Structural and Functional Aspects of *Bufo americanus* Spermatozoa: Effects of Inactivation and Reactivation

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**ABSTRACT** Very little is known about the effects of manipulating toad sperm activity in vitro, and such information is important in the development of a genetic resource bank for bufonid species. The specific objectives of this study were to: 1) identify the optimal inactivation and reactivation solutions for toad spermatozoa collected in urine; 2) establish the length of time toad spermatozoa can be exposed to an inactivation buffer and still resume motility upon reactivation; 3) evaluate the consequence of inactivation on specific sperm characteristics; and 4) characterize the sperm mitochondria vesicle (MV) and its relationship to motility. Reactivated sperm motility was similar after inactivation in either Simplified Amphibian Ringers (SAR) solution or DeBoer’s (DB) solution. Diluting the buffer by 80% with water provided the best method for reactivating sperm. Dilutions with NaCl solutions (10–50 mM) produced inferior results. SAR-inactivated spermatozoa could remain suspended up to 4 hr and still regain 25% of initial motility upon reactivation in water. Compared to the controls, sperm motility was greater ($P < 0.01$) over time for samples treated with SAR, although forward progression was significantly lower. Furthermore, SAR treatment resulted in sperm samples with a greater number of viable, morphologically normal, and intact MVs over time. Electron microscopy and fluorescent staining confirmed that the toad sperm’s MV contains a large number of active mitochondria with very few other cytoplasmic structures. Nearly all spermatozoa exhibiting motility had an intact MV, and dissociation of this structure was clearly related to motility loss. In conclusion, toad spermatozoa can be effectively inactivated and reactivated by varying the osmolality of the external solutions and, although sperm forward progression is reduced, all other characteristics are well maintained. Moreover, the increased number of spermatozoa with intact MV after inactivation suggests the process may help preserve this important structure. *J. Exp. Zool.* 295A:172–182, 2003. © 2003 Wiley-Liss, Inc.

Amphibian species worldwide are declining at an alarming rate due to habitat destruction, environmental pollution, ultraviolet exposure, and pathogenic agents (Daszak et al., '99; Morell, 2001; Kiesecker et al., 2001). In response to this crisis, numerous captive breeding programs have been established as a means of preserving these threatened or endangered amphibians; however, space and resource limitations restrict the number of genetically valuable animals that can be maintained in captivity. In addition, un-represented founders or under-represented lineages often exist in captive populations due to reproductive failure. To address these issues, studies have been initiated to explore the development of a genome resource bank for the preservation of amphibian gametes. Due to the poor success in freezing oocytes of most taxa studied to date (Shaw et al., 2000), our laboratory has concentrated on developing protocols for the cryopreservation of anuran spermatozoa. Initial attempts to cryopreserve American toad (*Bufo americanus*) sperm using protocols established for domestic mammalian species (Curry, 2000; Holt, 2000) have failed to provide high levels of post-thaw motility. Therefore, a better understanding of basic anuran sperm morphology and physiology is needed to facilitate the development of effective cryopreservation protocols and additional reproductive technologies.

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Toad species, within the *Bufo* genus, primarily practice external fertilization. Spermatozoa are immotile in the male reproductive tract until their release into a hypotonic aquatic environment, wherein the decrease in osmolality activates sperm to become motile. It is generally accepted that in fish (Morisawa et al., '83), urodèles (Hardy and Dent, '86), and especially mammals (Drevius and Eriksson, '66), exposure of spermatozoa to a hypoosmotic aqueous environment results in considerable damage to cellular structures. In mammals, such damage results in immediate death of the spermatozoa due to hypoosmotic swelling (Drevius and Eriksson, '66). In amphibians, the effects are delayed, and sperm retain their motility and fertilizing capacity for a short time in such environments. Although osmotic damage does not immediately kill urodele spermatozoa, the longevity of sperm motility is greatly reduced (Hardy and Dent, '86). Activated anuran spermatozoa diluted in water are motile for approximately 10 min in *Xenopus* (Inoda and Morisawa, '87; Bernardini et al., '87, '88) and *Bufo* (Obringer et al., 2000; Roth and Obringer, 2003) species. The rapid decrease in sperm motility imposes significant challenges for studying amphibian sperm in their natural, activated state. Furthermore, it complicates the development of sperm storage and cryopreservation protocols.

