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Egg Yolk-Supplemented Tris–Citric Acid Extender Improves the Prefreezing and Post-Thaw Sperm Quality Indices of Guinea Pig (*Cavia porcellus*) Epididymal Spermatozoa

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This study aimed to assess the suitability of egg yolk (EY) supplementation to a tris–citric acid–based extender on cryosurvival of guinea pig (*Cavia porcellus*) epididymal spermatozoa. Two synthetic-based extenders, tris–citric acid–glucose plus 20% EY (TCG-EY) and tris–citric acid–fructose (TCF) both with 5% glycerol, were compared. Thirty-two epididymides were recovered from 16 adult guinea pig males by gonadectomy, and then the sperm samples were retrieved by retrograde flushing using TCG-EY and TCF extenders for left or right epididymis, respectively. TCG-EY and TCF sperm samples were frozen in static liquid nitrogen vapors through a two-step cooling procedure. Before freezing, the percentage of progressive sperm motility and sperm with intact plasma and acrosome membranes from TCG-EY sperm samples were higher ($p < 0.05$) than those diluted with TCF. Post-thaw sperm kinematic variables and membrane integrity were drastically reduced ($p < 0.001$) compared with prefreezing samples, regardless of extender type. The post-thaw plasmatic and acrosome membrane integrity from TCG-EY sperm samples was higher ($p < 0.05$) than those from TCF samples. Except for the length, the morphometric head dimensions of sperm diluted with TCG-EY or TCF did not vary ($p > 0.05$) after the freezing–thawing process compared with the prefreezing samples. In conclusion, despite greater cell cryoinjury with both extenders, the EY supplementation exerted greater cell membrane protection before and after the freezing–thawing process. This research shows an in-depth analysis of guinea pig sperm cryopreservation; however, more studies are recommended.

Keywords: guinea pig, epididymal spermatozoa, cryopreservation, TCG, egg-yolk

Introduction

The preservation of the guinea pig (*Cavia porcellus*) genetic biodiversity is important as this species represents a native source of protein for many people in the Andean countries (e.g., Peru, Ecuador, Bolivia, and Colombia).[1] Guinea pig meat consumption contributes to food security and provides a small source of income for the population.[2,3] Food security approaches and Food and Agriculture Organization (FAO) guidelines stress the importance of preserving the genetic resources of these native species through germplasm banks.[4] In this sense, the optimization of production and preservation of their genetic diversity through assisted reproductive techniques (ART) is a priority.

Several efforts have been made to obtain guinea pig ejaculate semen by transrectal electroejaculation.[5–7] However, not all guinea pigs respond satisfactorily to electrical stimulation.[8] Epididymal retrieval of guinea pig spermatozoa (*postgonadectomy* or *postmortem*) has also been used with promising results.[9,10] Nevertheless, there is only one brief report on the cryopreservation of guinea pig sperm.[11]

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Tris–citric acid–glucose (TCG)[12–15] and tris–citric acid–fructose (TCF)–based[16,17] extenders have been used for freezing sperm from several animals. In some species, such as pigs[18] and tree shrews,[19] spermatozoa were frozen with both TCG and TCF extenders, and no significant differences were found in motility and membrane integrity. However, a study has shown that the use of fructose induces a more linear and less oscillatory motility pattern in dog spermatozoa compared with glucose.[20] To the best of our knowledge, there are no reports of the use of glucose or fructose added to the tris–citric acid–based medium before or after cryopreservation of guinea pig epididymal spermatozoa.

Cryopreservation of wild rodent epididymal sperm relative to guinea pigs (i.e., *Galea spixii and Spix's yellow-toothed cavy*) has previously been reported. In these studies, sperm from these rodents was successfully cryopreserved using a Tris-based extender supplemented with 10%–20% egg yolk (EY) and 6% glycerol.[12,16] EY provides the greatest benefit for sperm freezing, protecting cells against cold shock and preventing the loss of cholesterol and phospholipids from the sperm membranes (plasmalemma and acrosome) during the freezing–thawing process.[21,22] It is expected that supplementing TCG-based extenders with 20% EY could be also suitable for protecting sperm membranes of guinea pig during freezing–thawing procedure.

Changes in sperm head dimensions during cryopreservation might provide information about the sperm's sensitivity to the freezing–thawing process.[13] Guinea pig spermatozoa are characterized by big sperm heads (area: $43.02 \pm 3.03\ \mu m^2$)[6] and strong head-to-head agglutination.[23] These large head dimensions and cell-to-cell arrangement could make cryopreservation difficult in a conventional freezing with static liquid nitrogen (LN_2) vapors. Conventional slow freezing has a high initial cooling rate during the critical temperature range (−5°C to −25°C), which might provoke inefficient dehydration and lethal intracellular ice crystals formation.[24] Previous studies in wild and domestic ruminants[14,25,26] revealed that a conventional slow freezing protocol consisting in exposing straws to LN_2 vapors (i.e., at 5 cm above the surface of the LN_2) produced decelerating cooling rates (40°C/min from +5°C to −35°C, 17°C/min from −35°C to −65°C, and 3°C/min from −65°C to −85°C at 3°C/min) that caused major cryodamage to the sperm cells than two-step accelerating cooling rates (5°C/min from +5°C to −10°C, and 60°C/min from −10°C to −130°C). To avoid the formation of intracellular ice crystals, the use of low initial cooling rates has been proposed to achieve optimal dehydration.[14,25,26]

The aim of this study was to evaluate the effect of both tris–citric acid–glucose plus 20% EY (TCG-EY-) and TCF (as control) synthetic-based extenders before and after cryopreservation of guinea pig epididymal spermatozoa using a two-ramp cryo-box freezing-based system. The efficacy of two extenders was measured on kinematics parameters, the integrity of the plasma and acrosomal membranes, and changes in spermatozoa head morphometry in prefreezing and frozen–thawed samples.

Materials and Methods

All diluents and media were prepared in the Animal Reproduction Biotechnology Laboratory, University of Cuenca, Ecuador, using reagent-grade chemicals purchased from Sigma Chemical Co. (St. Louis, MO).

Animals and epididymal sperm collection

All animals were handled according to procedures approved by the Veterinary Science Committee, Faculty of Agricultural Sciences, University of Cuenca, and the research was performed following chapter 7.8 of the Terrestrial Animal Health Code-2019© OIE (July 8, 2019) regarding to protection of animals used in scientific experiments.

Testes were obtained after bilateral orchiectomy from 16 healthy and sexually mature guinea pigs aged 8–20 months and weighing 1100 ± 100 g. One month before surgery, all males were confined in individual floor cages of $1 \times 1 \times 0.7$ m in length, width, and height, respectively. Males were fed with a basal diet that included 250 g of green grass (mix of alfalfa [*Medicago sativa*] and Ryegrass [*Lolium perenne*]), and 35 g of concentrate (wheat-grain, minerals, and vitamins). Males were subjected to fasting for at least 8 hours. In brief, bilateral orchiectomies were performed under anesthesia with 9 mg/kg intramuscular tiletamine–zolazepam (Zoletil 100; Virbac, Mexico). After 5 minutes, the scrotal area was cleaned (shaved and disinfected) and surgery was performed. The right and left testicles were washed with saline and stored separately at room temperature until processing, within 1–2 hours after surgery. The testes and their intact epididymides were placed into sterile Ziploc® bags (properly labeled: left or right) at room temperature and transported (<1 hour) at Animal Reproduction Biotechnology Research Laboratory located in the ''Irquis'' of the University of Cuenca (3°04′48.1″S 79°04′31.0″W).

