Improved Quality of Cryopreserved Cheetah (*Acinonyx jubatus*) Spermatozoa After Centrifugation Through Accudenz

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ABSTRACT: Sperm cryopreservation, in combination with assisted reproductive techniques, is a valuable tool for the genetic management of endangered felids. However, the acrosome of the cheetah spermatozoon is especially sensitive to cryopreservation, with approximately 40% of spermatozoa experiencing acrosomal damage immediately after thawing and then another approximately 15% loss during the next 4 hours in vitro. Additionally, thawing causes a reduction in sperm motility by approximately 20% with another decrease of approximately 12% during subsequent incubation in vitro. We hypothesized that slow removal of glycerol from cryopreserved cheetah spermatozoa using an Accudenz gradient would improve acrosomal integrity, sperm motility longevity, and structural morphology. Accudenz was compared with traditional cheetah sperm processing methods for glycerol removal that involves washing, multistep resuspension, and swim-up processing. Electroejaculates (n = 21 total from 8 males) were washed in Ham F10 medium, and sperm pellets were resuspended in TEST-yolk buffer with 0% glycerol. Samples were cryopreserved in straws in 4% final glycerol, thawed, and assessed for percent intact acrosomes (% IA), percent motility (% M), and forward progressive status (FPS; scale, 0–5). Sperm motility index (SMI) was calculated as (% M + [FPS × 20]) ÷ 2. In study 1, glycerol removal by centrifugation through an Accudenz gradient (4%, 10%) was compared with traditional sperm washing (control) and multistep resuspension

protocols. At each time after centrifugation (hourly for 4 hours), % IA was improved ($P < .05$) for Accudenz (range, 36%–39%) compared with control (30%–33%) and multistep (29%–33%) treatments. In study 2, a modified Accudenz protocol was compared with traditional washing and was found to improve ($P < .05$) SMI (range, 52–64) compared with controls (range, 41–52) at each time postthaw after centrifugation. In study 3, swim-up processed sperm were compared with those treated by centrifugation through Accudenz and traditional sperm washing for improving sperm morphology. The percentage of structurally-normal sperm recovered postthawing increased ($P < .05$) for both the Accudenz (38%) and swim-up (33%) treatments compared with controls (21%). Percent IA and SMI also were improved ($P < .05$) for Accudenz (range, 39%–47% and 46–59, respectively) compared with controls (range, 26%–33% and 40–53, respectively). Results indicate that using Accudenz for glycerol removal from cryopreserved cheetah sperm mitigates the significant loss in sperm quality that occurs after freeze-thawing. This alleviation of cellular damage resulting from cryopreservation contributes to a more than 10% improvement in overall sperm motility and, more importantly, allows retention of 40% or more of sperm with intact acrosomes.

Key words: Felid, acrosome, cryopreservation, cryoprotectant, genome resource banking.

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The reproductive physiology of the cheetah in captivity and in nature has been intensively studied in the female (Howard et al, 1992, 1997; Wielebnowski et al, 2002) and male (Wildt et al, 1983, 1987, 1993; Pukazhenthi et al, 2001; Crosier et al, 2006, 2007). This species is especially interesting because of its erratic and

short estrous cycles (Brown et al, 1996) and its consistent ejaculation of high proportions (>75%) of abnormally shaped spermatozoa, a condition known as teratospermia (Wildt et al, 1983). Furthermore, the worldwide captive cheetah population, composed of approximately 1350 animals, is not self-sustaining because of poor reproductive success and a high incidence of cub mortality (approximately 20% [Marker, 2005]). For these reasons and because the species is considered threatened with extinction in the wild (Marker-Kraus and Kraus, 1995; Marker-Kraus et al, 1996; International Union for Conservation of Nature), more detailed studies are warranted, especially those that allow the species to be genetically managed and conserved.

The preservation of germplasm, especially spermatozoa, can assist with conserving genetic diversity in captive breeding programs for select wildlife species (Holt and Pickard, 1999; Wildt et al, 2001; Agca and

Critser, 2002). For the cheetah, the organized collection and storage of spermatozoa in a genome resource bank would allow genetic management of this species on a global scale potentially using a variety of assisted reproductive techniques (Wildt et al, 1997). Sperm already have been collected and frozen from a wild cheetah, transferred intercontinentally, and used via artificial insemination (AI) to produce offspring (Wildt et al, 1997; Howard and Wildt, 2009). Additionally, cryopreserved cheetah sperm have been used successfully for in vitro production of embryos in North America and Namibia (Crosier et al, unpublished data). If more effective, this global approach has the potential for large-scale infusions of new genetic material into the captive population without removing more cheetahs from the wild to maintain genetic diversity among zoological collections. The use of cryopreserved sperm for assisted breeding also overcomes the common problem of sexual incompatibility between male and female cheetahs (Caro, 1993; Wildt et al, 1993) and enables managers to move genetic material between institutions while eliminating the stresses associated with transporting live, wild animals (Wildt et al, 1997, 2001).

Cryopreserved cheetah sperm experience loss of membrane and acrosomal integrity in as many as 40% of cells following freeze-thawing using current laboratory protocols (Crosier et al, 2006). Spermatozoa typically are cryopreserved using the permeating cryoprotectant glycerol, which in turn, contributes to fluid flux and osmotic stress that can disrupt cell membrane integrity (Gao et al, 1995; Pukazhenthi et al, 2002). Most damage during cryopreservation is caused by cryoprotectant and water movement across sperm membranes during initial equilibration before freezing and again during thawing as the cryoprotectant is removed and the cell rehydrated. Spermatozoa from teratospermic animals, such as the cheetah, are especially vulnerable to osmotic (Pukazhenthi et al, 2001) and freezing-induced damage (Crosier et al, 2006). We have determined that the sperm acrosome in teratospermic felids is especially vulnerable to damage during cryopreservation and thawing (Crosier et al, 2006; Pukazhenthi et al, 2006).