To avoid challenges associated with collecting activated sperm, scientists have typically acquired amphibian sperm from testes macerates of euthanized animals. By suspending the testes macerates in a buffer mimicking the osmolality within the male reproductive tract, the sperm are held in an inactive state until used for fertilization. Two buffers commonly used for macerating testis are Simplified Amphibian Ringer’s solution (SAR) (Cabada, '75; Browne et al., '98, 2001) and DeBoer’s solution (DB) (Wolf and Hedrick, '71; Bernardini et al., '88; Yamasaki and Katagiri, '91). When frog or toad sperm are collected from testis macerates using either SAR or DBT, a significant reduction in the osmolality (<100 mosmol/kg) of the environment is required to initiate sperm motility (Inoda and Morisawa, '87; Browne et al., '98). Typically, this is accomplished by diluting the inactivated sperm sample with water. Although effective, the techniques involving euthanasia and testicular removal are inappropriate for endangered animals. As an alternative, reliable methods have been established for inducing spermiation in live toad (Obringer et al., 2000; Rowson et al., 2001) and frog (Waggener and Carroll, '98b) species using luteinizing hormone releasing hormone (LHRH). Spermic urine can be collected relatively noninvasively 5–7 hr after LHRH injection. Methods for manipulating (inactivating and reactivating) toad sperm motility after activation in urine and the effects such manipulations might have on sperm structural integrity have not been evaluated. Furthermore, osmotic damage to cellular structures after dilution into water versus low ionic strength solutions has not been assessed.

One structure that appears to be highly sensitive to changes in the external osmotic environment is a sac-like structure located at the posterior portion of the toad sperm nucleus, above the flagellum, that contains a high concentration of mitochondria and is referred to as the mitochondria vesicle (MV) (Roth and Obringer, 2003). A similar structure has been referred to as a protoplasmic bead in salamanders (Picheral, '79) and as an accessory cell in frogs (Waggener and Carroll, '98a). Waggener and Carroll ('98a) report that the presence of this structure in frog spermatozoa is associated with motility and that it appears to contain a nucleus. In toads, shortly after diluting spermatozoa into water, this structure often collapses, ruptures, and/or dissociates from the sperm head (unpublished observations), and damage to this structure may be associated with decreased motility. This structure has not been comprehensively studied in toads, nor has its relationship to motility been clearly defined.

In the present study, methods of inactivating and reactivating toad spermatozoa were tested to: 1) identify methods that optimize the recovery and duration of sperm motility; and 2) determine the effect of these processes on sperm viability and morphology. Furthermore, initial studies were undertaken to characterize the MV associated with toad spermatozoa and to determine the relationship between this structure and sperm motility.

**MATERIALS AND METHODS**

**Animals**

Male American toads were collected in the spring and summer from Northern Kentucky and adult males were identified by weight (25–48 g), nuptial pad, and the presence of blackened vocal sacs. Animals were housed in groups (n ≤ 8) in plastic enclosures (12"H × 15"W × 22"L) with aged tap water and cover supplied. Crickets, earthworms, or wax worms were provided three times a week, and the animals were maintained in
the laboratory at 22–25°C. All animal procedures were approved by the Center for Conservation and Research of Endangered Wildlife’s IACUC (protocol # 98-001).

Spermic urine was collected from live American toads 5 hr following intraperitoneal injection of LHRH (4 μg/20 μl dH₂O; L4513, Sigma Aldrich, St. Louis, MO) as previously described (Obringer et al., 2000). If a male toad was not producing sperm by 5 hr post-injection, then the animal was screened every half hour up to 7 hr. Immediately upon collection, spermatozoa in urine were evaluated for motility, and sperm samples exhibiting <50% motility were excluded from the study. For these studies, spermatozoa exhibiting flagella beating were considered motile, even if no forward progression was observed. Forward progressive velocity was based on a scale of 0–5, where 0 = no movement and 5 = rapid forward movement.

