Osmotic tolerance of rat spermatozoa and the effects of addition and removal of cryoprotectants on rat sperm motility, plasma membrane integrity and acrosome integrity

Wei Si, James D. Benson, Hongsheng Men, John K. Critser

University of Missouri

Comparative Medicine Center

Research Animal Diagnostic Laboratory

College of Veterinary Medicine

1600 East Rollins Street

Columbia, Missouri 65211

Address correspondence to:

John K. Critser, Ph.D.

Comparative Medicine Center

College of Veterinary Medicine

University of Missouri

1600 East Rollins Street, Room E-109

Columbia, Missouri 65211

Telephone: (573)-884-9469

Fax: (573)-884-7521

E-mail: critserj@missouri.edu

Abstract

Osmotic stress is an important factor that results in cell damage during sperm cryopreservation. The objective of this study was to determine isosmotic sperm cell volume and osmotic inactive volume, osmotic tolerance limits of rat sperm, and the effects of addition and removal of glycerol, ethylene glycol, propylene glycol or dimethyl sulfoxide on sperm function by analyzing motility, plasma membrane and acrosome integrity. Spermatozoa from Sprague Dawley and Fischer 344 rats were used to perform the experiments in this study. The isotonic mean sperm cell volumes of the two strains were 36.15 μm^3 and 36.98 μm^3 ,respectively. Rat spermatozoa behave as linear osmometers from 260 to 450 mOsm, and the osmotic inactive sperm volumes of the two strains were 81.4% and 79.8%, respectively. Rat sperm have very limited osmotic tolerances, and motility is substantially more sensitive to osmotic stress than plasma membrane and acrosome integrity. In a range of anisosmotic solutions (75-1200 mOsm), only spermatozoa treated with 260-375 mOsm were able to maintain motility equal to the control level after being returned to isosmotic conditions. In order to maintain 90% of pretreatment motility, spermatozoa of Sprague Dawley and Fischer 344 rat should be maintained within 95.6-102.6% and 95.6-1.3.1% of their isosmotic volume, respectively, during cryopreservation. The one step addition and removal of dimethyl sulfoxide showed the most deleterious effect on rat sperm motility, plasma membrane integrity and acrosomal integrity among the four cryoprotectants. These data characterizing rat sperm

osmotic behavior, osmotic and cryoprotectant tolerance will be helpful for designing cryopreservation protocols for rat sperm.

Introduction

The rat is one of the most popular and important animal models for biomedical research. During the past several years, many transgenic rat lines have been produced in different laboratories [Tesson, et al, 2005], and the completion of the full sequence of the rat genome [Gibbs et al, 2004] will be enable and enhance the production of new mutant and genetic engineered rat lines for biomedical research. Compared to the mouse, the rat has a larger body size, higher genetic diversity and in general more accurately represents many human pathologies [Canzian, 1997; Tesson, et al, 2005]. These biological characteristics make the rat an essential animal model for human disease investigation [Charreau et al, 1996; Tesson, et al, 2005].

The maintenance and breeding of animal model lines are costly. Spermatozoa cryopreservation can provide an efficient way to preserve valuable genetic resources. With the combination of assistant reproductive technologies such as *in vitro* fertilization (IVF), artificial insemination (AI) and intracytoplasmic sperm injection (ICSI), animal offspring can be produced using cryopreserved spermatozoa at relatively low cost and high efficiency.

However, in contrast to the success of mouse sperm cryopreservation, which was first reported in 1990 [Tada et al., 1990; Yokoyama et al., 1990;] and has been well developed in different mouse strains [Thornton et al., 1999], rat spermatozoa cryopreservation was not reported until recently [Nakatsukasa et al, 2001; 2003]. However, compared to fresh spermatozoa, the motility, pregnancy rate and litter size after intrauterine insemination with cryopreserved spermatozoa are much lower [Nakatsukasa et al, 2001]. Thus, further

development of protocols for rat spermatozoa cryopreservation is still needed to improve the overall efficiency and complete retention of fertilizing ability.

During spermatozoa cryopreservation, osmotic stress is an important cause of sperm cryoinjury [Meyers 2005]. Typical cryopreservation protocols require the addition of permeating cryoprotectant agents (CPA) before freezing and removal of CPA after thawing [Gao et al., 1997]. During the addition of CPA, cells are exposed to a hyperosmotic surrounding, causing the cell to shrink due to the intracellular water efflux through plasma membrane. The cell will then swell to slightly greater than isosmotic volume due to the influx of CPA and the reentry of water. On the other hand, during the removal of CPA, cells will swell due to the influx of extracelluar water, and then shrink to isotonic volume as CPA diffuses out the cells. Furthermore, during the process of freezing, the ice nucleation of extracelluar water will change the osmolality of unfrozen solution due to the increased concentration of solutes dissolved in the unfrozen water, which also will cause osmotic stress to cells [Mazur 1984]. When volume excursions extend beyond certain points, called osmotic tolerance limits, cell damage will occur. This osmotic tolerance of sperm cells varies among animal strains and species [Walters et al, 2005; Guthrie et al, 2002; Gilmore et al, 1998; Gao et al, 1995; Agca et al, 2005; Rutllant et al, 2003; Ball and Vo, 2001]. If the fundamental cryobiological properties of sperm cells including osmotic tolerance limits, isosmotic cell volume (V_{iso}) and osmotically inactive cell volume (V_b), hydraulic conductivity to water (L_p), the membrane permeability coefficient (P_{CPA}) and activation energies (E_a) are well understood, it is possible to design protocols for the addition and removal of cryoprotectant that will

minimize osmotic damage and also to compute the optimal cooling and warming rate to reduce the probability of intracellular ice formation [Mazur 1984, Critser and Mobraaten 2000]. Unfortunately for such an important animal model as the rat, to our knowledge there are no studies concerning the fundamental cryobiological properties of their spermatozoa, and little is known about the osmotic tolerance of rat sperm cells and the effects of CPA on rat sperm function. The objective of the present study was carried out to determine the osmotic behavior; the osmotic tolerance limits; and the effect of addition and removal CPA on the function of rat spermatozoa. For the first objective, an electronic particle counter was used to detect the change of rat sperm cell volume. For the second and third objectives, computer-assisted sperm motility analysis and flow cytometric analysis were used to access sperm motility, plasma membrane and acrosome integrity.

Materials and Methods

Animals

Mature male rats between 15-20 weeks old from Sprague Dawley and Fischer 344 genetic backgrounds (Harlan, Indianapolis, IN) were used as sperm donors in this study. All animals were maintained in accordance with the policies of the University of Missouri Animal Care and Use Committee, and the Guide for the Care and Use of Laboratory Animals.

Media Preparation

All chemicals were from Sigma Chemical (St. Louis, MO) unless otherwise stated. The DPBS medium (Gibco #14287-080; Invitrogen, Carlsbad, CA) was used in all

experiments. Hypotonic solutions (75, 150, 225, and 260mOsm) were prepared by diluting the isosmotic DPBS medium (290 mOsm) with MilliQ water, and hypertonic solutions (375, 450, 600, and 1200 mOsm) were made by adding appropriate amounts of sodium chloride to isosmotic DPBS medium. Osmolalities of the solutions were measured using a vapor pressure osmometer (VAPRO 5520, Wescor, Logan, UT) with an accuracy of ± 5 mOsm. CPA solutions were prepared by dissolving glycerol (Gly), ethylene glycol (EG), propylene glycol (PG) and dimethyl sulfoxide (DMSO) in isosmotic DPBS medium at a final concentration of 1M. Prior to the use, 2 mg /ml bovine serum albumin (BSA) were supplemented to all of the media and solutions.

Rat sperm collection

Male rats were euthanized and their cauda epididymides were excised. Epididymides were placed in a 35 mm dish containing 3 ml of DPBS medium supplemented with 2 mg/ ml BSA and dissected with eye scissors. Rat spermatozoa were allowed to swim out for 10 min at 37°C.

Sperm motility, plasma membrane and acrosome status analysis

A computer-aided semen analyzer (Hamilton Thorne IVOS v 12.2c, Beverly, MA) was used to analyze rat sperm motility. Flow cytometric analysis was used to assess sperm acrosome and plasma membrane status. Propidium iodide (PI) and Alexa Xuor-488-PNA (peanut agglutinin) conjugate (Molecular Probes, Eugene, OR) were used to determine rat sperm membrane and acrosome integrity respectively. The flow cytometric analysis followed the procedure described by Walters et al. [2005]. Briefly, rat spermatozoa were incubated with 0.4 µg/ml Alexa Xuor-488-PNA and 1 µM PI at 37 °C for 30 min, and

analyzed by FACS Scan (Beckman–Dickinson, San Jose, CA). The fluorophores were excited with the 488 nm line of the laser. Alexa Xuor-488 emission was collected with a 530 bandpass (BP) filter, and PI emission was collected with a 670 longpass (LP) filter. Ten thousand cells were analyzed from each sample.

