Toxicity of glycerol for the stallion spermatozoa: Effects on membrane integrity and cytoskeleton, lipid peroxidation and mitochondrial membrane potential

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Abstract

Glycerol is, to date, the most widely used cryoprotectant to freeze stallion spermatozoa at concentrations between 2% and 5%. Cryoprotectant toxicity has been claimed to be the single most limiting factor for the success of cryopreservation. In order to evaluate the toxic effects of the concentrations of glycerol used in practice, stallion spermatozoa were incubated in Biggers Whitten and Whittingham (BWW) media supplemented with 0%, 0.5%, 1.5%, 2.5%, 3.5%, and 5% glycerol. In two additional experiments, a hyposmotic (75 mOsm/kg) and a hyperosmotic (900 mOsm/kg) control media were included. Sperm parameters evaluated included cell volume, membrane integrity, lipid peroxidation, caspase 3, 7, and 8 activation, mitochondrial membrane potential, and integrity of the cytoskeleton. Glycerol exerted toxicity at concentrations ≥ 3.5% and the maximal toxicity was observed at 5%. The actin cytoskeleton was especially sensitive to glycerol presence, inducing rapid F actin depolymerization at concentrations over 1.5%. The sperm membrane and the mitochondria were other structures affected. The toxicity of glycerol is apparently related to osmotic and nonosmotic effects. In view of our results the concentration of glycerol in the freezing media for stallion spermatozoa should not surpass 2.5%.

Keywords: Stallion; Sperm; Glycerol; Actin cytoskeleton; Toxicity; Cryopreservation

1. Introduction

The equine industry is experiencing a steadily increasing interest in reproductive technologies [1,2]. Among them, sperm freezing is considered an especially interesting tool to increase international commerce of semen, and/or to preserve genetics from superior stallions [3,4]. In the near future, the probable introduction in the market of both refrigerated and frozen sexed-spermatozoa stresses the need of further research in stallion sperm cryobiology. It is remarkable that procedures to freeze mammalian spermatozoa were developed quasi-empirically nearly 60 years ago, and yet are essentially the same in use today [5–7]. Damage

during cryopreservation occurs according to the two factors hypothesis [8]; this theory states that cells cooled too rapidly are killed by formation of intracellular ice, while cells cooled too slowly are killed by long exposure to concentrated solutions resulting from the progressive phase-conversion of liquid water to ice [9]. To avoid this latter effect, cell permeable cryoprotectants (CPA) are incorporated in freezing extenders. Glycerol is such a CPA, which exerts its action retaining water within the cell, thus avoiding excessive dehydration due to the exposure to concentrated solutions. The higher the CPA concentration, the higher the protective effect [10]. On the other hand, CPAs as glycerol are considered toxic, a toxicity that has been claimed as being the single most limiting factor for the success of cryopreservation [11] and appears to be equally dose- and temperature-dependent.

In the equine species, semen is frozen using glycerol at concentrations ranging from 2% to 5% [4,12–14]. However, very few controlled studies have addressed the toxicity of different concentrations of glycerol [15–17]. The efficacy of CPAs is almost always based on comparing the prefreeze sperm motility with the immediate postthaw motility; an approach that does not consider the intrinsic damage that the CPA may have caused during the process. A CPA is ideal when, by penetrating the plasmalemma, does not dehydrate the cell osmotically, or stimulate freezing injury while not being toxic on substructures/functions at concentrations needed to prevent excessive dehydration, e.g., causing osmotic effects [18,19]. Noteworthy, has been pointed out that while CPA toxicity is well recognized in vitrification research, there is probably less awareness of the relevance of CPA toxicity on the part of those that use freezing as their method of cryopreservation [11].

Recent evidence [20] indicates that the main factor explaining cryoinjury in the equine species is an osmotic imbalance at thawing. This fact stresses the importance of minimizing cryoinjury by using a CPA that rapidly penetrates the plasmalemma during cooling and thawing. In addition, it has been proposed that damage after freezing and thawing can, in certain cases, be highly correlated with the concentration of permeating CPAs during freezing and thawing [21]. Therefore, to understand and significantly reduce freezing injury during cryopreservation of stallion spermatozoa in particular, we need to increase our knowledge on CPA toxicity.

The aim of this study was to evaluate the intrinsic toxicity of glycerol establishing a dose-related toxicity for equine spermatozoa.

2. Materials and methods

2.1. Reagents

Ethidium homodimer, 5,5=,6,6=–tetrachloro-1,1=,3,3= tetraethylbenzymidazolyl carbocianyne iodine (JC-1), Yo-Pro-1, BODIPY 581/591 C_{11}, and the caspase fluorescein isothiocyanate (FITC)-DEVD-FMK (caspase 3 and 7), and FITC-LETD-FMK (caspase 8) in situ markers were from Molecular Probes (Molecular Probes, Leiden, the Netherlands), PureSperm was from Nidacon, Mölndal, Sweden, FITC labeled phalloidin and all other reagents unless otherwise stated were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Semen collection

Semen (four ejaculates per stallion) was obtained from four Pure Spanish Stallions (PRE) individually housed at the Veterinary Teaching Hospital of the University of Extremadura, Cáceres, Spain. The stallions were maintained according to institutional and European regulations, and ejaculates were collected on a regular basis (two collections per week) during the 2010 breeding season, using a Missouri model artificial vagina with an inline filter to separate the gel fraction, lubricated and prewarmed to 45 °C to 50 °C. The collected ejaculate was immediately transported to the laboratory for evaluation and processing.