**Study 1: Comparison of inactivation and reactivation solutions**

In study 1, reactivated sperm motility was compared between samples inactivated in Simplified Amphibian Ringers solution (SAR; 113 mM NaCl, 1 mM CaCl₂, 2 mM KCl, 3.6 mM NaHCO₃, 220 mosmol/kg, pH 7.4) (Browne et al., ’98) and DeBoer’s solution (DB; 119 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 10 mM tris [hydroxymethyl] aminomethane [TRIS], 250 mosmol/kg, pH 7.5) (Bernardini et al., ’88). In addition, motility recovery was evaluated in SAR- and DB-treated samples by testing different reactivation solutions of varied ionic strengths. Sperm samples were collected (n = 7/treatment), evaluated for motility, and immediately inactivated by diluting samples (1:1) in SAR (2X) or DB (2X), and inactivation was confirmed by the absence of motility. To serve as a control, a sub-sample from each collection of spermic urine was diluted (1:1) with dH₂O such that the sperm remained activated. Diluted sperm samples were concentrated by centrifugation at 100 × g for 5 min, the supernatant discarded, and the sperm pellet resuspended in 100 μl of the corresponding buffer (SAR or DB, 1X) or dH₂O (control). Approximately 10 min after resuspension, inactivated sperm samples were reactivated by dilution (1:5) with dH₂O or solutions containing 10, 25, or 50 mM NaCl. The sperm samples were evaluated for motility immediately after reactivation and at 1, 3, 5, 10, 15, 30, 45, 60, 90, and 120 min post-reactivation. Both the proportion of sperm that became activated upon dilution and the duration of sperm motility served as criteria for the selection of the optimal buffer and reactivating solution to be used in studies 2 and 3. Percentage data in study 1 are shown as relative values ([absolute parameter value/absolute parameter value at time of collection] × 100) and reflect the parameter recovery rate after inactivation and reactivation. The absolute (raw) data is the percent of motile spermatozoa out of the total number counted.

**Study 2: Recovery of motility after an extended period of inactivation**

Study 2 was designed to evaluate the length of time toad spermatozoa could remain inactivated in SAR before losing a significant percentage of its recoverable motility. Upon collection, sperm samples (n = 6) were divided into two treatments. One aliquot was inactivated in SAR as described previously, while the second aliquot (control) was not inactivated nor diluted in dH₂O. SAR-treated sub-samples were reactivated at specific time points after inactivation (0, 5, 15, 30, 60, 120, and 240 min) by dilution in dH₂O and evaluated for sperm motility and forward progression. Sub-samples of the control were examined in parallel with the reactivated sub-samples of the treated aliquot.

**Study 3: Effects of inactivation on sperm characteristics**

Study 3 was designed to evaluate the effects of inactivation and reactivation on several toad sperm characteristics, specifically: sperm motility, forward progressive velocity, viability, morphology, and MV status. Both the immediate impact and long-term (up to 2 hr) effects of inactivation and reactivation were evaluated for these parameters. Sperm samples (n = 11) were inactivated by dilution (1:1) in SAR (2X). As a control, an aliquot from the original sperm sample was diluted (1:1) in dH₂O, allowed to remain activated, and examined in parallel with the inactivated treatments. Inactivated sperm samples were centrifuged and resuspended as described in study 1. Approximately 15 min after collection, sperm samples were reactivated by dilution in dH₂O and evaluated for sperm motility and forward progression immediately and at 1, 3, 5, 10, 15, 30, 45, 60, 90, and 120 min after reactivation. At 0, 5, 15, 30, 60, and 120 min after reactivation, a 15 μl aliquot of sperm was placed into 5 μl of 8% (v:v) glutaraldehyde and stored at 4°C for evaluation of
viability, morphology, and MV status. Viability was assessed using Hoescht 33258 (Sigma-Aldrich) as previously described (Obringer et al., 2000). In brief, 5 μl of sperm was mixed with 5 μl of Hoescht stain (5 μg/ml) for 5 min in the dark at room temperature. A wet mount was prepared and evaluated by fluorescent microscopy (× 400), and viability status for 100 sperm cells was determined. The morphology and MV status of 100 spermatozoa per slide was evaluated (× 400) by phase-contrast microscopy. Percentage data (motility, viability, morphology, and MV status) are expressed as relative values (see study 1) and reflect the parameter recovery rate after inactivation and reactivation, compared to the controls.

