ULTRASTRUCTURAL MODIFICATIONS OF RABBIT SPERMATOZOA INDUCED BY TWO FREEZING AND THAWING TECHNIQUES

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Abstract - The ultrastructural morphology of rabbit spermatozoa was analysed during the steps of freezing and thawing using either the techniques devised by ANDRIEU and COUROT (1976) or CHEN et al. (1989). The main cellular alterations resulted from osmotic problems. ANDRIEU and COUROT diluents are hyperosmotic and induce cell dehydration both before and during freezing. When thawed, spermatozoa are more sensitive to osmotic shock and many plasma membranes and acrosomes are damaged. CHEN et al. diluent is physically hyperosmotic, but biologically hypoosmotic. It induces membrane swelling and breakage before thawing. The cells are dehydrated during the freezing step and are rehydrated at thawing. Due to the higher hydration in the frozen stage, many ice crystal damages are present.
According to the used technique, cellular damages appear respectively before and during freezing (CHEN et al. technique) or during thawing (ANDRIEU and COUROT technique). The thawing temperatures (37°C or 45°C) are equivalent for main morphology but more plasma membranes are intact after thawing at 45°C.

INTRODUCTION

The results obtained after artificial insemination (AI) with frozen spermatozoa are still far from those raised with fresh semen. Among available methods, ANDRIEU and COUROT technique (1976) has been tested on numerous animals (COSTANTINI, 1989, THEAU and ROUSTAN, 1982). On the other hand, Chen et al technique has been claimed to be better for cell survival. FARGEAS (1995), reported that both techniques are equivalent when fertility and litter size are recorded. For these reasons, we have studied the ultrastructure of spermatozoa during all the steps of the two techniques from ejaculation to thawing, with the aims that understanding cellular modifications induced by techniques could be a starting point to improve the technology.

MATERIAL AND METHODS

Diluents

I *STRANZINGER et al., 1971* (670 mOsm/Kg H_2O)
Tris hydroxymethylaminomethane 3.03 g, DMSO 5 ml, egg yolk 20 %, citric acid 1 H_20, 1.67 g, D-glucose 1.25 g, streptomycin 0.1 g, specillin G 100000 IU, enough water for 100 ml.

II *NAGASE et al., 1964* (458 mOsm/Kg H_2O before glycerol)
Egg yolk 20 %, D-lactose 8.25 g, glycerol 1.3 %, streptomycin 0.1 g, specillin G 100000 IU enough water for 100 ml.

III *CHEN et al., 1989* (1161 mOsm/Kg H_2O)
Egg yolk 20 %, acetamide 4.73 g, raffinose 5.41g, D-lactose 4 g, D-glucose 2.25 g, streptomycin 0.1 g, specillin G 100000 IU, enough water for 100 ml.

Semen collection

Two successive ejaculates (10 mins. interval) were obtained from three adult Californian rabbits selected for high semen quality. Both ejaculates from each rabbit were pooled and processed for freezing according to ANDRIEU and COUROT (1976). Semen from the same rabbits, obtained one week later, were frozen using the CHEN et al. protocol (1989).

Freezing and thawing procedures

1-*ANDRIEU and COUROT* - Spermatozoa were diluted 1:3 in STRANZINGER et al. diluent at 35 °C, cooled to 22°C (room temperature) and cooled to 5°C in 3 hrs. They were rediluted 1:2 with NAGASE et al. medium, and equilibrated for 5 mins. after being aspirated in 0.5 ml straws. Freezing for 3 mins. in nitrogen vapours (-120°C), was followed by immersion in liquid nitrogen.
2-*CHEN et al.* - The spermatozoa were diluted 1:6 in CHEN et al. diluent at 35°C and cooled down to 5°C in 4 hrs. 0.5 ml straws were allowed to wait for 30 mins before freezing as above.
Thawing. Whatever the procedure, straws were thawed either at 37°C for 1 min, or at 45°C for 20 secs.

Electron microscopy

Samples obtained immediately after ejaculation, after dilution, after reaching 22°C, after equilibration, in the frozen state, and 5 mins. after thawing, were processed as following: Unfrozen cells were fixed with 4% glutaraldehyde in 0.175 M cacodylate/HCl buffer pH 7.3 at cell temperature. Fixation was continued for 1 hr. at room temperature or at 5°C according to the samples, and additionally for 3 days at 4°C. The cells washed in 0.3 M cacodylate/HCl buffer for 30 mins were post fixed with 2% osmium tetroxide in 0.175 M cacodylate buffer for 2 hrs at 20°C, dehydrated through an ethanol serie and embedded in EPON 812. Frozen cells were freeze substituted at -80°C for 4 days with 1% osmium tetroxide in acetone, rewarmed and embedded in EPON 812 according to COURTENS and PAQUIGNON (1985). Ultrathin sections, stained with uranyle acetate and lead citrate, were observed with a Philips CM10 electron microscope.