2.3. Semen processing

For each stallion, aliquots of 500×10^6 total spermatozoa were centrifuged at 800 × g for 10 min at room temperature in 35% colloid (PureSperm) suspension in order to remove contaminating cells and debris. Seminal plasma was then discarded and the sperm pellet resuspended to 100×10^6 spermatozoa in Biggers Whitten and Whittingham (BWW) media (91.06 mM NaCl, 4.78 mM KCL, 2.44 mM Mg SO_4, 1.17 mM KPO4, 21.0 mM HEPES, 5.5 mM glucose (anhydrous), 0.25 mM sodium pyruvate, 1.71 mM lactic acid hemicalcium salt, and 21.55 mM sodium lactate) at 320 mOsm/kg and pH 7.4. All media were filtered through a 0.45 μm filter. Aliquots of semen were extended in BWW media supplemented with 0% (control), 1.5%, 2.5%, 3.5%, and 5% glycerol vol/vol. Samples were incubated for up to 60 min at 37°C and then aliquots were taken for cell volume measurement and flow cytometry analysis. Albeit equine spermatozoa are customarily exposed to glycerol at 22 °C, we have chosen incubation at 37 °C, as done previously with human spermatozoa [22]. The rationale behind included the assurance of a maximal permeability of the stallion

spermatozoa to glycerol, to minimize osmotic shock and to better manifest the intrinsic toxicity of the CPA, separating osmotic effects from the intrinsic toxicity of glycerol [16]. Moreover, the major damage during cryopreservation is seen during thawing [20] and final observation of motility postthaw is customarily done at 37 °C to 38 °C. The osmolarities of the media were 320, 388, 492, 670, 750, and 1300 mOsm/kg, respectively, for final concentrations of glycerol of 0%, 0.5%, 1.5%, 2.5%, 3.5%, and 5%. All osmolarities were checked using a freeze-point digital micro-osmometer Type 5 R (Hermann Roebling Messtechnik, Berlin, Germany).

2.4. Determination of cellular volume

Cellular volume was measured using a Multisizer 3 Coulter Counter (Coulter Corporation, Inc., Miami, FL, USA) calibrated according to the Beckman Coulter Particle Characterization assay sheet (aperture Instrument Standard L5 for an aperture tube size of 50 μM reference 6 602 794 and calibration verified using standards L3, L5, and L10 references 6 602 793, 6 602 794, and 6 602 797), following the instructions of the manufacturer. Spermatozoa were suspended in BWW media and drawn through a cylindrical opening (2 to 60 μm) separating two electrodes between which a small electrical current flows. As each particle passes through the aperture, it displaces its own volume of conducting liquid, momentarily increasing the impedance of the aperture. This change produces a pulse that is digitally processed in real time and that is proportional to the tridimensional volume of the particle that produced it. The analysis of the pulses was digitally converted to volume (μm^3). For each sample 30 000 events were analyzed and, if present, particles below six and above 60 μm^3 were discarded.

2.5. Assessment of subtle sperm membrane changes and viability

Early sperm membrane changes and viability were determined as described in Peña, et al. [23] with modifications for adaptation to the equine species [22,24]. In brief, 1 mL of sperm suspension (5×10^6 spermatozoa/mL) was loaded with 3 μL of Yo-Pro-1 (25 μM) and 1 μL of ethidium homodimer-1 (1.167 mM) which was—after thorough mixing—incubated at 37 °C in the dark for 16 min. This staining distinguishes four sperm subpopulations. The first is the subpopulation of unstained spermatozoa. These spermatozoa are considered alive and without any membrane alteration. Another sperm subpopulation consists of Yo-Pro-1-positive cells emitting green fluorescence. It is has been demonstrated that in the early stages of apoptosis there is a modification of membrane permeability that selectively allows entry of some nonpermeable DNA-binding molecules [25]. This subpopulation groups spermatozoa which may show a shift to another physiological state or early damage, because membranes become slightly permeable during the first steps of injury, enabling Yo-Pro-1 but not ethidium homodimer to penetrate the plasma membrane [26]. None of these probes enters intact cells. Finally, two subpopulations of necrotic spermatozoa were easily detected, early necrotic, spermatozoa stained both with Yo-Pro-1 and ethidium homodimer (emitting both green and red fluorescence), and late necrotic spermatozoa, cells stained only with ethidium homodimer (emitting red fluorescence).

2.6. Staining for detection of lipid peroxidation

Lipid peroxidation (LPO) was measured using the probe BODIPY 581/591 C_{11}. A suspension of 2×10^6 spermatozoa/mL was loaded with the probe at a final concentration of 2 μM. The spermatozoa were then incubated at 37 °C for 30 min, washed by centrifugation to remove the unbound probe, and analyzed using a flow cytometer. Positive controls were obtained after addition of 80 μM ferrous sulfate to additional sperm suspensions [27].