**Study 4: Characterization of the MV and its relationship to motility**

Study 4 was divided into three separate objectives designed to characterize structural and functional aspects of the MV. First, electron microscopy (EM) was utilized to evaluate the cytoplasmic organelles contained within the MV. Spermic urine samples (n = 3) were centrifuged at 300 × g for 5 min, the supernatant discarded, and the sperm pellet resuspended in 500 μl of EM grade glutaraldehyde (2% v/v) in 0.1 M phosphate buffered saline (PBS, pH 7.4). Sperm samples were placed on ice and sent overnight to the University of Florida’s Interdisciplinary Center for Biotech Research (electron microscopy core facility) for immediate processing. In brief, samples were embedded in a 3% agarose pellet, washed and post-fixed with a 1% osmium tetroxide (in PBS) for 1 hr. After several washes in distilled water, samples were dehydrated in a graded ethanol series followed by 100% acetone. Following dehydration, samples were infiltrated in a graded epoxy resin series and polymerized in 100% epoxy resin (EmBED 812, Electron Microscopy Sciences, Fort Washington, PA) for two days at 60°C. Ultra thin sections (70 nm) were collected on 0.25% polyvinyl-coated 200-mesh copper grids, stained with 2% uranyl acetate, followed by Reynolds lead citrate and examined on a Hitachi H-7000 transmission electron microscope (Hitachi Scientific Instruments, Danbury, CT).

For the second objective, toad spermatozoa were inactivated and centrifuged as described. Sperm pellets were resuspended with 1 ml of SAR and incubated with 100 nM of MitoTracker Green (Molecular Probes, Eugene, OR), a mitochondrial probe, for 20 min at room temperature. Samples (n = 3) were then centrifuged and washed × 3 with 1 ml of SAR. Stained spermatozoa were mounted and the cellular location of mitochondria examined by fluorescent microscopy (excitation and emission maxima are 490 and 516 nm, respectively) at × 400 magnification. The mitochondrial probe described above can be used to detect activity by microscope observation, as metabolically active mitochondria can be seen moving within the MV.

The third objective was to determine the relationship between the MV and toad sperm motility. Toad (n = 6) spermatozoa were collected as described and left activated in spermic urine up to 4 hr. Individual spermatozoon (n = 100/slide) were randomly evaluated by light microscopy at 0, 1, 2, and 4 hr after collection and scored for the presence of MV and motility. Spermatozoa were placed into one of four quantitative classifications; motile + MV, motile − MV, nonmotile + MV, and nonmotile − MV.

**Statistical analysis**

Data were analyzed by a repeated measures two-way least-squares means analysis of variance (ANOVA) using Statview (SAS Institute Inc., '98). Analyses included the main effects of treatment and time, and the treatment by time interaction. A minimum of six samples from six different animals were evaluated in each experiment. Percentage data were subjected to arcsine transformation before analysis. Values are expressed as the means ± SEM, and differences were considered significant at P<0.05. Differences between means were evaluated by Fisher’s Protected Least Significant Difference (PLSD) test.

**RESULTS**

**Study 1: Comparison of inactivation and reactivation solutions**

The purpose of study 1 was two-fold: to find the optimum reactivation solution and to determine if the method of inactivation affects sperm motility recovery. Of several reactivation solutions tested, water induced the best (P<0.001) recovery of sperm motility (Fig. 1). SAR-treated sperm samples diluted 80% in water recovered > 50% of initial motility within the first minute of reactivation and maintained > 25% motility for up to two hours post-reactivation. In contrast, the best sperm motility obtained by reactivation in the other treatments was <25% at all times. Reacti-
Evolution of spermatozoa in different salt solutions of increasing ionic strength resulted in a dose-dependent inhibition of motility, with the 50 mM NaCl solution producing the lowest motility recovery of any treatment (<5%), presumably due to its higher osmolality (150 mosmol/kg). For sperm reactivated in water, there was no effect ($P > 0.05$) of inactivation buffer (SAR or DB) on sperm motility, although a treatment by time interaction ($P < 0.001$) was detected. This interaction indicated that spermatozoa inactivated in SAR and reactivated in water were better able to maintain motility over time compared to those inactivated in DB. Based on these results, SAR and water were chosen as the inactivation and reactivation solutions, respectively, in studies 2 and 3.

