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Cryopreservation of Boar Semen
III: Ultrastructure of Boar Spermatozoa Frozen Ultra-Rapidly at Various Stages of Conventional Freezing and Thawing

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Bwanga, C. O., H. Ekwall and H. Rodriguez-Martinez: Cryopreservation of boar semen. III: Ultrastructure of boar spermatozoa frozen ultra-rapidly at various stages of conventional freezing and thawing. Acta vet. scand. 1991, 32, 463–471. – Ejaculated boar spermatozoa subjected to a conventional freezing and thawing process, were ultra-rapidly fixed, freeze-substituted and examined by electron microscopy to monitor the presence of real or potential intracellular ice and the degree of cell protection attained with the different extenders used during the process. Numerous ice crystal marks representing the degree of hydration of the cells were located in the perinuclear space of those spermatozoa not in proper contact with the extender containing glycerol (i.e. prior to freezing). The spermatozoa which were in proper contact with the extenders presented a high degree of preservation of the acrosomes, plasma membranes as well as the nuclear envelopes. No ice marks were detected in acrosomes before thawing, indicating that the conventional assayed cryopreservation method provided a good protection against cryoinjury. The presence of acrosomal changes (internal vesiculization, hydration and swelling) in thawed samples however, raises serious questions about the thawing procedure employed.

cryopreservation; freeze-substitution; electron microscopy.

Introduction

The viability of frozen and thawed spermatozoa is lower in the pig than in other domestic species (*Polge* 1976, *Bwanga* 1991). Furthermore, freezing of boar semen is associated with reduced fertility due to decreased motility and membrane integrity (*Johnson et et al.* 1981, *Johnson* 1985). Cooling velocity, interacting with glycerol concentration, greatly affects sperm motility and acrosomal integrity when freezing boar semen (*Fiser* 1990). During each step in the cryopreservation protocol for boar semen, spermatozoa are exposed to osmotic stress (*Meryman* 1971, *Watson & Duncan* 1988), dehydration (*Mazur* 1984, 1985, *Courtens et al.* 1989) and formation of ice of variable dimensions (*Courtens & Paquignon* 1985), which account for the membrane and organelle defects that affect the final integrity of the cells. Most defects are located subcellularly at the acrosome and acrosomal region of the plasma membrane.

The use of programmable freezers has made it possible to obtain consistent cooling rates among large numbers of semen doses frozen simultaneously, and to design freezing curves optimozing the cooling rate (*Hofmo & Almlid* 1990, *Bwanga et al.* 1990). The use of various containers and optimal cooling rates have brought up promising methods for freezing boar semen (*Bwanga et al.* 1991a, 1991b), which do not solely depend upon the cooling velocities but also to the

composition of the diluents and their degree of dehydration and cryoprotection.

The use of proper semen extenders, adequate containers allowing the fast dissipation of latent heat of fusion, fast cooling rates and minimal supercooling contribute to the proper dehydration of the cells and the formation of microscopic intracellular ice-crystals, which do not disrupt the cell membrane (*Mazur* 1977, *Hammerstedt et al.* 1990).

As pointed out recently, a better knowledge of the effects of each of the steps of the conventional freezing technique, including the frozen state, is of utmost importance in enriching the understanding of the phenomena affecting the viability of the cells, provided that electron microscopy is used (*Courtens et al.* 1989).

The use of freeze-substitution, to examine cells that were fixed in the frozen state, allows the recording of intracellular ice in boar sperm (*Courtens et al.* 1989). Therefore, ultra-rapid cryofixation combined with cryosubstitution was hereby applied to monitor the degree of protection of the sperm heads attained by extenders used during the various steps of conventional freezing of boar semen in plastic bags.