Assisted reproductive techniques with cryopreserved sperm routinely use postthaw processing methods to concentrate cellular suspensions, remove egg yolk prior to intrauterine insemination, and select spermatozoa with good motility (Henkel and Schill, 2003). For example, sperm samples collected for AI in felids generally are washed (resuspended in medium and centrifuged) with the resulting pellet directly mixed with insemination medium to maximize sperm numbers (Howard et al, 1992, 1997). In comparison, cryopreserved felid sperm used for in vitro fertilization (IVF) usually are subjected to swim-up processing to enhance sperm motility and to improve the number of structurally normal cells available (Comizzoli et al, 2003). However, the swim-up technique reduces sperm concentration (Howard et al, 1990), which is of special concern in a species producing approximately 75% pleiomorphic cells in an average ejaculate. Maintaining cellular integrity also is critical, and we have evidence that multistep cryoprotectant addition and removal using a buffered solution is beneficial in felids (Pukazhenthi et al, 2002). Furthermore, we recently demonstrated that cheetah spermatozoa withstand incubation in glycerol for up to 60 minutes at room temperature before cryopreservation with little detrimental impact on postthaw acrosomal integrity (Crosier et al, 2006). This observation suggests that the majority of damage to cheetah sperm integrity occurs during cryoprotectant removal from cells after thawing.

It was logical that the next step toward enhancing the viability of thawed cheetah spermatozoa was to explore novel glycerol removal approaches for enhancing both motility and acrosomal integrity. Sperm centrifugation through a density gradient (eg, Percoll) is widely used in domestic livestock and humans for separating sperm from seminal fluid (McLean et al, 1998), separating motile from nonmotile cells (Sbracia et al, 1996), and removing cryoprotectant from thawed sperm samples (Long and Kulkarni, 2004). Recently, Accudenz (formerly known as Nycodenz) has gained popularity as an inert and chemically dense substance that prevents endotoxin precipitation (Sbracia et al, 1996). Accudenz also is noted for yielding sperm fractions with better motility longevity compared with those filtered through a Percoll gradient (Froman and McLean, 1996; King et al, 2000). Centrifugation through Accudenz gradients of increasing density has been shown to mitigate the damage arising from removing cryoprotectant from spermatozoa and facilitates a slower transfer of permeating cryoprotectant and water across cell membranes (Long and Kulkarni, 2004). For poultry spermatozoa, the use of Accudenz improved fertility after AI compared with a standard Percoll gradient (Long and Kulkarni, 2004).

All evidence to date strongly suggests that the most significant damage induced by contemporary cryopreservation protocols for cheetah spermatozoa is related to cryoprotectant removal after thawing. Further, we suspect that mitigating the osmotic stress associated with glycerol elimination after thawing cheetah spermatozoa would coincidentally enhance acrosomal integrity while maintaining sperm motility. Thus, the objective of the present study was to determine if postthaw sperm quality was improved significantly by processing through a gradient filtration compared with the current

protocols of traditional sperm washing, multistep resuspension, and swim-up processing. We hypothesized that use of an Accudenz gradient would effectively remove glycerol from thawed cheetah spermatozoa, which in turn, would enhance sperm motility and acrosomal integrity.

Materials and Methods

Animals and Semen Collection

All cheetah procedures were approved by the Institutional Animal Care and Use Committee of the Smithsonian's National Zoological Park. The sperm donors were 8 adults (ages, 2.5–9 years), all wild born and housed in outdoor enclosures (1 hectare per cheetah) at the Cheetah Conservation Fund (CCF) near Otjiwarongo, Namibia, or the Naua Naua Game Farm (a licensed Namibian facility) in Outjo, Namibia. Animals were fed a combination of donkey, horse, and game species (1.5–3 kg of meat and bone/day; 6 or 7 days/week) with daily vitamin and mineral supplementation with the commercially available CAL-SUP Powder (5 g/cheetah/day; Bayer Co, Isando, South Africa) or a 1:1 mixture of Feli-Cal and Feli-Vit (5 g total/cheetah/day; Kyron Laboratories, Benrose, South Africa). Animals also received organ meat (mixture of heart, liver, and/or lung) once per week. One individual that recently had been caught on nearby farmland and maintained at CCF for 1 week contributed a single ejaculate before release back to the wild. The other 7 males contributed a total of 20 ejaculates (1–3/male). Our recent findings have determined that there is no impact of diet or being wild caught vs housed in captivity on seminal traits (Crosier et al, 2007).

Anesthesia was induced in all animals using a combination of tiletamine hydrochloride and zolazepam (4–6 mg/kg body weight; Telazol; Fort Dodge Laboratories, Fort Dodge, Iowa) (Wildt et al, 1987; Crosier et al, 2007) or a combination of medetomidine hydrochloride (1.5–2 µg/kg body weight; Domitor; Pfizer Inc, La Jolla, California) and tiletamine hydrochloride/zolazepam (2 mg/kg body weight; Telazol; Fort Dodge Laboratories) (Crosier et al, 2007) administered intramuscularly. Testicular length and width were measured using laboratory calipers and then converted to total testes volume per male (Howard et al, 1990). A rectal probe of 1.6 or 1.9 cm in diameter with 3 longitudinal electrodes and an electrostimulator (P.T. Electronics, Boring, Oregon) were used to provide a total of 80 stimuli (at a low voltage of 2–5 volts) over a 30-minute interval (Howard, 1993). Ejaculates (n = 21) were collected in sterile prewarmed collection vials as previously described (Wildt et al, 1983; Howard, 1993; Crosier et al, 2006, 2007).

Each raw ejaculate was examined for volume, and an aliquot (3 µL) was immediately assessed for sperm percent motility (% M) and forward progressive status (FPS; scale, 0–5 with a 5 rating equivalent to rapid, straightforward progression [Howard, 1993]) using a prewarmed microscope slide and coverslip. A sperm motility index (SMI) was calculated using the formula (% M + [FPS × 20]) ÷ 2 (Howard et al, 1990). A 15-µL aliquot of raw semen was fixed in 100 µL of 0.3% glutaral-dehyde in phosphate-buffered saline (pH 7.4, 340 mOsm) (Pukazhenthi et al, 2000) for assessing sperm morphology. A 15-µL aliquot of raw semen also was fixed in 500 µL of 4% paraformaldehyde for evaluating acrosomal integrity. The remainder of the raw ejaculate was diluted 1:1 (vol/vol) with sterile Ham F10 culture medium (HF10; Irvine Scientific, Santa Ana, California) supplemented with 20 mM HEPES, 5% (vol/vol) fetal calf serum (Irvine Scientific), pyruvate (1 mM), L-glutamine (2 mM), 10 000 IU/mL penicillin, 10 mg/mL streptomycin, and 20 mg/mL neomycin (Sigma Chemical Co, St Louis, Missouri). The final handling medium had an osmolarity of approximately 285 mOsm. Sperm concentration was determined using a hemocytometer (Wildt et al, 1983; Howard, 1993; Crosier et al, 2006).