**Study 2: Recovery of motility after an extended period of inactivation**

Study 2 was designed to test how long toad spermatozoa could remain in an inactive state and still regain motility upon reactivation. Results indicate that toad spermatozoa can be held inactive for extended periods of time without experiencing a large drop in recovered motility (Fig. 2A). Even at 4 hr, spermatozoa inactivated in SAR could be reactivated, achieving 25% of initial motility. However, these sperm exhibited no forward progression (Fig. 2B). Inactivation and reactivation resulted in an immediate and significant reduction ($P < 0.01$) in sperm motility and forward progression relative to the spermic urine control. The recovery rate for these parameters (motility and forward progression) decreased the longer a sample was held inactive at a rate that paralleled that for similar parameters measured in the spermic urine control (Fig. 2). Spermatozoa left in urine could maintain a high level of motility and forward progression for up to 4 hr.

**Study 3: Effects of inactivation on sperm characteristics**

Study 3 evaluated the effects of SAR-inactivation on sperm motility, forward progression, viability, morphology, and MV status. Although motility appeared higher in SAR-treated samples compared to control samples that were not inactivated (Fig. 3A), there was no difference ($P$...
viability began to decrease after 30 min in the control, viability was maintained at a constant rate in SAR-treated samples, resulting in a 40% difference between treatments at 2 hr post-reactivation (Fig. 4A). The fraction of sperm that were morphologically normal with intact MV also was greater ($P < 0.01$) in SAR-treated samples compared to the control (Fig. 4B,C). Perhaps most importantly, SAR-treatment preserved the spermatozoon's MV throughout the 2 hr evaluation period, whereas the controls showed a time-dependent loss of this structure.

Fig. 3. Sperm motility (A) and forward progression (B) over time (0–120 min) after inactivation in SAR and reactivation in water (●) versus the diluted control (○). Data are expressed as means ± SEM.

Fig. 4. Sperm viability (A), morphology (B), and mitochondrial vesicle status (C) over time (0–120 min) after inactivation in SAR and reactivation in water (●) versus the diluted control (○). Data are expressed as means ± SEM.
Study 4: Characterization of the mitochondria vesicle and its relationship to motility

The mitochondrial vesicle of toad spermatozoa is an auxiliary structure typically oblique to the sperm head but not completely surrounding it. This vesicle differs from a cytoplasmic droplet that is typically associated with the flagellum on mammalian spermatozoa. Located near the posterior region of the head, the vesicle can vary in size, being barely discernable to occupying up to 2/3 the length of the sperm head. Yet, this auxiliary structure rarely extends onto the flagellum. Transmission electron microscopy revealed this vesicle contains a large number of mitochondria, smooth endoplasmic reticulum, and numerous empty vacuoles, possibly representing exhausted storage granules (Fig. 5A,B). There are also numerous organelles enclosed by a double membrane. These organelles are not mitochondria or nuclei (Fig. 5C,D) and were not identifiable by our laboratory.

Staining of the mitochondrial vesicle with MitoTracker Green revealed a large number of mitochondria associated with this vesicle and, furthermore, indicated that mitochondria were not found in other locations within the spermatozoa (Fig. 6). The stained mitochondria found within the vesicle were subjectively observed to be very active, exhibiting a substantial amount of movement as determined by fluorescent microscopy.

