Effects of various conditions of semen storage on the acrosin system of human spermatozoa

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Summary. Stability of the human sperm acrosin system (major components: non-zymogen acrosin, proacrosin and acrosin inhibitor) was studied under various conditions of semen storage used clinically or in the laboratory. Freezing at -196°C caused a profound decrease in total acrosin content and in the amount of this enzyme present in zymogen form (proacrosin), but resulted in some increase in non-zymogen acrosin. Acrosin inhibitor did not appear to be significantly affected by this treatment. No relationship was present between the decreases in sperm motility induced by freezing to -196°C and the alterations in total acrosin, proacrosin and non-zymogen acrosin.

Storage of whole semen at -20°C had deleterious effects on all the components of the acrosin system measured except for non-zymogen acrosin. Major decreases in the total acrosin, proacrosin and acrosin inhibitor occurred after only 1 day at -20°C and continued slowly thereafter. Whole semen kept at room temperature for up to 24 h after ejaculation did not show any significant changes in the sperm acrosin system. Seminal plasma did not have a detrimental or stabilizing effect on acrosin and proacrosin when spermatozoa were kept at room temperature. However, removal of seminal plasma and re-suspension of spermatozoa in 0-9% NaCl resulted in the liberation of a significant amount of the acrosin inhibitor from the spermatozoa and the apparent activation of some of the proacrosin to acrosin.

Introduction

The acrosomal serine proteinase, acrosin (EC 3.4.21.10), is important for fertilization, presumably by enabling sperm penetration through the zona pellucida of the ovum and/or by causing or aiding the sperm acrosome reaction (reviewed by McRorie & Williams, 1974; Zaneveld, Polakoski & Schumacher, 1975; Meizel, 1978). Like that of other animal species, most of the acrosin associated with human spermatozoa is present in the zymogen form called proacrosin (Polakoski, Zahler & Paulson, 1977; Tobias & Schumacher, 1977; Goodpasture, Polakoski & Zaneveld, 1980). An inhibitor of acrosin is also associated with human spermatozoa (Zaneveld, Dragoje & Schumacher, 1972; Zaneveld, Schumacher, Fritz, Fink &

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Jaumann, 1973; Goodpasture et al., 1980). The non-zymogen acrosin, proacrosin and acrosin inhibitor of spermatozoa will be referred to as the sperm acrosin system although there may be other components in this system.

Human semen can be preserved by freezing at $-196^\circ C$ but there is an associated decrease in the fertilizing capacity of the spermatozoa (Behrman & Sawada, 1966; Behrman & Ackerman, 1969; Steinberger & Smith, 1973; Ansbacher, 1978; Quinlivan, 1979) which has been attributed to decreased sperm motility, loss of midpiece metabolic enzymes required for motility, and aberrations in sperm morphology. Little is known regarding the effect of freezing at $-196^\circ C$ on the acrosin system, and we therefore investigated whether freezing of human spermatozoa to this temperature has any deleterious effects on the acrosin system.

Large volumes of fresh human semen for research purposes are often difficult to obtain, and semen samples are frequently kept in a laboratory freezer (approximately $-20^\circ C$) before study. The second objective of the present study was to determine the effect of storage at this temperature on the sperm acrosin system.

Finally, because of the time required to bring human ejaculates to the clinic, as well as the organization and timing of certain procedures, semen is often kept for hours at room temperature before it is used for such clinical procedures as artificial insemination, in-vitro fertilization, and semen analysis, or for experimentation. During this period, changes may occur in the acrosin system as they are known to take place in other seminal characteristics (Mann, 1964), or seminal plasma may have a protective, stabilizing effect on the acrosin system. Therefore, the third objective of this study was to determine whether the storage of human spermatozoa at room temperature has an effect on the acrosin system and whether seminal plasma can modify any of these changes.

Materials and Methods

Materials

Benzamidine HCl was purchased from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin; $\alpha$-N-benzoyl-$L$-arginine ethyl ester (BAEE) from Sigma Chemical Company, St Louis, Missouri; Spectrapor 1 membrane dialysis tubing from Fisher Scientific Company, Chicago, Illinois (manufactured by Spectrum Medical Industries, Inc., Los Angeles, California); and sucrose from Mallinckrodt, Inc., St Louis, Missouri.

