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RESEARCH ARTICLE

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Outlining adequate protocols for Lidia bull epididymal storage and sperm cryopreservation: use of glycerol, dimethylformamide and N-acetylcysteine

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Abstract

The Lidia bovine breed is an important hallmark of the Spanish cattle industry. Bulls are selected based upon aggressiveness and epididymal sperm cryopreservation is the way to obtain and store their genetics. There are not specifically designed protocols yet to perform Lidia bull sperm cryopreservation. The present study aimed to determine if a tris-fructose-citrate-egg yolk (20% v/v; TFY) extender supplemented with 7% glycerol (TFY1) or 3.5% glycerol plus 3.5% dimethylformamide (DMF; TFY2) are suitable media for cryopreservation of epididymal Lidia bull sperm. Moreover, the effect of N-acetylcysteine (NAC), a potent antioxidant, was evaluated. The epididymis were stored at 4°C for 24, 48, 72 or 96 h, and both freezing media were tested as such or supplemented with 1 or 2.5 mM of NAC. Our data demonstrated that post-thaw viability was well maintained (TFY1: 50.8% ± 1.9 at 24 h and 52.4% ± 0.8 at 96 h and TFY2: 52.6% ± 1.6 at 24 h and 56.1% ± 1.8 at 96 h; mean % ± SEM; $p>0.05$) as also were total and progressive sperm motility, high mitochondrial membrane potential, ROS production, DNA status and acrosomal intactness of Lidia bull sperm up to 96 h of epididymal storage, all extender variations being similar ($p>0.05$). In conclusion, the use of TFY medium supplemented either with 7% glycerol alone or the combination of 3.5% glycerol and 3.5% DMF were equally safe choices for epididymal Lidia bull sperm cryopreservation, and NAC addition did not significantly improve sperm post-thaw quality.

Additional keywords: Fighting bull; epididymal sperm; sperm freezing extenders; antioxidants; cooling.

Abbreviation used: ART (assisted reproductive technique); CPA (cryoprotective agent); DFI (DNA fragmentation index); DMF (dimethylformamide); HDS (high DNA stainability); H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate); hMMP (high mitochondrial membrane potential); NAC (N-acetylcysteine); PI (Propidium iodide), ROS (reactive oxygen species); TF (tris-fructose); TFY (tris-fructose-egg yolk); TFY1 (TFY + 7% of glycerol, v/v); TFY2 (TFY + 3.5% of glycerol + 3.5% of DMF, v/v).

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Introduction

The Lidia bovine breed or fighting bull is an autochthonous breed of the Iberian Peninsula. The cows and the sires are selected based upon temperament and aggressiveness, disregarding their reproductive performance. In addition, the descendants are not allowed to breed and are not tested until they reach the

fighting ring, thus increasing the lineage inbreeding (Canon *et al.*, 2008). The use of assisted reproductive techniques (ARTs) is considered as an important tool to obtain offspring or preserve the genetics of a particular bull killed during the bullfight (Katska-Ksiazkiewicz *et al.,* 2006). Usually, bullrings are far from ART facilities and thus, transport and cooled storage of the epididymis become necessary to allow for sperm harvesting (Malcotti *et al.*, 2012). Fortunately, epididymal sperm can be successfully cryopreserved and used for artificial insemination or *in vitro* production of embryos (Martins *et al.*, 2007; Lopes *et al.*, 2015), although the maximum refrigerated epididymal storage time still needs to be determined in the Lidia bovine breed.

One of the most damaging events occurring during sperm refrigerated storage and freezing-thawing cycles is an increased production of reactive oxygen species (ROS), which overwhelms the sperm's antioxidant defenses (Nichi *et al.*, 2007). This oxidant/antioxidant imbalance results in DNA damage (Gürler *et al.*, 2016), plasma membrane lipid peroxidation and cytoskeletal alterations (Agarwal *et al.*, 2014), among other injuries. Two strategies have been followed to partially alleviate the detrimental effect of ROS: a) antioxidant addition to freezing extenders and b) the use of alternative cryoprotective agents (CPAs) of potentially lower toxicity.

Regarding antioxidants, a wide variety have been tested as supplements to freezing extenders (Fernández-Santos *et al.*, 2009a). Among them, N-Acetylcysteine (NAC) has demonstrated to alleviate glutathione (GSH) depletion and free radical formation during oxidative stress (Wu *et al.*, 2006), and has yielded satisfactory results in different species (Ciftci *et al.*, 2009; Mata *et al.*, 2012; Pérez *et al.*, 2015). However, until now, there is only one report regarding NAC addition to extenders used for freezing Lidia bull epididymal sperm, showing that sperm kinematics remain unchanged after prolonged epididymal cooled storage (Matilla *et al.*, 2017).