Material and methods

Semen from 4 Swedish Yorkshire boars, housed at the Department of Obstetrics and Gynaecology, was used. The boars had previously been used in the university experimental farm with good pregnancy results. The age of the boars averaged 2 years. The filtered sperm-rich fraction (*Einarsson* 1971) of the ejaculate (3 ejaculates/boar) was collected by the glowed-hand method (*Hancock & Hovell* 1959) into pre-warmed (37° C) thermos flasks. The volume of the split-ejaculate was measured and the cell concentration determined with a photometer (Electrolux Mecatronix AB, Sweden) and later confirmed with the aid of a Bürker chamber. Sperm motility was determined by direct observation at 37° C in a microscope equipped with phase contrast optics (250 ×) and the evaluation of acrosomal integrity was done by examination with phase contrast microscopy (1000 ×) in formol-saline fixed samples. Sperm viability (progressive motility and acrosome integrity) was estimated on fresh semen shortly after dilution with Beltsville Thawing Solution (BTS) (*Pursel & Johnson* 1975), and at various stages of the cooling procedure and post-thawing.

Freezing and thawing

Semen for freezing was required to have at least 70 % motile spermatozoa. The semen was processed and frozen by a modified procedure (*Bwanga et al.* 1990) based on the straw freezing method originally described by *Westendorf et al.* 1975). Briefly, shortly after collection, the semen was diluted 1:3 with BTS (extender I), both at 30° C, in 250 ml centrifuge flasks and allowed to cool at 15° C for 3 h. Thereafter, the diluted semen was centrifufed (800 × g for 10 min), the supernatant skipped and the concentrated semen rediluted (1:1 at 15° C) with Extender II (80 ml of 11 % Lactose (0,15 M) solution and 20 ml egg yolk). The diluted semen was gently mixed, the sperm concentration counted and the necessary amount of Extender II added to acquire a concentration to be frozen of 1×10^9 spermatozoa/ml. The flasks were again placed in the centrifuge set at 5° C, and the diluted semen allowed to cool down for 2 h. Extender III (89.5 ml of extender II, 1.5 ml Orvus Es Paste (OEP), 9.0 ml glycerol) was then added, 2 parts of semen: 1 part of extender, at 5° C. The final diluted semen was manually put into 10 × 5 cm plastic bags made up of Teflon[R] FEP-film (Dupont, Switzerland, 0.12 mm thick) and

sealed with a thermo-sealer (Auto-Seal 102, Nitech AB, Sweden). The bags were horizontally placed into the chamber of the programmable biological freezing machine (PTC-200, Planer Products Ltd., England) on a metallic net and frozen using a freezing program (5° C to –6° C with 3° C/min, 1 min at –6° C, and a further decrease from –6° C to –100° C with 20° C/min freezing rate) which produces a short freezing plateau (dissipation of the latent heat of fusion) of less than 1 min, and a very rapid post-crystallization freezing rate (*Bwanga et al.* 1991a). The frozen bags were stored in liquid N_2 until thawing. Thawing was done by immersion into circulating water at 50° C for 25 sec and, immediately after thawing, 0.5 ml of semen was added to 4.5 ml of BTS at room temperature. For motility evaluation, triplicates (sub-samples) of each sample were incubated at 37° C and the samples examined after 30 min of incubation.

Freeze-quenching and freeze-substitution

A small drop (0.15 μl) of the suspension of spermatozoa either freshly ejaculated (U), after dilution in BTS (extender I, 20–30° C) (**2**), after equilibration (at 5° C in extender II) (**3**), prior to freezing (at 5° C in extender III) (**4**) and immediately after thawing/redilution in BTS (20° C) (**5**) was placed into a thin plastic spacing ring and impact-frozen against a highly polished solid copper surface chilled with liquid N_2 to –190° C using a Reichert-Jung MM80[R] (Reichert-Jung, Austria) automatic metal-mirror slam-freezer. The frozen samples were then transferred to and stored in liquid N_2 until freeze-substituted. A 1 % solution of osmium tetroxide in dry-acetone was solidified at –196° C and the frozen-quenched droplets were placed on top of the frozen media. The vials with the samples were then transferred onto crushed dry ice (–80° C) in a thick-walled styro-foam box and allowed to slowly warm up for 70 h inside a refrigerator (+4° C) in order to get a fixation/slow substitution rate. Afterwards, each sample was repeatedly rinsed in acetone to remove excess osmium tetroxide, followed by a 3 h infiltration of a mixture of Spurr's low viscosity epoxy plastic resin and acetone (1:1). Finally, the samples were equilibrated into pure Spurr's epoxy resin for 24 h and then transferred into pure newly made epoxy plastic, polymerized at +70° C for 9 h. Thin sections were cut on a Reichert[R] ultramicrotome with a diamond knife, counterstained with uranyl acetate and lead citrate and examined in a Philips 420 TEM electron microscope at 80 kV.