2.7. Detection of active caspases 3, 7, and 8

The caspase FITC-DEVD-FMK (caspase 3 and 7) and FITC-LETD-FMK (caspase 8) in situ markers were used to detect active caspases. These cell-permeable specific caspase inhibitor peptides are conjugated to FITC and binds covalently to active caspases 3, 7, and 8 serving as in situ markers for apoptosis. A sample of 5×10^6 spermatozoa were suspended in 1 mL of PBS, and—after adding 1 μL of FITC-DEVD-FMK or FITC-LETD-FMK (5 mM)—the suspensions were incubated at room temperature (22 °C to 25 °C) in the dark for 20 min. After incubation, the spermatozoa were washed with PBS, followed by the addition of 1 μL of ethidium homodimer (1.167 mM) (Molecular Probes) to detect membrane damage. Flow cytometry and fluorescence microscopy were conducted within 10 min.

2.8. Evaluation of mitochondrial membrane potential (ΔTm)

The lipophilic cationic compound JC-1 (Molecular Probes) has the unique ability to differentially label mitochondria with low and high membrane potential. In

mitochondria with high membrane potential, JC-1 forms multimeric aggregates emitting in the high orange wavelength of 590 nm, when excited at 488 nm. At the same excitement wavelength (488 nm), however, JC-1 forms monomers within mitochondria with low membrane potential, emitting in the green wavelength (525 to 530 nm). For staining,a3 mM stock solution of JC-1 was prepared in DMSO. From each sperm suspension, 5×10^6 spermatozoa were placed in 1 mL of PBS and stained with 0.5 µL JC-1 stock solution. The samples were incubated at 38 °C in the dark for 40 min before flow cytometric analysis.

2.9. Fluorescence staining of actin filaments

The protocol for fluorescence staining of actin filaments was modified after protocols previously published both for spermatozoa [28] and somatic cells [29]. Samples were washed by centrifugation at 800 × g for 30 sec, seminal plasma was removed and corresponding aliquots were resuspended in BWW and incubated up to 1 h at 37 °C. To each aliquot glycerol was added at a final concentration (vol:vol) of 0%, 0.5%, 1.5%, 2.5%, 3.5%, and 5%, respectively, additionally a hyperosmotic (900 mOsm/kg) and a hyposmotic (75 mOsm/kg) control was also included. Spermatozoa were then fixed in 4% formaldehyde in PBS for 15 min at room temperature and cells were washed twice in PBS (800 × g for 30 sec), and permeabilized using 0.2% (vol/vol) Triton X-100 solution in PBS for 5 min at room temperature. After permeabilization cells were again washed in PBS, a blocking solution (5% BSA in PBS) added, and cells further incubated for 30 min at room temperature. Finally, cells were washed in PBS and stained with 1 µM FICT-labeled phalloidin (stock solution made in 1% BSA [vol/wt] in PBS) and incubated for 60 min at room temperature in the dark. After incubation the cells were collected by centrifugation at 800 × g for 30 sec and resuspended in PBS adjusting the cell concentration to 100×10^6 spermatozoa per mL. Fifteen microliters of the sperm suspension were spread on poly-l-lysine coated slides and examined with a confocal microscope.

2.10. Flow cytometry

Flow cytometric analyses were carried out with a Coulter EPICS XL (Coulter Corporation, Inc.) equipped with standard optics, an argon-ion laser (Cyonics, Coherent, Santa Clara, CA, USA) performing 15 mW at 488 nm and EXPO 2000 software. Subpopulations were divided by quadrants, and the frequency of each subpopulation was quantified. Nonsperm events (debris) were gated out based on the forward scatter and side scatter dot-plot by drawing a region enclosing the cell population of interest. Forward and sideways light scatter were recorded for a total of 10 000 events per sample. Samples were measured at flow rate of 200 to 300 cells per sec. Green fluorescence was detected in FL1 (525 nm band pass filter), red fluorescence was detected in FL3 (620 nm band pass filter), and orange fluorescence in FL2 (570 nm band pass filter).

2.11. Confocal laser microscopy

Sperm samples were observed under a spectral inverted confocal laser microscope (Bio Rad MRC 1024, Hercules, CA, USA). The localization of the FICT-labeled phalloidin emission was at 510 nm. Samples were excited with an argon ion laser emitting at 488 nm.

2.12. Statistical analysis

The main effects considered were time of incubation and concentration of glycerol, the interaction of both effects was not significant. Comparisons were made using multivariate analysis of variance using one-way ANOVA models followed by Tukey's post test, when data were distributed normally. When data were not normally distributed the nonparametric Mann-Whitney *U* test was used to directly compare pairs of values. Analyses were performed using SPSS Version 15.0 for Windows (SPSS, Inc., Chicago, IL, USA). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect of glycerol on sperm volume

Low concentrations of glycerol had no effect on sperm volume, but when spermatozoa were exposed to concentrations of glycerol of 2.5%, a significant increase in sperm volume was observed ($P < 0.05$). At this concentration, the osmolality of the solution was 670 mOsm/kg. After 1 h of incubation, cell volume was also significantly increased by a concentration of 1.5% glycerol in the media (Table 1).

3.2. Effect of glycerol on subtle sperm membrane changes and viability

A significant decrease ($P < 0.05$) in sperm membrane integrity was observed when spermatozoa were incubated in presence of 5% glycerol (Table 2). The effect was already evident at the beginning of the incubation period. This impairment in the integrity of the

Table 1
Cell volume in mm^3 (mean ± SD) of stallion spermatozoa incubated in BWW media supplemented with different concentrations of glycerol.