Glycerol is the most commonly added cryoagent to bovine sperm freezing media (Forero *et al.*, 2012; Almeida *et al.*, 2017) although it increases plasmalemmal permeability and induces the disruption of the actin cytoskeleton in equine sperm (Macias *et al.*, 2012). Recently, in the horse, alternative cryoagents such as dimethylformamide (DMF) have been tested with great success (Alvarenga *et al.*, 2005). The lower molecular weight of amides compared to glycerol improves sperm membrane permeation thus decreasing the pre-cooling interval (Squires *et al.*, 2004; Alvarenga *et al.*, 2005). In addition, DMF has been demonstrated to enhance equine sperm post-thaw quality when used as single CPA (Olaciregui *et al.*, 2014) or combined with glycerol (Morillo *et al.*, 2012). However, the use of DMF as single CPA at 3% (v/v) in bull ejaculates has been shown to detrimentally affect sperm cells (Forero *et al.*, 2012; Martins *et al.*, 2015). Accordingly, this study was designed to evaluate the effect of glycerol alone (7%; v/v) or a combination of 3.5% glycerol and 3.5% DMF (v/v/v) in presence or absence of N-acetylcysteine (1 mM or 2.5 mM) to the freezing extender of Lidia bull epididymal sperm stored at 4°C for 24, 48, 72 and 96 h.

Material and methods

Reagents and media

All the reagents were purchased from Sigma-Aldrich (Barcelona, Spain) unless otherwise stated. The base medium was composed of tris-fructose-citrate (TF) consisting of tris (250 mM), citric acid (86.9 mM), fructose (36 mM), 1 mg/mL penicillin and 0.5 mg/mL gentamicine in sterile Milli-Q water (Chaudhari *et al.*, 2015). Base freezing medium was composed of TF added with 20% egg yolk (v/v; TFY) (van Wagtendonk-de Leeuw *et al.*, 2000) and the following cryoprotectants: 7% glycerol (v/v; referred as TFY1) or 3.5% glycerol and 3.5% DMF (v/v; referred as TFY2). Furthermore, N-acetylcysteine was added to each extender and TFY1 or TFY2 devoid of NAC were considered as controls (TFY1-C and TFY2-C respectively). Both extenders were supplemented with 1 or 2.5 mM of NAC, resulting in three groups per freezing medium.

Sperm harvesting and processing

Sperm were collected from the epididymis of 17 Lidia Spanish bulls aged 3-4 years from June to September of 2016. Testes with attached epididymis were obtained post-mortem from the bulls at the bullring. Immediately after removal, the testis were placed into plastic bags in an isothermal box at 4ºC and shipped overnight to the laboratory. Once at the laboratory, the epididymis were separated from the testis, as described by Yu & Leibo (2002) and stored in the fridge (4ºC) prior processing at four time points: 24 h, 48 h, 72 h and 96 h. The connective tissue was carefully dissected, and the cauda epididymis was straightened to allow for flushing extender passage. A 20G needle-attached to a 10 mL plastic syringe was used to flush the cauda epididymis of each bull using 5 mL of pre-warmed (37ºC) buffered TF (Chaudhari *et al.*, 2015). Each sample obtained was aspirated using a Pasteur plastic pipette and transferred to a 15 mL tube. Sperm was then centrifuged at 600 × g for 10 min, the supernatant was discarded and the pellets

were diluted in 1.5-2 mL of TF at room temperature (22-25ºC). Sperm concentration was determined using a Neubauer counting chamber. Each sample was divided into six groups, one per treatment, and then centrifuged at 600 × g for 10 min. The supernatant was discarded and the freezing medium (TFY1 or TFY2) was slowly added to reach a final concentration of 100 × 10^6 sperm/mL in the presence or absence of NAC (no addition, 1 or 2.5 mM). Once the extender was added, subjective total motility was assessed; sperm samples with total sperm motility below 40% were discarded. The diluted semen was packed into 0.25 mL French straws at room temperature and closed using an ultrasound sealer (Ultrasound Welding Machine, Vitrolife, Sweden). The straws were placed horizontally in a rack, and preserved in the fridge at 4°C for 2 h; then, the straws were located 4 cm above liquid nitrogen vapors for 20 min, seeded and subsequently plunged into liquid nitrogen (Forero *et al.*, 2012). The straws were stored for at least 1 month prior analysis. Thawing was achieved by immersing the straws for 1 min in a water bath set at 37°C. After thawing, 50 µL of each sample were resuspended with an equal volume of TF, subjected to a short spin (MiniSpin®, eppendorf) for 10 s, the supernatant was discarded and the pellet was resuspended in 90 µL of pre-warmed TF to achieve final concentration of 50 × 10^6 sperm/mL.

Motility assay

All samples were examined using a CASA system (ISAS®, ProiserR+D, Paterna, Valencia, Spain). Two microliters of sperm were placed in a pre-warmed motility chamber of 20 µm depth (Leja®, Nieuw-Vennep, The Netherlands). Sperm motility was assessed with a Nikon Eclipse 50i microscope equipped with a ×10 negative-phase contrast objective and a heated stage at 38°C. Analysis was based on the examination of 25 consecutive digitalized images obtained from 3 fields and at least 200 sperm per sample were analyzed; total and progressive motility were measured (Amann & Waberski, 2014).

