at 37°C in a humidified atmosphere of 5% CO\(_2\) in air for 15 to 30 min.

**Pronase Treatment.** Two- to eight-cell pig embryos were transferred from microdrops of WM with 1.5% BSA to culture dishes containing .5 ml of 5.0% pronase in WM. Embryos were incubated in the enzyme solution for 3.0 min at 37°C. When 3.0 min had elapsed, embryos were immediately recovered from the pronase solution and washed by serial transfer through three microdrops of WM with 1.5% BSA. The effect of pronase on the zonae pellucidae of treated embryos was evaluated by phase contrast microscopy (x200) and zona pellucida morphology was recorded as: (1) absent, indicating that the zona pellucida had completely dissolved; (2) stretched, indicating that the zona pellucida had only thinned and (3) intact, indicating that no observable change had occurred due to the pronase treatment (figure 1, A through D). Enzyme-treated embryos were then allocated to an ongoing culture project.

Following enzyme treatment, each animal was coded as to the type of zona pellucida morphology displayed by its embryos. Embryos were coded as possessing: (1) — only intact zonae pellucidae, (2) — intact and stretched zonae pellucidae, (3) — only stretched zonae pellucidae, (4) — stretched and absent pellucidae and (5) — only absent zonae pellucidae. Also, the number of embryos belonging to a particular cell stage was recorded and expressed as a fraction of the total number of embryos collected from each animal. For example, a gilt in which three four-cell and seven eight-cell embryos were collected was assigned the following fractions: .30 (four-cell) and .70 (eight-cell). The fractions of embryo cell stages were then tabulated and means were calculated from all animals in each of the coded zona pellucida morphology groups.

**Statistical Methods.** Chi-square analysis was used to evaluate the effect of cell stage on pronase-induced zona pellucida morphology (Steel and Torrie, 1960). The mean fractions of embryos of a particular cell stage for pigs with (1) only intact, (2) intact and stretched, (3) only stretched, (4) stretched and absent and (5) only absent zona pellucidae were tested by a one-way analysis of variance, and differences were determined by Least Significant Difference procedures (Steel and Torrie, 1960). Correlation-regression analysis with day of collection (X) and coded zona pellucida morphology (Y) was conducted to determine if a significant relationship existed between the two variables (Steel and Torrie, 1960).

**Results**

Five hundred and fifty embryos were collected from 690 ovulations in six sows and forty-two gilts, for a recovery rate of 79.7% and an ovulation rate of 14.4/animal (table 1). A total of 372 two- to eight-cell embryos were treated with 5.0% pronase in WM for 3.0 min. The remaining 178 embryos not treated consisted of the following cell stages: one-cell, morula, degenerate and two- to eight-cell

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\(^5\)Fisher Scientific Co., 2761 Walnut Ave., Tustin, CA 92680.
Figure 1. The effect of pronase treatment on four-cell embryos collected 48 to 72 h after mating. (A) Untreated. (B) Intact zona pellucida. (C) Stretched zona pellucida. (D) Absent zona pellucida. X 370.

TABLE 1. DISTRIBUTION OF CELL STAGES IN SOW AND GILT EMBRYOS COLLECTED AT SLAUGHTER BETWEEN D 1 AND 5 AFTER NATURAL MATING

<table>
<thead>
<tr>
<th>Cell stage</th>
<th>Day of embryo collection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1 or degenerate</td>
<td>16</td>
</tr>
<tr>
<td>2 to 3</td>
<td>54</td>
</tr>
<tr>
<td>4 to 5</td>
<td>45</td>
</tr>
<tr>
<td>6 to 8</td>
<td>9</td>
</tr>
<tr>
<td>Morula</td>
<td>0</td>
</tr>
<tr>
<td>No. of embryos</td>
<td>124</td>
</tr>
<tr>
<td>No. of pigs</td>
<td>12</td>
</tr>
</tbody>
</table>

*one synchronized, superovulated gilt bred artificially, from which 31 embryos were collected, did not show estrus and is not included in the table.
TABLE 2. INCIDENCE OF INTACT, STRETCHED AND ABSENT ZONAE PELLUCIDAE IN TWO- TO EIGHT-CELL PIG EMBRYOS TREATED WITH PRONASE

<table>
<thead>
<tr>
<th>Cell stage</th>
<th>Intact</th>
<th>Stretched</th>
<th>Absent</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 to 3</td>
<td>58ad</td>
<td>11ae</td>
<td>4af</td>
<td>73</td>
</tr>
<tr>
<td>4 to 5</td>
<td>114bd</td>
<td>65be</td>
<td>54bd</td>
<td>233</td>
</tr>
<tr>
<td>6 to 8</td>
<td>16cd</td>
<td>7ad</td>
<td>43ce</td>
<td>66</td>
</tr>
<tr>
<td>Totals</td>
<td>188</td>
<td>83</td>
<td>101</td>
<td>372</td>
</tr>
</tbody>
</table>

a,b,c Values in the same column without common superscripts differ (P<.05).
d,e,f Values in the same row without common superscripts differ (P<.05).

embryos used as controls in an ongoing culture project.