	Glycerol					
	0%	0.5%	1.5%	2.5%	3.5%	5%
T0	21.3 ± 2.30	21.9 ± 2.94**	21.9 ± 2.37**	23.6 ± 2.65*	23.5 ± 2.63**	22.0 ± 2.60**
T1	22.1 ± 3.45	23.1 ± 3.56**	24.6 ± 4.18*	24.8 ± 3.57*	24.3 ± 2.70**	24.5 ± 2.50**

The osmolality of each solution was, respectively 322, 388, 492, 670, 750 and 1300 mOsm/kg.
BWW, Biggers Whitten and Whittingham; T0, measurement after 10 min of incubation at 37 °C; T1, measurement after 60 min of incubation at 37 °C.
Within a row values differ statistically; * $P < 0.05$; ** $P < 0.01$.

membrane was due to an increase in the percentage of necrotic (ethidium+) spermatozoa, because neither the percentages of early (Yo-Pro+/Eth—) nor late apoptotic (Yo-Pro+/Eth+) increased.

3.3. Lipid peroxidation

Lipid peroxidation was low (below 2%) in all the samples studied at any time of incubation (Table 3) and exposure of stallion spermatozoa to glycerol did not change the percentage of spermatozoa showing peroxidation of their membrane lipids.

3.4. Activation of caspases 3, 7, and 8

Because it has been demonstrated that cryopreservation induces caspase activity [30], and in order to determine whether this effect is attributable to the intrinsic toxicity of glycerol or to osmotic stress, spermatozoa were incubated in presence of glycerol and also in a hypo- or hyperosmotic solution. Glycerol did not induce activation of any of the caspases studied. The only change observed in our study was a significant increase in the activation of caspase 8 and caspase 3 and 7 in dead (ethidium+) cells when spermatozoa were incubated at 900 mOsm/kg (Table 4).

3.5. Effect of glycerol on mitochondrial membrane potential (ΔTm)

Incubation of spermatozoa in glycerol supplemented BWW produced a significant increase in the percentage of sperm mitochondria showing high ΔTm, when exposed to 2.5% glycerol compared with 5%, however this was not statistically different from the control (Table 5).

3.6. Effect of glycerol on sperm actin cytoskeleton

Previous research has demonstrated that hypertonic shock induces a reorganization of the actin cytoskeleton [28] so we decided to study if glycerol may cause a similar effect. For this we incubated stallion spermato-

Table 2
Membrane integrity (mean % ± SD) and early membrane changes of stallion spermatozoa incubated in BWW media supplemented with different concentrations of glycerol.

	Glycerol					
	0%	0.5%	1.5%	2.5%	3.5%	5%
Live						
T0	64.8 ± 6.44[a]	62.9 ± 5.51[a]	61.6 ± 9.86[a]	61.7 ± 7.88[a]	58.4 ± 8.49[a]	52.2 ± 12.50[b]
T1	62.8 ± 7.78[a]	61.6 ± 9.86[a]	61.4 ± 10.73[a]	58.5 ± 8.49[a]	58.2 ± 11.60[a]	49.9 ± 11.26[b]
Yo-Pro+						
T0	5.5 ± 6.79	4.3 ± 3.19	4.5 ± 4.92	3.6 ± 3.81	3.0 ± 2.47	4.7 ± 5.72
T1	5.0 ± 6.30	2.3 ± 2.48	2.6 ± 2.57	3.4 ± 3.38	3.3 ± 2.84	3.1 ± 2.83
Yo-Pro+Eth+						
T0	5.9 ± 3.87	4.8 ± 2.30	4.7 ± 3.27	4.7 ± 2.92	4.3 ± 3.15	5.5 ± 4.56
T1	5.9 ± 5.46	3.5 ± 2.38	6.2 ± 10.11	4.6 ± 3.39	4.6 ± 3.22	4.5 ± 2.90
Eth+						
T0	23.7 ± 6.51[a]	27.8 ± 4.83[a]	27.3 ± 9.39[a]	29.9 ± 4.83[a,b,c]	34.1 ± 7.47[b,a]	37.6 ± 11.43[b]
T1	26.3 ± 8.06[a]	32.5 ± 7.92[a]	29.7 ± 10.31[a]	34.6 ± 7.85[a,b]	33.9 ± 8.69[b]	42.5 ± 10.79[b]

Within a row values with different superscripts differ statistically; [a-c] $P < 0.05$. The osmolality of each solution was, respectively 320, 388, 492, 670, 750, and 1300 mOSm/Kg.
BWW, Biggers Whitten and Whittingham; T0, measurement after 10 min of incubation at 37 °C; T1, measurement after 60 min of incubation at 37 °C.

Table 3
Lipid peroxidation (LPO) (mean ± SD) of stallion spermatozoa incubated in BWW media supplemented with different concentrations of glycerol.

	Glycerol					
	0%	0.5%	1.5%	2.5%	3.5%	5%
LPO-T0	1.7 ± 1.75	1.0 ± 0.74	1.2 ± 1.69	0.8 ± 0.50	0.6 ± 0.49	0.8 ± 0.67
LPO-T1	2.0 ± 1.85	1.2 ± 1.07	1.1 ± 1.02	1.1 ± 1.16	1.0 ± 0.85	1.3 ± 1.59

The osmolality of each solution was, respectively 320,388, 492, 670, 750, and 1300 mOSm/Kg.
BWW, Biggers Whitten and Whittingham; T0, measurement after 10 min of incubation at 37°C; T1, measurement after 60 min of incubation at 37°C.

zoa in increasing concentrations of glycerol and also in hypotonic alternatively hypertonic solutions. Glycerol resulted in a concentration-dependent dramatic decrease in actin-F, while hypertonicity resulted in a redistribution of actin-F fluorescence toward the post-acrosomal region (Fig. 1).