Cauda epididymides and vas deferens were dissected from each testis and placed in a dry Petri dish at room temperature. Epididymal sperm samples were collected by retrograde flushing by administering 1 mL of TCG-EY medium (313.7 mM Tris, 104.7 mM citric acid, 30.3 mM glucose, 0.54 mM streptomycin, 2.14 mM penicillin, plus 20% EY [v/v]; pH: 7.16 and osmolality: 353 mOsm/kg) for left epididymis and 1 mL of TCF medium (313.7 mM Tris, 104.7 mM citric acid, 30.3 mM fructose, 0.54 mM streptomycin, 2.14 mM penicillin; pH: 7.2 and osmolality: 315 mOsm/kg) for right epididymis. For that, the cauda epididymides were cleaned and the vas deferens cannulated at a distance of 2–3 cm from the cauda epididymides with a 25G × 16 mm needle (Nipro® Medical Corp., Buenos Aires, Argentina) connected to a 1 mL syringe filled with each medium. The medium was then introduced into the epididymal tube by using manual pressure from the syringe. When the major part of the epididymal tube was filled, three small cuts were made in the terminal part of the epididymal tube in the cauda epididymides. The fluid that emerged from the cut tubules with retrograde flushing was collected in a Petri dish and then recovered by pipetting into a 1.5 mL Eppendorf tube.[15,27] The percentage of motile sperm and the quality of motility were initially evaluated via a phase contrast microscope (Nikon Eclipse; Nikon Instruments, Inc., New York, NY) by warming an aliquot of retrieved samples at 37°C for 5 minutes. Only those epididymal sperm samples with a sperm motility value of >60%, and a score of >2 on a motility scale of 0 (lowest) to 5 (highest), were used in the subsequent experimental work.

A total of 32 epididymal samples from left ($n = 16$) and right ($n = 16$) epididymides were used in this experiment.

The volume of each epididymal sperm sample was measured by pipetting content using a graduate micropipette (Boeco, Germany). The sperm concentration was evaluated using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany).

Sperm processing and freezing

The conventional freezing procedure was carried out in two steps.[15] The first step consisted in taking the first aliquot of each left or right epididymal spermatozoa sample and diluted with TCG-EY or TCF extender (glycerol free), respectively, at a concentration of 100×10^6 spermatozoa/mL and then equilibrating for 1 hour at 5°C. The second step consisted of slowly adding a second cooled extender (5°C) made of TCG-EY or TCF plus 10% glycerol (v/v) (Sigma G9012, St. Louis, MO) to each first aliquot sample in a volume equal (1:1) and equilibrating for 1 hour more. Thereby, the final volume of these samples before conventional freezing reached a final concentration of 50×10^6 spermatozoa/mL and 5% glycerol (final osmolarity of freezing mediums: 1268 and 1057 mOsm/kg for TCG-EY and TCF extenders, respectively).

Samples were manually loaded into 0.25 mL IVM French straws (L'Aigle Cedex, France) and sealed with polyvinyl alcohol (Sigma P8136, St. Louis, MO). At this point, the kinematic parameters and status of membranes of pre-freezing spermatozoa samples were evaluated before the freezing process. Straws were frozen using two ramps placed inside a Styrofoam cryo-box of $30\times29\times31$ cm of length, width, and height, respectively, which contained 1.7 L of LN_2 (up to 2 cm of height). Thirty minutes before freezing, the LN_2 was placed inside the cryo-box to stabilize the vapors generated by the nitrogen. Straws were exposed horizontally to LN_2 vapors and placed in the first ramp at 24 cm above the LN_2 surface for 3 minutes, and then placed in a second lower ramp at 10 cm for 2 minutes more above the LN_2.

Finally, straws were plunged in LN_2 to cool to −196°C and kept for 3 months. The freezing rate pattern was as follows: from +5°C to −118°C at 39°C/min and from −118°C to −148.2°C at 15°C/min; they were then plunged into the LN_2 to reach −196°C. This procedure was standardized to ensure such cooling and checked using a Digital Thermometer® (6802 II Bestdo, Shanghai, China) with a 2K-type Thermocouple Sensor of fine wire resistant to freezing. The thermocouple sensor was introduced to a 0.25 mL straw containing the freezing medium. When the temperature inside the straws approached 5°C, the freezing procedure was started. Consequently, the thermocouple inside the straw registered the temperature drop (°C) over time (seconds) up to the immersion into LN_2. The thermometer registered the occurrence of ice nucleation, and estimated the duration of the subsequent dissipation of the latent heat of fusion (Fig. 1).

A total of 89 straws were frozen from two diluents: TCG-EY ($n=47$) and TCF ($n=42$). All frozen samples were thawed after 2 months by placing the straws in a water bath at 37°C for 30 seconds. The contents were poured into dry 1.5 mL Eppendorf tubes and incubated for 5 minutes at 37°C. Kinematic parameters, status of sperm membranes, and sperm head morphometry were subsequently evaluated (see sperm quality assessment section).