Results

After ultra-rapid freezing and freeze-substitution of the boar spermatozoa, ice-crystals could be easily identified in the ultra-thin sections (Figs. 1–10). In some cells, irrespective of the considered group, the chromatin was not totally compact, and crystals were observed in nuclear "holes" (Fig. 3).

In samples frozen directly from the ejaculate, some of the spermatozoa situated near the freezing front showed swelling of the supra-acrosomal plasma membrane (Fig. 1). Other cells did not exhibit such a swelling but on the other hand marks from ice crystals were visible occupying the peri-nuclear space (Figs. 2 and 3).

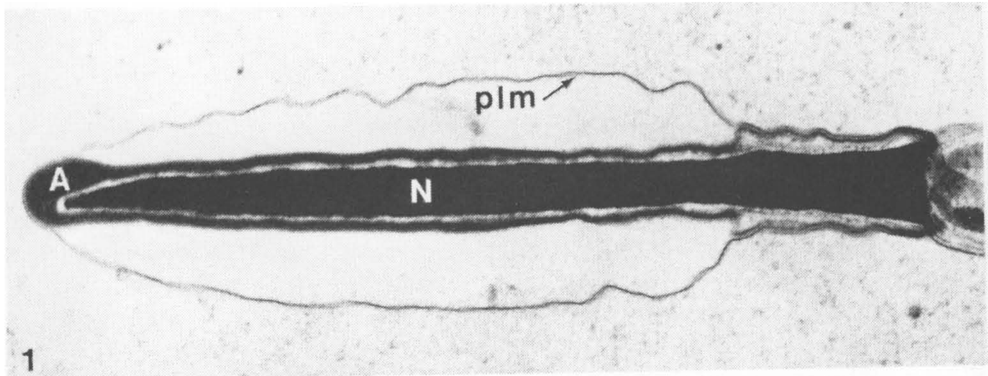
The spermatozoa sampled after dilution in extender I (BTS) or during equilibration (extender II) showed similar amounts of ice crystals, mostly located in the perinuclear space, but also within the acrosome (Figs. 4 and 5). Prior to freezing however, 2 groups of cells could be identified. Those which were surrounded by a very thin layer of extender III (containing glycerol and OEP) presented similar marks after the presence of ice crystals, mostly perinuclear, particularly

located at the concave side of the sperm head (Fig. 6). The other category of cells was constituted of spermatozoa completely surrounded by the extender, showing a very well preserved morphology and almost devoid of ice, i.e. dehydrated (Fig. 7). After ultra-rapid freezing and freeze-substitution of thawed/rediluted spermatozoa, ice crystals were either absent (Fig. 9) or observed both in the acrosomes and in the perinuclear spaces (Fig. 8). Many acrosomes were swollen to different degrees, ranging from single dilations to complete disruption. Intra-acrosomal vesicles (Fig. 10) seemed to originate from invaginations of the outer acrosomal membrane.