Experimental Design

Experiment 1. Osmotic Behavior of Sprague Dawley and Fischer 344 rat spermatozoa

A modified Coulter counter (ZM model; Coulter Electronics Inc. Hialeah, FL) with a 50 µm standard-resolution aperture tube was used to measure cell volume as previous description [Gilmore et al., 1995]. Sperm cell suspension samples from five rats (n = 5) were analyzed for each strain, and all of the measurements were performed at 22°C. Isosmotic cell volume was determined by adding 100 µl of sperm cell suspension to 15 ml of isosmotic DPBS. Osmotically driven cell volume responses and the osmotically inactive cell volume (V_b) were determined by adding 100 µl of sperm cell suspension to 15 ml of aniosmotic DPBS (260, 375 and 450 mOsm, respectively). The final concentration of sperm cells in the 15 ml isosmotic DPBS and aniosmotic DPBS solutions was 1×10^5 cells/ml. The volume change of sperm cells was recorded kinetically during the shrink or swell period and the final cell volumes were determined after equilibration, and cell volumes were measured in triplicate of each sample. Mean sperm cell volumes exposed to isosmotic and aniosmotic solutions were calculated with 5 µm diameter spherical styrene beads (Beckman Coulter Corporation, Miami, FL). The coulter

counter was interfaced to a microcomputer using a CSA-1S interface (The Great Canadian Computer Company, Edmonton, Canada), and the cell volume data were analyzed using custom software.

To determine the osmotically inactive cell volume of rat sperm and whether rat sperm behaves as linear osmometers, sperm volume at isotonic and anisosmotic conditions were fitted to the Boyle van't Hoff relationship:

$$\frac{V}{V_{iso}} = \frac{M_{iso}}{M}\left[1 - \frac{V_b}{V_{iso}}\right] + \frac{V_b}{V_{iso}}$$

Where V is the cell volume at osmolality M, V_{iso} is the cell volume at isosmolality (M_{iso}), and V_b is the osmotically inactive cell volume.

Experiment 2. Sprague Dawley and Fischer 344 rat sperm osmotic tolerance limits

Sperm suspensions collected from Sprague Dawley rats (n = 5) or Fischer 344 rats (n = 5) were used for this experiment, and all of the experiments were performed at 22°C. Sperm solutions (3×10^7 cells/ml) in 20 µl aliquots were added to nine 1.5 ml Eppendorf centrifuge tubes containing 500 µl DPBS solutions with different osmolalities (75, 150, 225, 260, 290, 375, 450, 600 and 1200 mOsm). Spermatozoa were equilibrated in these DPBS solutions with different osmolalities for 5 min, and the sperm motility of each treatment was evaluated immediately. The anisosmotic treated sperm solutions were then returned to near isosmolality (290-300 mOsm) by adding appropriate amounts of DPBS solutions at other corresponding osmolalites [Willoughby et al., 1996; Walters et al., 2005]. Sperm solutions were equilibrated at room temperature for 5 min and the motility

of each treatment was evaluated immediately. At the same time, a sample of each treatment group was taken for immediate membrane and acrosome integrity assessment.

Experiment 3. Effects of one-step addition and removal of CPAs on Sprague Dawley and Fischer 344 rat sperm motility, plasma membrane integrity and acrosome integrity

Glycerol, EG, DMSO and PG were investigated here to empirically test the sensitivity of rat spermatozoa to CPA, and all of the experiments were performed at 22°C. Sperm samples were collected from Sprague Dawley rats (n=5) or Fischer 344 rats (n=5). Aliquots of 20 μL sperm cells suspension (3×10^7 sperm/mL) were added to five 1.5 ml Eppendorf centrifuge tubes containing 200 μL isosmotic DPBS medium and DPBS solutions containing 1 M Gly, EG, PG and DMSO, respectively. Spermatozoa were equilibrated at room temperature for 5 min. Then, the motility of sperm treated with DPBS and various CPA was assessed immediately. The removal of CPA was performed by adding 1ml isosmotic DPBS in one-step to the tubes, and sperm solutions were equilibrated for 5 min. Then, sperm motility of each treatment group was evaluated again immediately. At the same time, sperm sample of each treatment group was taken for immediate membrane and acrosome integrity assessment.

Statistical analysis

All data are expressed as mean ± SEM. Analysis of variance using the General Linear Model univariate procedure of the SPSS software (SPSS INC., Chicago) was used to determine the effect of osmolality on rat sperm volume and the effect of osmolality, CPA

and genetic background on sperm motility, plasma membrane integrity and acrosome integrity. The Tukey multiple comparison test was employed, and a *p* value of less than 0.05 was considered to be statistically significant.

Results

Experiment 1

Osmotic Behavior of Sprague Dawley and Fischer 344 rat spermatozoa

The mean isosmotic cell volume of rat spermatozoa determined by Coulter counter was 36.15 ± 0.23 μm^3 for the Sprague Dawley strain, 36.98 ± 0.13 μm^3 for the Fischer 344 strain. Analysis of cell volume in the various osmotic conditions indicate that rat sperm were linear osmometers in the range of 260-450 mOsm (r^2 = 0.97 in Sprague Dawley rat; r^2 = 0.92 in Fischer 344 rat). The inactive cell volume (V_b) of Sprague Dawley rat and Fischer 344 was 81.4% and 79.8% of their isosmotic volume, respectively. No significant difference was observed for isosmotic cell volumes and V_b values between the two strains (p>0.05). Data presented as a Boyle van't Hoff plot relationship is shown in Figure 1.

Experiment2

Osmotic tolerance limits of Sprague Dawley and Fischer 344 rat spermatozoa

Osmotic tolerance of maintenance of motility of Sprague Dawley and Fischer 344 rat spermatozoa

The effects of the anisosmotic treatments on spermatozoa motility, normalized to the isosmotic treatment, are shown in Figure 2. A significant main effect of osmolality was found on spermatozoa motility ($p<0.05$), but the effect of genetic background was not

significant ($p>0.05$). As shown in Figure 2A, the motility of Fischer 344 rat spermatozoa in the anisosmotic conditions (75, 150, 225, 450, 600 and 1200 mOsm, respectively) was decreased significantly compared to the motility in isosmotic DPBS medium (290 mOsm) ($p<0.05$). After returning to isosmotic condition, the motility of spermatozoa treated with 225 and 450 mOsm solution was partly recovered ($p<0.05$). However, the motility of spermatozoa treated with 75, 150, 600 and 1200 mOsm solutions did not change after being returned to an isosmotic condition ($p>0.05$), and few sperm cells maintained motility after the exposure. No statistical differences of sperm motility were found among the spermatozoa treated with 260, 290, and 375 mOsm solutions both in aniosmotic condition and after being returned to an isosmotic condition ($p>0.05$).

Similar to Fischer 344 rat spermatozoa, as shown in Figure 2B, the motility of Sprague Dawley rat spermatozoa in anisosmotic conditions (75, 150, 225, 375, 450, 600 and 1200 mOsm, respectively) was decreased significantly compared to the motility of spermatozoa in isosmotic DPBS (290 mOsm) ($p<0.05$). After returning to an isosmotic condition, only the motility of spermatozoa treated with 375 mOsm solution fully recovered to the level of spermatozoa treated with isosmotic solution, but the motility of spermatozoa treated with 75, 150, 225, 450, 600 and 1200 mOsm solutions did not change significantly ($p>0.05$), and almost all of the spermatozoa lost motility after the exposure to 75, 150, 600 and 1200 mOsm solutions. No statistical differences of sperm motility were found between the spermatozoa treated with 260 mOsm solution and isosmotic medium both in aniosmotic condition and after returned to isosmotic condition ($p>0.05$).

Osmotic tolerance of maintenance of membrane and acrosome integrity of Sprague Dawley and Fischer 344 rat spermatozoa

The effects of anisosmotic treatments on Fischer 344 rat and Sprague Dawley rat spermatozoa membrane and acrosome integrity, normalized to the isosmotic treatment, are shown in Figure 3. A significant main effect of osmolality was found on plasma membrane integrity and acrosome integrity ($p<0.05$), but the effect of genetic background was not significant ($p>0.05$). As shown in Figure 3A, the plasma membrane integrity of Fischer 344 rat spermatozoa exposed to anisosmotic solutions (75, 150, 225, 260, 375, 450, 600 and 1200 mOsm) and the acrosome integrity of spermatozoa exposed to 75 and 150 mOsm DPBS solutions was significantly decreased ($p<0.05$) after being returned to isosmotic condition compared to the isosmotic treatment. Similar to Fischer 344 rat, as shown in Figure 3B, the plasma membrane integrity of Sprague Dawley rat spermatozoa exposed to anisosmotic DPBS solutions (75, 150, 225, 450, 600 and 1200 mOsm) and the acrosome integrity of spermatozoa exposed to 75 and 150 mOsm was significantly decreased ($p<0.05$) after being returned to isosmotic condition when compared to the isosmotic treatment. No significant difference of plasma membrane integrity and acrosome integrity was found among the spermatozoa treated with 260 and 375 mOsm DPBS solutions and the isosmotic medium in Sprague Dawley rat spermatozoa ($p>0.05$).