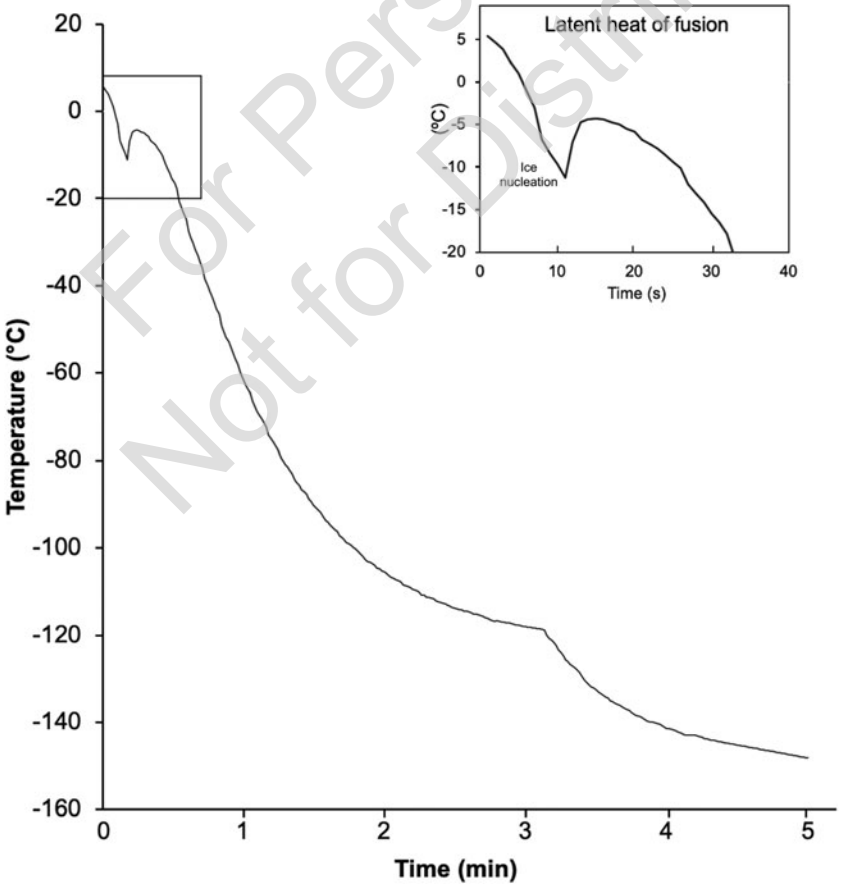

FIG. 1. Time series of measured temperature inside straws (T_{in}) during the freezing protocol. The time (seconds) and temperature at which latent heat of fusion (ice nucleation) occurs within straws are shown in the small box.

Sperm quality assessment

Before sperm quality analysis, the fresh, prefrozen, and post-thawed samples were gently pipetted because the spermatozoa exhibited some degree of head stacking. Kinematic parameters in prefreezing and post-thawed spermatozoa samples were determined using a CASA system (Sperm Class Analyzer, SCA-Evolution 2018, v.6.4.0.99 software; Microptic S.L., Barcelona, Spain), coupled to a phase contrast microscope (Nikon Eclipse Ci-E; Nikon Instruments, Inc.; negative phase contrast [Ph1] with green filter) with the following settings: 25 frames/s, head area 20–70 μm^2, velocity limit for slow sperm 10 μm/s, velocity limit for medium sperm 50 μm/s, velocity limit for fast sperm 70 μm/s, and minimal straightness for progressive spermatozoa 80%. All samples were adjusted to a concentration of 25×10^6 spermatozoa/mL using the same mediums (TCG-EY or TCF, glycerol free) warmed to 37°C.

In brief, the 5-μL aliquot of sperm samples was loaded into a warmed (37°C) slide and covered with a coverslip. A minimum of three fields and 200 sperm tracks were evaluated at 100× magnification. The following kinematic parameters were analyzed: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity index (LIN, %), straightness index (STR, %), oscillation index (WOB, %), beat-cross frequency (BCF, Hz), and amplitude of lateral head displacement (ALH, μm).

The integrity of plasma and acrosome membranes in prefreezing and post-thawed sperm samples were analyzed by fluorescence microscopy, counting 200 cells, using a combination of propidium iodide (PI; Sigma Chemical Co.) and fluorescein isothiocyanate–conjugated peanut agglutinin (PNA-FITC; Sigma Chemical Co.).[15] A total of 200 sperms per slide were examined, and 4 subpopulations of cells were quantified into percentages: (1) intact plasma membrane with intact acrosome (PI–/PNA–), (2) intact plasma membrane with damaged acrosome (PI–/PNA+), (3) damaged plasma membrane with intact acrosome (PI+/PNA–), and (4) damage plasma membrane and acrosome (PI+/PNA+). In addition, the total proportion of cells presenting an intact plasma membrane equivalent to live sperm (Total IP: [PI–/PNA–] + [PI–/PNA+]) and intact acrosomal membrane (Total IA: [PI+/PNA–] + [PI–/PNA–]) were calculated.

Spermatozoa head measurements and midpiece area in prefreezing and post-thawed spermatozoa samples were assessed by morphometry module ASMA (automated sperm morphometry analysis) of CASA-SCA® system coupled to a contrast phase microscope (Nikon Eclipse Ci–L; Nikon Instruments, Inc.). Smears of sperm samples stained with SpermBlue® stain were observed at 60× magnification, and at least 100 spermatozoa were per sample examined.[15] The following morphometric head dimensions were registered: length, width, area, perimeter, elongation, ellipticity, rugosity, and regularity.

Statistical analysis

Data are given as mean ± SEM. Statistical significance was considered as $p < 0.05$. Values for sperm variables showing a skewed distribution (as determined by the Shapiro–Wilks test) were arcsine (percentages values) or Log10 (numeric values) transformed. Factorial ANOVA and Tukey *post hoc* multiple comparison tests were used to examine the effects of ''*extender type*'' (TCG-EY and TCF), ''*spermatozoa type*'' (prefreezing and post-thawed), and its interaction on kinematic parameters, the integrity of plasma and acrosome membranes, and morphometric of head and midpiece. All calculations were made using STATISTICA for Windows v.12.0 software (StatSoft, Tusla, OK).

Results

The freezing curve used to freeze the epididymal spermatozoa is given in Figure 1. Ice nucleation (*seeding*) occurred at −11.2°C (at 11 seconds), and the dissipation of the latent heat of fusion finished at 27 seconds with a total duration of 16 seconds. Strong heads stacking (10 to 15 cells) of spermatozoa were observed in fresh samples after retrograde flushing; however, in prefreezing and post-thawed samples, less sperm heads stacking (2–4 cells) was observed with or without acrosomes detachment (Fig. 2). The status of sperm membranes (assessed with fluorescent markers PI/PNA-FITC) in different categories according to their integrity or damage is given in Figure 3.

Significant interaction were observed between *extender type* × *spermatozoa type* on PM ($p < 0.05$) and in the following fluorescence categories regarding the status of sperm membranes ($p < 0.001$): intact plasma membrane with intact

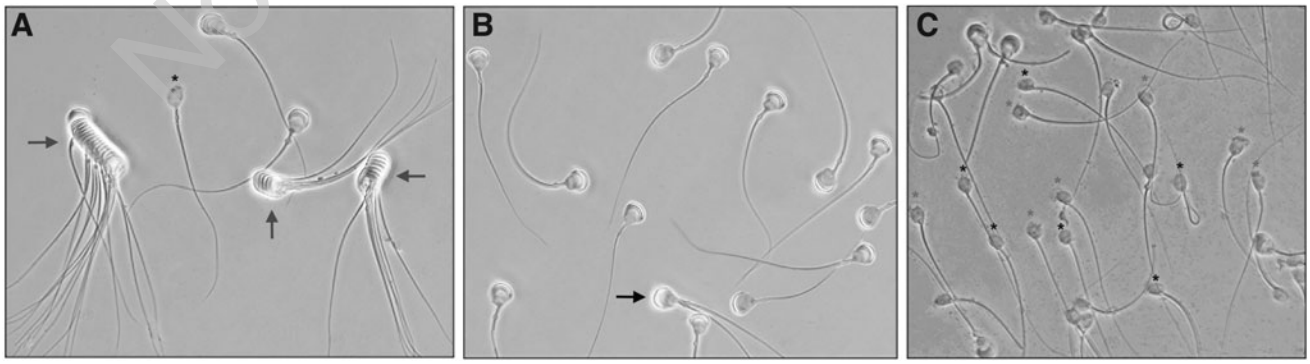

FIG. 2. Images of epididymal guinea pig spermatozoa (fixed with 2% glutaraldehyde) observed in a phase contrast microscope (×400). **(A)** Strong heads stacking (10–15 cells) of spermatozoa in fresh samples after retrograde flushing (*arrows*). **(B)** Separated spermatozoa after pipetting; notice some two-headed stacking (*arrow*). **(C)** Spermatozoa with festooned or detached acrosomes (*asterisks*).