Collection and treatment of spermatozoa; acrosin assay techniques

Semen samples were obtained from healthy donors by masturbation. After complete liquefaction, ejaculates were pooled or used individually, depending on the type of experiment. Benzamidine was always used at a final concentration of 0.05 M. This compound is a synthetic proteinase inhibitor which prevents the activation of proacrosin to acrosin (Zahler & Polakoski, 1977; Goodpasture et al., 1980).

The method used for extraction of the spermatozoa and measurement of the acrosin system has been found to be optimal for human spermatozoa (Goodpasture et al., 1980). Briefly, benzamidine was added to the semen immediately after collection, seminal plasma was removed by centrifugation through 1 M sucrose containing benzamidine (6000 g, 4°C, 15 min; Zahler & Polakoski, 1977) and the sperm pellet was re-suspended in 10% glycerol containing benzamidine. After removing a small aliquot to determine the sperm count in a white blood cell haemocytometer, the suspension was acidified to pH 2.8 with 1 M HCl and kept at 4°C for at least 12 h. The spermatozoa were removed from the extracts by centrifugation at 27000 g for 30 min. The extracts were dialysed with Spectrapor 1 membrane tubing against 1 mM HCl (pH 3.0) to remove the benzamidine.
The amounts of total acrosin, acrosin in proacrosin form, non-zymogen acrosin, and inhibited acrosin after zymogen activation were measured by testing the acrosin activity of the extracts before and after activation of the proacrosin, and before and after acidification of the activated extracts to pH 3-0 (activation takes place at pH 8-0) (Goodpasture et al., 1980). Acrosin activity was determined by adding 0.1 ml of the sample to 2.9 ml 0.05 M-Tris buffer, pH 8-0, containing 0.05 M-CaCl₂, and 0.5 mM-BAEE as substrate, and measuring the rate of change in absorbance at 253 nm (BAEE hydrolysis) at 25°C with a recording spectrophotometer. A molar absorbance difference of 1150 M⁻¹ cm⁻¹ was used to convert the change in absorbance to nmol BAEE hydrolysed (Whitaker & Bender, 1965): 1 m.i.u. of activity was defined as that amount of acrosin hydrolysing 1 nmol BAEE/min at 25°C. The term total acrosin refers to the sum of non-zymogen acrosin and proacrosin. The amount of acrosin inhibitor in the sample could be estimated from the amount of acrosin inhibited after proacrosin activation (before acidification to pH 3) because stoichiometric inhibition occurs until approximately 88% of acrosin is inhibited (Zaneveld et al., 1973). Thereafter much more inhibitor is necessary for the same extent of inhibition.

Freezing at −196°C

After complete liquefaction, individual ejaculates were divided into two equal portions. One of the portions served as control and was processed as follows. The percentage of motile spermatozoa was determined, and the semen was immediately treated with benzamidine and centrifuged over 1 M-sucrose containing benzamidine. The spermatozoa were extracted and tested for the acrosin system. The second portion of each ejaculate was frozen as customary for sperm banks (Sherman, 1963), except that slower cooling rates were used. Semen was treated with glycerol to 10% final concentration, and drawn into 0.5 ml capacity plastic straws which were then sealed. The contents of the straws were frozen by suspending the straws in a sealed styrofoam box floating on liquid nitrogen. After 15 min, the lid of the box was removed and the open box remained on the liquid nitrogen for an additional 15 min. The straws were then submerged in liquid nitrogen. The approximate cooling rates provided by this procedure were, respectively, 2-3 3-3 and 300°C/min (until the contents of the straws reached −196°C). Straws were stored in a standard cryobiologic container. After 1 month, they were thawed in a 37°C water bath. The percentage of motile spermatozoa was determined for each thawed semen sample. The samples were treated with benzamidine, centrifuged individually over 1 M-sucrose containing benzamidine and the extracted spermatozoa were tested for the acrosin system.