Experiment 3

Addition and removal of cryoprotectant to Sprague Dawley and Fischer 344 rat spermatozoa

A significant main effect of cryoprotectant was found on sperm motility, plasma membrane integrity and acrosome integrity ($p<0.05$), but the effect of genetic background was not significant ($p>0.05$). The effect of addition and removal of 1 M Gly, DMSO, EG and PG on sperm motility of Sprague Dawley and Fischer 344 rat, normalized to the isosmotic treatment, is shown in Figure 4. Compared to the isosmotic treatment, the one-step addition of 1M Gly, DMSO, EG, and PG did not affect sperm motility in both strains ($p>0.05$). However, after the 5-fold dilution using isosmotic DPBS medium, the sperm motility treated with Gly, EG, and PG was slightly decreased compared to the value of isosmotic treatment in each strain ($p<0.05$), the abrupt decrease of motility was found in DMSO treatment group, and almost no sperm maintained motility after the dilution ($p<0.05$).

The effect of addition and removal of 1 M Gly, DMSO, EG and PG on the sperm plasma membrane and acrosome integrity of Sprague Dawley and Fischer 344, normalized to the isosmotic treatment, is show in Figure 5. After the addition and removal of 1 M Gly, EG and PG, sperm membrane and acrosome integrity were not significantly different compared to the treatment with isosmotic DPBS medium in both strains ($p>0.05$). However, the sperm plasma membrane integrity and acrosome integrity of spermatozoa exposed to 1M DMSO were significant decreased in each strain after the 5-fold dilution using isosmotic DPBS medium ($p<0.05$) .

Discussion

Osmotic behavior of rat spermatozoa

Due to the unique size and morphology between spermatozoa from different species, the parameters of sperm surface-volume ratio and sperm osmotic inactive water volume are important to study the water and CPA transport and the intracellular ice formation during spermatozoa freezing. In this study, the relationship between the change of rat sperm cell volume and the osmolality of surrounding medium was determined. The mean isotonic sperm cell volume of Sprague Dawley and Fischer344 rats measured by Coulter Counter is 36.15 ± 0.23 μm^3 and 36.98 ± 0.13 at 22 °C, respectively. Since no previous attempt has been achieved to measure rat sperm cell volume, we can not compare our result to other studies. However, the rat sperm cell volume determined in this study is smaller than mice (ICR mouse: 56.0 μm^3, B6C3F1 mouse: 53.0 μm^3) [Willoughby et al., 1996], but larger than boar (26.3 μm^3) [Gilmore et al., 1996], bull (23.5 μm^3) [Guthrie et al., 2002], equine (24.4 μm^3) [Pommer et al., 2002], rhesus monkey (27.7 μm^3) [Agca et al., 2005] and human spermatozoa (28.2 μm^3) [Gilmore et al., 1995] determined by using Coulter counter method.

The Boyle van't Hoff relationship and the osmotic response of rat spermatozoa showed that rat sperm behaved as linear osmometers in the range of 260 to 450 mOsm. The anisosmotic treatments out of this osmolality range were excluded because the sperm membrane integrity was less than 50% as determined by experiment 2. The spermatozoa osmotically inactive volume (V_b) of Sprague Dawley rat and Fischer 344 rat was 81.4% and 79.8%, respectively, which is much greater than that of mouse (60.7%) [Willoughby et al., 1996], boar (67.4%) [Gilmore et al., 1996], human (50%) [Gilmore et al., 1995],

bull (61%) [Guthrie et al., 2002], stallion (70.7%) [Pommer et al., 2002] and rhesus monkey (51%) [Agca et al., 2005]. In other words, the amount of unbound water in rat spermatozoa that can be lost during freezing is only 18.6% for Sprague Dawley rat and 20.2% for Fischer 344 rat of the cell volume, which is the smallest osmotically active volume ratio we are aware of based on the published data of different species.

Osmotic tolerance of rat spermatozoa

Prior to this study, little information was available regarding osmotic responses of rat spermatozoa. In the present study, we demonstrated that aniosmotic osmotic stress decreased rat sperm motility; the extent of damage to sperm plasma membrane corresponds with the aniosmotic stress levels. The high sperm motility (>90%) of both of the two rat strains can be maintained only in the osmotic range of 250-380 mOsm after return to isosmotic condition. After exposure to these solutions of 75, 150, 600 and 1200 mOsm, almost all spermatozoa lost motility (less than 5% were motile) and the motility did not improve subsequent to returning to isosmotic conditions. In comparison, mouse spermatozoa maintain about 55% motility after exposure to a 150mOsm hyposmotic treatment followed by a return to isosmotic conditions, and maintain 20% motility after exposure to a 600mOsm hyperosmotic treatment followed by a return to isosmotic conditions [Willoughby et al., 1996]. Previous studies indicated that human sperm should be kept between 75% and 110% of the normal isosmotic volume in order to obtain > 90% motility [Gao et al., 1995], and mouse sperm volume excursions should be kept between 90% and 103% of the normal isosmotic volume in order to maintain > 90% motility, and kept between 76% and 124% of the normal isosmotic volume in order to maintain > 80%

motility [Willoughby et al., 1996]. In the present study, in order to maintain > 90% motility, Sprague Dawley and Fischer 344 rat sperm volume must be kept between 95.6% and 102.6% and 95.3% and 103.1% of the normal isosmotic volume, respectively, as calculated from the linear osmometric behavior of rat spermatozoa shown in Figure 2. When the Sprague Dawley and Fischer 344 sperm volume excursion exceeded 95.2% and 103.3%, and 94.8% and 104.1% of the normal isosmotic volume, respectively, the motility abruptly dropped to 80% of motility at isosmotic condition. The present study demonstrates that rat spermatozoa appear to have a very limited osmotic tolerance based on motility assessment compared to mouse [Walters et al., 2005] [Willoughby et al., 1996], bull [Guthrie et al., 2002], stallion [Pommer et al., 2002] [Ball andVo 2001], rhesus monkey [Rutllant et al., 2003] and human spermatozoa [Gao et al., 1995], but similar to boar spermatozoa [Gilmore et al., 1998].

Similar to the decrease of sperm motility, plasma membrane integrity of rat sperm declined rapidly at hyposmotic and hyperosmotic conditions. Although almost all of the spermatozoa lost motility (less than 5% were motile) after exposure to hyperosmotic solution (600 and 1200 mOsm), the percentage of spermatozoa with intact plasma membrane remained at 20-40%. Similar responses to hyperosmotic conditions have been reported in spermatozoa of human [Gao et al., 1995], bull [Liu and Foote, 1998], boar [Gilmore et al., 1996], stallion [Ball and Vo, 2001] and mouse [Willoughby et al., 1996; Walters et al., 2005]. This indicates that besides the damage to sperm plasma membrane, other factors such as the alteration of the bioenergetic status of spermatozoa or damage to the axonemal elements contribute to the reduction in motility [Watson, 1995]. Compared

to the decline of motility and plasma membrane integrity, sperm acrosome of the both rat strains was less sensitive to the aniosmotic treatment, especially to the hyperosmotic condition. Compared to isosmotic treatment, the significant decrease of acrosome integrity was only found in hyposmotic treatements (75 and 150 mOsm) in each of strain. The relative higher resistance to hyperosmotic treatments of sperm acrosome was also found in mouse spermatozoa [Walters et al., 2005].

Effect of addition and removal of 1 M cryoprotectant

Glycerol, EG, PG and DMSO are the most widely used penetrating cryoprotectants for cell cryopreservation. Addition of cryoprotectants to sperm can cause sperm cell volume excursions in response to the influx and efflux water and cryoprotectant. In previous reports, the rapid one-step addition of CPA resulted in loss of motility and viability of human [Gao et al., 1995] [Gilmore et al., 1997], bull [Guthrie et al., 2002], equine [Ball and Vo 2001], and mouse [Phelps et al., 1999] spermatozoa. However, when multi-step addition and removal strategies were applied, the motility and viability loss could be avoided or decreased [Gao et al., 1995] [Phelps et al., 1999]. In the present study, after one-step addition of 1 M glycerol, DMSO, ethylene glycol and propylene glycol to rat spermatozoa, no significant difference was found on the motility between the four cryoprotectans and isosmotic DPBS treatment. However, after the removal of CPA, the motility of spermatozoa treated with Gly, EG or PG decreased slightly compared to the motility of isosmotic treatment. While, DMSO showed the most detrimental effect on rat sperm motility after abrupt dilution, and almost all of the spermatozoa lost motility. The similar response of spermatozoa to DMSO was also found in bull spermatozoa: the sperm

motility was abruptly dropped from 63.9% to 6.4% after the removal of 1 M DMSO [Guthrie et al., 2002].