RESULTS

Ejaculated spermatozoa

They displayed usual rabbit sperm morphology. Mostly, the perinuclear space contained typical blisters (see Figures. 1-3 and COURTENS et al., 1976) and the plasma membranes were slightly elevated from the underlying structures of the heads. They were used as control to evaluate modifications induced by the techniques.

Technique 1 (ANDRIEU and COUROT)

From the first dilution to 5°C - The dilution in an hyper osmotic medium (1067 mOs) resulted immediately in the close application of the plasma membrane on underlying structures (Figure 1). This was especially impressive above mitochondria. The plasma membrane was invaginated in the neck and the main piece in about 10 % of spermatozoa. In few cells, the nuclear redundant envelope was observed close to the postacrosomal lamina, instead of being in the neck as usual. In one animal, 5-10% of flagella were coiled and up to 10 % cells displayed reacted acrosomes at 5°C.
Addition of glycerol - One animal had spermatozoa with micro folded plasma membranes. It also displayed numerous plasma membrane alterations after thawing. For all animals, *de novo* appearing membranous layers were observed in the perinuclear space (5% of the cells). In few spermatozoa, the acrosomal content was fragmented within an intact acrosomal membrane.
After freeze substitution - Whatever the technique, ANDRIEU and COUROT or CHEN et al., the coarse structure of the frozen straws was similar. Large crystals of water were surrounded by veins of frozen diluent containing the cells (see Fig. 4). Few spermatozoa were found in pure ice. The veins were generally thin (< 1µm) and were often broken. Since some material remained between the broken surfaces, it seems possible that breakage occurred at the time of freezing due to physical forces.
In ANDRIEU and COUROT technique, most of spermatozoa were intact. The plasma membranes were closely applied to the cells (Fig. 3) and few acrosomes were swollen. All flagella were strongly dehydrated. The mitochondria displayed irregular shapes and no space was present between the coarse fibbers. Ice was present inside microtubules of the axonemes in some occasions.
Thawing for 1 Min. at 37°C - Only 30 % of thawed spermatozoa displayed normal morphology. The unbroken plasma membranes were lifted out from the heads and from most flagella, revealing an hypoosmotic condition. The altered cells had broken plasma membranes and defective acrosomes, acrosome reactions or acrosome misshaping. The invaginations of the plasma membranes, which were formed immediately after the first dilution of ejaculated spermatozoa, were still present. Typical defects due to ice crystals, such as lifting of the perinuclear substance and ruptures of microtubules, were present.

Thawing for 30 secs. at 45°C - The same defects were present, but fewer plasma membranes and acrosomes were affected (Figure 5). On the other hand, more crystal damages were observed in all parts of the cells. In two animals 60 % of plasma membranes were intact *vs.* 25 % in the animal displaying problems at the time of glycerolisation.

Technique 2 (CHEN et al.)

Immediately after dilution - The plasma membranes were moderately elevated from the flagella and were severely detached from the underlying head structures (Figure 2).
During cooling from 2°C to 5°C - The plasma membranes were normally applied on flagella, but were still elevated from sperm heads. In two animals they were broken in less than 30 % of cells, but in one animal 50 % of sperm heads were affected. 15-30 % of acrosomes were ruptured in all animals.
Freeze substituted - Veins of frozen diluent were less dehydrated and displayed more microcrystalline ice than those obtained after the ANDRIEU and COUROT technique. In frozen cells, acrosomes were either normal, or swollen or filled with ice. Ice was also evident in some flagella and cytoplasmic droplets.
The most impressive finding was however the extensive lowering of sperm volume. The plasma membranes, closely stuck to the frozen diluent, were separated from the rest of the spermatozoa by a thick layer of ice (Figure 4). The central cell structures could be largely dehydrated.
Thawing for 1 min at 37°C - Plasma membranes were broken in 80-90 % of spermatozoa, mostly around the heads and mid pieces. Most cells were affected by ice crystal damages (Figure 6), mostly in acrosomes (40-50 % of acrosomes were destroyed or displayed acrosomal reactions). Ice ghosts were present in few mitochondria.
Thawing for 30 secs. at 45°C - More plasma membranes (40 %) were intact than after thawing at 37 °C. Crystal damages were equivalent in distribution.