Freezing at −20°C

Pooled ejaculates, each consisting of 3–11 samples, were divided into a number of portions, one of which was used as the control specimen which was treated with benzamidine, centrifuged through 1 M-sucrose containing benzamidine, and the sperm extract tested for the acrosin system. Other portions were put into sealed plastic containers and placed in a −20°C freezer where they were kept for 1, 7, 14 or 125 days. Samples were thawed at room temperature, and processed and tested for the acrosin system as above.

Storage at room temperature

Pooled semen was kept at room temperature and aliquots were removed at various times between 30 min and 24 h after ejaculation. Each aliquot was immediately treated as above for extraction, and tested for the acrosin system. Because it was difficult to obtain more than 2 semen samples that had been ejaculated within 5 min of each other, this part of the study was carried out with pooled samples consisting of 2 ejaculates each.
To determine the alterations in the acrosin system of spermatozoa after being separated from seminal plasma, between 4 and 13 ejaculates were pooled and suspended evenly. The acrosin system was tested in the usual way before treatment. The remaining semen was divided into several samples and the seminal plasma separated from the spermatozoa as described above. The spermatozoa were re-suspended to the original semen volume in 0-9% (w/v) NaCl or in seminal plasma, and the suspensions were maintained at room temperature. At various intervals between 0 and 240 min after re-suspension, aliquots of the suspensions were removed and treated immediately with benzamidine. Seminal plasma suspensions were centrifuged through sucrose (see above), and NaCl suspensions were centrifuged at 600 g for 5 min to separate spermatozoa. The resultant sperm pellets were extracted and tested for the acrosin system.

**Statistical analysis**

For experiments carried out at room temperature and −20°C, statistical analyses were performed for each component of the acrosin system of spermatozoa kept under different conditions for various times, by applying one-way analysis of variance and the Duncan Multiple Range Test to the data. Significance was accepted at $P < 0.05$. For the experiments involving frozen semen (−196°C), the components of the acrosin system were compared between test and control samples from the same ejaculates by means of the paired $t$ test. Significance was accepted at $P < 0.05$.

**Results**

**Freezing at −196°C**

Approximately 50% of the total amount of sperm acrosin was lost (Table 1). The difference between control and frozen portions of semen samples was even greater when 4 pairs of samples that showed negligible losses were excluded. All semen samples contained significantly less proacrosin after freezing at −196°C, the overall decrease being 3-fold. The 4 samples that showed negligible loss in total acrosin activity exhibited 2–3-fold decreases in proacrosin activity. By contrast, the non-zymogen acrosin activity increased after cryopreservation (Table 1). Overall, the increase was 3–4-fold less than the decrease in proacrosin, although in the 4 samples with negligible total acrosin change, non-zymogen acrosin increased to the same extent that the proacrosin levels decreased, suggesting that the proacrosin was converted to acrosin in these samples.

**Table 1. Effects of freezing at −196°C on the human acrosin system**

<table>
<thead>
<tr>
<th>Acrosin system (mi.u./10^9 spermatozoa)</th>
<th>Untreated semen</th>
<th>Semen kept at −196°C for 1 month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total acrosin</td>
<td>130 ± 74</td>
<td>72 ± 32*</td>
</tr>
<tr>
<td>Non-zymogen acrosin</td>
<td>14 ± 12</td>
<td>37 ± 18**</td>
</tr>
<tr>
<td>Proacrosin</td>
<td>116 ± 65</td>
<td>35 ± 28**</td>
</tr>
<tr>
<td>(as % of total acrosin)</td>
<td>(89 ± 6)</td>
<td>(43 ± 25)**</td>
</tr>
<tr>
<td>Acrosin inhibited after zymogen activation</td>
<td>104 ± 68</td>
<td>64 ± 25*</td>
</tr>
<tr>
<td>(% of total acrosin inhibited)</td>
<td>(84 ± 18)</td>
<td>(87 ± 13)</td>
</tr>
</tbody>
</table>

Values are mean ± s.d. for 16 samples; values in parentheses are mean ± s.d. % calculated from the original percentages obtained in each individual experiment.

Significantly different from untreated part of the same semen samples:

* $P < 0.01$, ** $P < 0.001$ (paired $t$ test).
With the exception of one ejaculate, each semen sample showed a decrease in the overall sperm motility after freezing at $-196^\circ$C, the decrease averaging 34%. The one ejaculate in which no motility decrease was observed did, however, show a decrease in both total sperm acrosin and proacrosin. No relationship was present between the decrease in motility and the changes in total acrosin, proacrosin and non-zymogen acrosin.