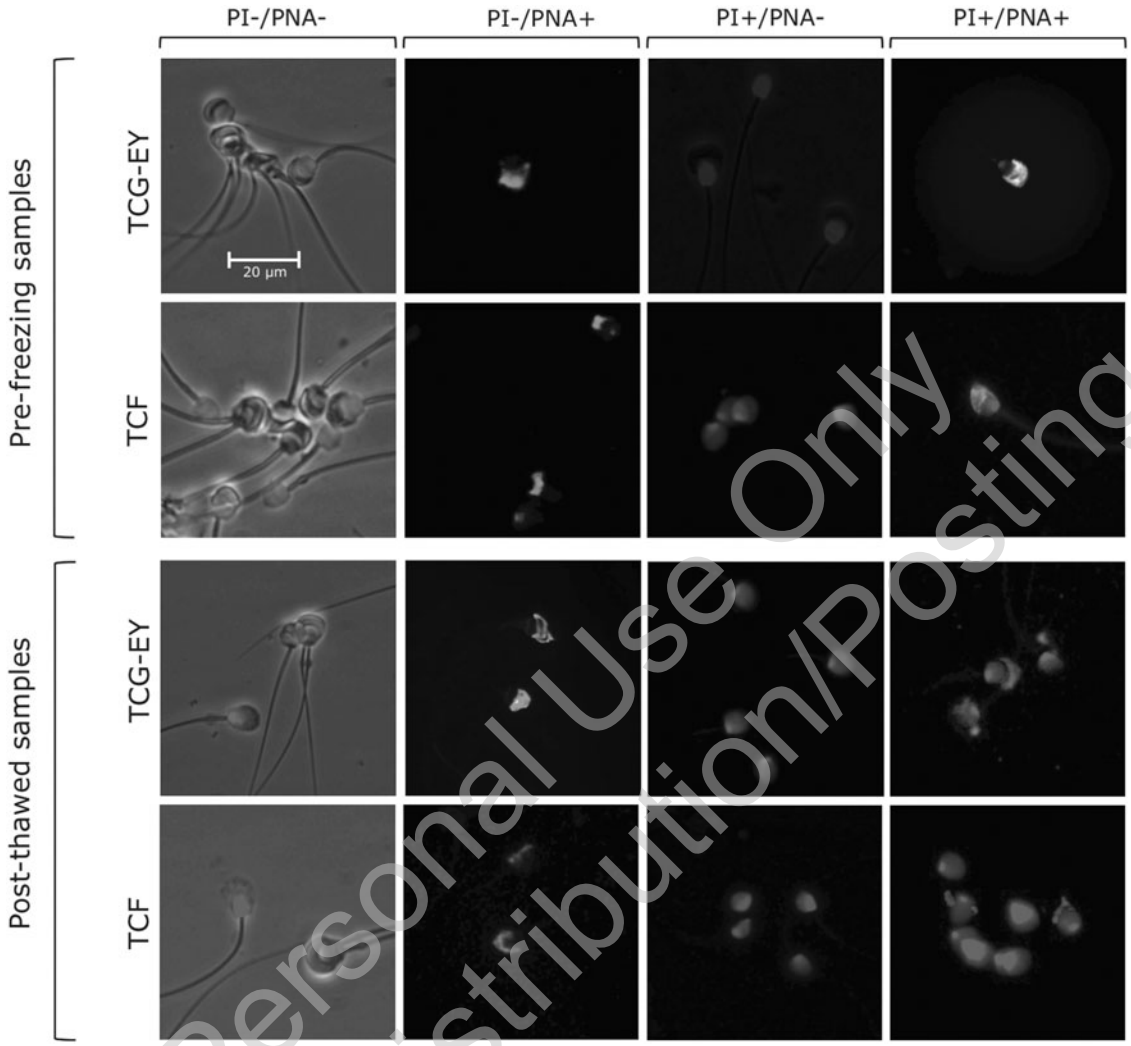

FIG. 3. Prefreezing and post-thawed guinea pig sperm samples stained with an association of *PI* and fluorescein isothiocyanate–conjugated PNA (×400). PI–/PNA–, sperm with intact plasma membrane with intact acrosome (nonstained cells); PI–/PNA+, sperm with intact plasma membrane with damaged acrosome; PI+/PNA–, sperm with damaged plasma membrane with intact acrosome; and PI+/PNA+, sperm with damage plasma membrane and acrosome. PI, propidium iodide; PNA, peanut agglutinin; TCF, tris–citric acid–fructose; TCG-EY, tris–citric acid–glucose plus 20% egg yolk.

acrosome (PI–/PNA–), damaged plasma membrane with intact acrosome (PI+/PNA–), damaged plasma membrane and acrosome (PI+/PNA+), and total spermatozoa with intact acrosome (total IA). Unlike progression ratio parameters (i.e., STR, LIN, and WOB indexes), the kinematic values and the percentages of spermatozoa with intact plasma membrane and acrosome (PI–/PNA–), total percentage of spermatozoa with intact plasma membrane (total IP) and acrosome (IA) from prefreezing spermatozoa samples were drastically affected by the freezing process owing to a severe reduction ($p < 0.001$) after thawing, regardless of the extender type.

In the *prefreezing analysis,* the percentages of spermatozoa with PM and with intact plasma membrane and acrosome (PI–/PNA–) were greater ($p < 0.05$) in samples diluted with the TCG-EY extender than those diluted with the TCF extender. Moreover, lower undesirable percentages of spermatozoa with damaged plasma membrane and intact acrosome (PI+/PNA–) and with both plasma membrane and acrosome damaged (PI+/PNA+) were found in samples diluted with TCG-EY than those diluted with TCF ($p < 0.01$). The other kinematic parameters and categories of the status of sperm membranes in prefreezing samples did not vary between extenders ($p > 0.05$) (Table 1).

In the *post-thaw analysis*, the percentage of spermatozoa with intact plasma membrane and acrosome (PI–/PNA–), total percentage of spermatozoa with intact plasma membrane (total IP), and total percentage of spermatozoa with intact acrosome (total IA) were higher ($p < 0.05$) in spermatozoa samples frozen with TCG-EY than in those frozen with TCF. Likewise, the post-thaw percentage of spermatozoa with plasma membrane and acrosome damaged (PI+/PNA+) were lower ($p < 0.001$) with TCG-EY than those with TCF extender. However, the TCF extender produced lower percentages of spermatozoa with damaged plasma membrane and intact acrosome (PI+/PNA–) than those with TCG-EY extender ($p < 0.01$) (Table 1).