4. Discussion

In this study, we determined the effect of glycerol on the physiology of stallion spermatozoa, in order to characterize its toxic concentration at maximal temperature (37 °C) and also to investigate potential mechanisms of sperm damage. Glycerol was toxic to stallion spermatozoa when present at concentrations ≥ 3.5%, being especially detrimental for the sperm membrane. The osmolality of the solution containing 3.5% glycerol was 670 mOsm/kg, indicating that stallion spermatozoa were very sensitive to glycerol considering human spermatozoa started to experience cellular damage when the osmolality of the solution containing this cryoprotectant was above 3000 mOsm/kg [16]. This fact may indicate that, for the stallion spermatozoa, glycerol toxicity is largely independent of the osmotic effects, in fact previous results from our laboratory confirm this statement, because the stallion spermatozoa were resistant to osmolalities of 900 mOsm/kg [31].

Table 4
Caspase 3, 7, and 8 activation (mean % ± SD) of stallion spermatozoa incubated for 1 h at 37 °C in BWW media supplemented with different concentrations of glycerol.

Treatment	Caspase 8 + dead cells	Caspase 3 and 7+ dead cells
Control	41.9 ± 12.81[a]	21.7 ± 12.64[a,b]
Glycerol 0.5%	33.3 ± 15.59[a]	26.4 ± 22.56[a,b]
Glycerol 1.5%	42.1 ± 14.87[a]	25.9 ± 15.89[a,b]
Glycerol 2.5%	37.4 ± 18.40[a]	23.6 ± 10.47[a,b]
Glycerol 3.5%	30.1 ± 15.71[a]	37.8 ± 14.08[a,b]
Glycerol 5%	40.6 ± 13.98[a]	15.71 ± 17.50[b]
Control 75 mOsm/kg	47.8 ± 8.74[a]	18.2 ± 16.33[b]
Control 900 mOsm/kg	68.0 ± 17.88[b]	41.5 ± 23.52[a]

Within a column values with different superscripts differ statistically; [a,b] $P < 0.01$. The osmolality of each solution was, respectively 388, 492, 670, 750, and 1300 mOSm/Kg. A hyposmotic control (75 mOsm/kg) and a hypertonic control (900 mOsm/kg) were also included.
BWW, Biggers Whitten and Whittingham.

Glycerol may induce cellular damage through two distinct mechanisms, either a physical-osmotic effect and/or a biochemical one [18,19]. The permeability of the sperm membrane to glycerol will control the osmotic damage, and glycerol is considered less permeable than other CPAs, such as amides [32]. To determine in our experiment whether the major damage induced by glycerol was of osmotic nature, we measured the osmolality of the solutions containing glycerol and the cellular volume of the spermatozoa incubated in each concentration of the cryoprotectant. Low concentrations of glycerol did not induce changes in sperm volume, but glycerol at 2.5% after 10 min and 1.5% after 60 min of incubation induced an increase of sperm volume. Values of glycerol above 2.5% did not induce change in cell volume; this effect may be related to the low permeability of glycerol resulting in similar osmolalities across the cell membrane. The osmolality of the solutions containing 1.5% and 2.5% glycerol were, respectively 388 and 490 mOsm/kg and thus instead of the registered increase in cell volume, a slight decrease should have been expected if only the osmolality was taken into account. This paradox is explained by the ability of glycerol to retain water within the cell, with glycerol at 1.5% only inducing changes in volume after 1 h of incubation, could be that glycerol does not reach an immediate equilibrium due to its relatively low permeability in stallion spermatozoa. Data from human spermatozoa indicates that when exposed to glycerol, spermatozoa shrink because of dehydration and then increase in volume as the glycerol permeates and water concomitantly re-enters the cell [33]. One possible explanation for our findings is that

Table 5
Mitochondrial membrane potential (ΔTm) (mean % ± SD) of stallion spermatozoa incubated in BWW media supplemented with different concentrations of glycerol.

	Glycerol					
	0%	0.5%	1.5%	2.5%	3.5%	5%
High ΔTm						
T0	33.2 ± 12.55	31.7 ± 12.41	33.2 ± 13.29	36.2 ± 12.67[a]	33.0 ± 9.33	26.2 ± 11.99[b]
T1	27.2 ± 13.57	23.6 ± 11.03	27.1 ± 14.89	30.1 ± 13.59	27.9 ± 12.17	22.2 ± 10.89

Within a row values with different superscripts differ statistically; [a,b] $P < 0.01$. The osmolality of each solution was respectively 388, 492, 670, 750, and 1300 mOSm/Kg.
BWW, Biggers Whitten and Whittingham; T0, measurement after 10 min of incubation at 37°C; T1, measurement after 60 min of incubation at 37°C.