Activated toad spermatozoa released into urine consisted primarily of three populations: motile + MV, nonmotile + MV, and nonmotile − MV. The majority of toad sperm released at urination was of the category motile + MV and numbered

![Fig. 5. Transmission electron micrographs of cross-sections through the mitochondrial vesicle of toad spermatozoa. This structure is found oblique to the sperm nucleus (N) and contains large numbers of mitochondria (m), its primary organelle. Also identified are smooth endoplasmic reticulum (SER), unknown double membrane organelles (arrows in panel C and D), and several empty vacuoles. Bars represent 1 micron.](image-url)
significantly greater ($P < 0.01$) than the other two categories (Fig. 7). In contrast, spermatozoa without a MV were rarely motile. By 4 hr after collection, there was a shift in cell types present with reduced numbers of motile + MV cells ($P < 0.01$) and an overall increase ($P < 0.01$) in immotile spermatozoa. The number of motile spermatozoa observed at 4 hr after collection was similar to that observed in study 2 (Fig. 2).

**DISCUSSION**

Results from this study indicate that toad spermatozoa collected in the urine can be inactivated and reactivated with a 50–60% recovery in sperm motility. A comparison between two commonly used buffers for suspending testes mace-rates, SAR (Cabada, '75; Browne et al., '98, 2001) and DB (Wolf and Hedrick, '71; Bernardini et al., '88; Yamasaki and Katagiri, '91), indicated no
differences in recovery of sperm motility after inactivation in either solution. However, the ability of SAR to better maintain sperm motility over time suggests that SAR would be more appropriate for long-term storage of toad spermatozoa. Although SAR and DB are relatively similar, bicarbonate is one component present only in SAR. Bicarbonate has been shown to have a stimulatory effect on mammalian sperm motility (Eddy and O’Brien, ’94) and may be similarly affecting toad spermatozoa.

Of the different reactivation solutions tested, only water induced optimal recovery of motility for both SAR and DB inactivated spermatozoa. This dilution (1:5) in water is similar to that reported for activating frog spermatozoa obtained from testis macerates (Wolf and Hedrick, ’71; Hollinger and Corton, ’80). Surprisingly, our initial hypothesis that dilution into a low ionic salt solution would improve motility recovery by preventing osmotic shock was rejected. An 80% dilution of the inactivating buffer with water provided an adequate level of ions to prevent osmotic shock, yet was low enough in osmolality to initiate sperm motility. Increasing the ionic strength to slightly more than 10 mM NaCl resulted in a significant decline in sperm motility with higher concentrations showing a dose-dependent inhibition. In contrast, preliminary studies showed that the dilution of inactivated samples with a larger volume of water (> 1:5) reduced recovery of motility (unpublished results), perhaps due to a greater osmotic shock. These results reflect what occurs when toads reproduce naturally. During external fertilization, sperm are released into the surrounding water and experience a significant reduction in osmolality, which in turn results in a rapid decline of sperm motility, with most becoming immotile in 10–15 min. Taken together, these data indicate that toad sperm motility after inactivation is dependent upon a balance between reducing the osmolality of the medium and maintaining a sufficient amount of an osmoticum. Interestingly, the resumption of sperm motility in SAR treated samples was slightly delayed when diluted in water and did not reach peak levels until 10 min after reactivation, possibly due to a delayed metabolic response in remobilization.

The ability to suspend toad spermatozoa in SAR for up to 4 hr and still recover about 30% of initial motility suggests that it may be possible to store inactivated spermatozoa for some time prior to fertilization. However, there are limits to what toad spermatozoa can tolerate, since no motility was recovered after incubation in SAR overnight at room temperature. In a previous study, Wolf and Hedrick (’71) reported that frog spermatozoa obtained from testes macerates can be held in DB for up to 24 hr at 0–4°C and still exhibit high levels of fertilization. Therefore, it is possible that American toad sperm storage will be more successful long-term if held at cooler temperatures, as has been shown for Marine toads (Browne et al., 2001).

Unfortunately, the inactivation/reactivation process resulted in an immediate loss of sperm forward progressive velocity even after only 15 min of inactivation, and samples inactivated for 4 hr failed to exhibit any forward movement after reactivation. Similar studies on sperm inactivation and reactivation have been performed in fish (Christen et al., ’87; Perchec et al., ’95), a taxa that may be a useful model for understanding sperm function in toad species, as both practice external fertilization and produce sperm that become motile in response to similar cues. Fish spermatozoa exhibit a relatively short duration of movement (Billard, ’78), primarily due to a low oxidative phosphorylation capacity of their mitochondrion that leads to a rapid decrease in ATP (Christen et al., ’87). It is possible that the recovery of toad sperm forward progression over time decreases due to the continuing depletion of ATP stores, such that even though the spermatozoa...
zoa can exhibit flagella beating, the propagation of a wave is not strong enough to initiate forward movement. Research is currently underway in our laboratory investigating the effects of various energy substrates and protein sources on the recovery of motility and forward progression of inactivated toad spermatozoa.