Only the length of the guinea pig sperm head was affected by the freezing–thawing procedure, reducing this value

Table 1. Sperm Quality Variable Values (Mean ± Standard Error of Mean) for Epididymal Guinea Pig Spermatozoa Before (Prefreezing) and After Freezing (Post-Thawing) Process Using Both Tris–Citric Acid–Glucose Plus 20% Egg Yolk or Tris–Citric Acid–Fructose Extenders

	Prefreezing		*Post-thawed*	
Parameters	*TCG-EY* (n = *16*)	*TCF* (n = *16*)	*TCG-EY* (n = *47*)	*TCF* (n = *42*)
TM (%)	72.2 ± 3.43^{a}	65.7 ± 4.20^{a}	11.2 ± 1.41^{c}	7.8 ± 1.27^{c}
PM (%)*	32.7 ± 4.34^{a}	22.9 ± 3.20^{b}	1.6 ± 0.39^{d}	1.6 ± 0.55^{d}
VCL (μm/s)	67.0 ± 4.86^{a}	56.6 ± 4.16^{a}	14.5 ± 2.75^{c}	15.2 ± 2.64^{c}
VSL (μm/s)	22.0 ± 1.37^{a}	18.4 ± 1.28^{a}	8.3 ± 0.83^{c}	8.7 ± 1.04^{c}
VAP (μm/s)	41.6 ± 2.45^{a}	34.7 ± 2.38^{a}	6.9 ± 1.31^{c}	5.8 ± 1.38^{c}
STR (%)	35.1 ± 1.40	33.3 ± 1.01	17.6 ± 1.75	12.0 ± 1.65
LIN (%)	54.4 ± 1.40	52.7 ± 1.02	15.9 ± 2.01	14.2 ± 2.66
WOB (%)	63.2 ± 1.14^{a}	61.3 ± 1.25^{ab}	32.5 ± 2.16^{ab}	34.5 ± 2.19^{b}
ALH (μm)	3.27 ± 0.22^{a}	2.88 ± 0.19^{a}	3.95 ± 2.49^{c}	1.52 ± 0.16^{c}
BCF (Hz)	4.49 ± 0.22^{a}	4.22 ± 0.17^{a}	2.2 ± 0.33^{c}	1.32 ± 0.18^{c}
PI–/PNA– (%)	77.6 ± 2.57^{a}	70.5 ± 3.59^{b}	14.8 ± 0.70^{c}	7.4 ± 0.43^{d}
PI–/PNA+ (%)	2.2 ± 0.52^{a}	3.8 ± 0.96^{a}	0.37 ± 0.22^{b}	0.00
PI+/PNA– (%)**	14.5 ± 1.51^{d}	19.1 ± 2.83^{c}	49.0 ± 2.21^{a}	32.5 ± 1.75^{b}
PI+/PNA+ (%)**	5.8 ± 0.97^{d}	6.5 ± 1.21^{c}	35.9 ± 2.14^{b}	60.1 ± 1.90^{a}
Total IP (%)	79.7 ± 2.22^{a}	74.3 ± 3.09^{a}	15.2 ± 0.73^{c}	7.4 ± 0.43^{d}
Total IA (%)**	92.1 ± 1.34^{a}	89.7 ± 2.03^{a}	63.8 ± 2.20^{c}	39.9 ± 1.90^{d}

Different letters in the same row between spermatozoa and extender types differ: $^{ab}p < 0.05$; $^{a–c;\ b–d}p < 0.001$; $^{a–d}p < 0.0001$. *Significant interaction between *extender type* × *spermatozoa type:* $*p < 0.05$; $**p < 0.001$.

ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; IA, total sperm with intact acrosome; IP, total sperm with plasmalemma integrity equivalent to viability; LIN, linearity index; PI–/PNA–, intact plasma membrane/intact acrosome; PI–/PNA+, intact plasma membrane/damaged acrosome; PI+/PNA+, damaged plasma membrane/intact acrosome; PI+/PNA+, damaged plasma membrane/damaged acrosome; PM, progressive motility; STR: straightness; TCF, tris–citric acid–fructose; TCG-EY, tris–citric acid–glucose plus 20% egg yolk; TM, total motility; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, wobble.

($p < 0.05$) compared with fresh samples. The other morphometric dimensions of the head were affected neither by the freezing process nor by extender type ($p > 0.05$) (Table 2).

Discussion

This research shows an in-depth evaluation of cryopreservation of guinea pig spermatozoa. The results indicate that EY-supplemented tris–citric acid extender improved the integrity of plasmatic and acrosomal membranes in prefreezing and post-thawed spermatozoa samples. The kinematic parameters of prefreezing (post-equilibration) reached desirable values that could be used in ARTs such as IA or *in vitro* fertilization as demonstrated by Cañón-Beltrán et al.[28] Nevertheless, despite these outcomes, cryosurvival rates after freezing–thawing remained very low.

The limited or scarce information published on cryopreservation of guinea pig spermatozoa shows that using an extender based on Tes/Tris/EY plus 15% glycerol, 10% Ficoll PM70, and 2% urea produced a motility and acrosome integrity of 10% and 50%, respectively, after the freezing–thawing process.[11] However, the authors did not provide more detail (e.g., EY concentration, freezing curves). Certainly, high concentrations of glycerol have a negative effect on sperm membrane integrity.[29] It has also been reported as harmful when it was added to extenders at temperatures >30°C, as well as at concentrations >6%.[30] Our results demonstrated that 5% glycerol added to the TCG-EY extender produced a similar cryogenic response

Table 2. Morphometric Head Dimensions (Mean ± Standard Error of Mean) of Prefreezing and Frozen-Thawed Guinea Pig Epididymal Spermatozoa with Tris–Citric Acid–Glucose Plus 20% Egg Yolk and Tris–Citric Acid–Fructose Extenders

	Prefreezing		*Frozen–thawed*	
Variables	*TCG-EY* (n = *16*)	*TCF* (n = *16*)	*TCG-EY* (n = *40*)	*TCF* (n = *35*)
L (μm)	7.9 ± 0.14^{a}	7.7 ± 0.32^{a}	7.1 ± 0.39^{b}	7.0 ± 0.46^{b}
W (μm)	6.9 ± 0.09	6.6 ± 0.29	6.4 ± 0.79	6.2 ± 0.46
A (μm^2)	47.0 ± 1.46	46.0 ± 1.36	42.0 ± 0.81	43.0 ± 0.97
p (μm)	24.8 ± 0.63	24.7 ± 0.74	26.4 ± 0.86	25.2 ± 0.82
Elongation	1.2 ± 0.01	1.2 ± 0.01	1.1 ± 0.12	1.2 ± 0.02
Ellipticity	0.1 ± 0.01	0.1 ± 0.00	0.2 ± 0.14	0.1 ± 0.01
Rugosity	1.3 ± 0.02	1.3 ± 0.02	1.2 ± 0.05	1.3 ± 0.01
Regularity	0.9 ± 0.01	0.9 ± 0.01	0.9 ± 0.00	0.9 ± 0.00

Different letters in the same row between spermatozoa and extender types differ: $^{ab}p < 0.05$.