The phenomenon that spermatozoa respond to cryoprotectants differently has been revealed in different species. For example, 1 M glycerol was more deleterious to human sperm motility than 1 M ethylene glycol [Gilmore et al., 1997]. Removal of DMSO resulted in only a 10% recovery of bull spermatozoa motility, and was much more harmful compared to glycerol and EG [Guthrie et al., 2002]. The addition and removal of EG appeared to have the least detrimental effect on equine sperm motility and viability, while glycerol decreased motility and damaged the membrane most among the cryoprotectants used [Ball and Vo 2001]. The addition and removal of 1 M glycerol resulted in more motility loss than that of 1 M EG on mouse spermatozoa [Phelps et al., 1999]. On the other hand, the only CPA that resulted in motility loss on rhesus monkey spermatozoa was 1 M PG [Agca et al., 2005]. The different responses of sperm addition and removal of cryoprotectants has been explained by the different P_{CPA} coefficient among the cryoprotectants [Gilmore 1995].

Compared to the reduction of motility and the loss of plasma membrane and acrosome integrity caused by sodium chloride at high osmolality (1200 mOsm) in experiment 2, 1 M glycerol, EG and PG caused much reduced detrimental effects on rat sperm function, which might be ameliorated by a high permeability to these CPAs through the plasma membrane—allowing rapid equilibration. No report is available about the hydraulic

conductivity in the presence of glycerol, DMSO, ethylene glycol and propylene glycol or the membrane permeability coefficients of these cryoprptectants of rat sperm. We attempted to use Coulter counter to determine these parameters, but the kinetic change of rat sperm cell volume occurred too rapidly to monitor using Coulter counter measurements—even after measurements were made at low supra-zero temperatures, a similar situation to bull spermatozoa when same technology was applied [Guthrie et al., 2002]. In the future, alternative method or technology must be applied to determine permeability coefficients. One such method is a concentration-dependent self-quenching entrapped fluorophore technique which can capture rapid cell volume changes in the presence of permeable cryoprotectants [Curry et al., 2000; Chaveiro et al., 2004].

In summary, the present study determined the osmotic behavior, osmotic tolerance limits of two different rat strains and the effect of addition and removal permeable cryoprotectants on rat sperm motility, plasma membrane integrity and acrosome integrity. The results showed that: 1) the isosmotic volume of Sprague Dawley and Fischer 344 rat sperm are 36.15 ± 0.23 μm3 and 36.98 ± 0.13 μm^3 and 81.4% and 79.8% osmotic inactive volume, respectively at 22 °C; 2) the spermatozoa of both strains exhibited a linear osmotic response in the range 260- 450 mOsm; 3) rat spermatozoa appear to have a very limited osmotic tolerance, osmotic stress can result in loss of sperm motility, as well as damage to the plasma membrane and acrosome; 4) the addition and removal of 1 M glycerol, EG and PG slightly decreased rat sperm motility but did not affect plasma membrane and acrosome integrity, but the addition and removal of DMSO caused

detrimental effect on rat sperm motility as well as plasma membrane and acrosome integrity. These results will be helpful for developing a protocol for rat spermatozoa cryopreservation with the combination of rat sperm membrane hydraulic conductivity and the permeability coefficients of cryoprotectants at various temperatures.

Reference

Agca Y, Mullen S, Liu J, Johnson-Ward J, Gould K, Chan A, Critser J. Osmotic tolerance and membrane permeability characteristics of rhesus monkey (Macaca mulatta) spermatozoa. *Cryobiology*. 2005;51:1-14.

Ball BA, Vo A. Osmotic tolerance of equine spermatozoa and the effects of soluble cryoprotectants on equine sperm motility, viability, and mitochondrial membrane potential. *J Androl*. 2001;22:1061-1069.

Canzian F. Phylogenetics of the laboratory rat Rattus norvegicus. *Genome Res*. 1997;7: 262–267.

Charreau B, Tesson L, Soulillou JP, Pourcel C, Anegon I. Transgenesis in rats: technical aspects and models. *Transgenic Res*. 1996;5:223-234.

Chaveiro A, Liu J, Mullen S, Woelders H, Critser JK. Determination of bull sperm membrane permeability to water and cryoprotectants using a concentration-dependent self-quenching fluorophore. *Cryobiology*. 2004;48:72-80.

Critser JK, Mobraaten LE. Cryopreservation of murine spermatozoa. *ILAR J*. 2000;41:197-206.

Curry MR, Kleinhans FW, Watson PF. Measurement of the water permeability of the membranes of boar, ram, and rabbit spermatozoa using concentration-dependent self-quenching of an entrapped fluorophore. *Cryobiology*. 2000;41:167-173.

Gao DY, Liu J, Liu C, McGann LE, Watson PF, Kleinhans FW, Mazur P, Critser ES, Critser JK. Prevention of osmotic injury to human spermatozoa during addition and removal of glycerol. *Hum Reprod*. 1995;10:1109-1122.

Gao DY, Mazur P, Critser JK. Fundamental cryobiology of mammalian spermatozoa. In: Karow AM, Critser JK, eds. Reproductive Tissue Banking: Scientific Principles. San Diego: Academic Press; 1997:263–328.

Gibbs RA, Weinstock GM, Metzker ML, Muzny DM, Sodergren EJ, Scherer S, Scott G, Steffen D, Worley KC, Burch PE, Okwuonu G, Hines S, Lewis L, DeRamo C, Delgado O, Dugan-Rocha S, Miner G, Morgan M, Hawes A, Gill R, Celera , Holt RA, Adams MD, Amanatides PG, Baden-Tillson H, Barnstead M, Chin S, Evans CA, Ferriera S, Fosler C, Glodek A, Gu Z, Jennings D, Kraft CL, Nguyen T, Pfannkoch CM, Sitter C, Sutton GG, Venter JC, Woodage T, Smith D, Lee HM, Gustafson E, Cahill P, Kana A, Doucette-Stamm L, Weinstock K, Fechtel K, Weiss RB, Dunn DM, Green ED, Blakesley RW, Bouffard GG, De Jong PJ, Osoegawa K, Zhu B, Marra M, Schein J, Bosdet I, Fjell C, Jones S, Krzywinski M, Mathewson C, Siddiqui A, Wye N, McPherson J, Zhao S, Fraser CM, Shetty J, Shatsman S, Geer K, Chen Y, Abramzon S, Nierman WC, Havlak PH, Chen R, Durbin KJ, Egan A, Ren Y, Song XZ, Li B, Liu Y, Qin X, Cawley S, Cooney AJ, D_Souza LM, Martin K, Wu JQ, Gonzalez-Garay ML, Jackson AR, Kalafus KJ, McLeod MP, Milosavljevic A, Virk D, Volkov A, Wheeler DA, Zhang Z, Bailey JA,

Eichler EE, Tuzun E, Birney E, Mongin E, Ureta-Vidal A, Woodwark C, Zdobnov E, Bork P, Suyama M, Torrents D, Alexandersson M, Trask BJ, Young JM, Huang H, Wang H, Xing H, Daniels S, Gietzen D, Schmidt J, Stevens K, Vitt U, Wingrove J, Camara F, Mar Alba M, Abril JF, Guigo R, Smit A, Dubchak I, Rubin EM, Couronne O, Poliakov A, Hubner N, Ganten D, Goesele C, Hummel O, Kreitler T, Lee YA, Monti J, Schulz H, Zimdahl H, Himmelbauer H, Lehrach H, Jacob HJ, Bromberg S, Gullings-Handley J, Jensen-Seaman MI, Kwitek AE, Lazar J, Pasko D, Tonellato PJ, Twigger S, Ponting CP, Duarte JM, Rice S, Goodstadt L, Beatson SA, Emes RD, Winter EE, Webber C, Brandt P, Nyakatura G, Adetobi M, Chiaromonte F, Elnitski L, Eswara P, Hardison RC, Hou M, Kolbe D, Makova K, Miller W, Nekrutenko A, Riemer C, Schwartz S, Taylor J, Yang S, Zhang Y, Lindpaintner K, Andrews TD, Caccamo M, Clamp M, Clarke L, Curwen V, Durbin R, Eyras E, Searle SM, Cooper GM, Batzoglou S, Brudno M, Sidow A, Stone EA, Payseur BA, Bourque G, Lopez-Otin C, Puente XS, Chakrabarti K, Chatterji S, Dewey C, Pachter L, Bray N, Yap VB, Caspi A, Tesler G, Pevzner PA, Haussler D, Roskin KM, Baertsch R, Clawson H, Furey TS, Hinrichs AS, Karolchik D, Kent WJ, Rosenbloom KR, Trumbower H, Weirauch M, Cooper DN, Stenson PD, Ma B, Brent M, Arumugam M, Shteynberg D, Copley RR, Taylor MS, Riethman H, Mudunuri U, Peterson J, Guyer M, Felsenfeld A, Old S, Mockrin S and Collins F. Genome sequence of the Brown Norway rat yields insights into mammalian evolution. *Nature*. 2004;428: 493–521.