SAR-treatment appeared to have a stabilizing or protective effect on sperm cell membranes because greater numbers of viable and morphologically normal spermatozoa were maintained over time in SAR-treated samples versus control spermic urine samples. SAR-treated samples also contained more spermatozoa with intact MVs compared to spermic urine controls. These data indicate that addition of a permeating osmoticum to toad spermatozoa preserves cell integrity and is likely due to stabilization of cellular membranes and/or organelles from the damaging effects of osmotic shock. Marian et al. ('93) have shown that osmotic shock induces a modification of membrane permeability and structural changes with possible reorganization of the lipid bilayer of the plasma membrane after transfer of fish spermatozoa to fresh water, and it is quite possible that a similar phenomenon is occurring with toad spermatozoa.

Electron microscopic evaluations of the MV attached to toad spermatozoa confirmed results from our previous preliminary studies, indicating that mitochondria are the primary cytoplasmic organelles. The MV also contains what appears to be smooth endoplasmic reticulum. Most interesting was the presence of several undetectable organelles containing a double membrane within the MV. These structures often were empty and appeared to be vacuoles, or their cellular contents could not be distinguished from the rest of the granular cytoplasmic content of the MV. A previous study by Waggener and Carroll ('98a) in frogs described this double-membrane structure as a nucleus; hence, they classified this vesicle as an accessory cell associated with the spermatozoa. In contrast, our studies revealed several of these structures within the MV and they were not electron dense, as most cell nuclei appear when evaluated by electron microscopy. Moreover, they varied in size, often appearing much smaller than mitochondria. Finally, when toad spermatozoa were stained with Hoechst 33342, a fluorescent nuclear stain, the stain was not incorporated into the MV of either dead or live spermatozoa. Together, these findings indicate that the MV of toad spermatozoa does not contain a nucleus; rather, this organelle represents an as yet unidentified cytoplasmic structure not previously described in mammalian spermatozoa.

MitoTracker staining proved useful for evaluating the mitochondria within the toad sperm MV. Although the spatial distribution of mitochondria within the MV was fairly even, they continuously moved, shifting position throughout the vesicle. Movement of mitochondria has previously been shown to be associated with microtubule organization (Blerkom, '91) and may be involved with concentrating ATP or specific ions to certain regions in the MV. How ATP is translocated to the flagellum from this structure is unclear, and toad sperm may present an interesting model for the study of ATP shuttle molecules, like phosphocreatine (Robitaille et al., '87; Tombes et al., '87), and their interaction with dynein ATPases. The extended duration of toad sperm motility when maintained in urine or hypotonic buffers suggests that these cells have a high mitochondrial activity, similar to that of mammalian (Jeulin and Soufir, '92) and sea urchin (Christen et al., '83) spermatozoa, but dissimilar to that of fish spermatozoa (Christen et al., '87; Perchec et al., '95).

The presence of a MV on mature toad sperm released into the urine appears to be required for sperm motility. Our data indicated that once the integrity of the MV is disturbed (dissociation or rupture), sperm motility is immediately terminated. In addition, toad sperm motility decreased as the number of nonmotile spermatozoa without MV increased over time. These findings are similar to those reported for Lepidobatrachus laevis (Waggener and Carroll, '98a). Preliminary experiments on sperm freezing in our laboratory suggest that this structure is extremely sensitive to the cryopreservation protocols tested. The MV of most spermatozoa were damaged or lost in post-thaw samples; hence, sperm were immotile. Clearly, a better understanding of the relationship of the MV to motility and the stabilization of this structure will facilitate the development of successful cryopreservation protocols.

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**LITERATURE CITED**