A, area; L, length; P, perimeter; W, wide.

(e.g., motility and acrosome integrity) than to those obtained by Eder et al.[11]

The sperm membrane fluidity is related to the high cryosurvival of epididymal spermatozoa owing to their lipid composition,[31] and chemical and physical differences in unsaturated fatty acids, phospholipids, and cholesterol.[32] Cold shock resistance is generally better in species with sperm membranes with a higher greater cholesterol–phospholipid ratio.[33] EY prevents the loss of cholesterol and phospholipids from the sperm membranes[21,34] conferring protection against cold shock during the freezing process.[22] Membrane integrity, along with PM, in samples recovered with the tris-citric acid-based medium supplemented with EY, suggests certain protection during the flushing retrograde process at room temperature.[35] This protection is due to the rearrangement and stabilization of the sperm membrane, facilitated by the presence of phospholipids in EY.[36] The phospholipids (like those contained in the EY) have been reported to be involved in the acrosome reaction of guinea pig sperm.[37] In this study, however, EY provided greater protection of the acrosome after cryopreservation.

Two studies on cryopreservation of spermatozoa from Spix's yellow-toothed cavies (*G. spixii* Wagler, 1891), a wild relative of the domestic guinea pig (family *Caviidae*), showed the positive effects in EY-supplemented Tris and ACP®-116c (powdered coconut water) extenders.[12,16] In the same way, the present results determined that 20% EY added to the TCG extender protected both plasma and acrosome membranes of guinea pig spermatozoa before and after the freezing process; despite this protection, the post-thaw motility and viability were <15%.

Guinea pigs are mammals in which the form of the sperm head is not determined by the shape of the nucleus, as in other species, but by an unusually large acrosome.[38] The stacking of sperm heads of guinea pig originating from the epididymis causes certain agglutinations (2–15 heads) that hinder displacement (motility).[23] Gentle pipetting manages to separate the head spermatozoa, as was evidenced in this study; however, adverse iatrogenic effects related to mechanical manipulations of the samples can cause detachment of the acrosomes. Agglutination of spermatozoa (stacking heads) in the domestic guinea pig is mediated by a protein (WH-30) that is present in testicular sperm.[39] In the guinea pig, acrosomal stacking in rouleaux may be related to the protection of fragile acrosomes that develop, during their epididymal passage, the ability to be lost upon stimulation with an ionophore.[40]

It has been suggested that agglutinate sperm in rouleaux could facilitate the survival of the associated spermatozoa in the female during the transit of the reproductive tract (vagina, cervix, or uterus).[41] From this viewpoint, we hypothesized the occurrence of two events that drastically affected sperm cryosurvival during the freezing process: (1) probably, heads stacking hindered the flow and efflux of sperm water across their plasmatic membranes during cellular dehydration, provoking intracellular ice crystals formation lethal to cells; and (2) during the separation of the sperm heads by gently pipetting, the plasma membranes suffered damage, enough to deteriorate during freezing.

This study showed that the freezing–thawing process with both extenders reduced only the head length; however, the elongation and ellipticity, and the other morphometric head dimensions were not altered by cryopreservation, irrespective of extender used. Our results of head dimensions of spermatozoa from prefreezing are consistent with those reported by Cabeza et al.[6] Other reports that used other methods of measuring the head of guinea pig sperm show different sizes from those observed in this study.[7,9] It is known that osmotic stress determines changes in the head dimensions of epididymal spermatozoa.[13] In addition, the shape and size of the sperm head may influence their susceptibility to osmotic damage during the freeze–thaw process,[38] especially in sperm more heterogeneous populations.[42] Variations in sperm head shape and volume/area may be responsible for variations in the velocity of water exchange across the plasma membrane during freezing–thawing.[43]

Sperm with small heads show high cryoresistance regardless of motility, viability, and acrosome integrity.[44] Since guinea pig spermatozoa have large head dimensions (length: 7.9 μm; width: 6.9 μm; and area: 47.0 μm^2), it is likely to expect low cryo-survival rates or significant cellular cryoinjuries. Other studies have determined that cryopreserved epididymal sperm suffer a reduction in the size of the head compared with prefreezing values.[42,44] It has been suggested that over-condensation of sperm chromatin, plasma, and acrosome membrane injuries, as well as acrosome loss and damage in the cell cytoskeleton, could cause a reduction in the head size.[5,45] The findings of this study concerning the morphometric sperm head dimensions demonstrated that the samples frozen with both extenders probably suffered minimal osmotic stress during cryopreservation, disrupting sperm membranes that affected only the length.

Conclusion

The TCG extender supplemented with 20% EY increased cryoprotection of membranes of guinea pig epididymal spermatozoa compared with TCF extender in prefreezing and post-thawed samples. Nevertheless, despite high cell cryoinjuries after the freezing–thawing process, the TCG-EY–based extender produced both low kinematics and integrity of the plasma and acrosomal membranes. Further studies on cooling rates, freezing protocols, and additives are recommended.

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References

1. Mínguez Balaguer C, Calvo Capilla A, Zeas Delgado VA, et al. A comparison of the growth performance, carcass traits, and behavior of Guinea pigs reared in wire cages and floor pens for meat production. Meat Sci 2019;152:38–40; doi: 10.1016/J.MEATSCI.2019.02.012