Although the amounts of inhibited acrosin decreased significantly after freezing at $-196^\circ$C, the percentages of total acrosin inhibited remained the same or increased slightly (Table 1). At these rather high inhibitor levels, the reaction with acrosin no longer occurs stoichiometrically and the lower amounts of inhibited acrosin could have been due to the decrease in acrosin without loss of inhibitor. On an individual basis, 11 of the semen samples possessed high levels of inhibited acrosin ($96 \pm 6\%$ of total) before freezing and 5 samples possessed lower amounts ($61 \pm 12\%)$. After freezing, the samples with the greater amount of inhibited acrosin showed no change ($94 \pm 8\%$ inhibition), whereas the samples with the lower amounts showed a significant increase ($81 \pm 8\%$ inhibition; $P < 0.05$), presumably because of the decreased amounts of acrosin present, whereas the amount of inhibitor changed little or not at all. Although the amount of acrosin in the other 11 samples also decreased, this was not expressed in terms of inhibited acrosin because of the excess inhibitor necessary to cause acrosin inhibition at these high inhibition levels. The results indicate that no or only small alterations occur in acrosin inhibitor after freezing at $-196^\circ$C.

**Freezing at $-20^\circ$C**

There was a large decrease in total acrosin and in proacrosin after storage at this temperature (Table 2). The major change occurred after only 1 day although slight decreases took place thereafter. By contrast, the amount of non-zymogen acrosin increased after 1 day, gradually continuing until 125 days of freezing when 2-fold higher amounts were present compared to the control samples. The increase in non-zymogen acrosin was very small in relation to the decrease in proacrosin.

**Table 2.** The acrosin system of human spermatozoa frozen at $-20^\circ$C in whole semen (pooled from 3–11 donors)

<table>
<thead>
<tr>
<th>Acrosin system (mIU/10^7 spermatozoa)</th>
<th>Duration at $-20^\circ$C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Total acrosin</td>
<td>85 ± 3*</td>
</tr>
<tr>
<td>Non-zymogen acrosin</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>Proacrosin</td>
<td>79 ± 3*</td>
</tr>
<tr>
<td>(as % of total acrosin)</td>
<td>(93 ± 1)*</td>
</tr>
<tr>
<td>Acrosin inhibited after zymogen activation (%) of total acrosin inhibited</td>
<td>76 ± 3*</td>
</tr>
<tr>
<td></td>
<td>(89 ± 2)*</td>
</tr>
</tbody>
</table>

Values are mean ± s.d. for 4 samples; values in parentheses are mean ± s.d. % calculated from the original percentages obtained in each individual experiment.

* Values significantly different from those of all other groups, $P < 0.001$ (Duncan’s MRT).
† Value significantly different from those of all other groups, $P < 0.01$ (Duncan’s MRT).
‡ Value significantly different from that at 1 day ($P < 0.001$), 7 days ($P < 0.01$), and 14 days ($P < 0.05$) (Duncan’s MRT).
§ Value significantly different from that at 1, 7 and 14 days ($P < 0.001$ for each) (Duncan’s MRT).

The amounts of acrosin inhibitor also decreased: the amount of inhibited acrosin and the percentage of acrosin inhibited were reduced. The largest change was observed after 1 day and gradual decreases occurred thereafter.
Storage at room temperature

There was no change in the total acrosin content between 30 min and 6 h after ejaculation (Table 3). A slight decrease in total acrosin content was observed between 6 and 24 h after ejaculation, but this was not significant. Similarly, no change occurred in the amount of non-zymogen acrosin, proacrosin or acrosin inhibitor.