Gilmore JA, Du J, Tao J, Peter AT, Critser JK. Osmotic properties of boar spermatozoa and their relevance to cryopreservation. *J Reprod Fertil*. 1996;107:87-95.

Gilmore JA, Liu J, Gao DY, Critser JK. Determination of optimal cryoprotectants and procedures for their addition and removal from human spermatozoa. *Hum Reprod*. 1997;12:112-118.

Gilmore JA, Liu J, Peter AT, Critser JK. Determination of plasma membrane characteristics of boar spermatozoa and their relevance to cryopreservation. *Biol Reprod*. 1998;58:28-36.

Gilmore JA, McGann LE, Liu J, Gao DY, Peter AT, Kleinhans FW, Critser JK. Effect of cryoprotectant solutes on water permeability of human spermatozoa. *Biol Reprod*. 1995;53:985-995.

Guthrie HD, Liu J, Critser JK. Osmotic tolerance limits and effects of cryoprotectants on motility of bovine spermatozoa. *Biol Reprod*. 2002;67:1811-1816.

Kedem O, Katchalsky A. Thermodynamic analysis of the permeability of biological membranes to non-electrolytes. *Biochim Biophys Acta*. 1958;27:229-246.

Liu Z, Foote RH. Osmotic effects on volume and motility of bull sperm exposed to membrane permeable and nonpermeable agents. *Cryobiology*. 1998;37:207-218.

Mazur P. Freezing of living cells: mechanisms and implications. *Am J Physiol*. 1984;247:C125-142.

Meyers SA. Spermatozoal response to osmotic stress. *Anim Reprod Sci*. 2005;89:57-64.

Nakatsukasa E, Inomata T, Ikeda T, Shino M, Kashiwazaki N. Generation of live rat offspring by intrauterine insemination with epididymal spermatozoa cryopreserved at -196 degrees C. *Reproduction*. 2001;122:463-467.

Nakatsukasa E, Kashiwazaki N, Takizawa A, Shino M, Kitada K, Serikawa T, Hakamata Y, Kobayashi E, Takahashi R, Ueda M, Nakashima T, Nakagata N. Cryopreservation of spermatozoa from closed colonies, and inbred, spontaneous mutant, and transgenic strains of rats. *Comp Med*. 2003;53:639-641.

Phelps MJ, Liu J, Benson JD, Willoughby CE, Gilmore JA, Critser JK. Effects of Percoll separation, cryoprotective agents, and temperature on plasma membrane permeability characteristics of murine spermatozoa and their relevance to cryopreservation. *Biol Reprod*. 1999;61:1031-1041.

Pommer AC, Rutllant J, Meyers SA. The role of osmotic resistance on equine spermatozoal function. *Theriogenology*. 2002;58:1373-1384.

Rutllant J, Pommer AC, Meyers SA. Osmotic tolerance limits and properties of rhesus monkey (Macaca mulatta) spermatozoa. *J Androl*. 2003;24:534-541.

Tada N, Sato M, Yamonoi J, Mizorgi T, Kasai K, Ogawa S. Cryopreservation of mouse spermatozoa in the presence of raffinose and glycerol. *J Reprod Fertil*. 1990;89:511-516.

Tesson L, Cozzi J, Menoret S, Remy S, Usal C, Fraichard A, Anegon I. Transgenic modifications of the rat genome. *Transgenic Res*. 2005;14:531-546.

Thornton CE, Brown SD, Glenister PH. Large numbers of mice established by in vitro fertilization with cryopreserved spermatozoa: implications and applications for genetic resource banks, mutagenesis screens, and mouse backcrosses. *Mamm Genome*. 1999;10:987-992.

Walters EM, Men H, Agca Y, Mullen SF, Critser ES, Critser JK. Osmotic tolerance of mouse spermatozoa from various genetic backgrounds: acrosome integrity, membrane integrity, and maintenance of motility. *Cryobiology*. 2005;50:193-205.

Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod Fertil Dev*. 1995;7:871–891.

Willoughby CE, Mazur P, Peter AT, Critser JK. Osmotic tolerance limits and properties of murine spermatozoa. *Biol Reprod*. 1996;55:715-727.

Yokoyama M, Akiba H, Katsuki M, Nomura T. Production of normal young following transfer of mouse embryos obtained by in vitro fertilization using cryopreserved spermatozoa. *Exp Anim*. 1990;39:125-128.

Zhou Q, Renard JP, Le Friec G, Brochard V, Beaujean N, Cherifi Y, Fraichard A, Cozzi J. Generation of fertile cloned rats by regulating oocyte activation. *Science*. 2003;302:1179.

Figure Legends

Figure 1. Bolyle-van't Hoff plot of Fischer 344 rat (Figure 2A) and Sprague Dawley rat (Figure 2B) spermatozoa derived from equilibration volumes (mean ± SEM) exposed to DPBS solutions of seven different osmolalities: 260, 290, 375 and 450 mOsm. The *y* intercept indicates the osmotically inactive water volume (V_b), which are 79.8% and 81.4% of the isosmotic volume of Fischer 344 rat and Sprague Dawley rat sperm respectively.

Figure 2. The percent normalized progressive motility (mean ± SEM) of spermatozoa from Fischer 344 rat (Figure 3A) and Sprague Dawley rat (Figure 3B) that were abruptly exposed to different osmotic conditions and abruptly returned to isosmotic conditions.

Figure 3. The percent normalized membrane and acrosome integrity (mean ± SEM) of spermatozoa from Fischer 344 rat (Figure 4A) and Sprague Dawley rat (Figure 4B) that were abruptly exposed to different osmotic conditions and abruptly returned to isosmotic conditions.

Figure 4. The percent normalized progressive motility (mean ± SEM) of spermatozoa from Fischer 344 rat (Figure 5A) and Sprague Dawley rat (Figure 5B) exposed to 1 M glycerol, dimethyl sulfoxide, ethylene glycol, or propylene glycol, and after dilution. *Significantly different than the isosmotic DPBS solution treated sperm.

Figure 5. The percent normalized plasma membrane integrity and acrosome integrity (mean ± SEM) of spermatozoa from Fischer 344 rat (Figure 6A) and Sprague Dawley rat (Figure 6B) exposed to 1 M glycerol, dimethyl sulfoxide, ethylene glycol, or propylene

glycol, and after dilution. *Significantly different than the isosmotic DPBS solution treated sperm.

