Cryopreservation of Atlantic Croaker Spermatozoa: Evaluation of Morphological Changes

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ABSTRACT The spermatozoon of the Atlantic croaker (Micropogonias undulatus) is a primitive type in that it lacks an acrosome. The kidney-shaped head has a diameter of about 1.5 μm and is occupied by a granular and electron-dense nucleus. The short midpiece contains 3 spherical mitochondria and encircles the basal body of the flagellum but is separated from it. The flagellum consists of the typical 9 + 2 axoneme and surrounding plasma membrane but lacks a lateral ridge. Spermatozoa of Atlantic croaker diluted in either NaCl or sodium citrate solutions with or without DMSO were examined with the electron microscope before freezing in liquid nitrogen and after thawing. Damage following cryopreservation appeared to be greater to the mitochondria, plasma membrane, and 9 + 2 axoneme than to the nucleus. The incidence of postthaw damage in spermatozoa diluted with NaCl solutions containing DMSO was remarkably lower than that with either pure NaCl solutions, pure sodium citrate solutions, or sodium citrate solutions containing DMSO.

Teleost spermatozoan morphology varies considerably (Mattei, '70; Stein, '81), and differences in the ultrastructure of fish spermatozoa have been useful for the inference of phylogenetic relationships among species (Mattei and Mattei, '74; Afzelius, '79; Baccetti et al., '84). Very little, however, is known of the reproductive biology of male sciaenids, and there is little published information on the morphology of sciaenid spermatozoa.

Although there are many papers on how to preserve fish semen by cryogenic methods (Stoss, '83), there has been little study of changes in morphology which might occur during freezing–thawing. Recently, it has been shown that cryopreservation is associated with spermatozoan structural change, such as deformed acrosome and rupture of membranes in a variety of different animals, including fowl (Bakst and Sexton, '79), bull, boar, ram (Healey, '69; Watson, '81), and rainbow trout (Gwo '82; Billard, '83). The damage appears to correspond well to reduced spermatozoan motility and fertility. No attempt has yet been made to correlate the degree of observed damage with the ability of fish spermatozoa to fertilize. Billard ('83) observed ultrastructural changes in rapidly frozen rainbow trout spermatozoa; these changes included swelling of the head and midpiece, and rupture of plasma membrane.

The present work was designed to characterize the ultrastructure of Atlantic croaker spermatozoa, to investigate possible causes of low motility and reduced fertility due to cryopreservation, and to infer possible mechanisms of damage during the freezing and thawing process. Experiments carried out previously (Gwo et al., '91) revealed that 1) no fertilization was obtained when spermatozoa of Atlantic croaker were frozen without the addition of dimethyl sulfoxide (DMSO) to the extender; 2) 1% NaCl extender containing 15% DMSO was a suitable diluent for freezing, whereas 3% sodium citrate extender with 15% DMSO was not; and 3) a freezing rate at −30°C/min was optimum. All 3 findings were exploited to examine possible ultrastructural changes between pre- and postfreezing.

MATERIALS AND METHODS

From preliminary fertilization studies (Gwo et al., '91), it was found that 1% sodium chloride was a suitable extender for Atlantic croaker spermatozoa, whereas 3% sodium citrate was not. The semen sample was a pooled collection that appeared to be of high quality. Only sperm showing vigorous movement upon microscopic examination were used. Immediately following the collection procedure, the semen sample was divided into 5 smaller samples (3–6 grams each). Throughout the experiment, semen and semen-extender mixtures were kept con...
continually immersed in ice water. One sample served as a control. Semen to be frozen was collected and diluted in 2 extenders (see below) and DMSO, and was drawn up into 0.25 ml straws. The straws were frozen in a programmable, controlled rate Bio-Cool freezer (FTS systems, Inc., Stone Ridge, NY). The freezing rate was measured using a thermocouple (Newport Electronics, Inc., Santa Ana, CA) inserted into straws; the freezing rate was expressed as the average rate of temperature decline between 25 and −80°C. The second sample was added to 1% sodium chloride with DMSO. One third of this second sample was frozen to −196°C with a freezing rate of −30°C/min, one third was frozen to −196°C with a freezing rate of −400°C/min, and one third was not frozen and served as a control. The third sample was added to 3% sodium citrate with DMSO and was processed in the same way as the second sample. The fourth sample was added to 1% sodium chloride, and the fifth sample was added to 3% sodium citrate. Half of the fourth sample was frozen to −196°C with a freezing rate of −30°C/min, and one half was not frozen and served as a control. The processing procedure for the fifth sample was identical to that of the fourth sample. The ratio of extender to DMSO to semen was 0.75:0.15:0.10 by volume for both the second and third samples, while the ratio was 0.90:0.0:0.10 for the fourth and fifth samples.