2. Avilés DF, Martínez AM, Landi V, et al. The Guinea Pig (*Cavia Porcellus*): An Andean resource of interest as an agricultural food source. Anim Genet Resour 2014;55:87–91; doi: 10.1017/S2078633614000368
3. Maass BL, Chauca-Francia L, Chiuri WL, et al. From 'cuy' in South America to 'cavy' in Sub-Sahara Africa: Advancing development through South-South Cooperation. Advancing development through South-South Cooperation. In: Paper presented at the Tropentag 2016 Conference on Solidarity in a Competing World—Fair Use of Resources, Vienna, Austria, 19–21 September 2016. University of Göttingen: Göttingen, Germany.
4. HLPE. Sustainable agricultural development for food security and nutrition: what roles for livestock? A report by the High Level Panel of Experts on Food Security and Nutrition of the Committee on World Food Security, Rome. 2016. Available from: http://www.fao.org/3/a-i5795e.pdf [Last accessed: September 7, 2022].
5. Benavides F, Sutovsky P, López V, et al. Semen parameters of fertile Guinea pigs (*Cavia Porcellus*) collected by trasnrectal electroejaculation. Anim Multidiscip Digit Publ Inst 2020;10(5):767; doi: 10.3390/ani10050767
6. Cabeza U, Ordóñez C, Meza A, et al. Morphological and morphometric characterization of guinea pig (*Cavia Porcellus*) sperm [In Guarani]. Spermova 2020;10(2):94–101; doi: 10.18548/aspe/0008.14
7. Paredes M, Guevara J, Mantilla J, et al. Features of the semen and reproductive performance of native guinea pigs and improved in reciprocal crosses [In Spanish]. Spermova 2020;10(1):11–17; doi: 10.18548/aspe/0008.02
8. Freund M. Interrelationship among the characteristics of Guinea-pig semen collected by electro-ejaculation. Reproduction 1969;19(3):393–403; doi: 10.1530/JRF.0.0190393
9. Rosales C, Guevara G, Perea F, et al. Morphometry of the male gonad and sperm of native and improved guinea pigs (*Cavia porcellus*) from southern Ecuador [In Guarani]. Rev Investig Vet del Perú 2021;32(2):e17988; doi: 10.15381/RIVEP.V32I2.17988
10. Yanagimachi R, Suzuki F. A further study of lysolecithin-mediated acrosome rection of Guinea pig spermatozoa. Gamete Res 1985;11(1):29–40; doi: 10.1002/mrd.1120110104
11. Eder S, Müller K, Jewgenow K, et al. P1.6 Cryopreservation of Guinea pig spermatozoa—Challenges and first success. Reprod Biol 2013;13:24; doi: 10.1016/j.repbio.2013.01.014
12. Silva AM, Praxedes ECG, Campos LB, et al. Epididymal sperm from Spix's yellow-toothed cavies sperm successfully cryopreserved in tris extender with 6% glycerol and 20% egg yolk. Anim Reprod Sci 2018;191:64–69; doi: 10.1016/J.ANIREPROSCI.2018.02.009
13. O'Brien E, Esteso MC, Castaño C, et al. Effectiveness of ultra-rapid cryopreservation of sperm from endangered species, examined by morphometric means. Theriogenology 2019;129:160–167; doi: 10.1016/j.theriogenology.2019.02.024
14. Esteso MC, Toledano-Díaz A, Castaño C, et al. Effect of two cooling protocols on the post-thaw characteristics of Iberian ibex sperms. Cryobiology 2018;80:12–17; doi: 10.1016/j.cryobiol.2018.01.003
15. Galarza DA, Landi G, Mejía E, et al. Cryopreservation of dog epididymal spermatozoa by conventional freezing or ultra-rapid freezing with nonpermeable cryoprotectant. Cryobiology 2021;103:15–21; doi: 10.1016/j.cryobiol.2021.10.002
16. Moreira SS, da Silva A, Paz AL, et al. Cryopreservation of Spix's yellow-toothed cavy epididymal sperm using Tris- and Coconut water-based extenders supplemented with egg yolk or Aloe vera. Cryobiology 2021;99:40–45; doi: 10.1016/J.CRYOBIOL.2021.01.016
17. Esteso MC, Soler AJ, Fernández-Santos MR, et al. Functional significance of the sperm head morphometric size and shape for determining freezability in Iberian red deer (*Cervus elaphus hispanicus*) epididymal sperm samples. J Androl 2006;27(5):662e70; doi: 10.2164/jandrol.106.000489
18. Hu JH, Li QW, Chen XY, et al. The cryoprotective effect on frozen-thawed boar semen of egg yolk low density lipoproteins. Asian Australas J Anim Sci 2006;19(4):486–494; doi: 10.5713/ajas.2006.486
19. Ping SH, Wang CY, Tang WR, et al. Effects of some extenders and monoamines on sperm cryopreservation in tree shrews (*Tupaia belangeri*). Zool Res 2012;33(1):19–28; doi: 10.3724/sp.j.1141.2012.01019
20. Rigau T, Farré M, Ballester J, et al. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. Theriogenology 2001;56(5):801–815; doi: 10.1016/S0093-691X(01)00609-4
21. Manjunath P, Nauc V, Bergeron A, et al. Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of Hen's egg yolk. Biol Reprod 2002;67(4):1250–1258; doi: 10.1095/BIOLREPROD67.4.1250
22. Mocé E, Blanch E, Tomás C, et al. Use of cholesterol in sperm cryopreservation: Present moment and perspectives to future. Reprod Domest Anim 2010;45(SUPPL. 2):57–66; doi: 10.1111/J.1439-0531.2010.01635.X
23. Fawcett D, Hollenberg R. Changes in the acrosome of Guinea pig spermatozoa during passage through the epididymis. Zeitschrift für Zcllforsch 1963;60:276–292.
24. Mazur P. Freezing of Living Cells: Mechanisms and implications. Am J Physiol Physiol 1984;247(16):125–142; doi: 10.1152/ajpcell.1984.247.3.c125
25. Galarza D, López-Sebastián A, Santiago-Moreno J. Effectiveness of two-step accelerating cooling rate on post-thaw characteristics of ram sperm. Reprod Domest Anim 2019;54:113.
26. Galarza DA, López-Sebastián A, Woelders H, et al. Two-step accelerating freezing Protocol yields a better motility, membranes and DNA integrities of thawed ram sperm than three-steps freezing protocols. Cryobiology 2019;91:84–89; doi: 10.1016/j.cryobiol.2019.10.007
27. Santiago-Moreno J, Astorga RJ, Luque I, et al. Influence of recovery method and microbial contamination on the response to freezing–thawing in ibex (*Capra pyrenaica*) epididymal spermatozoa. Cryobiology 2009;59(3):357–362; doi: 10.1016/j.cryobiol.2009.09.012
28. Cañón-Beltrán K, Cajas YN, González E, et al. Acquisition of fertilization competence in guinea pig spermatozoa under different capacitation protocols. Theriogenology 2023;198:231–240; doi: 10.1016/j.theriogenology.2022.12.042
29. Yotov S. Effect of TFC-based extenders with Soybean lecithin and/or low concentration of glycerol on the quality of goat chilled-stored semen. Int J Curr Microbiol Appl Sci 2015;4(3):752–761.
30. Colas G. Effect of initial freezing temperature, addition of glycerol and dilution on the survival and fertilizing ability of deep-frozen ram semen. J Reprod Fertil 1975;42(2):277–285; doi: 10.1530/jrf.0.0420277