Discussion

After an initial cooling and formation of extracellular ice microcrystals at about –5° C, cooling at variable rates affects both the rate of water movement out of the cell and the extent of intracellular ice formation (*Mazur* 1984, 1985). Thus, when the temperature remarks –196° C, the sperm intracellular and extracellular ice differ according to the cooling rate. An appropriate rate of cooling/freezing, results in dehydration, leaving the cell in a tightly packed and restraining environment dominated by the effects of high solution and ice crystals (*Hammerstedt et al.* 1990). If cooling is slow, the cell will be able to loose water rapidly enough by exosmosis to concentrate the intracellular solutes sufficiently to eliminate supercooling and mainten the chemical potential of intracellular water in equilibrium with that of extracellular water, so that the cell dehydrates and does not freeze intracellularly. However, if the cell is cooled too rapidly, it is not able to lose water fast enough to maintain equilibrium; it becomes increasingly supercooled and eventually attains equilibrium by freezing intracellularly (*Mazur* 1985). The size and position of these crystals will determine the degree of cell damage and of sperm viability at thawing. A good example of the effects of rapid cooling/freezing is shown in the present study, where the degree of dehydration in the perinuclear area was large enough to leave ice crystal marks representing cell damage. The nuclei however, were in all cases, almost dehydrated (except for nuclear "holes"), confirming previous studies (*Livoland* 1984, *Courtens et al.* 1989). Results obtained during freezing (*Bwanga,* unpublished data) indicated that minimal damage occurred during the supercooling