Sperm Cryopreservation

Each ejaculate diluted in HF10 was centrifuged (Mini-Spin; Eppendorf, Hamburg, Germany) for 8 minutes at $100 \times g$ and the supernatant aspirated from the sperm pellet. The resulting sperm pellet was resuspended in TEST-yolk buffer (TYB refrigeration medium; Irvine Scientific) containing 0% glycerol at ambient temperature. A modified TYB containing 8% glycerol (vol/vol) was prepared as a 2:1 mixture of the "freezing medium − TYB with 12% glycerol" (Irvine Scientific) and "refrigeration medium without glycerol." Each sample was placed in a water bath (350 mL at ambient temperature) and slow cooled (approximately 3.5 hours) to 5°C. After reaching this temperature, TYB plus 8% glycerol was added in 3 aliquots over 30 minutes (adding one-fourth volume and waiting 15 minutes, adding one-fourth volume and waiting 15 minutes, and then adding the remaining half volume). Samples were loaded into 0.25-mL straws containing 80 to 120 µL of sperm suspension with an average final sperm concentration of $67.8 \pm 5.3 \times 10^6$ motile cells/mL (range, 30–120×10^6 motile cells/mL) and frozen over liquid nitrogen (LN) using a 2-step cryomethod (Crosier et al, 2006). Briefly, straws were placed 7.62 cm above LN for 1 minute, then 2.54 cm above liquid for an additional 1 minute, and then plunged into LN.

Study 1: Efficacy of an Accudenz Gradient for Glycerol Removal

Cryopreserved straws (n = 9 ejaculates total from 5 males) were thawed individually for 10 seconds in air followed by immersion in a 37°C water bath for 30 seconds. Each straw was dried, and its contents emptied into a sterile Eppendorf tube (1–2 straws/tube; approximately 150 µL of suspension total). The thawed aliquot immediately was assessed for % M and FPS, and 10 µL was fixed to subsequently determine percent intact acrosomal membranes (% IA). Remaining sperm suspension in the Eppendorf tube then was divided equally (40–50 µL of sperm suspension/aliquot) among 3 processing treatments: 1) sperm washing (control), 2) multistep resuspension, or 3) Accudenz (Accurate Chemical and Scientific Corp, Westbury, New York). These treatments were selected to represent different rates of glycerol removal from cryopreserved sperm (Pukazhenthi et al, 2002). The control

treatment (traditional method for handling cryopreserved cheetah sperm [Howard, 1990]) represented "rapid" glycerol removal in that a single aliquot of culture medium (HF10) was pipetted into the sample followed by centrifugation to pellet the sperm. The multistep treatment represented "moderate" glycerol removal through its slow addition (in multiple steps) of a greater volume of medium (1400 µL) compared with the control (300 µL). The third treatment (Accudenz gradient, which separates cells in suspension based on density [Sbracia et al, 1996]) represented the first examination of the influence of a gradient approach on a carnivore ejaculate and specifically from a species well known for its low sperm density, prevalence of malformed cells, and acrosomal sensitivity to cooling and thawing (Pukazhenthi et al, 2001; Crosier et al, 2006).

For the control treatment, sperm aliquots in TYB were diluted with 300 µL of HF10 added dropwise over 1 minute. Samples in HF10 were centrifuged (8 minutes at 100 × g), the supernatant removed, and the sperm pellet resuspended in 200 µL of HF10 added dropwise over 1 minute. For the multistep treatment, sperm aliquots in TYB were slowly diluted (added in 7 equal 200-µL aliquots over 3 minutes) with 1400 µL of HF10. The sample in HF10 then was centrifuged (8 minutes at 100 × g), the supernatant removed, and resulting pellet resuspended in 200 µL of HF10 added dropwise over 1 minute. For the Accudenz treatment, a modification of the technique described by Long and Kalkarni (2004) was used. The gradient was created by layering 100 µL of 10% (wt/vol) Accudenz in HF10 underneath 500 µL of 4% (wt/vol) Accudenz in HF10 in an Eppendorf tube. The thawed sperm sample in TYB was layered on top of the Accudenz gradient and centrifuged (8 minutes at 100 × g). After centrifugation, the entire suspension formed 3 distinctive layers: the top layer, predominantly composed of TYB; intermediate containing a high concentration of motile sperm; and bottom pellet of less motile cells. The intermediate layer was removed, diluted in 1 mL of HF10, and centrifuged a second time (8 minutes at 100 × g) to remove residual Accudenz. The resulting pellet was resuspended in 200 µL of HF10 added dropwise over 1 minute. Each sample from all treatments was assessed for % M, FPS, and % IA postdilution (precentrifugation), postcentrifugation, and at hourly intervals for 4 hours (while being maintained in Eppendorf tubes at a constant ambient temperature and protected from light).

Study 2: Efficacy of a Single Centrifugation With the Accudenz Gradient for Improving Sperm Motility

Cryopreserved straws (n = 6 ejaculates total from 5 males) were thawed (as in study 1; 3 of 5 donors were the same males as in study 1). In this study, the contents of 2 straws per ejaculate were emptied into a sterile Eppendorf tube (approximately 150 µL of suspension total) and immediately evaluated for % M, FPS, and % IA. The remaining sperm suspension then was divided equally (approximately 75 µL of suspension per aliquot) between 2 treatments, the control procedure and the Accudenz gradient. The control treatment was modified slightly to include an initially larger volume of HF10 (1 mL [compared with 300 µL for study 1]) to allow more thorough glycerol removal. Specifically, a sperm aliquot in TYB

(approximately 75 µL) was diluted slowly with 1 mL of HF10 added dropwise over 1 minute followed by centrifugation (8 minutes at 100 × g) and supernatant removal. The resulting sperm pellet was resuspended in 200 µL of HF10 added dropwise over 1 minute and then evaluated.

The Accudenz protocol was modified to include only a single centrifugation step (to eliminate the detrimental effects of a second centrifugation on sperm motility; see study 1 results). The Accudenz gradient was prepared as in study 1, and then each thawed sample in TYB (approximately 80 µL) was layered on top of the gradient and the tube centrifuged (8 minutes at 100 × g). The intermediate layer (approximately 80 µL) was removed and resuspended with fresh HF10 to a total volume of 200 µL. All samples were assessed for % M, FPS, and % IA immediately postthaw, postdilution before centrifugation, postcentrifugation, and hourly for 4 hours.