| Table 3. The acrosin system of spermatozoa in whole semen at room temperature |
|--------------------------------------------------|--------|--------|--------|--------|--------|
| Acrosin system (mi.u./10^7 spermatozoa)          | Time at room temperature after ejaculation |
|                                                  | 30 min | 1 h    | 2 h    | 6 h    | 24 h   |
| Total acrosin                                    | 105 ± 18 | 102 ± 14 | 106 ± 15 | 106 ± 18 | 95 ± 17 |
| Non-zymogen acrosin                             | 8 ± 3   | 6 ± 3   | 9 ± 3   | 7 ± 3   | 7 ± 3   |
| Proacrosin                                       | 97 ± 18 | 96 ± 16 | 97 ± 12 | 99 ± 15 | 88 ± 19 |
| (as % of total acrosin)                         | (93 ± 2) | (94 ± 3) | (92 ± 2) | (93 ± 2) | (92 ± 3) |
| Acrosin inhibited after zymogen activation       | 93 ± 16 | 90 ± 18 | 91 ± 21 | 94 ± 20 | 86 ± 18 |
| (% of total acrosin inhibited)                   | (90 ± 2) | (88 ± 4) | (87 ± 3) | (90 ± 3) | (89 ± 3) |

Values are mean ± s.d. for 3 samples of 2 pooled ejaculates (see text); values in parentheses are mean ± s.d. calculated from the original percentages obtained in each individual experiment.

Spermatozoa separated from seminal plasma were examined over a shorter period because, under normal conditions, washed spermatozoa are only maintained for a short time before treatment, but the observations were more frequent. When centrifuged spermatozoa were re-suspended in 0.9% NaCl no alterations in the total amount of acrosin took place (Table 4). However, the amount of non-zymogen acrosin increased significantly after centrifugation and continued to increase thereafter while the amount of proacrosin decreased. The decreases in proacrosin were approximately equal to the increases in non-zymogen acrosin, implying that proacrosin activation to acrosin was taking place. The total amount of proacrosin activation was only small, however, amounting to 14% over the 4-h period.

| Table 4. The acrosin system of centrifuged spermatozoa incubated in 0.9% NaCl |
|--------------------------------------------------|--------|--------|--------|--------|--------|
| Acrosin system (mi.u./10^7 spermatozoa)          | Control | Time (min) in saline |
|                                                  |        | 0      | 30     | 60     | 90     | 120    | 240    |
| Total acrosin                                    | 100 ± 18 | 103 ± 23 | 102 ± 19 | 102 ± 18 | 104 ± 19 | 107 ± 20 | 104 ± 17 |
| Non-zymogen acrosin                              | 8 ± 3*  | 15 ± 7* | 20 ± 4  | 21 ± 6  | 22 ± 4  | 23 ± 5  | 23 ± 4  |
| Proacrosin                                       | 92 ± 16 | 88 ± 18 | 82 ± 16 | 81 ± 12 | 82 ± 16 | 84 ± 16 | 81 ± 15 |
| (as % of total acrosin)§                         | (92 ± 2)* | (86 ± 3)* | (81 ± 2)* | (79 ± 2)* | (78 ± 3)* | (78 ± 5)* | (78 ± 4)* |
| Acrosin inhibited after zymogen activation       | 87 ± 17‡ | 67 ± 15 | 65 ± 9  | 64 ± 9  | 67 ± 10 | 71 ± 11 | 65 ± 9  |
| (% of total acrosin inhibited)                   | (87 ± 3)‡ | (65 ± 3)‡ | (65 ± 4)‡ | (63 ± 3)‡ | (64 ± 3)‡ | (66 ± 3)‡ | (63 ± 2)‡ |

Values are mean ± s.d. for 5 observations on ejaculates pooled from 4-13 donors; values in parentheses are the mean ± s.d. % calculated from the original percentages obtained in each individual experiment.

* Values significantly different from those at 0 (P < 0.05), 30 (P < 0.01) and 60, 90, 120 and 240 min (P < 0.001) (Duncan's MRT).

† Values significantly different from those at 90, 120 and 240 min (P < 0.05) (Duncan's MRT).

‡ Values significantly different from those of all other groups (P < 0.001) (Duncan's MRT).

§ Values with different superscript letters are significantly different at P < 0.01, except for the 0 and 30 min values which were different at P < 0.05 (Duncan's MRT).