After 1 day of storage, frozen samples were thawed at room temperature. Spermatozoa from each treatment were fixed in 2% glutaraldehyde in phosphate buffer (0.12 M) for 1 hr at room temperature, postfixed in 1% osmium tetroxide in the same phosphate buffer, and embedded in low viscosity Spurr resin. Ultrasections were stained with 2% uranyl acetate followed by lead citrate and were examined under a transmission electron microscope (TEM) and photographed. At least 30 spermatozoa from each sample were examined utilizing photoprints obtained with the EM. Particular attention was given to damage of the plasma membrane, mitochondria, axial tubules, and nuclear material.

Fertility of spermatozoa from samples 1–5 and subsamples was tested using 1 batch of ovulated eggs freshly stripped from 1 or more (usually 1) females of Atlantic croaker. A 0.25 ml sample of treated semen was mixed with about 5,000 eggs in a plastic beaker for 1 min before adding seawater. The fertilization rate was determined just prior to hatching. For Atlantic croaker at 25°C, this was 24 hr after fertilization.

RESULTS

Ultrastructure of the Atlantic croaker spermatozoa

The primitive teleost spermatozoon is relatively small, has a more or less rounded or conical head, contains a nucleus, a basal midpiece, and a few mitochondria, and has a tail consisting of a long, free flagellum (Franzen, '70). Most fishes have primitive spermatozoa and discharge them into the water for external fertilization (Franzen, '70). Spermatozoa of ovoviviparous teleosts differ from those of fishes with external fertilization by having a more elongated and compressed head and an elongated midpiece that may be longer than the head length (Nicander, '70; Ginzburg, '72; Gardiner, '78). The spermatozoa of Atlantic croaker fall into the category of primitive spermatozoa. The 3 main parts (head, midpiece, and flagellum) will be described separately.

Head

The nucleus is kidney shaped with an average diameter of 1.5 μm (Fig. 1A). The chromatin consists of many clumps of dense granules and is contained within a typical double nuclear membrane. The undulating nuclear envelope and plasma membranes run tightly along the anterior side of the nucleus. No acrosomes were present (Fig. 1A). Figures 1B and 1D indicates 2 profiles of the indented nucleus.