Study 3: Efficacy of the Accudenz Gradient Compared With Swim-up Processing for Enhancing Sperm Morphology

Cryopreserved straws (n = 6 ejaculates total from 6 males) were thawed (as in studies 1 and 2; 3 of 6 donors were the same males as in studies 1 and 2). To allow more comparative examinations, the contents of 2 or 3 straws were emptied into a single Eppendorf tube (approximately 240 µL of suspension total) and then assessed immediately for % M, FPS, and % IA. The collective content of each tube was divided equally (approximately 80 µL of suspension per aliquot) among 3 processing treatments: the control (washing) procedure, swim-up, and the Accudenz gradient. The control and Accudenz treatments were identical to those described for study 2. For swim-up, a thawed sample was resuspended in 1 mL of HF10 and centrifuged (8 minutes at 100 × g), the supernatant removed, and the sperm pellet overlaid gently with 60 µL of HF10. Sperm were allowed to swim-up into the medium for 30 minutes after which 50 µL of the supernatant was removed and resuspended in 150 µL of HF10 for a final volume of 200 µL. An aliquot (20 µL) was removed to determine sperm structural morphology and concentration immediately after swim-up processing. Samples from all treatments were assessed for % M, FPS, and % IA immediately postthaw, following the allocated swim-up time (30 minutes), and at hourly intervals for 4 hours.

Assessment of Sperm Morphology and Acrosomal Integrity

Sperm morphology was evaluated using phase-contrast microscopy at ×1000 (Wildt et al, 1987; Howard, 1993; Crosier et al, 2007). Sperm were classified as normal or as having 1 of the following abnormalities: 1) head anomalies, including microcephalic, macrocephalic, and bicephalic or tricephalic; 2) acrosomal aberrations, including missing, loose, or damaged acrosomal membrane; 3) midpiece deviations, including abnormal or missing midpiece, bent midpiece with retained cytoplasmic droplet, or bent midpiece with no droplet; 4) flagellar irregularities, including tightly coiled flagellum, bent flagellum with retained cytoplasmic droplet, bent flagellum with no droplet, biflagellate or triflagellate,

retained proximal cytoplasmic droplet, or retained distal droplet; and 5) other abnormalities, including spermatid, bent neck and detached head, or detached flagellum (Crosier et al, 2007). If an individual spermatozoon had an abnormality of either the head or acrosome in addition to an anomaly of either the midpiece or flagellum, the cell was classified as having a head or acrosomal defect. Similarly, if an individual sperm had both a midpiece as well as a flagellar abnormality, the cell was classified as having a midpiece defect.

Sperm samples were evaluated for acrosomal integrity using a modified Coomassie staining technique (Larson and Miller, 1999; Crosier et al, 2006). Briefly, aliquots fixed in 4% paraformaldehyde were centrifuged for 8 minutes at 2000 × g and the supernatant discarded. Resulting sperm pellets were washed twice with 500 μL of 0.1 M ammonium acetate (pH 9.0) and the final pellet resuspended in approximately 50 μL of the ammonium acetate solution. This sperm suspension was smeared onto a single microscope slide and allowed to dry at ambient temperature. Each slide was flooded with Coomassie stain (0.22% of Coomassie Blue G-250; Fisher Biotech, Springfield, New Jersey) for 90 seconds, rinsed with deionized water, dried at ambient temperature, and permanently preserved by placing a coverslip over a drop of mounting medium (Krystalon; EM Science, Gibbstown, New Jersey). For each sample, either 100 or 200 spermatozoa (dependent on sperm concentration) were assessed individually for acrosomal integrity using bright-field microscopy at ×1000. Each spermatozoon was categorized as having an intact or nonintact acrosome, and the number of sperm per sample with intact membranes was converted to a percentage (Crosier et al, 2006). Sperm considered to have intact acrosomes displayed uniform staining overlying the entire acrosomal region (Figure 1A) compared with patchy or no staining for cells with damaged or missing acrosomes, respectively (Figure 1B).

Statistical Analysis

All percentage data for sperm motility and intact acrosomal membranes were arcsine-transformed before analysis. Based on previous reports that postthaw acrosomal integrity of cheetah spermatozoa is positively correlated with the percentage of normal spermatozoa in the original sample (Crosier et al, 2006), the final statistical models contained the main effects of treatment with the covariate of percent normal sperm. This improved the resulting R^2 values by approximately 50% compared with those in which the model included only the main effect of treatment. Means were separated using Duncan's multiple-range test and were considered statistically different at $P < .05$. All data were analyzed using general linear model procedures (SAS, 2002). Results are reported as means ± SEM unless otherwise stated.

Results

Overall Ejaculate Characteristics and Sperm Morphology

Cheetah testes volume, seminal volume, and ejaculate traits, including sperm motility and structural morphology (Table 1), were comparable to previous reports for
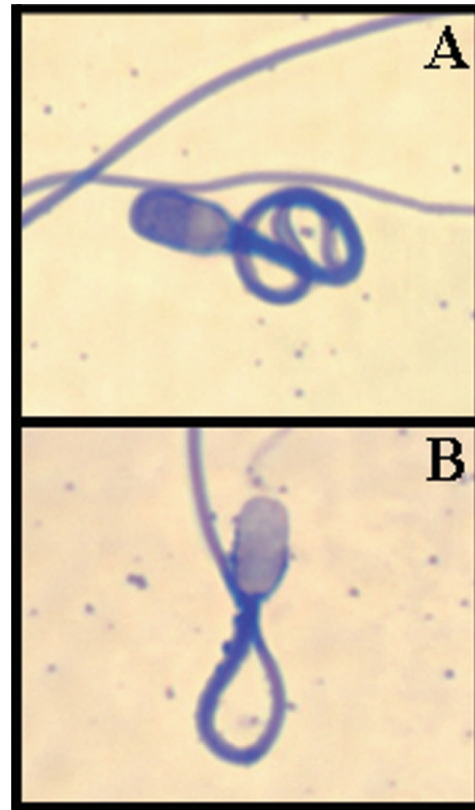

Figure 1. Coomassie blue staining of cheetah spermatozoa for assessment of acrosomal integrity. (**A**) Spermatozoa with intact acrosomal membranes exhibit uniform purple staining overlying the acrosomal region. (**B**) Spermatozoa with nonintact acrosomes have clear or patchy staining patterns.

this species (Wildt et al, 1983, 1993; Crosier et al, 2007). Male cheetahs produced ejaculates containing a low sperm density (approximately 36×10^6 cells/mL) and a high proportion of motile (approximately 75%) and structurally abnormal (approximately 80%) spermatozoa. Furthermore, a high proportion (approximately 87%) of spermatozoa retained intact acrosomes (Table 1).