The amount and percentage of inhibited acrosin, and thus the amount of inhibitor, were significantly lower at all times (Table 4), the main decrease occurring immediately after re-suspension in the saline.
When centrifuged spermatozoa were re-suspended in seminal plasma (Table 5), the results were similar to those obtained after re-suspension in saline with the exception of the amount of inhibited acrosin which showed no change.

Table 5. The acrosin system of centrifuged spermatozoa incubated in seminal plasma

<table>
<thead>
<tr>
<th>Acrosin system (mi.u./10^7 spermatozoa)</th>
<th>Time (min) in seminal plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (control)</td>
</tr>
<tr>
<td>Total acrosin</td>
<td>103 ± 19</td>
</tr>
<tr>
<td>Non-zymogen acrosin</td>
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<td>Proacrosin</td>
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<tr>
<td>(as % of total acrosin)</td>
<td>(94 ± 2)†</td>
</tr>
<tr>
<td>Acrosin inhibited after zymogen activation</td>
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</tr>
<tr>
<td>(% of total acrosin inhibited)</td>
<td>(87 ± 3)</td>
</tr>
</tbody>
</table>

Values are mean ± s.d. for 4 observations on ejaculates pooled from 4–13 donors; values in parentheses are mean ± s.d. % calculated from the original percentages obtained in each experiment.
* Value significantly different from those at 120 and 240 min (P < 0.05) (Duncan’s MRT).
† Value significantly different from those at 120 and 240 min (P < 0.01) (Duncan’s MRT).

Discussion

The results show that different methods of storage of human spermatozoa have different effects on the acrosin system. Storage of semen at room temperature does not cause any alterations in the acrosin system up to 24 h, freezing at –196°C causes decreases in the amounts of total acrosin and proacrosin, and freezing at –20°C causes changes in all the components of the acrosin system tested. Clinical procedures are therefore best performed with spermatozoa maintained as whole ejaculates at room temperature, at least in regard to the acrosin system. For an accurate analysis of the major components of the acrosin system, human semen should not be frozen for any length of time.

It cannot be stated with certainty why these changes occur. The harsher the treatment, i.e. the more membrane damage that was presumably induced, the greater the alterations. Proacrosin is believed to be a membrane-bound molecule, and non-physiological alterations in the plasma and acrosomal membranes of non-human spermatozoa can cause zymogen activation (Zahler & Polakoski, 1977; Brown & Harrison, 1978). The same appears to be true for human spermatozoa because washing resulted in the activation of proacrosin although to a much smaller extent than that reported for non-human spermatozoa. Induction of the acrosome reaction in guinea-pig spermatozoa causes the activation and release of approximately half of the proacrosin and total acrosin (Goodpasture, Reddy & Zaneveld, 1981). Freezing and thawing can inactivate human acrosin in solution (J. C. Goodpasture & L. J. D. Zaneveld, unpublished): the acrosin activity lost from the frozen human spermatozoa can sometimes be recovered in the surrounding seminal plasma, but at other times only a portion or none of the acrosin could be found. Finally, after freezing, the amounts of non-zymogen acrosin increased as the proacrosin levels decreased. In general, the increase in non-zymogen acrosin was much less than the decrease in proacrosin, but 4 samples frozen to –196°C showed identical increases and decreases, respectively. Thus, it would appear that, as the sperm membranes are altered or broken by the freeze–thaw procedures, varied amounts of proacrosin are activated to acrosin, after which some of the acrosin is released and/or denatured, the quantity depending on the
fertilizing capacity of deep-frozen human semen may be due, in part, to a loss or inactivation of acrosin from spermatozoa. This would explain why certain human semen samples showing normal post-thaw motility are still less fertile. The fact that the spermatozoa of 4 men showed negligible decreases in total sperm acrosin amounts (although significant zymogen activation occurred) indicates that the spermatozoa of certain men may be more resistant to the type of damage that results in the loss and/or denaturation of acrosin. No relationship was observed between the loss of sperm motility in the individual semen samples after cryopreservation and the changes in total enzyme activity, non-zymogen acrosin or proacrosin. Therefore, sperm immotility after freezing to $-196^\circ$C does not appear to be related to aberrations in the sperm acrosin system. The analysis of the acrosin system after deep freezing may therefore be an additional tool for screening semen for its potential fertilizing capacity.

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References


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