31. White IG. Lipids and calcium uptake of sperm in relation to cold shock and preservation: A review. Reprod Fertil Dev 1993;5:639e65; doi: 10. 1071/RD9930639
32. Ladha S. Lipid heterogeneity and membrane fluidity in a highly polarized cell, the mammalian spermatozoon. J Membr Biol 1998;165:1e10; doi: 10.1007/s002329900415
33. Meyers SA. Spermatozoal response to osmotic stress. Anim Reprod Sci 2005;89:57e64; doi: 10.1016/j.anireprosci.2005.06.026
34. Bergeron A, Manjunath P. New insights towards understanding the mechanisms of sperm protection by egg yolk and milk. Mol Reprod Dev 2006;73(10):1338–1344; doi: 10.1002/MRD.20565
35. Shannon P, Curson B. Effect of egg yolk levels on the fertility of diluted bovine sperm stored at ambient temperatures. New Zealand J Agric Res 2012;26(2):187–189; doi: 10.1080/00288233.1983.10427058
36. Quinn PJ, Chow PYW, White IG. Evidence that phospholipid protects ram spermatozoa from cold shock at a plasma membrane site. Reproduction 1980;60(2):403–407; doi: 10.1530/JRF.0.0600403
37. Fleming AD, Yanagimachi R. Effects of various lipids on the acrosome reaction and fertilizing capacity of Guinea pig spermatozoa with special reference to the possible involvement of lysophospholipids in the acrosome reaction. Gamete Res 1981;4(4):253–273; doi: 10.1002/MRD.1120040402
38. Morris TJ. A Comparison of Acrosomal Proteinase Activity in the Male and Female Reproductive Tracts of the Guinea Pig. Virginia Commonwealth University; 1975; doi: 10.25772/0W76-RG43
39. Cooper TG, Weydert S, Yeung CH, et al. Maturation of epididymal spermatozoa in the nondomesticated guinea pigs *Cavia aperea* and *Galea musteloides*. J Androl 2000; 21(1):154–163; doi: 10.1002/j.1939-4640.2000.tb03285.x
40. Hunnicutt GR, Koppel DE, Myles DG. Analysis of the process of localization of fertilin to the sperm posterior head plasma membrane domain during sperm maturation in the epididymis. Dev Biol 1997;191(1):146–159; doi: 10.1006/dbio.1997.8700
41. Martan J, Shepherd BA. Spermatozoa in rouleaux in the female guinea pig genital tract. Anat Rec 1973;175(3):625–629; doi: 10.1002/ar.1091750309
42. Esteso MC, Fernández-Santos MR, Soler AJ, et al. The effects of cryopreservation on the morphometric dimensions of Iberian red deer (*Cervus elaphus hispanicus*) epididymal sperm heads. Reprod Domest Anim 2006;41(3): 241–246; doi: 10.1111/j.1439-0531.2006.00676.x
43. Yániz JL, Soler C, Santolaria P. Computer assisted sperm morphometry in mammals: A review. Anim Reprod Sci 2015;156:1e12; doi: 10. 1016/j.anireprosci.2015.03.002
44. Hidalgo M, Rodríguez I, Dorado JM. The effect of cryopreservation on sperm head morphometry in florida male goat related to sperm freezability. Anim Reprod Sci 2007;100(1–2):61–72; doi: 10.1016/J.ANIREPROSCI.2006.07.003
45. Santiago-Moreno J, Esteso MC, Pradiee J, et al. Giant panda (*Ailuropoda melanoleuca*) sperm morphometry and function after repeated freezing and thawing. Andrologia 2016;48(4):470–474; doi: 10.1111/AND.12468

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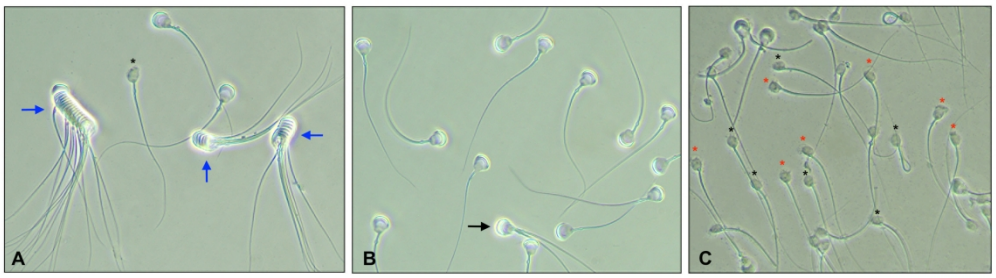

Fig. 2. Images of epididymal guinea pig spermatozoa (fixed with 2% glutaraldehyde) observed in a phase contrast microscope (x400). A, Strong heads stacking (10 to 15 cells) of spermatozoa in fresh samples after retrograde flushing (blue arrows). B, Separated spermatozoa after pipetting; notice some 2-headed stacking on the (black arrow). C, Spermatozoa with festooned (red asterisks) or detached (black asterisks) acrosomes.

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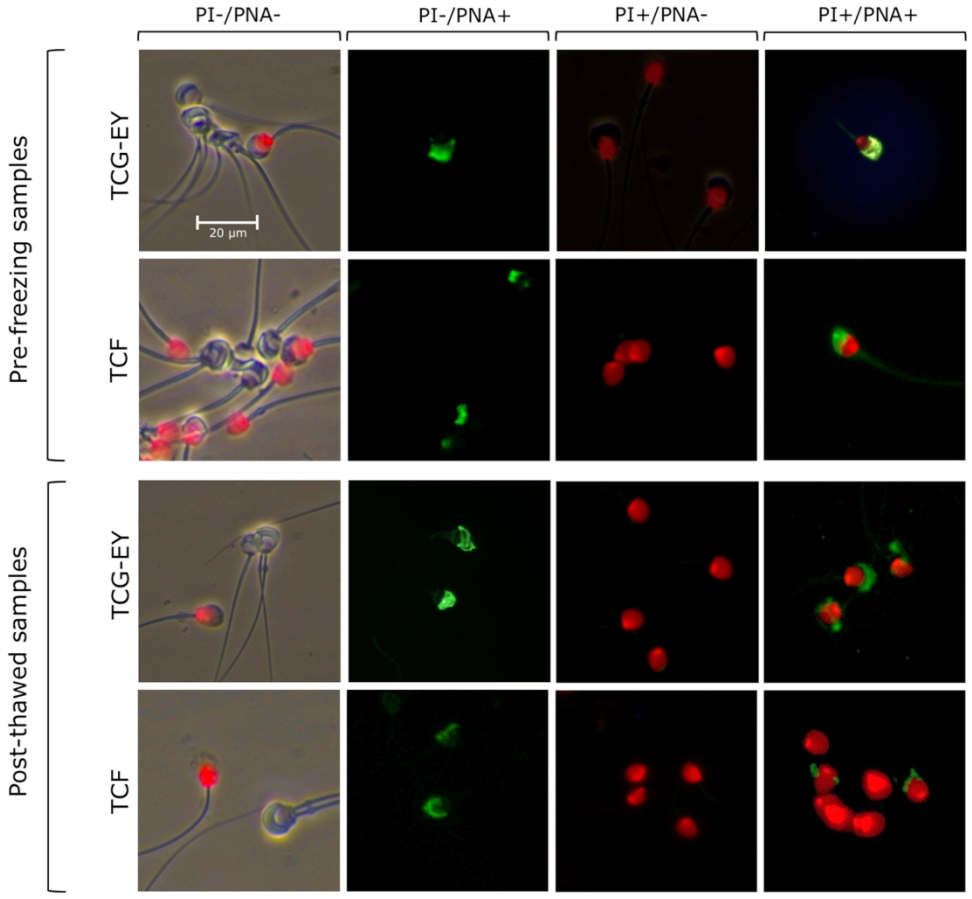

Fig. 3. Pre-freezing and post-thawed guinea pig sperm samples stained with an association of propidium iodide (PI) and fluorescein isothiocyanate-conjugated peanut agglutinin (PNA) (x400). PI-/PNA-, sperm with intact plasma membrane with intact acrosome (non-stained cells); PI-/PNA+, sperm with intact plasma membrane with damaged acrosome (green); PI+/PNA-, sperm with damaged plasma membrane with intact acrosome (red); and PI+/PNA+, sperm with damage plasma membrane and acrosome (red/green)

149x138mm (300 x 300 DPI)