Study 1: Efficacy of an Accudenz Gradient for Glycerol Removal

Cryopreserved cheetah sperm samples processed for glycerol removal using the control, multistep, or Accudenz approaches maintained similar ($P > .05$) sperm motility (SMI, approximately 60) through the washing (postcentrifugation) step after thawing (Figure 2A). Compared with controls, sperm subjected to multistep resuspension experienced a reduced ($P < .05$) SMI both immediately after resuspension (precentrifugation) as well as after centrifugation (Figure 2B). The SMI was similar ($P > .05$) between controls and Accudenz for all time points except an approximately 10% reduction for the latter treatment at 4 hours (Figure 2C).

Table 1. *Ejaculate and testicular characteristics of wild-born Namibian cheetahs*[a]

Trait	Mean ± SEM	Minimum	Maximum
Testicular volume, cm^3	11.1 ± 0.7	7.1	16.9
Seminal volume, mL	3.3 ± 0.2	1.6	4.7
% M	74.2 ± 1.6	70.0	90.0
Sperm FPS[b]	3.4 ± 0.1	3.0	4.0
Sperm motility index[c]	70.7 ± 1.4	65.0	85.0
Sperm concentration, ×10^6/mL	36.0 ± 4.9	4.9	80.5
Total motile sperm/ejaculate, ×10^6	82.8 ± 12.6	16.1	236.7
Intact sperm acrosomes, %	86.6 ± 1.3	71.5	96.0
Morphologically normal sperm, %	19.9 ± 2.1	7.0	38.0
Morphologically abnormal sperm, %	80.1 ± 2.1	62.0	93.0
Head defects	22.6 ± 2.1	4.0	38.0
Midpiece defects	36.5 ± 2.5	19.0	55.0
Flagellar defects	14.5 ± 2.4	3.0	36.0
Other defects[d]	6.5 ± 0.9	2.0	15.0

Abbreviations: % M, percent sperm motility; FPS, forward progressive status.

[a] n = 21 ejaculates total from 8 males.

[b] Scale, 0–5 with 5 being the most rapid, forward progression.

[c] (% M + [FPS × 20]) ÷ 2

[d] Other abnormalities include spermatid, bent neck, detached head, or detached flagellum.

Processing sperm using the traditional washing protocol (ie, control approach) caused significant acrosomal membrane damage. Between the assessment immediately postthaw and postcentrifugation, approximately 20% of spermatozoa lost intact acrosomes (from 54% with intact acrosomes to 37%) with another 8% loss over the next 4 hours (Figure 3). This pattern of acrosomal loss in the controls was mimicked ($P > .05$) by the multistep method (from 36% to 29% from postcentrifugation through 4 hours postthaw). Separation of sperm via an Accudenz gradient mitigated this damage and improved ($P < .05$) acrosomal integrity at every evaluation postcentrifugation compared with the other 2 treatments (Figure 3).

Study 2: Efficacy of a Single Centrifugation With the Accudenz Gradient for Improving Sperm Motility

Postthaw SMI was not different ($P > .05$) between control and Accudenz treatments immediately after centrifugation (Table 2). However, centrifugation through Accudenz improved ($P < .05$) the SMI at every subsequent time of evaluation (1–4 hours; Table 2) compared with controls. Consistent with study 1, the percent of intact acrosomal membranes following thawing was improved ($P < .05$) at every time point after centrifugation for Accudenz treatment compared with controls (Table 2).

Study 3: Efficacy of the Accudenz Gradient Compared With Swim-up Processing for Enhancing Sperm Morphology

Compared with the control, both swim-up and Accudenz processing increased ($P < .05$) the proportion of structurally normal sperm recovered (Table 3). Additionally, the proportion of head anomalies was reduced ($P < .05$) for Accudenz samples compared with both the control and swim-up treatments. However, there was no difference ($P > .05$) among treatments in the proportion of midpiece, flagellar, or ''other'' deformities observed (Table 3).

Because the control treatment involved pelleting the entire sperm population after washing, the concentration of spermatozoa was highest ($P < .05$) for this treatment compared with either swim-up or Accudenz (Table 3). The total number of motile sperm was higher ($P < .05$) for the control compared with swim-up samples but was comparable ($P > .05$) to Accudenz (Table 3). Because of the increase in percent normal sperm from both the swim-up and Accudenz treatments, there was no difference ($P > .05$) among groups in number of total motile normal sperm recovered (Table 3).

For sperm motility, the SMI was comparable ($P > .05$) for all treatments and time points through 1.5 hours of postthawing (Figure 4). However, centrifugation through Accudenz resulted in superior ($P < .05$) sperm motility compared with control and swim-up treatments at 2.5 and 3.5 hours postthaw. There was 1 time point (2.5 hours after Accudenz) at which there was an improvement ($P < .05$) in sperm acrosomal morphology (8%–10%), a difference that was not sustained for the 2 later evaluation periods (Table 4).