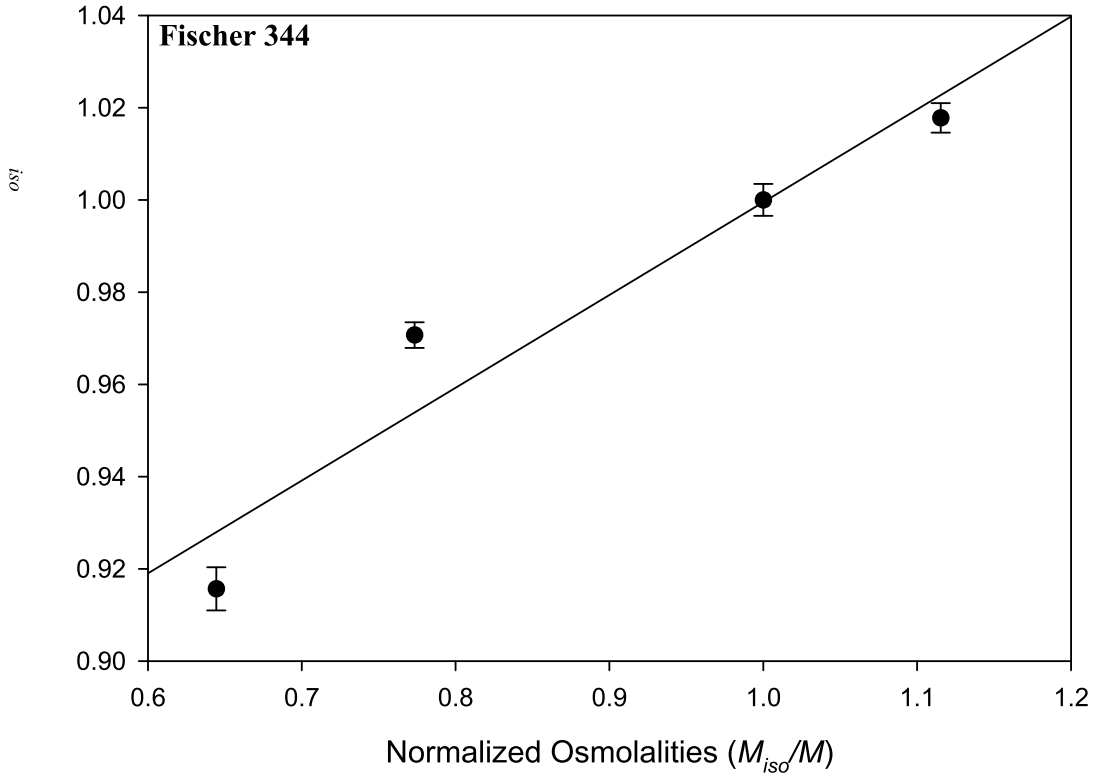

Fischer 344
1.04
1.02
1.00
0.98
0.96
0.94
0.92
0.90
0.6
0.7
0.8
0.9
1.0
1.1
1.2
Normalized Osmolalities (M_{iso}/M)
iso

Fig. 1A

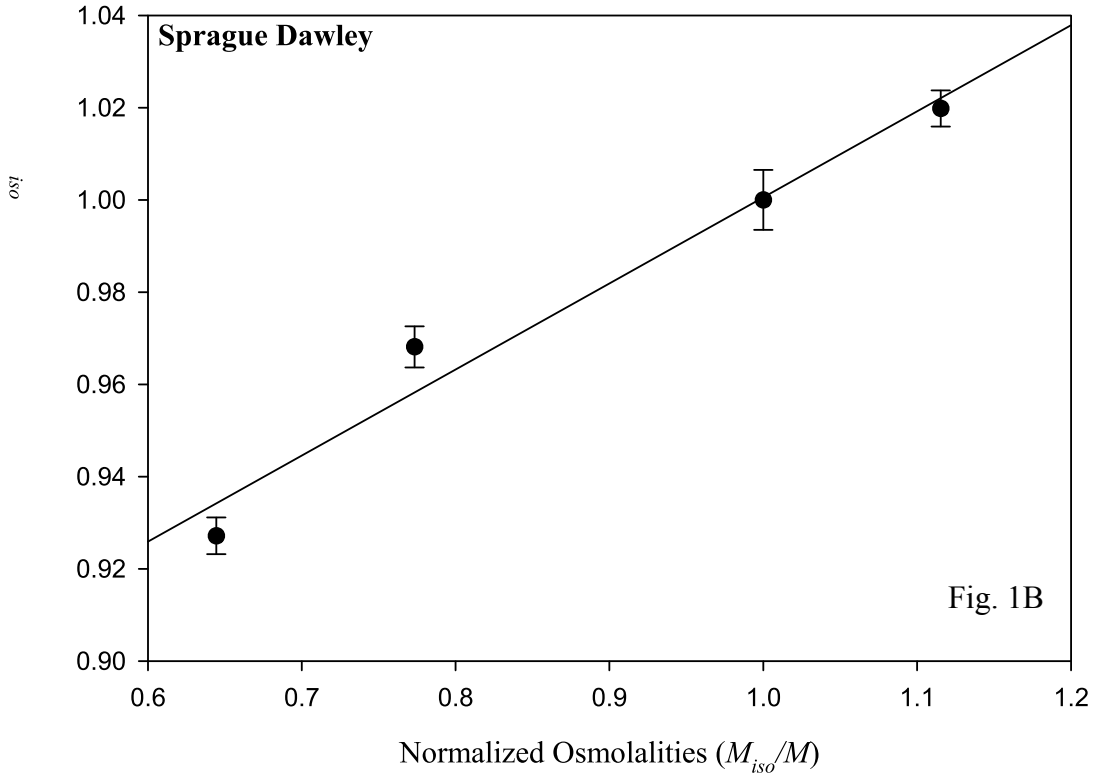

Sprague Dawley
1.04
1.02
1.00
0.98
0.96
0.94
0.92
0.90
0.6
0.7
0.8
0.9
1.0
1.1
1.2
Normalized Osmolalities (M_{iso}/M)
Fig. 1B

Fig. 2A

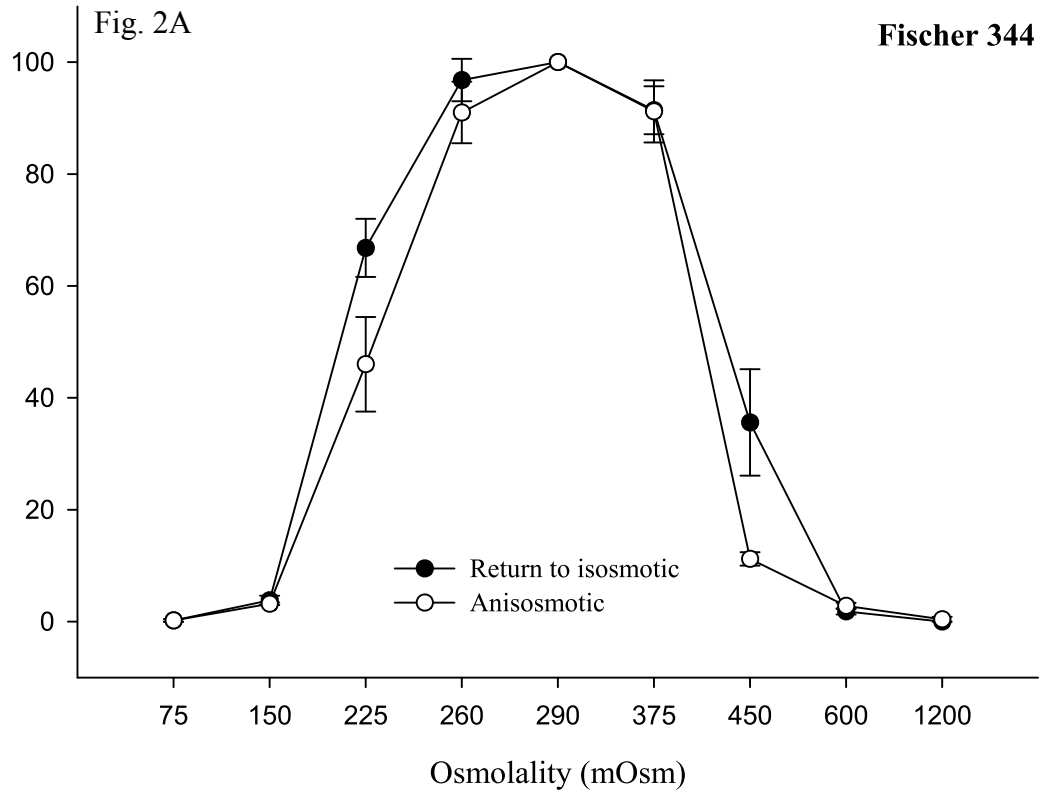
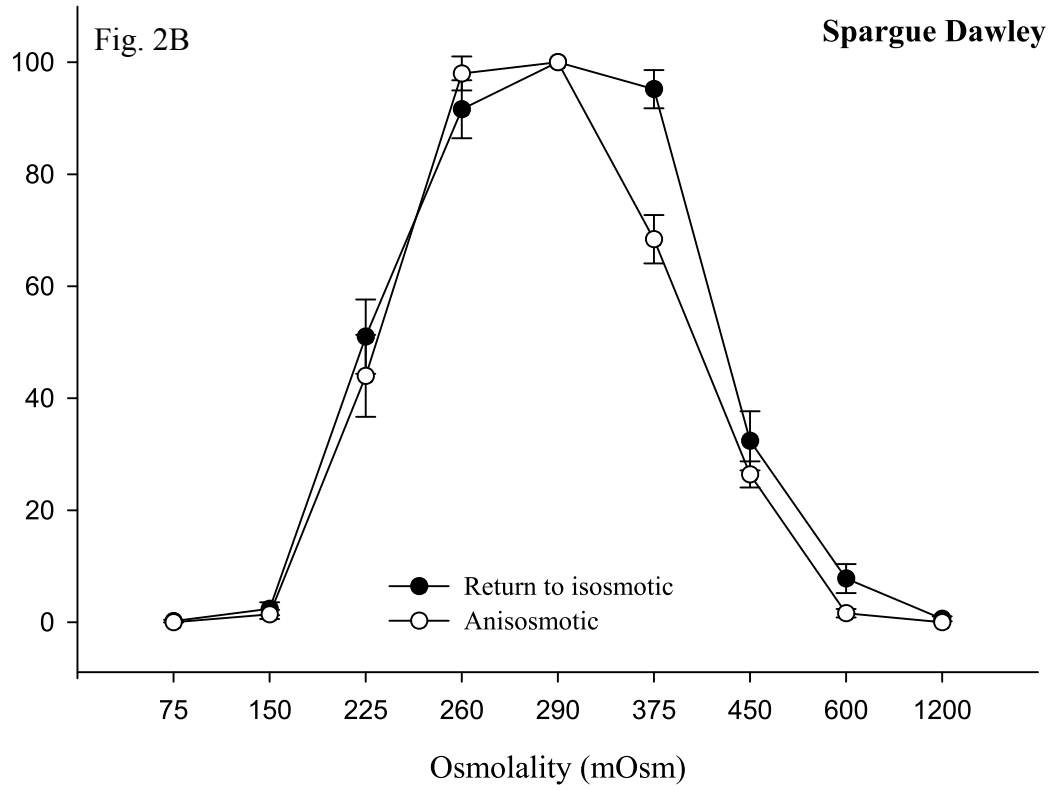