DISCUSSION

The sperm ultrastructural morphology was largely modified from ejaculation to thawing by both freezing techniques. In the ANDRIEU and COUROT technique, spermatozoa were dehydrated before and probably during freezing. Minor alterations of membranes occurred during the addition of glycerol, and main cell damages appeared after thawing. This later point could result from a hypoosmotic shock. In the CHEN et al. technique, most spermatozoa were swollen before freezing, as if the diluent was hypoosmotic. However, the plasma membranes over the sperm heads were more sensitive to osmosis than over the flagella. Cell dehydration occurred at the time of thawing, but was not rapid enough to be totally effective. The numerous large ice crystals present between the plasma membranes and the other structures are suspected to be a large source of cellular injuries. No further swelling of cells was observed at thawing. The diluents presently used have different biological osmotic properties. The first ones (ANDRIEU and COUROT), have a composition similar to numerous classical diluents used in other mammals. They were devised first to dehydrate the cells prior to freezing, at a time it was believed that the main alterations of spermatozoa were due to ice. And actually, they do. However, at thawing time, the cells behave as if they were diluted in a medium with low concentration. Since this is false, we speculate that due to strong dehydration, they were enriched in different elements and reached a higher potential osmolarity. A similar behaviour is classical in boar sperm (COURTENS and PAQUIGNON, 1985). In this specie, the intracellular concentration of ionisable elements and the potential osmolarity at thawing are increased 3 times (COURTENS et al., 1989) and the spermatozoa also explode at the time of thawing. Since situations look similar, it is tempting to propose that most of problems encountered with the ANDRIEU and COUROT technique owe to cellular increase in salt concentration prior to and during thawing. Such high concentrations modify both the conformations of proteins and the relations between nucleoproteins and DNA (COURTENS et al., 1989), a situation which does not favour fertility performances in the rabbit (COURTENS et al., 1994). The addition of glycerol in one step seems also deleterious at least in some animals. The micro folded plasma membranes might result first from a hypoosmotic shock in spermatozoa which, being already dehydrated, are suurounded by a second diluent with a lower molarity. After penetration of glycerol in cells, a new osmotic state is obtained, and the expanded membranes tend to return to their original position, close to the underlying structures. This part of the technique could probably be improved by the addition of glycerol to cells in several steps. Whether sensitivity to glycerol could explain differences in freezing capacities observed between animals is speculative. However, we have observed this behaviour in 1/3 rabbits and the concerned animal also had much numerous membrane damages after thawing. The second diluent (CHEN et al.) was devised to improve the results obtained so far in 1989. It adds some interesting tips and features such as the dilution of spermatozoa in one step, and the avoidance of

cell dehydration prior to freezing. However, in our hands, dilution in CHEN et al. medium immediately resulted in cell swelling due to an appearant biological hypotonicity. Therefore, most of membrane damages occured prior to freezing, and the spermatozoa with intact membranes were so hydrated at the time of freezing that intracellular ice volume was far from being acceptable. One curious observation is the difference in osmotic behaviour of the plasma membrane over the sperm heads as compared to that over the flagella. It seems that owing to its location, the plasma membrane does not follow the same osmosis rules. No clear explanation is proposed.

In conclusion; both techniques need improvements to cryopreserve the morphology of spermatozoa. An alternative could be to use a first dilution in a medium with biological osmotic properties intermediate between those of the two above techniques.