Figures 1 to 10. Electron micrographs of ultra-thin sections of boar sperm heads ultrarapidly frozen and freeze-substituted from the ejaculate, during the equilibration process and after conventional thawing. **1:** Ejaculate showing a sperm (16700 ×) with normal morphology besides the distended plasmalemma (**plm**), **N** = nucleus and **A** = acrosome. **2:** In other sperms, ice crystal marks (**arrowa**) were present between the nucleus and the acrosome (36000 ×) or **3:** have disrupted the acrosome (20000 ×), * = seminal plasma. **4:** Sperm head diluted in BTS (35000 ×) showing major ice crystal marks that distended the acrosome, the perinuclear space and disrupted the plasmalemma. :After being diluted in extender II for 2 h, ice crystals were clearly present in the perinuclear space (**arrows,** 21000 ×). **6:** Being equilibrated in extender III, with addition of glycerol and the detergent OEP, sperm heads not surrounded by the extender at the moment of freezing showed the presence of ice crystal marks particularly on the concave face (**arrows**) while the other face is clearly dehydrated (18700 ×). **7:** Entirely surrounded by extender medium, the sperm head appears intact, dehydrated and free from ice crystals (35000 ×). When ultra-rapidly frozen, sperm thawed in BTS medium show various degrees of ice crystal formation (perinuclearly, as in **8,** 34500 ×) to intact structures (**9,** 33000 ×) as well as from swollen acrosomes (**8**), to the presence of intra-acrosomal vesicles (Vin **10,** 25000 ×).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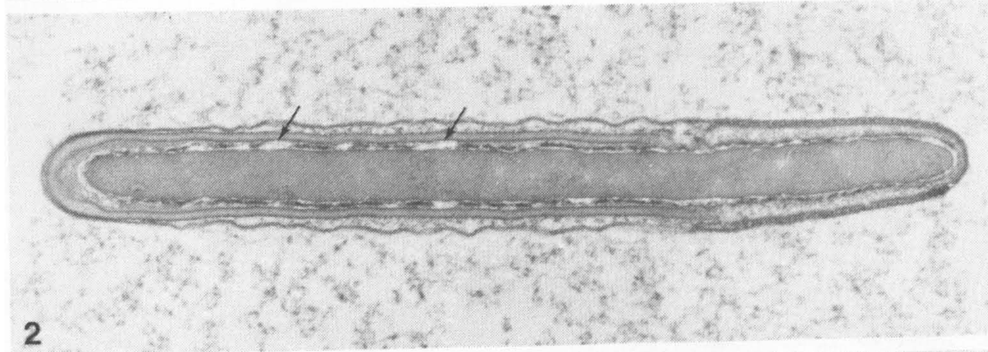
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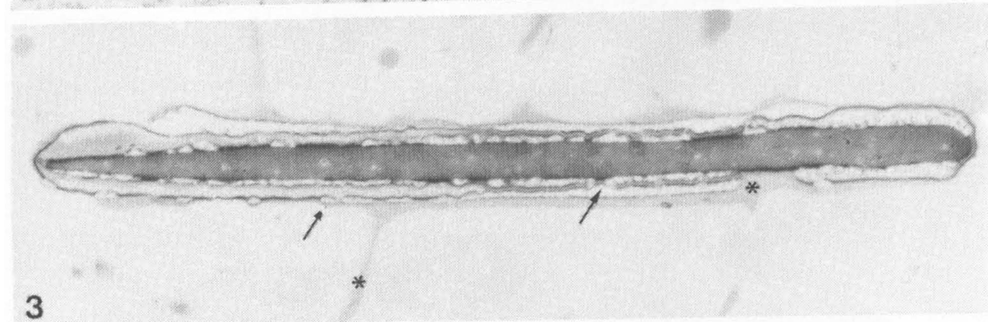
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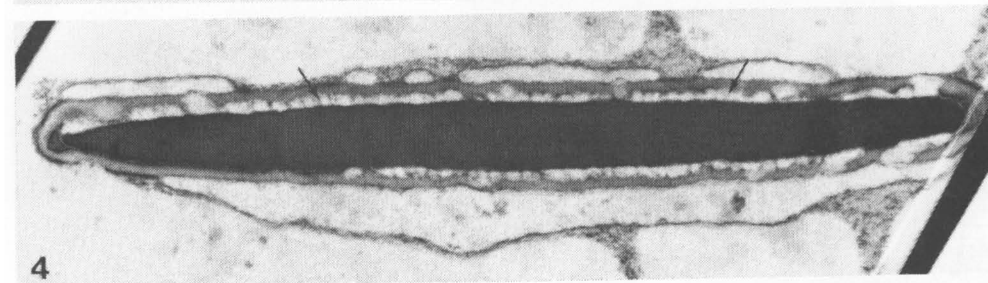
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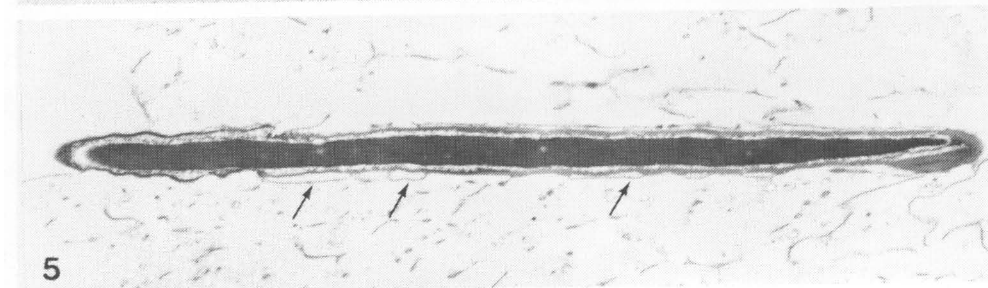
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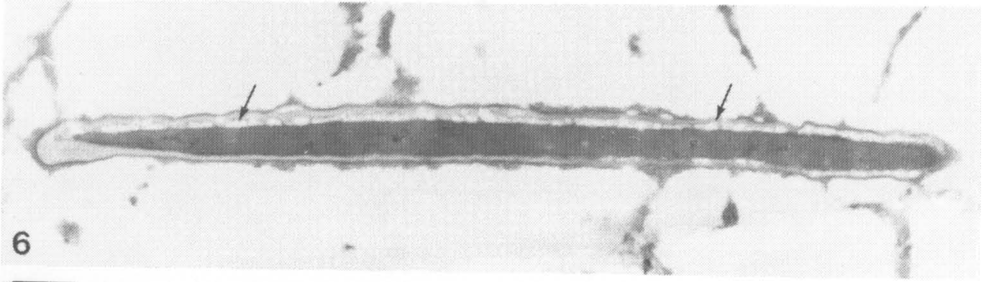
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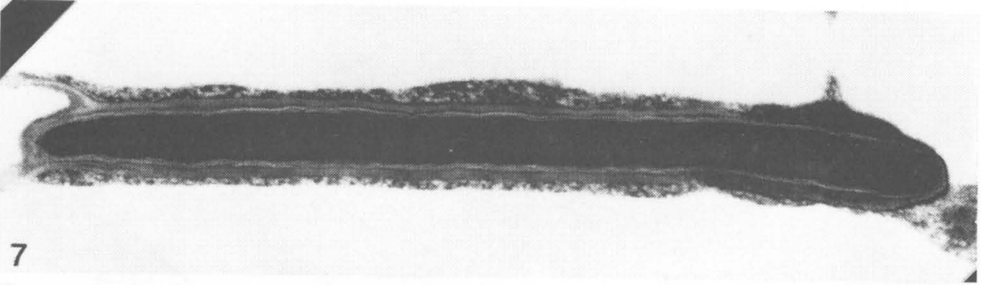
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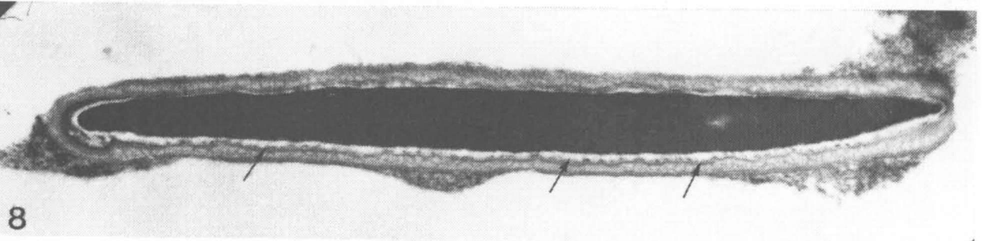
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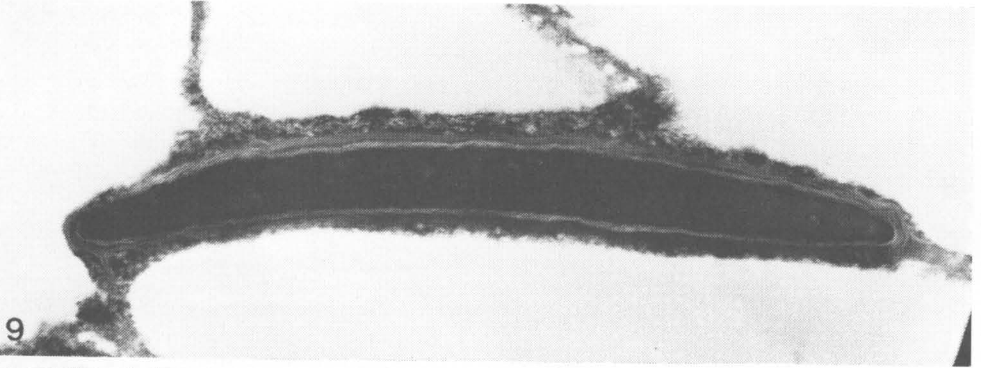
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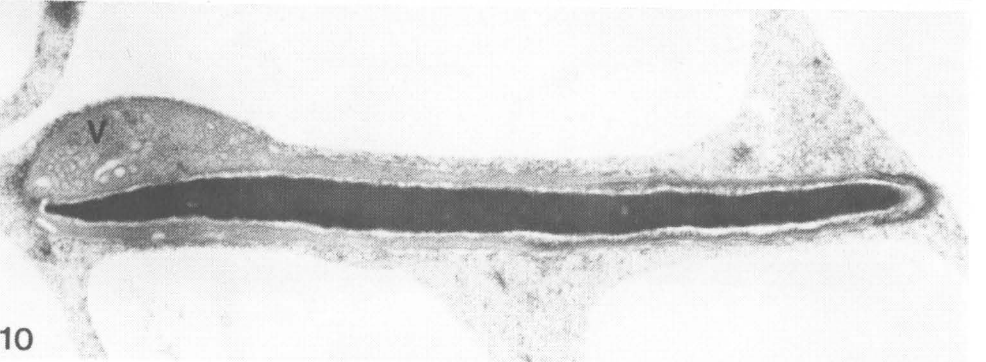
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phase. In addition, no ice crystals were consistently present in the acrosomes of cells prior to thawing.