Fischer 344
100
80
60
40
20
0
Return to isosmotic
Anisosmotic
75
150
225
260
290
375
450
600
1200
Osmolality (mOsm)

Fig. 2B


Spargue Dawley
100
80
60
40
20
0
Return to isosmotic
Anisosmotic
75
150
225
260
290
375
450
600
1200
Osmolality (mOsm)

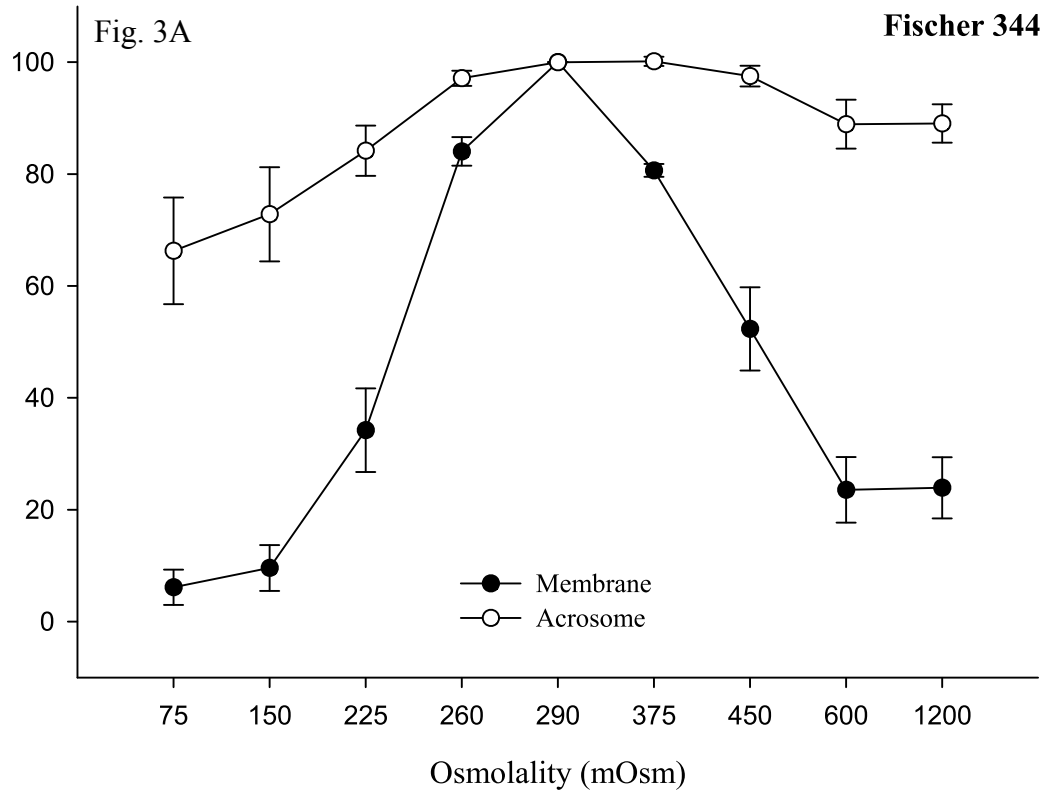


Fig. 3A
Fischer 344
100
80
60
40
20
0
Membrane
Acrosome
75
150
225
260
290
375
450
600
1200
Osmolality (mOsm)

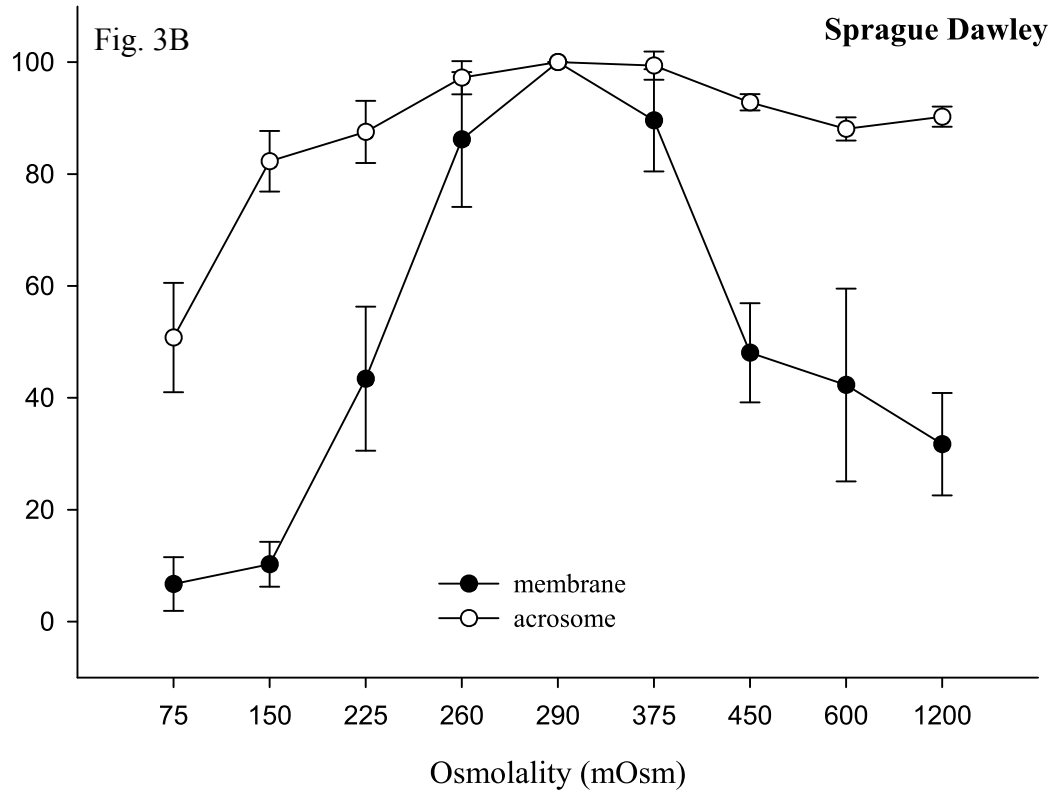


Fig. 3B
Sprague Dawley
100
80
60
40
20
0
membrane
acrosome
75
150
225
260
290
375
450
600
1200
Osmolality (mOsm)