Midpiece

The midpiece of the Atlantic croaker spermatozoon contains mitochondria (chondrisome), vesicles, the centriolar complex (a proximal and a distal centriole), and several types of unknown cytoplasmic inclusions (Fig. 1A–1C). Three complete mitochondria, each bounded by an outer and an inner membrane, are located posterior to the nucleus and surround the flagellar canal (cytoplasmic canal, postnuclear canal, plasmic canal, and invagination) on 3 sides (Fig. 1A and 1C). The spheroidal mitochondria exhibit transverse or concentric cristae and contain a medium electron-dense matrix (Fig. 1C). The mitochondrial collar encircles the flagellum and is completely separated from it by the flagellar canal (Fig. 1C). Vesicles and inclusions are irregularly distributed inside the cytoplasm (Fig. 1B and 1C). A direct sheet runs along between the cytoplasmic canal membrane and the outer layer of the mitochondria, and forms triple concentric structures (Fig. 1H). The triple concentric structures extend from the posterior end of the...
Fig. 1. Photographs of fresh (nondiluted, unfrozen) spermatozoa taken using transmission electron microscope. A: Longitudinal section through a spermatozoon showing the kidney shaped nucleus (n) with granular chromatin contents. Mitochondria (m) surrounding the proximal region of the axoneme. Note the transition region (a*) of the flagellum (f) and double nuclear membranes (arrowhead). × 49,000. Bar length is 0.25 μm. B: Cross section through the caudal region of nucleus near the site of flagellar attachment of spermatozoan head showing 2 profiles of the indented nucleus (n). Vesicles and inclusions are irregularly distributed inside the cytoplasm. × 50,000. Bar length is 0.25 μm. C: Cross section through the midpiece of spermatozoon showing the flagellar canal (*) surrounded by 3 profiles of mitochondria (m) bounded by their outer and inner membranes (arrowhead). The looped plasma membrane (pm) is visible. × 70,000. Bar length is 0.25 μm. D: Cross section through a spermatozoan head showing that the proximal centriole (pc) is located within the nuclear pit (np) and a dense
body (arrowhead) is present between the proximal centriole and the nucleus (n). \( \times 49,000 \). Bar length is 0.25 \( \mu \text{m} \). E: Longitudinal section through a spermatozoan nucleus and midpiece showing the relationship of flagellum to the centriole complex consisting of a proximal (pc) and a distal centriole (dc). The anterior part of the distal centriole (dc) is surrounded by a partial osmiophilic ring (or). The proximal centriole (pc) shows the typical 9 triples of microtubules. \( \times 52,000 \). Bar length is 0.25 \( \mu \text{m} \). F: Longitudinal section through a spermatozoan head passing through a plane parallel to the proximal centriole illustrating the 90° relationships between the proximal (pc) and distal (dc) centrioles. \( \times 40,000 \). Bar length is 0.25 \( \mu \text{m} \). G: Longitudinal section perpendicular to the section in Fig. F showing osmiophilic filaments (arrowhead) connecting with both the proximal centriole (pc) and distal centriole (dc). \( \times 70,000 \). Bar length is 0.25 \( \mu \text{m} \). H: Cross section of the flagella showing the 9 + 2 organization of the axoneme (a). Note the triple concentric structure (arrowhead). \( \times 111,000 \). Bar length is 0.25 \( \mu \text{m} \).
Fig. 2. Photographs of postthaw spermatozoa taken using transmission electron microscope. A: Spermatozoa showing several profiles of axoneme (a). The plasma membrane is ruptured from axoneme. Note that an axonemal cross section is enclosed in the plasma membrane near the middle nucleus. No recognizable mitochondria are observed. No changes in the nuclei (n) of the 3 spermatozoa are recognized. × 31,000. Bar length is 0.25 μm. B: Spermatozoa showing the ruptured plasma membrane and no apparent midpiece, but with nuclei (n) still resent. Some microtubules of the 9 + 2 axoneme (a) have lost their orientation or separated into pieces. × 41,000. Bar length is 0.25 μm. C: Spermatozoa showing that the 9 + 0 region of an axoneme (a*) at the level of the distal centriole and 2 deformed 9 + 2 axonemes (a) at the level of the flagellum. × 44,000. Bar
length is 0.25 μm. D: Spermatozoa showing that the plasma membrane is displaced, the midpiece is not recognizable, but nuclei (n) are still present. × 41,000. Bar length is 0.25 μm. E: Spermatozoa showing no midpiece and that the plasma membrane (pm) appears swollen and ruptured. The nucleus (n) appears intact. × 41,000. Bar length is 0.25 μm. F: The plasma membrane (pm) of a spermatozoon has become wrinkled. Mitochondria (m) are swollen with a reduction in density of the cristae. There are no visible changes of chromatin structures in the nucleus (n). × 52,000. Bar length is 0.25 μm. G: Both the plasma membrane (pm) and mitochondria membrane (mm) of a spermatozoon are ruptured. Mitochondria (m) exhibit vacuolization and cristae are disrupted. × 52,000. Bar length is 0.25 μm.
midpiece collar to the anterior end of the flagellar canal (Fig. 1A).

A proximal centriole lies anterior to the distal centriole and is located within the nuclear pit (implantation fossa, hilus, fossa, indentation, concavity, recess, ultranuclear canal) (Fig. 1D and 1F). Lying within the nuclear pit and above the proximal centriole is a dense body (the axial body, extension).

An osmiophilic ring, embedded in electron-sense material, surrounds the anterior part of the distal cavity, recess, ultranuclear canal) (Fig. 1D and 1F). Lying within the nuclear pit and above the proximal centriole is a dense body (the axial body, extension).

The Atlantic croaker spermatozoon has only a single flagellum (Fig. 1A and 1C). In the transition or proximal region, no central tubules are present in the midpiece and the doublets are still subdivided. The flagellum is of the 9 triplets + 0 type, consisting of 9 double outer tubules and no central tubule (Fig. 1A and 1B). A classic 9 + 2 organization (9 + 2 axial complex of the axoneme consisting of 9 double outer tubules and 2 single central tubules) is observed distal to this region (Fig. 1A, 1C, and 1H). The axoneme is surrounded by a plasma membrane (Fig. 1H).