human spermatozoa is very permeable to glycerol [15], so this change in cellular volume occurs in a short period, while the more impermeable membrane of the stallion spermatozoa causes this process to occur much more slowly and thus causing more damage. It has been hypothesized that the actin cytoskeleton is damaged during cryopreservation [34,35] and indirect evidence has been described in ram spermatozoa [36]. In order to further determine if glycerol may damage the sperm cytoskeleton the percentage of F-actin was evaluated. In order to increase the permeability of the stallion spermatozoa to glycerol we incubated the cells at 37 °C instead of at room temperature, an approach that might have helped to characterize the biochemical toxicity of glycerol over the actin cytoskeleton. Such effect would had been imposed through actin F depolymerization,

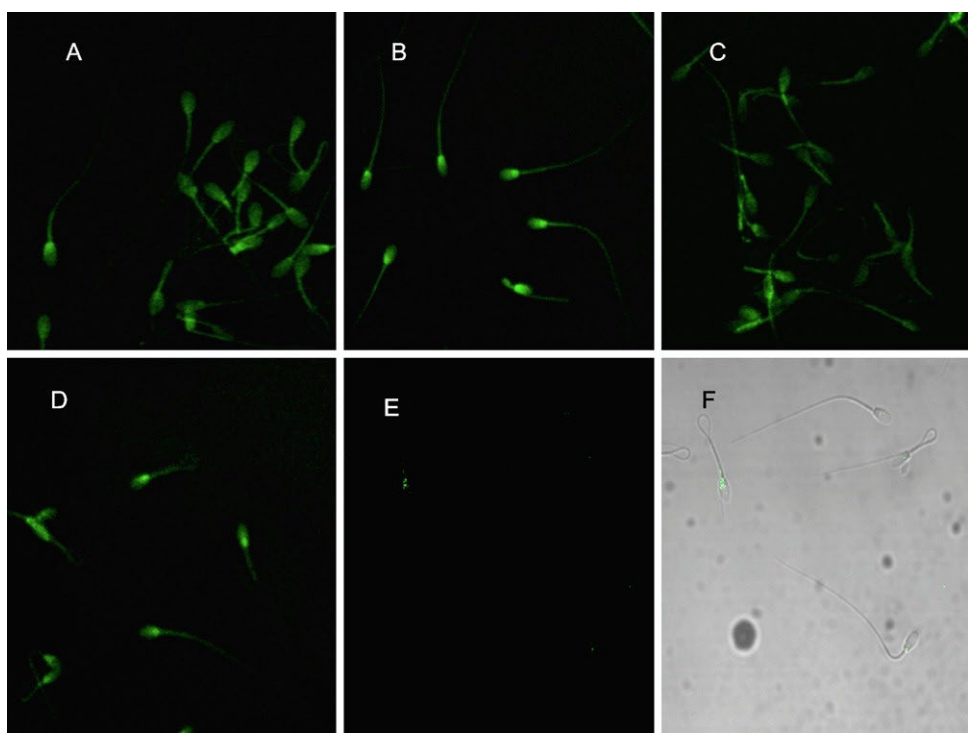

Fig. 1. Falloidin-FICT fluorescence (actin-F) of stallion spermatozoa incubated for 1 h at 37 °C in Biggers Whitten and Whittingham (BWW) media supplemented with different concentrations of glycerol (0%, 0.5% 1.5%, 2.5%, 3.5%, 5%). A hypertonic control (900 mOsm/kg) (B) and a hyposmotic control (75 mOsm/kg) (C) were also included. Hyperosmolality induced the redistribution of F-actin to the postacrosomal region as seen in (B). (**A**) Isosmotic 0% glycerol (control), while glycerol at concentrations ≥ 1.5% induced F-actin depolymerization; (D) 0.5% glycerol, (E) 1.5% glycerol, (F) transmission image combined with laser illumination 1.5% glycerol. Images are representative of 8 identical experiments.

while hyperosmolarity induced a redistribution of F-actin fluorescence in a similar way as previously described in monkey spermatozoa [28]. Probably, the toxicity of glycerol is due to its action on the cytoskeleton; an assumption backed by two independent studies. First, it has been suggested that the cytoskeleton acts as a cell volume sensor and also offers protection against the deleterious effect of excessive shrinkage [37], and second, that the toxicity of glycerol on human erythrocytes is by making red cells susceptible to osmotic stress at thawing [38]. However the low number of stallions used in our experiment does not allow us to exclude a stallion effect, it is well known the stallion-to-stallion variability in sperm characteristics, but anyway an interesting hypothesis arises if susceptibility of the cytoskeleton would explain differences in freezeability.