The numbers of pronase-treated embryos with intact, stretched or absent zonae pellucidae are presented in table 2. The proportion of two- to three-cell embryos with intact zonae pellucidae was greater (P<.05) than the proportion of either four- to five- or six- to eight-cell embryos, and the number of four- to five-cell embryos with intact zonae pellucidae was greater (P<.05) than the number of six- to eight-cell embryos with intact zonae pellucidae was lower (P<.05) than that of four- to five-cell embryos and the proportion of four- to five-cell embryos with absent zonae pellucidae was smaller (P<.05) than the number of six to eight-cell embryos.

At the two- to three-cell stage, the incidence of intact zonae pellucidae was greater (P<.05) than that of stretched or absent and the incidence of stretched zonae pellucidae was greater (P<.05) than that of absent zonae pellucidae (table 2, figure 2). The proportion of embryos with stretched zonae pellucidae was greater (P<.05) than that of either two- to three- or six to eight-cell embryos, with no significant difference between the latter. The proportion of two- to three-cell embryos with absent zonae pellucidae was lower (P<.05) than that of four- to five-cell embryos and the proportion of four- to five-cell embryos with absent zonae pellucidae was smaller (P<.05) than the number of six to eight-cell embryos.

![Figure 2](image.png)

**Figure 2.** Percentages of two- to three-, four- to five- and six- to eight-cell embryos with intact, stretched or absent zonae pellucidae following pronase treatment.

![Figure 3](image.png)

**Figure 3.** Percentages of pronase-treated embryos with intact, stretched and absent zonae pellucidae at the two- to three-, four- to five and six- to eight-cell stages.
TABLE 3. MEAN FRACTIONS OF PIG EMBRYOS CODED AS CONTAINING (1) ONLY INTACT, (2) INTACT AND STRETCHED, (3) ONLY STRETCHED, (4) STRETCHED AND ABSENT AND (5) ONLY ABSENT PRONASE-INDUCED ZONAE PELLUCIDAE

<table>
<thead>
<tr>
<th>Zona pellucida morphology</th>
<th>No. of pigs</th>
<th>Mean fraction of embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 or degenerate</td>
</tr>
<tr>
<td>Only intact</td>
<td>20</td>
<td>.08c</td>
</tr>
<tr>
<td>Intact and stretched</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Only stretched</td>
<td>9</td>
<td>.04c</td>
</tr>
<tr>
<td>Stretched and absent</td>
<td>4</td>
<td>0c</td>
</tr>
<tr>
<td>Only absent</td>
<td>12</td>
<td>.16c</td>
</tr>
</tbody>
</table>

None of the pigs observed had embryos with both intact and absent zonae pellucidae.

Not included in analysis of variance because there was only one animal in this group.

Means in the same column without common superscripts differ (P<.05).

at the four- to five-cell stage with stretched zonae pellucidae was greater (P<.05) than the proportion of either absent or intact, with no significant difference between the latter. More (P<.05) six- to eight-cell embryos has absent zonae pellucidae than either intact or stretched zonae pellucidae, with no significant difference between the latter.

The mean fractions of embryo cell stages collected from pigs coded as (1) only intact, (2) intact and stretched, (3) only stretched, (4) stretched and absent and (5) only absent zonae pellucidae following pronase treatment are presented in table 3. No significant difference was observed in the fraction of one-cell or degenerate embryos among pigs with the various zonae pellucidae. However, the fraction of two- to three-cell embryos was greater (P<.05) in pigs with only intact zonae pellucidae than in those with absent zonae pellucidae. No significant difference in the fraction of two- to three-cell embryos was observed among pigs with only intact, only stretched and stretched and absent zonae pellucidae, or between pigs with only stretched, stretched and absent and only absent zonae pellucidae. The fraction of four- to five-cell embryos did not differ significantly among pigs with only intact, only stretched and stretched and absent zonae pellucidae; however, the fraction was lower (P<.05) for pigs with only absent zonae pellucidae. The fraction of six- to eight-cell embryos was greater (P<.05) in pigs with only absent zonae pellucidae than in those with either only intact or only stretched zonae pellucidae, but was not significantly different from the fraction in pigs with stretched and absent zonae pellucidae. Morulae were not observed in pigs with only intact, only stretched or stretched and absent zonae pellucidae; however, the fraction of morulae was greater (P<.05) in pigs with only absent zonae pellucidae.

The analysis of variance regression for the coded zona pellucida morphology on day of embryo collection is presented in table 4. Day

**P<.01.
of collection was determined as the time between slaughter and the last day of breeding for naturally mated pigs (d 0). Significance in the regression analysis indicated that variation in day of embryo collection contributed to variation in the pronase-induced zona pellucida morphology. The correlation coefficient of day of embryo collection vs coded zona pellucida morphology was .79 and highly significant as determined by Student's t-test.