The 2 central tubules are surrounded by a central sheath (Fig. 1H). There is no clear indication of a Y-shaped bridge (Y-link structure, Y-shaped electron-dense body) (Fig. 1C and 1H). Each of the 9 doublets consist of subfibers A and B (Fig. 1H). Two dynein arms arise from subfiber A of each doublet and extend toward the next fiber. Structures similar to radial spokes are just visible (densities that may represent them; Figs. 1C,1H). Neither lateral ridge (lateral extension, sidefin, peripheral sheet), dense fibers, nor vesicles were present in the flagellum of Atlantic croaker spermatozoa (Fig. 1A, 1C, and 1H).

The effect of cryopreservation on the ultrastructure of Atlantic croaker spermatozoa

Observation using the light microscope

Changes in neither motility nor morphology of Atlantic croaker spermatozoa were observed when fresh semen was diluted before freezing with the 3% sodium citrate extender with or without 15% DMSO or with the 1% NaCl extender with or without 15% DMSO. Motility after thawing was observed only when the diluent was the 1% NaCl extender containing 15% DMSO and the freezing rate was 30°C/min. Coagulation of thawed semen occurred in samples of undiluted semen and in samples which contained either 3% sodium citrate extender with or without 15% DMSO, or in 1% NaCl extender without 15% DMSO. Viewed under the light microscope, their spermatozoa were observed to be clumped and the seminal plasma coagulated. In contrast, when semen was diluted in 1% NaCl extender with 15% DMSO and frozen rapidly (−400°C/min) or slowly (−30°C/min), postthaw spermatozoa dispersed evenly without the formation of clumped aggregates.

Observation using the electron microscope

In order to make comparisons of cryodamage in the ultrastructure of Atlantic croaker spermatozoa that underwent different treatments, the head, midpiece, and tail regions of spermatozoa are considered separately. Of the 6 treatment groups studied, 2 general patterns of freeze–thaw damage were observed. The patterns of ultrastructural change induced by using either undiluted (neat), fresh semen diluted in 3% sodium citrate extender with or without 15% DMSO or fresh semen diluted in 1% NaCl extender without DMSO frozen at the −30°C/min freezing rate resembled each other, but differed from those either rapid freezing (−400°C/min) or slow freezing (−30°C/min) of spermatozoa diluted in 1% NaCl extender with 15% DMSO (Fig. 2). The first pattern was characterized by clumping of spermatozoa, whereas spermatozoa in the second pattern remained dispersed. These 2 patterns are considered below separately.

Pattern 1. Clumping of Spermatozoa following freeze–thaw. The ultrastructural appearance of spermatozoa frozen to −196°C and later thawed are shown for 1) undiluted semen (Fig. 2A), 2) semen diluted in 3% sodium citrate extender with 15% DMSO and frozen at either −30°C/min (Fig. 2B) or −400°C/min (Fig. 2C), 3) semen diluted in 1% NaCl extender without DMSO and frozen at a −30°C/min freezing rate to −196°C (Fig. 2D), and 4) semen diluted in 3% sodium citrate extender without DMSO and frozen at a −30°C/min freezing rate to −196°C (Fig. 2E).

Head. The plasma membrane seemed to become loose and swollen from spermatozoa (Fig. 2A–2E). A wide space appeared between the plasma membrane and the nuclear surface. Several axonemal cross sections (9 + 2 structures) were enclosed in
the head along with the nucleus by a single plasma membrane, though no changes in the nucleus were recognized.

**Midpiece.** In the majority of spermatozoa, at least 25 of 30, the midpiece was no longer recognizable (Fig. 2A–2E). A few distinguishable mitochondria were misshaped and displaced, and had lost most of their cristae.

**Tail.** The plasma membranes appeared swollen and ruptured (compare Fig. 2A–2E to Fig. 1A). None of the axonemal cross sections were surrounded by a plasma membrane. Several cross sections of the axoneme were found close to each other without their plasma membrane. Some 9 + 2 structures were deformed and even separated into pieces. These observations suggested that either the axoneme retracts and coils spirally, or the plasma membrane swells along length of the axoneme and the axoneme coils during freeze–thaw.

Postthaw spermatozoa appeared to have undergone drastic morphological changes. Some of the more commonly observed changes were rupture and vesiculation of the plasma membrane, breakage of the nuclear envelope, outflow of nuclear chromatin, lack of distinct mitochondria, and loss of the flagellum by detachment from the spermatozoan head. It should be emphasized that the most extensive changes involved change to cellular membranes, including those of the mitochondria and flagellum.