Fig. 4A

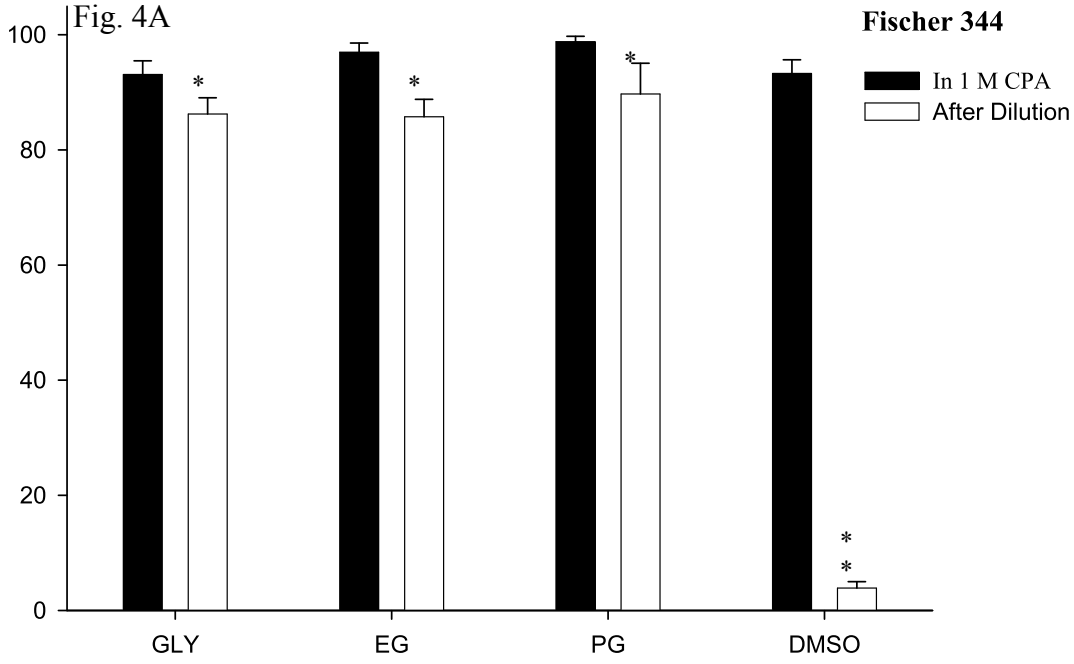

Fischer 344
In 1 M CPA
After Dilution
100
80
60
40
20
0
GLY
EG
PG
DMSO

Fig. 4B

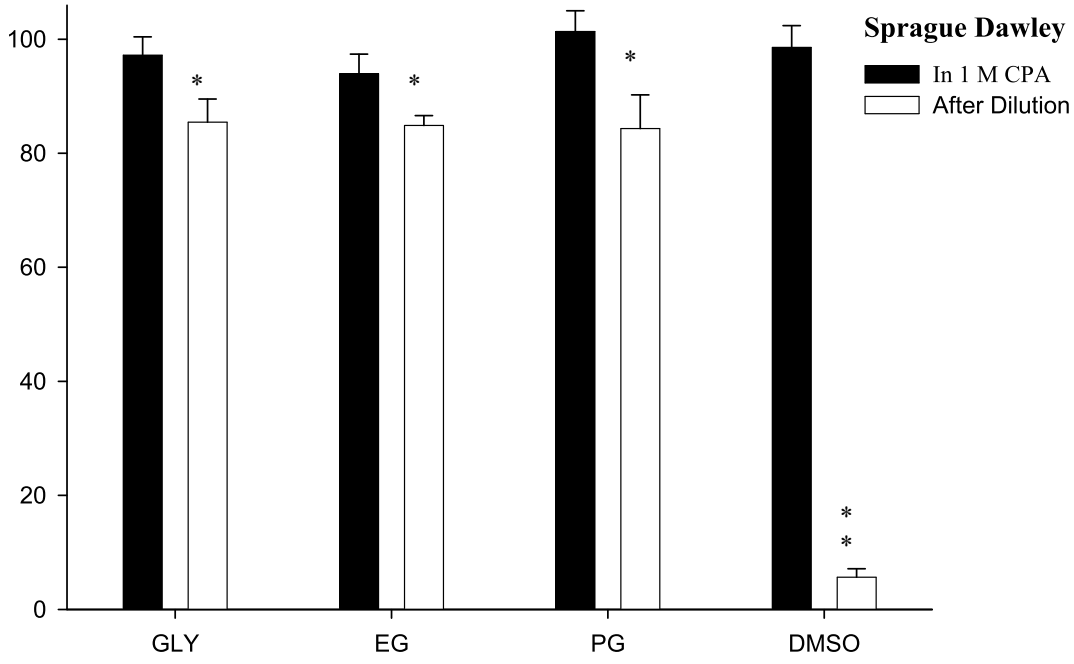

Sprague Dawley
In 1 M CPA
After Dilution
100
80
60
40
20
0
GLY
EG
PG
DMSO

Fig. 5A

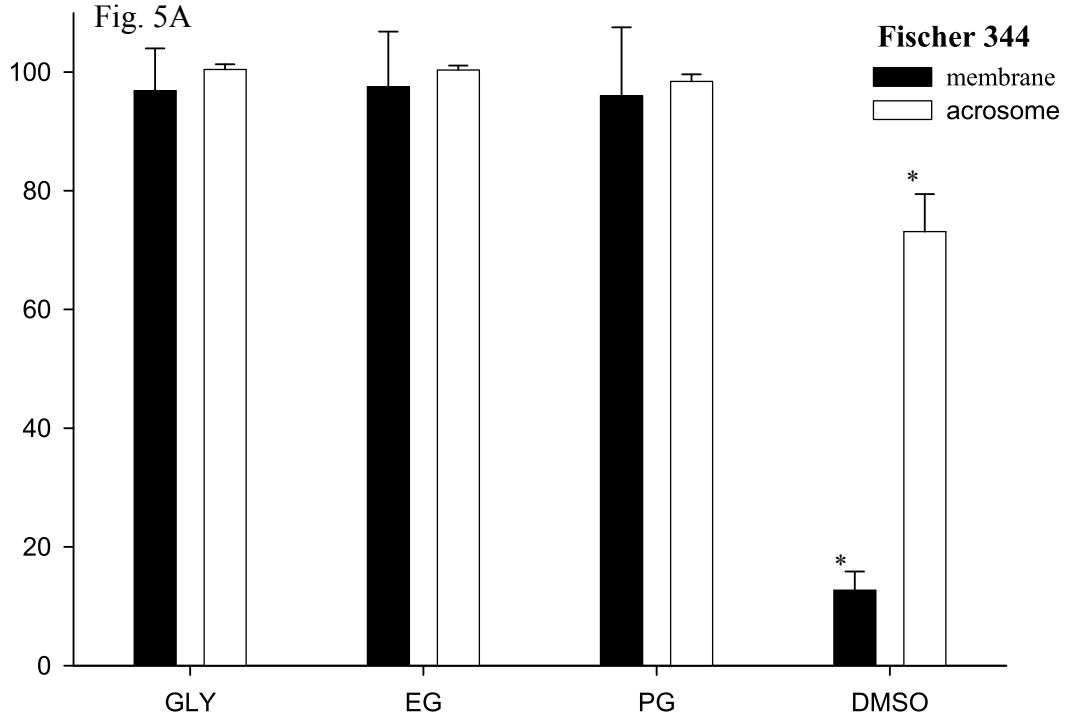
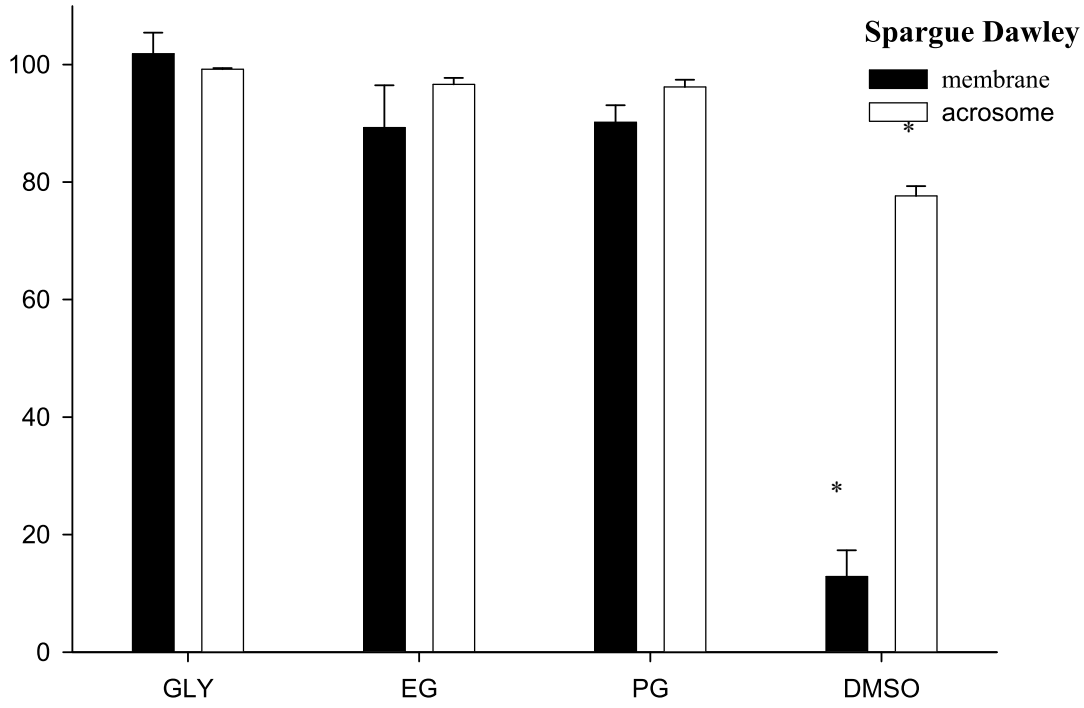

Fischer 344
membrane
acrosome
100
80
60
40
20
0
*
*
GLY
EG
PG
DMSO

Fig. 5B


Spargue Dawley
membrane
acrosome
100
80
60
40
20
0
*
*
GLY
EG
PG
DMSO