As indicated before, glycerol induced cellular damage, because a significant decrease in the percentage of intact membranes was observed in samples incubated in presence of 5% glycerol, but also concentrations of glycerol of 3.5% resulted in a significant increase in the percentage of necrotic (ethidium+) spermatozoa. Differences between 3.5% and 5% glycerol can also be explained by differences in osmolality (750 vs. 1300 mOsm/kg, respectively). The damage was not oxidative because lipid peroxidation was not affected by any of the concentrations of glycerol tested. The mitochondria of stallion spermatozoa were affected by incubation in presence of 5% glycerol after 1 h of incubation, this may represent that glycerol diffuses slowly or a major resistance of these organelles to glycerol toxicity. Also this may explain the lack of lipid peroxidation in our experiment, but another possible explanation relies in the ability of glycerol to scavenge the hydroxyl radical [39]. Sperm mitochondria have been characterized as the main source of reactive oxygen species in spermatozoa [40,41], and also to be among the sperm structures most sensitive to cryopreservation [22,24,42–44], but glycerol toxicity does not seem to be a major factor involved in mitochondrial damage. Finally, we studied the activity of caspases 3, 7, and 8 which, however, were not increased at any of the glycerol concentrations studied. Because cryopreservation [24,45] induces activation of caspases, we also studied the effect of a hyperosmotic and of a hyposmotic milieu on caspase activation. Bare exposition to high osmolality induced a significant increase in the percentage of dead (ethidium+) spermatozoa showing caspase activity. Interestingly, the osmotic shock induced a great increase of caspase 8+ cells, which may indicate that the extrinsic pathway of apoptosis is present in stallion spermatozoa. Recent data in somatic cells indicates that osmotic stress may induce caspase 8 activation [46]. Activation of caspase 8 occurs during human sperm cryopreservation [47], but not in bovine sperm cryopreservation [48]. In view of our results, the toxicity of glycerol explains some of the damage induced by cryopreservation.

In sum, we studied the toxicity of glycerol on stallion spermatozoa. This CPA damages sperm membranes when present at concentrations above 3.5%. The results suggest that this damage is related to an osmotic effect but also is due to the intrinsic toxicity of glycerol upon the actin cytoskeleton that may compromise the capacity of the spermatozoa to cope with osmotic stress. In view of our results, glycerol should be added to the extended stallion semen at concentrations at or below 2.5% vol/vol.

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References

[1] Aurich J, Aurich C. Developments in European horse breeding and consequences for veterinarians in equine reproduction. Reprod Domest Anim 2006;41:275–9.
[2] Allen WR. The development and application of the modern reproductive technologies to horse breeding. Reprod Domest Anim 2005;40:310–29.
[3] Bruemmer JE. Collection and freezing of epididymal stallion sperm. Vet Clin North Am Equine Pract 2006;22:677–82.
[4] Loomis PR, Graham JK. Commercial semen freezing: individual male variation in cryosurvival and the response of stallion sperm to customized freezing protocols. Anim Reprod Sci 2008; 105:119–28.
[5] Mazur P, Leibo SP, Seidel GE Jr. Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions. Biol Reprod 2008;78:2–12.
[6] Samper JC, Morris CA. Current methods for stallion semen cryopreservation: a survey. Theriogenology 1998;49:895–903.
[7] Woods EJ, Benson JD, Agca Y, Critser JK. Fundamental cryobiology of reproductive cells and tissues. Cryobiology 2004;48: 146–56.
[8] Mazur P. Cryobiology: the freezing of biological systems. Science 1970;168:939–49.
[9] Mazur P. Freezing of living cells: mechanisms and implications. Am J Physiol 1984;247:C125–42.