**Pattern 2.** Spermatozoa remaining dispersed following freeze–thaw. The ultrastructural changes of spermatozoa diluted in 1% NaCl extender with DMSO and frozen at both rapid (−400°C/min) and slow (−30°C/min) freezing rates to −196°C and later thawed are shown in Figures 2F and 2G, respectively.

**Head.** Although the plasma membrane was usually broken and became wrinkled, no effect of the freeze–thaw process on the nucleus was evident (Fig. 2F and 2G).

**Midpiece.** The mitochondria membranes were broken (Fig. 2G). Mitochondria were swollen, with a corresponding decrease in density of the matrix (Fig. 2F and 2G). Cristae were disrupted and only occasionally were normal profiles seen. For severely damaged spermatozoa, mitochondria exhibited extensive vacuolization with a disappearance of most of the cristae.

**Tail.** The general 9 + 2 pattern of the axoneme was unchanged (Fig. 2F and 2G), but usually the fibers were much less distinct than seen in normal spermatozoa. The membrane was totally detached in a majority of the spermatozoa.

The “treated” spermatozoa, usually, gave an appearance of disorganization of organelles. The plasma membrane and coil of mitochondria were more disorderly than in normal spermatozoa. Examination using a transmission electron microscope showed a considerable range in type and extent of morphological changes within the same postthaw sample of spermatozoa, ranging from no apparent damage to virtual disintegration of the spermatozoa. Therefore, it seems justified to conclude that most spermatozoa showed varying and often pronounced change, even when subjected to the optimal method of freezing.

**DISCUSSION**

The morphology of spermatozoa was about the same in diluted and undiluted semen before freezing, and changes in spermatozoan ultrastructure were rarely observed before freezing in either isotonic 1% NaCl extender with or without 15% DMSO or in 3% sodium citrate extender with or without 15% DMSO. Only a slight swelling of the plasma membrane was observed in a few spermatozoa suspended in these diluents. The lack of morphological changes prior to freezing observed in Atlantic croaker semen diluted with either 1% NaCl extender containing 15% DMSO or 3% sodium citrate with or without 15% DMSO may be due to the brief period, about 3–5 min, of exposure to an acceptable extender, proper concentrations of both extender and DMSO, and the low dilution ratio. Drastic morphological changes were found only in freeze–thaw spermatozoa.

Freezing and thawing caused structural damage to all types of spermatozoan organelles examined except the nucleus: The damage appeared greatest to the mitochondria, plasma membrane, and the 9 + 2 flagellar structure. Billard ('83) found that the primary site of freeze–thaw damage to rainbow trout spermatozoa was the plasma membrane and mitochondria. He also showed greater clumping of chromatin of rainbow trout spermatozoa following slow freezing. However, no such phenomenon was observed in Atlantic croaker spermatozoa. The nucleus has been shown to be remarkably stable to freezing and thawing, and freezing and thawing is generally not considered to be mutagenic (Ashwood-Smith and Farrant, '80). This is supported by the lack of observable morphological aberration in offspring following cryopreservation of semen from striped bass, common carp, seabream, and tilapia (Mocarski, '76; Kerby et al., '85; Kurokura et al., '86; Koldras and Bieniarz, '87; Rana and McAndrew, '89).

It is well known that the function of the mitochondria is to supply energy, normally provided by
adenosine triphosphate (ATP), to the cell. In the case of spermatozoa, the major energy requirements are for movement and osmoregulation (Gibbons, '82). ATP passes distally by diffusion to the flagellum, which is composed of 9 pairs of fused peripheral microtubules (doublets) and 2 separated central microtubules (singlets; Gibbons, '82). One of each doublet (the A tubule) carries 2 dynein arms, which contain an ATPase (flagellar ATPase protein dynein). Inactive dynein, upon activation by hydrolysis of ATP from mitochondria, induces a shearing force which, with the participation of radial spokes and nexin links, results in movement of the flagellum (Gibbons, '82; Nelson, '85). The profound structural changes observed in certain of the above experiments apparently account for the loss of motility of the Atlantic croaker spermatozoa after freezing and thawing. From examination of data, we infer that the mechanism of damage during freezing and thawing is due to the lack of recognizable mitochondria, distortion of the 9 + 2 structure (axoneme), or the clumping of the flagella, and make freeze—thaw spermatozoa nonmotile and infertile (Fig. 2A—2E). Moreover, the damaged mitochondria and the broken plasma membranes that occurred in the 1% NaCl extender with 15% DMSO (Fig. 2F and 2G) may be the primary reason for the strongly reduced motility of spermatozoa following both rapid and slow freezing. The general structure of the 9 + 2 structure of the flagellum was unchanged, but some minor alterations occurred and it seems natural to believe that these in some way are partially responsible for the loss of motility.