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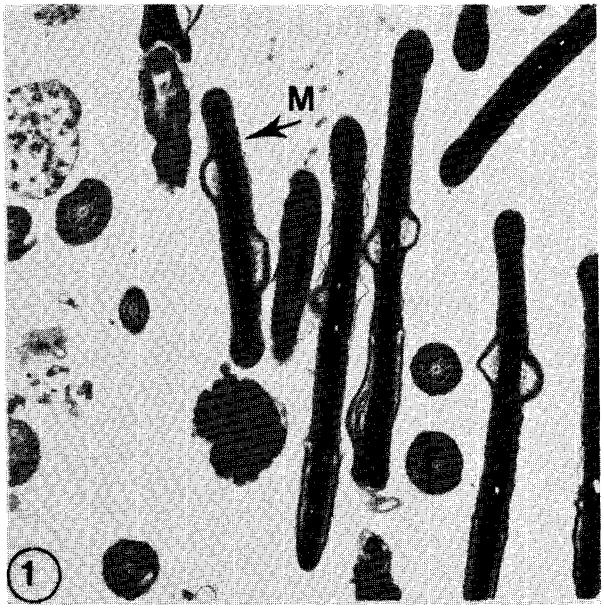
Modifications ultrastructurales des spermatozoïdes de lapin, induites par deux techniques de congélation-décongélation - L'ultrastructure des spermatozoïdes de Lapin est analysée à toutes les étapes des techniques de congélation-décongélation par les techniques de Andrieu and Courot (1976) et Chen et al. (1989). Les altérations morphologiques principales semblent résulter de problèmes osmotiques. Les dilueurs d'Andrieu et Courot, hypertoniques, induisent une déshydratation des cellules avant et pendant la congélation. Au cours de la décongélation, les spermatozoïdes, deviennent plus sensibles au choc osmotique et beaucoup subissent des dommages au niveau de leurs membranes et de leurs acrosomes. Le dilueur de Chen et al. semble immédiatement biologiquement hypo-osmotique et induit des ruptures de membranes avant la congélation. Les cellules se déshydratent essentiellement pendant celle-ci, et retrouvent leur volume après dégel. Toutefois, la plus grande hydratation avant congélation est un facteur favorable à la formation de glace entre la membrane plasmique et les structures sous-jacentes. Selon la technique utilisée, les lésions cellulaires apparaissent respectivement avant et pendant (technique de Chen et al.), ou après la congélation (technique Andrieu et Courot). Dans les deux cas, des lésions dues à la glace sont évidentes. Les deux techniques de décongélation utilisées (37°C, 1 min. ou 45°C, 20 secs.) ne modifient pas sensiblement l'aspect général des cellules après décongélation, mais moins de membranes sont lésées par la seconde technique.

Legends for figures : *Figures. 1-3-5 Andrieu and Courot technique.* **(1)** Immediately after dilution, spermatozoa display few modifications as compared to ejaculate. The plasma membranes (M) are adjusted close to the underlying structures. (F = flagellum) Mag. = 8000. **(3)** Frozen spermatozoon (freeze-substitution). The plasma membrane (M) is close to the head. The diluent (Dil) is microcrystalline. (Ac = acrosome, b = blister of perinuclear substance, N = nucleus) Mag. = 35000. **(5)** Spermatozoon thawed at 45°C. The plasma membrane (M) is elevated from the rest of the head. (EY = egg yolk) Mag. = 20000.