Discussion

Although structure and function of the entire cheetah spermatozoon have been extensively studied (Pukaz-henthi et al, 2001; Crosier et al, 2006, 2007), the
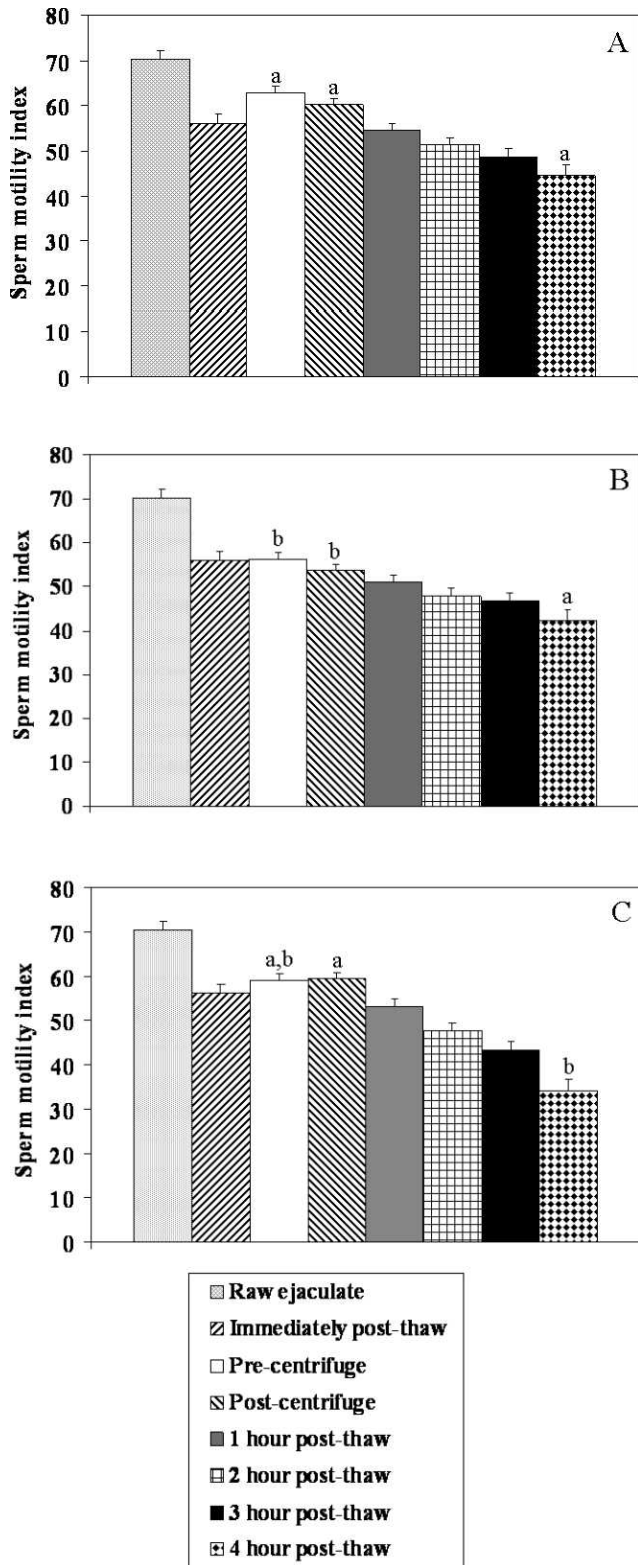
Figure 2. Cheetah sperm motility index for (**A**) washed (control), (**B**) multistep, and (**C**) Accudenz treatments. Among treatments and within each time point, bars with different superscripts differed at $P <$ .05 (study 1).

acrosome has received little attention. This structure deserves consideration because of its crucial role in fertilization (Patrat et al, 2000; Rodriguez-Martinez and Barth, 2007) and its sensitivity to osmotic changes that occur during standard sperm cryopreservation and thawing processes (Pukazhenthi et al, 2002; Crosier et al, 2006). We do know from recent investigations that acrosomal damage to cheetah spermatozoa does not result from glycerol exposure before freezing because membrane disruption is equally severe in cells sustaining short- vs long-term glycerol exposure (Crosier et al, 2006). Therefore, currently there is an approximately 35% loss in acrosomal integrity immediately upon thawing with another 15% decline over the next 4 hours from conventional removal of permeating cryoprotectant from spermatozoa. We speculated that using the density gradient, Accudenz, might mitigate the severe acrosomal damage typically incurred by cheetah sperm when glycerol is removed postthawing. We discovered that processing cheetah sperm through this synthetic density gradient essentially eliminated the loss of the latter 15% of damaged cells arising during the traditional approach. Furthermore, using Accudenz to remove glycerol significantly improved the longevity of spermatozoal motility in vitro. Thus, an Accudenz filtration gradient appeared to allow approximately 40% or more of thawed sperm to retain intact acrosomes while improving overall sperm motility by at least 10%.

Standard protocols for cryopreserving felid sperm rely on adding glycerol slowly to cells before freezing, either at room temperature or after cooling (Pukazhenthi et al, 2001, 2006; Crosier et al, 2006). Intracellular water is replaced by glycerol, and upon thawing, the reverse occurs. During glycerol removal, spermatozoa swell in response to a rapid influx of extracellular water, resulting in extreme volume excursions, which can cause cell membrane disruptions (Noiles et al, 1993). Cheetah spermatozoa are not particularly sensitive to osmotic changes during equilibrium prefreeze but are highly vulnerable to injury during the thawing and resuspension processes. The density gradient, Accudenz, appeared to slow the movement of water and/or cryoprotectant across the membranes, thereby maintaining higher levels of acrosomal integrity compared with conventional 1-step or multistep removal protocol. In this context, our findings were similar to an earlier study of poultry spermatozoa (Sbracia et al, 1996), demonstrating for the first time the applicability of Accudenz to carnivore sperm, even in a species that usually produces extraordinary numbers of pleiomorphic spermatozoa.

Other commonly used separation gradients (ie, Percoll, PureSperm, IxaPrep) function in much the same way as Accudenz by creating layers of increasing
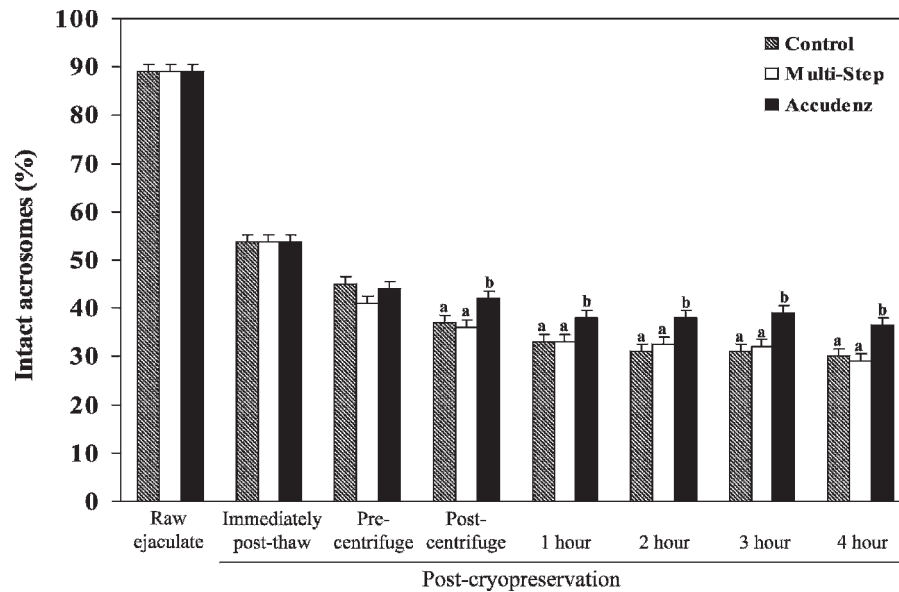
Figure 3. Postthaw acrosomal integrity of cheetah spermatozoa subjected to washed (control), multistep resuspension, or Accudenz treatments. Among treatments and within each time point, bars with different superscripts differed at $P < .05$ (study 1).