Xia et al. ('88) reported that the degree of ultrastructural integrity of the chicken spermatozoa can be an indicator of semen quality and fertilizing ability. They found a positive correlation between fertility and the percentages of normal mitochondria, nuclei, midpieces, an perforatoria of unfrozen spermatozoa. However, with the freeze—thaw semen, their correlation coefficients were significant \( P < 0.05 \) for the midpiece (0.69) and nucleus (0.71) only.

Fertilization may depend on the presence of only slight damage to at least some spermatozoa in the freeze—thaw Atlantic croaker spermatozoa, because freezing and thawing of semen selects for resistant spermatozoa, screening out the nonviable and defective ones, with only the strongest spermatozoa, although weakened by the cold and adverse osmotic and pH conditions, still retaining fertility after thawing. No attempt has been made to correlate the damaged part, its degree of damage, and the frequency of damaged spermatozoa to the fertilization rate. In order to estimate their percentages and to test the above hypothesis, more extensive quantitative studies involving large numbers of spermatozoa will be necessary.

Exposure of unprotected spermatozoa to freezing results in morphological and biochemical derangements that render them irreversibly immotile and infertile. DMSO protected the viability of the Atlantic croaker spermatozoa during freezing. One of the effects of DMSO appears to be protection of individual spermatozoa from coagulation. This effect disappeared totally once semen were diluted in 3% sodium citrate extender with 15% DMSO and frozen either slowly or rapidly. This aspect requires further investigation. It has been proposed that cell damage from freezing might largely be caused by an osmotic pressure gradient (Meryman, '70) or solution effect (Mazur, '65). Mazur ('65) proposed that for any given cell type there is an optimum temperature-change rate during freezing, above and below which cell survival declines. At rates higher than the optimum, cell death is caused by the formation of intracellular ice, while at less than optimum rates, increased solute concentration, the changing of pH as buffer salts reach their solubilities, the increasing cellular dehydration, the diminishing fluid volume, and the rising cryoprotectant concentration may all contribute to damage and loss of viability.

It seems that biochemical changes, perhaps some sort of irreversible destabilization of cell membranes, renders the cells susceptible to lowered temperature and to mechanical trauma (Mazur, '65). DMSO may reduce or eliminate these biochemical effects by its colligative ability to reduce shrinkage of cells. This may minimize lethal intracellular ice formation at low temperatures and prevent lethal damage from concentrated solute (Ashwood-Smith and Farrent, '80). Spermatozoa have a complex and rather rigid substructure, comprised mostly of a condensed nucleus in the head region and mitochondria, and motile flagellar components in the tail. Watson and Duncan ('88) proposed that freezing spermatozoa strongly resist cell shrinkage during dehydration. Atlantic croaker spermatozoa frozen and thawed in sodium citrate solution had increased visible morphological changes and significantly decreased \( P < 0.05 \) fertility compared to those frozen and thawed in sodium chloride solution, even though the 2 extenders were of the same osmolarity. Therefore, different solutes in the extenders produce different degrees of deformity and fertilization rates. A high concentration of solutes in the medium may occur due to ice crystallizing during the freezing procedure (Mazur, '65; Meryman, '70). It is pos-
sible that an increased concentration of sodium citrate and sodium chloride occurred during our experiments. If this happened, an increase in sodium citrate concentration is much more damaging than an increase in sodium chloride. Consequently, the dramatic morphological changes during freezing and thawing may have been related to a rise in the concentration of sodium citrate in the diluent. Sneed and Clemens ('56) have shown that sodium citrate caused spermatozoa to clump in common carp semen following freezing to -75°C and thawing. There is a species-specific relationship between different extenders and various fish species, as discussed in a previous experiment (Gwo et al., '91). Sodium citrate is harmful to the structural integrity of both Atlantic croaker and common carp spermatozoa stored at low temperatures of -75 or -196°C (Sneed and Clemens, '56).

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