Cryoprotectants classified as penetrating (e.g. Glycerol) can cause a dehydration of the cell by osmotically induced water egress, according to their process of cell entrance which is dependent upon normal membrane structure (*Hammerstedt et al.* 1990). Cells to be frozen are therefore submitted to various cell volume changes to which they are entitled to adjust, in order to survive the process. The first volume adjustment of the process occurs in response to the addition of cryoprotectant to cells in isotonic extenders. An initial fast shrinkage associated with osmotically driven egrees of intracellular water is expected, which will be followed by a slower return to the original volume as the penetrating cryoprotectant enters. A second volume adjustment occurs when the extracellular water freezes, reflecting the outward movement of water in response to high concentrations of extracellular salts as a consequence of the freezing of extracellular water. As will be discussed later, thawing yields analogous but opposite volume changes. During cooling/ freezing, dehydration will result in a diminution of cell volume, solely substantial in the mid-piece and tail regions but it is minimal in the head of the sperm (*Courtens et al.* 1989, *Hammerstedt et al.* 1990).

Recovery of the hydrated state, during thawing, requires plasticity of the cell. Since the addition of 1 M glycerol (as used in semen preservation) would result in egress of osmotically responsive cell water to yield a cell at about 50 % of its isotonic volume (added to the effect of the hypertonic conditions during freezing); thawing into non-glycerol containing extenders means that water enters the cell more rapidly than the glycerol can exit through the membrane, and as a result; the sperm must expand dramatically, sometimes with a 2 fold overall increase in volume, before returning to its original isoosmotic volume (*Hammerstedt et al.* 1978). The plasmalemma can barely accomodate these large changes (reduction and expansion) in volume, particularly at the enterior (head) region. The present results sustain this reasoning. Furthermore, damage further results if the thawing rate chosen is inappropriate, i.e., too rapid a thaw rate, results in unbalanced rates of egress of cryoprotectant and influx of water, while too slow a thaw rate results in recrystallization of microcrystals of intracellular water with the resultant damage to the cellular organelles (*Amann & Pickett* 1987). These damages were well illustrated in the present study.

The present results clearly showed that the conventional freezing method applied does provide proper conditions for successful dehydration during freezing. However, the thawed cells suffered major deleterious changes, particularly at the anterior head level. Whether or not this implies that the composition of the plasma membrane of the sperm entering the process had changed and it was unable to circumvent the dramatic changes during thawing remains to be proven and should be the aim of further research.

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Sammanfattning

Djupfrysning av galtspermier.
III: Ultrastrukturen hos galtspermier, som frysts med ultrahög hastighet vid olika stadier av den konventionella infrysning- upptinings proceduren.

Elakulerade galtspermier, som utsatts för konventionell infrysning och upptining, fixerades med ultrarapid hastighet, fryssubstituerades och undersöktes elektronmikroskopiskt för bestämning av närvaron av verklig eller potentiell intracellulär is och graden av cellskada uppkommen under processen. Tallrika spår av iskristaller, representerande graden av hydrering i cellerna, var lokaliserade til perinukleära utrymmet hos de spermier som inte var i tillräcklig kontakt med den glyceroltillsatta spädningsvätskan. De spermier, som var i tillräcklig kontakt med den glyceroltillsatta spädningsvätskan, visade i hög grad intakta akrosomer, plasmamembraner och kärnmembraner. Inga spår av iskristaller upptäcktes i akrosomerna före upptining, vilket visar att den konventionella djupfrysningsmetoden gav ett gott skydd mot fryskador. Förekomsten av akrosomskador (inre vesikulering, hydrering, avsvällning) i upptinade prover, reser allvarliga frågor om den använda tiningsproceduren.

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