Discussion

The results suggest that the variable zona pellucida morphology observed in pig embryos treated with pronase is dependent on both day of collection and embryo cell stage. The present study, conducted with the pigs, is in agreement with that of Trounson and Moore (1974) with sheep. Trounson and Moore (1974) reported that "Protease" treatment of 2- to 3-d embryos failed to solubilize the zona pellucida completely while 4- to 6-d embryos possessed zonae pellucidae that readily dissolved. The authors concluded that either aging of the embryo or a component of the uterine environment was responsible for the varied solubility effects. Although Moor and Cragle (1971) attributed the difference in zona pellucida solubility to individual animal variation, all embryos were recovered at 72 h after estrus and displayed marked variability in response to pronase. Sheep embryos collected at this time and exposed to pronase by Trounson and Moore (1974) demonstrated similar variation in zona pellucida solubility. It is conceivable that 3 d after estrus represents a period in which the zona pellucida, because of an embryo, uterine or combined influence, undergoes a transition in morphology identifiable by a decreased resistance to pronase. Individual animal variation may contribute to temporal differences in onset of the solubility change, which would explain the variability in zona pellucida solubility displayed by 3-d sheep embryos. Sheep embryos collected at this time have eight to 16 cells and are entering the uterus (Moore, 1977). If a uterine effect is indeed responsible for the transition in zona pellucida solubility, then the time at which embryos enter the uterus and are exposed to that environment would further explain the variation observed. Such an effect would support the observations from the present study in which pig embryos at the four- to five-cell stage were almost equally distributed among the three categories of zona pellucida morphology (figures 2 and 3). Pig embryos enter the uterus approximately 48 h after ovulation and would be exposed to the uterine environment at the four- to five-cell stage (Polge, 1977). Consequently, solubility of the pig zona pellucida at the four-cell stage may depend on whether the embryos are oviductal or uterine and, if uterine, on the length of time in utero.

Although the theory that a uterine factor acts on the zona pellucida has been proposed, this study did not determine the source of an agent(s) capable of exerting such an effect. Denker (1977) has reported that the tropho-blast cells of the rabbit blastocyst undergo an increase in enzymatic activity of a specific endopeptidase (coined blastolemmase) at 6 d postcoitum. Bergstrom (1972) reported that hatching in the mouse, the process by which the blastocyst escapes from the zona pellucida, is due to expansion of the blastocoele and not gradual lysis. On the basis of their observations in mice, Orsini and McLaren (1967) suggested that loss of the zona pellucida is due to both the hatching process and a factor produced by the estrogen-sensitized uterus. Mintz (1971) has suggested the presence of an estrogen-dependent uterine lysin that dissolves the mouse zona pellucida and acts as an implantation-initiating factor.

The chemical nature of a uterine factor capable of exerting a solubility change in the zona pellucida is as yet undetermined; however, Brun and Psychoyos (1972) have speculated that, in the rat, a transient acidosis in the uterine environment may be responsible. Rosenfeld and Joshi (1977) have isolated a specific uterine fluid peptidase in the rat that has considerable affinity for the zona pellucida.

The presence of uterine factors weakening the zona pellucida to allow blastocyst escape in the hatching process would explain the limited degree of hatching observed in embryos cultured in vitro from domestic animals (for a review, see Wright and Bondioli, 1981). Although the incidence of blastocyst formation increases with advanced cell stage, many of the blastocysts developing in vitro fail to initiate and complete the hatching process, especially blastocysts developing from embryos in the early cleavage stages. Continued exposure to uterine factors may be necessary for hatching in vivo, consequently, removal of the embryo
from the uterus would prevent interaction with the zona pellucida.

Morphological observations of pig embryos made by Lindner and Wright (1978) demonstrated that expanded blastocysts obtained in vivo were significantly larger than in vitro. Also, the zona pellucida was twice as thick in in vitro cultured-expanded blastocysts as in vivo blastocysts, although the difference was not statistically significant (Lindner and Wright, 1978). The size differences in expanded blastocysts and zona pellucida thickness may be due to a structurally weakened zona pellucida which allows the in vivo blastocyst to expand to a greater size and to stretch the zona pellucida thinner. Lindner and Wright (1978) speculated that an enzymatic factor is involved in hatching on the basis of the observation that zonae pellucidae of expanded blastocysts never regain their preexpansion thickness. The data presented strongly suggest that the pronase-induced zona pellucida morphology observed represents time-dependent changes in zona pellucida solubility. In addition, a factor of either uterine or embryo origin, or both, has been implicated in the alteration of the solubility of the pig embryo zona pellucida, as evaluated by a variable pronase-induced zona pellucida morphology.

Literature Cited