osmolarity. Since the withdrawal of Percoll from use in human assisted reproduction in 1996, alternative nontoxic density gradients, including Accudenz, have been investigated. This gradient has been popularized for use in human and domestic poultry sperm samples, both to remove seminal plasma from fresh ejaculates as well as to remove cryoprotectants from thawed suspensions (Sbracia et al, 1996; King et al, 2000; Long and Kulkarni, 2004). This is important for use in human intrauterine AI procedures because Accudenz does not have to be removed completely from a sperm sample before AI or other assisted reproductive techniques. In the present study, we were initially interested in determining if using Accudenz on thawed cheetah sperm influenced membrane integrity as well as motility over time. Therefore, the objective of study 1 was to

thoroughly remove Accudenz from each sperm sample by 2 centrifugation steps and resuspending the pellet in fresh, gradient-free medium. This approach obviously exerted additional mechanical stress that did not accelerate motility decline nor did it improve overall sperm motility. Because Accudenz is nontoxic to cells (Sbracia et al, 1996), we then investigated a less intensive centrifugation protocol that involved direct removal of the interphase gradient layer containing the highest proportion of motile and acrosome-intact sperm. The result was improved sperm motility as well as acrosomal integrity, which agreed with earlier reports in human sperm samples (Sbracia et al, 1996).

Although securing significant populations of acrosome-intact sperm postthawing is important for AI, we also envision a role for embryo technologies in the

Table 2. *SMI and % IA of cryopreserved cheetah sperm (study 2; means ± SEM)*[a]

	SMI		% IA	
	Washed	Accudenz	Washed	Accudenz
Raw ejaculate	71.3 ± 3.0	71.3 ± 3.0	84.7 ± 2.9	84.7 ± 2.9
Postcryopreservation				
Immediately postthaw	52.5 ± 2.1	52.5 ± 2.1	47.7 ± 3.4	47.7 ± 3.4
Postcentrifugation	58.3 ± 3.8	62.9 ± 4.7	34.0 ± 3.5	39.5 ± 4.5
1 h postthaw	51.7 ± 2.1[b]	64.2 ± 1.9[c]	32.3 ± 2.2[d]	46.7 ± 4.0[e]
2 h postthaw	47.5 ± 2.5[b]	60.0 ± 2.0[c]	32.4 ± 2.5[d]	46.4 ± 4.0[e]
3 h postthaw	43.3 ± 2.5[b]	54.0 ± 2.7[c]	30.1 ± 2.4[d]	46.4 ± 3.4[e]
4 h postthaw	40.8 ± 3.0[b]	52.0 ± 3.1[c]	29.6 ± 3.8[d]	53.7 ± 2.2[e]

Abbreviations: % IA, percent intact acrosomes; SMI, sperm motility index.

[a] n = 6 ejaculates total from 5 males.
[b,c] Within rows, SMI values with different superscripts differ at $P < .05$.
[d,e] Within rows, % IA values with different superscripts differ at $P < .05$.

Table 3. *Structural morphology and concentration of cryopreserved cheetah sperm (study 3; means ± SEM)*[a]

Trait	Treatments		
	Washed	Swim-up	Accudenz
Raw ejaculate			
Morphologically normal sperm, %	21.8 ± 5.0	21.8 ± 5.0	21.8 ± 5.0
Postcryopreservation			
Morphologically normal sperm, %	20.7 ± 1.7[b]	33.0 ± 1.7[c]	37.6 ± 1.9[c]
Morphologically abnormal sperm, %	79.3 ± 1.7[b]	67.0 ± 1.7[c]	62.4 ± 1.9[c]
Head defects	18.5 ± 1.5[b]	19.5 ± 3.0[b]	9.6 ± 2.3[c]
Midpiece defects	26.0 ± 3.0	21.0 ± 3.1	28.8 ± 4.1
Flagellar defects	27.7 ± 3.4	23.8 ± 3.4	20.0 ± 3.0
Other defects[d]	7.5 ± 1.8	2.7 ± 1.0	3.8 ± 1.1
Sperm concentration, $\times 10^6$/mL	29.5 ± 8.0[b]	3.4 ± 0.5[c]	8.1 ± 4.0[c]
Total motile sperm, $\times 10^6$	2.9 ± 1.0[b]	0.4 ± 0.1[c]	1.0 ± 0.6[b,c]
Total motile normal sperm, $\times 10^6$	0.6 ± 0.2	0.1 ± 0.03	0.4 ± 0.2

[a] n = 6 ejaculates total from 6 males.
[b,c] Within rows, values with different superscripts differ at $P < .05$.
[d] Other abnormalities include spermatid, bent neck, detached head, or detached flagellum.

genetic management of the cheetah. We recently made significant progress in producing cheetah embryos in vitro (Crosier et al, unpublished), a process that also requires membrane-intact, motile spermatozoa. IVF in felids has traditionally relied on the use of the swim-up technique, which is based on the concept that the most motile spermatozoa will actively swim from a centrifuged seminal pellet into an overlying medium that, in turn, serves as a resource for the inseminate (Howard et al, 1990; Henkel and Schill, 2003). This approach works well for felid IVF, producing sperm samples with improved motility and normal morphology and decreased debris compared with controls (Howard et al, 1990). The disadvantage is that swim-up processing results in recovery of low numbers of spermatozoa. The present study revealed that, although swim-up processing and exposure to the Accudenz gradient produced reduced sperm concentrations compared with the traditional washing protocol, both of these treatments significantly improved the proportions of recoverable normal spermatozoa and in similar proportions. We envision that the Accudenz gradient might be the most practical application for assisted breeding in cheetahs that could benefit from IVF. Our most recent studies determined that as many as 40% of cheetah oocytes matured in vitro formed cleaved embryos after insemination with as few as approximately 1.0×10^5 motile sperm/mL (Crosier et al, unpublished results). Extrapolating to findings from study 3 here, we recovered an average of 4.0×10^5 total motile sperm with intact acrosomal membranes from each 80-μL aliquot of TYB exposed to Accudenz (Table 4). Therefore, given an average of approximately 80×10^6 total motile sperm in a raw sample (Table 1) and sperm recovery postthaw, it

is potentially possible to produce up to 60 doses of high-quality IVF inseminates from a single cheetah ejaculate.