Figs. 2-4-6 Chen et al. technique. **(2)** Immediately after dilution, the plasma membranes (M) are largely elevated from the sperm heads, forming bubbles. Mag. = 7000. **(4)** Frozen spermatozoon (freeze substituted). The plasma membrane (M) is stuck to the frozen diluent. The sperm head is compressed or dehydrated to such an extent that a large ice space is present between the membrane and the other structures (thin arrows). The picture also displays the classical structure of a frozen staw: Very large ice crystals are surrounded by veins of diluent containing most of frozen cells. Mag. = 10000. **(6)** Spermatozoa thawed at 37°C. Ice crystal damages (I) are present between the plasma membrane and the acrosome (Ac). Mag. = 15500.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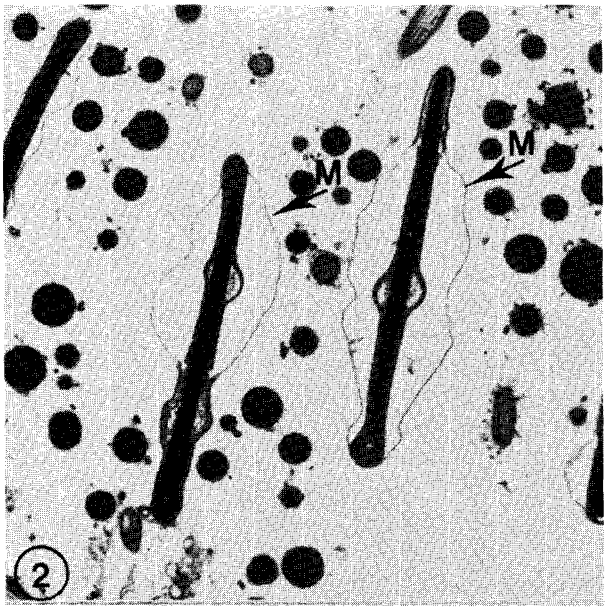
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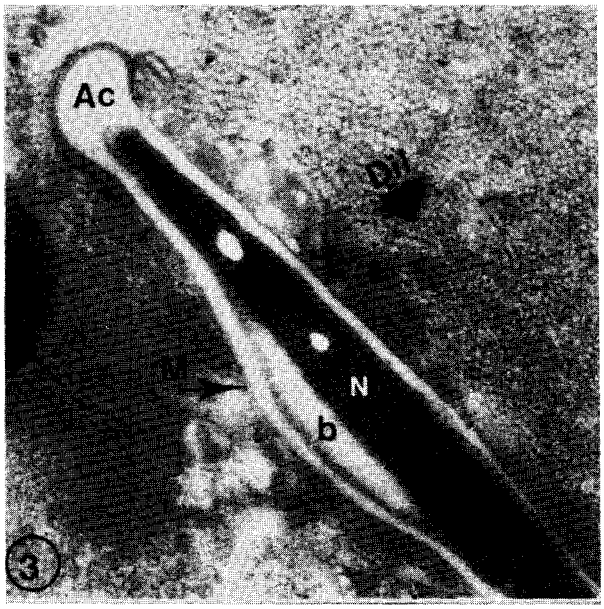
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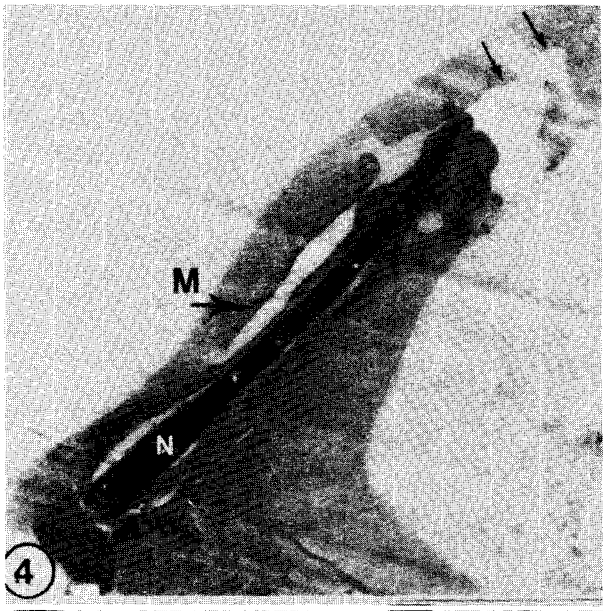
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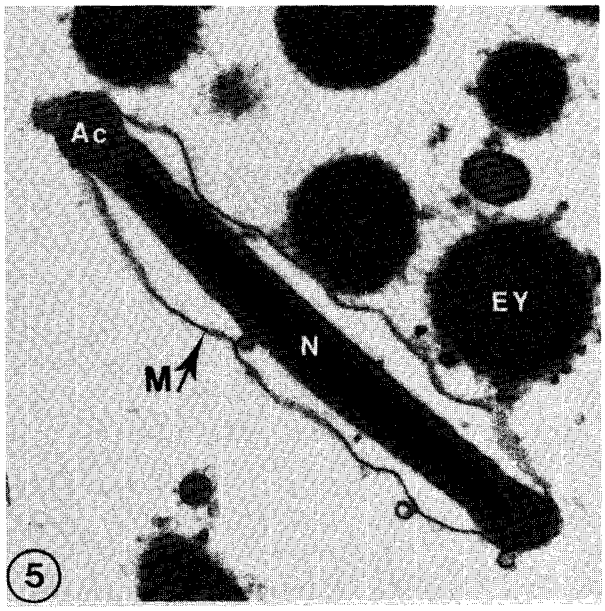
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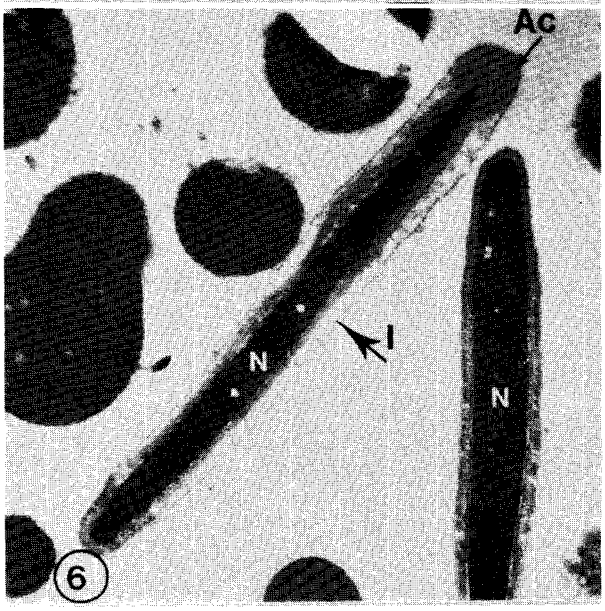
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