[10] Mazur P, Kleinhans FW. Relationship between intracellular ice formation in oocytes of the mouse and Xenopus and the physical state of the external medium–a revisit. Cryobiology 2008; 56:22–7.
[11] Fahy GM. Cryoprotectant toxicity neutralization. Cryobiology 2010;60:S45–53.
[12] Vidament M, Ecot P, Noue P, Bourgeois C, Magistrini M, Palmer E. Centrifugation and addition of glycerol at 22 degres C instead of 4 degrees C improve post-thaw motility and fertility of stallion spermatozoa. Theriogenology 2000; 54:907–19.
[13] Vidament M, Yvon JM, Couty I, Arnaud G, Nguekam-Feugang J, Noue P, et al. Advances in cryopreservation of stallion semen in modified INRA82. Anim Reprod Sci 2001; 68:201–18.
[14] Loomis PR, Amann RP, Squires EL, Pickett BW. Fertility of unfrozen and frozen stallion spermatozoa extended in EDTA-lactose-egg yolk and packaged in straws. J Anim Sci 1983;56: 687–93.
[15] Gao DY, Mazur P, Kleinhans FW, Watson PF, Noiles EE, Critser JK. Glycerol permeability of human spermatozoa and its activation energy. Cryobiology 1992;29:657– 67.
[16] Gao DY, Ashworth E, Watson PF, Kleinhans FW, Mazur P, Critser JK. Hyperosmotic tolerance of human spermatozoa: separate effects of glycerol, sodium chloride, and sucrose on spermolysis. Biol Reprod 1993;49:112–23.
[17] 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;22:1061–9.
[18] Fahy GM. The relevance of cryoprotectant "toxicity" to cryobiology. Cryobiology 1986;23:1–13.
[19] Fahy GM, Levy DI, Ali SE. Some emerging principles underlying the physical properties, biological actions, and utility of vitrification solutions. Cryobiology 1987;24:196 –213.
[20] Morris GJ, Faszer K, Green JE, Draper D, Grout BW, Fonseca F. Rapidly cooled horse spermatozoa: Loss of viability is due to osmotic imbalance during thawing, not intracellular ice formation. Theriogenology 2007;68:804 –12.
[21] Fahy GM, Karow AM Jr. Ultrastructure-function correlative studies for cardiac cryopreservation. V. Absence of a correlation between electrolyte toxicity and cryoinjury in the slowly frozen, cryoprotected rat heart. Cryobiology 1977;14: 418 –27.
[22] Ortega Ferrusola C, González Fernández L, Morrell JM, Salazar Sandoval C, Macías García B, Rodríguez-Martinez H, et al. Lipid peroxidation, assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes. Reproduction 2009; 138:55– 63.
[23] Peña FJ, Saravia F, Johannisson A, Walgren M, Rodríguez-Martínez H. A new and simple method to evaluate early membrane changes in frozen-thawed boar spermatozoa. Int J Androl 2005;28:107–14.
[24] Ortega-Ferrusola C, Sotillo-Galán Y, Varela-Fernández E, Gallardo-Bolaños JM, Muriel A, González-Fernández L, et al. Detection of "apoptosis-like" changes during the cryopreservation process in equine sperm. J Androl 2008;29:213–21.
[25] Ormerod MG, Sun XM, Snowden RT, Davies R, Fearnhead H, Cohen GM. Increased membrane permeability of apoptotic thymocytes: A flow cytometric study. Cytometry 1993; 14:595– 602.
[26] Idziorek T, Estaquier J, De Bels F, Ameisen JC. YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. J Immunol Methods 1995;185:249 –58.
[27] Aitken RJ, Wingate JK, De Iuliis GN, McLaughlin EA. Analysis of lipid peroxidation in human spermatozoa using BODIPY. Mol Hum Reprod 2007;13:203–11.
[28] Correa LM, Thomas A, Meyers SA. The macaque sperm actin cytoskeleton reorganizes in response to osmotic stress and contributes to morphological defects and decreased motility. Biol Reprod 2007;77:942–53.
[29] Redondo PC, Harper MT, Rosado JA, Sage SO. A role for cofilin in the activation of store-operated calcium entry by de novo conformational coupling in human platelets. Blood 2006; 107:973–9.
[30] Ball BA. Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. Anim Reprod Sci 2008;107:257– 67.
[31] Macias Garcia B, Miro Moran A, Gonzalez Fernandez L, Ortega Ferrusola C, Morillo Rodriguez A, Gallardo Bolanos JM, et al. The mitochondria of stallion spermatozoa are more sensitive than the plasmalemma to osmotic induced stress: role of c-Jun N-terminal kinase (JNKs) pathway. J Androl 2011, in press. DOI:10.2164/jandrol.110.011957.
[32] Glazar AI, Mullen SF, Liu J, Benson JD, Critser JK, Squires EL, et al. Osmotic tolerance limits and membrane permeability characteristics of stallion spermatozoa treated with cholesterol. Cryobiology 2009;59:201– 6.
[33] Du J, Kleinhans FW, Mazur P, Critser JK. Human spermatozoa glycerol permeability and activation energy determined by electron paramagnetic resonance. Biochim Biophys Acta 1994; 1194:1–11.
[34] Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev 1995;7:871–91.
[35] Watson PF. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 2000;60 – 61:481–92.
[36] Holt WV, North RD. Cryopreservation, actin localization and thermotropic phase transitions in ram spermatozoa. J Reprod Fertil 1991;91:451– 61.
[37] Burg MB, Ferraris JD, Dmitrieva NI. Cellular response to hyperosmotic stresses. Physiol Rev 2007;87:1441–74.
[38] Rall WF, Mazur P, Souzu H. Physical-chemical basis of the protection of slowly frozen human erythrocytes by glycerol. Biophys J 1978;23:101–20.
[39] Miller JS, Cornwell DG. The role of cryoprotective agents as hydroxyl radical scavengers. Cryobiology 1978;15:585– 8.
[40] Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA, Aitken RJ. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. J Clin Endocrinol Metab 2008;93:3199 –207.
[41] Koppers AJ, Garg ML, Aitken RJ. Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. Free Radic Biol Med 2010;48:112–9.
[42] Pena FJ, Rodriguez Martinez H, Tapia JA, Ortega Ferrusola C, Gonzalez Fernandez L, Macias Garcia B. Mitochondria in mammalian sperm physiology and pathology: A review. Reprod Domest Anim 2009;44:345–9.
[43] Peña FJ, Johannisson A, Wallgren M, Rodriguez Martinez H. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreserva-

tion of different fractions of the ejaculate. Anim Reprod Sci 2003;78:85–98.

[44] Peña FJ, Johannisson A, Wallgren M, Rodríguez-Martínez H. Assessment of fresh and frozen-thawed boar semen using an annexin-V assay: a new method of evaluating sperm membrane integrity. Theriogenology 2003;60:677– 89.

[45] Ortega-Ferrusola C, García BM, Gallardo-Bolaños JM, González-Fernández L, Rodríguez-Martinez H, Tapia JA, et al. Apoptotic markers can be used to forecast the freezeability of stallion spermatozoa. Anim Reprod Sci 2009;114:393–403.

[46] Xia Y, Wang J, Liu TJ, Yung WK, Hunter T, Lu Z. C-Jun downregulation by HDAC3-dependent transcriptional repression promotes osmotic stress-induced cell apoptosis. Mol Cell 2007;25:219–32.

[47] Wundrich K, Paasch U, Leicht M, Glander HJ. Activation of caspases in human spermatozoa during cryopreservation–an immunoblot study. Cell Tissue Banking 2006;7:81–90.

[48] Martin G, Cagnon N, Sabido O, Sion B, Grizard G, Durand P, et al. Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. Hum Reprod 2007;22:380 – 8.