The Accudenz gradient approach also has been touted as a means for restoring sperm cells to a chemically defined environment, including allowing the removal of 98% of seminal plasma proteins (McLean et al, 1998). Novel approaches to cheetah conservation have involved the development of a genome resource bank (ie, an organized frozen repository including germplasm) for the explicit purpose of improved genetic management by moving sperm and embryos rather than whole, living animals (Wildt et al, 1997; Holt and Pickard, 1999). It is well known that the cheetah appears vulnerable to infectious diseases related no doubt to the species' lack of genetic diversity and homozygosity at the level of the major histocompatibility complex (O'Brien et al, 1983, 1985). For other species, the transmission of pathogens generally occurs via seminal plasma rather than the sperm cell itself, which resists incorporating viral genetic material (Couto et al, 2004). Therefore, it is worth noting that passing cheetah sperm through a concentration gradient to remove virtually all seminal plasma has the added benefit of substantially lessening (or totally eliminating) the potential of disease transmission as sperm samples begin to be transported intercontinentally to benefit genetic management. Further studies designed to investigate the efficacy of Accudenz for removing infectious agents are warranted.

In conclusion, this investigation confirmed that the cheetah spermatozoon is highly sensitive to the processes of thawing and cryoprotectant removal, which cause a more than 35% reduction in cells with intact acrosomes and more than 15% decline in cellular motility. Traditional postthaw processing, including
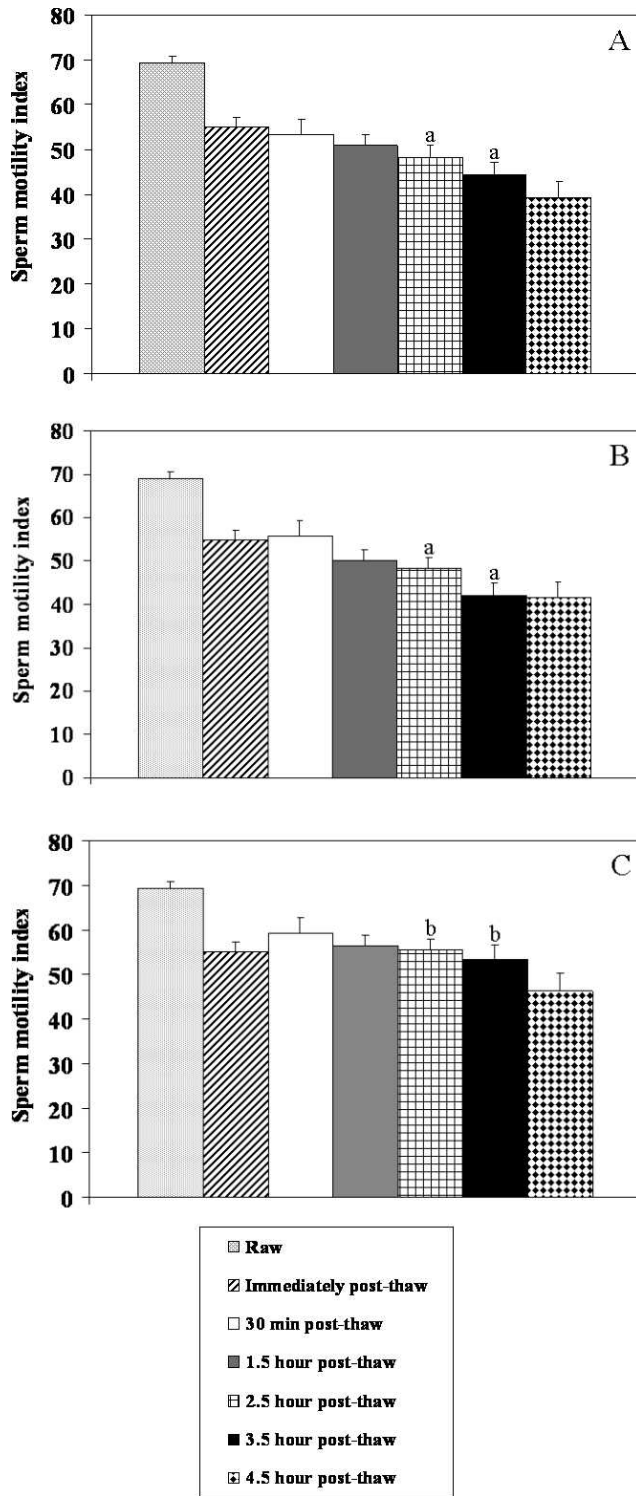
Figure 4. Cheetah sperm motility index for (**A**) washed (control), (**B**) swim-up, and (**C**) Accudenz treatments. Among treatments and within each time point, bars with different superscripts differed at *P* < .05 (study 3).

Table 4. *Acrosomal integrity of cryopreserved cheetah sperm (study 3; means* ± *SEM)*[a]

	Intact acrosomes, %		
	Washed	Swim-up	Accudenz
Raw ejaculate	86.8 ± 1.6	86.8 ± 1.6	86.8 ± 1.6
Postcryopreservation			
Immediately postthaw	44.4 ± 7.2	44.4 ± 7.2	44.4 ± 7.2
30 min postthaw	31.9 ± 5.7	32.8 ± 10.1	40.6 ± 7.4
1.5 h postthaw	30.1 ± 5.6	37.3 ± 9.4	39.3 ± 6.5
2.5 h postthaw	32.5 ± 6.3[b]	34.1 ± 9.6[b]	42.1 ± 7.3[c]
3.5 h postthaw	33.0 ± 7.2	32.8 ± 10.4	39.8 ± 7.1
4.5 h postthaw	26.2 ± 3.0	32.3 ± 9.5	46.8 ± 8.1

[a] n = 6 ejaculates total from 6 males.
[b,c] Within rows, values with different superscripts differ at *P* < .05.

the use of swim-up separation, fails to reduce this damage that appears related to the removal of glycerol from the spermatozoon. However, it is clear that most of the postthaw damage can be mitigated by Accudenz gradient processing, which alleviates a significant portion of the injurious effects of glycerol permeating the cell membrane. The benefit is largely expressed by the retention of acrosomal integrity in a higher proportion of spermatozoa. The biological impact of this improved quality factor now can be assessed in our contemporary IVF studies geared to eventually applying cryopreserved sperm to enhanced reproduction and genetic management.

Acknowledgments

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