Flow cytometry

All fluorescence signals of labeled sperm were analyzed by flow cytometry (ACEA NovoCyte™; ACEA Biosciences, Inc., San Diego, CA, USA) using the ACEA NovoExpress™ software. A minimum of 10000 sperm were examined for each assay at a minimum flow rate of 300 cells/s. The sperm population was gated using forward and side scatter light signals to exclude debris and aggregates. The fluorophores were excited with a 200 mV argon ion laser operating at 488 nm.

Mitochondrial potential status

The metachromatic dye JC-1 (Thermo Fisher, Madrid, Spain) differentiates between mitochondria with high and low mitochondrial membrane potential (MMP). When MMP is high (hMMP), the JC-1 molecules form aggregates that emit in the orange wavelength while JC-l monomers emitting green fluorescence are detected when the MMP is low (Garner & Thomas, 1999). From each sperm sample, 5 µL were diluted in 245 µL of isotonic buffered diluent (PBS) containing 0.9 µM of JC-1 and was incubated at 38°C for 30 min prior evaluation.

Acrosomal status

The sperm acrosomal status was assessed using peanut agglutinin conjugated with fluorescein thiocyanate PNA-FITC as a marker for acrosomal integrity and propidium iode for viability (PI, component B of sperm viability kit, Thermofisher, Madrid, Spain). Aliquots of 5 µL of each sperm sample (100 × 10^6 cells/mL) were incubated at 38ºC in the dark for 5 min with 1 µg/mL PNA-FITC and 6 µM PI in 45 µL of isotonic buffered diluent. Then, 200 µL of isotonic buffered diluent were added to each sample and mixed before flow cytometry analysis (Hurtado de Llera *et al.*, 2013).

Reactive oxygen species production

The cell-permeant 2',7'-dichlorodihydrofluorescein diacetate or H_2DCFDA (Thermo Fisher, Madrid, Spain) is a non-fluorescent probe that becomes de-esterified intracellularly and turns into highly fluorescent 2´,7´-dichorofluoresecein upon oxidation by ROS; cell viability was simultaneously assessed by detection of fluorescence of the non-permeant probe PI. The protocol used is a modification of the one described by Macias *et al.* (2015). In brief, aliquots of 5 µL of each semen sample (100 x 10^6 cells/mL) were diluted in 245 µL of isotonic buffered diluent and incubated for 30 min with 20 µM of H_2DCFDA. In the last 2 min, PI was added (final concentration, 6 µM). Results represent the percentage of living cells producing ROS.

Sperm chromatin status

The SCSA technique (Evenson *et al.*, 2002) is based on the metachromatic stain acridine orange; this probe exhibits green fluorescence when combined with double-stranded DNA, and red when combined with single-

stranded DNA (denatured). Sperm were diluted in TNE buffer (0.15 M NaCl, 0.01 M TrisHCl, 1 mM EDTA; pH 7.4) to reach a final concentration of 2-4 × 10^6 sperm/mL). Samples were flash frozen in LN_2 and stored at -80°C until analysis. For the analysis, the samples were thawed on crushed ice and 200 µL were transferred to a cytometry tube. Then, 400 µL of an acid detergent solution consisting of 80 mM HCl, 0.15 M NaCl and 0.1% Triton X-100 at a 1.2 pH were added. Exactly, 30 s after the acid-detergent solution addition, 1.2 mL of staining solution were added (6 µg/mL of acridine orange chromatographically purified (Polysciences, Warrington, PA, USA) in a buffer containing 37 mM citric acid, 126 mM Na_2HPO_4, 1.1 mM disodium EDTA and 150 mM NaCl; pH 6). Sample acquisition was carried out with the CellQuestv.3 software (Becton Dickinson, Franklin Lakes, NJ, USA). Flow cytometry data (FCS files) were processed by using the R statistical environment with the Bioconductor flow Core package. The DNA fragmentation index (DFI) was calculated for each spermatozoon as the ratio of red fluorescence with respect to total fluorescence (red + green) and expressed as a percentage. The percentage of sperm with high fragmentation index (%DFI) was calculated as the percentage events with DFI>25%. The high DNA stainability index (HDS) was obtained as the percentage of sperm with green fluorescence intensity above channel 600 (0–1023 channels).

Statistical analysis

Data were tested for normality using a Shapiro–Wilk test and the obtained results are represented as mean ± standard error of the mean (SEM). Groups were compared using an ANOVA on ranks due to their non-Gaussian distribution. When statistically significant differences were found, a Dunn's post-hoc test was used to compare groups. All statistical analyses were performed using Sigma Plot software version 12.3 for Windows (Systat Software, Chicago, IL, USA). Differences among values were considered as statistically significant when $p<0.05$.

Results

Total and progressive motility analysis

An apparent decrease in total motility was observed after prolonged storage. Statistically significant differences were observed in progressive motility between TFY1-C at 24 h and TFY1-C at 96 h (16.2 ± 0.6 *vs.* 2.8 ± 0.4; mean % ± SEM; $p<0.05$). Although, values obtained for total motility did not vary significantly between TFY1-C at 24 h and TFY2-C at 96 h (33.1 ± 1.4 *vs.* 15.3 ± 2.2; mean % ± SEM). Additionally, NAC addition or the use of glycerol or the combination of glycerol and DMF did not significantly affect total or progressive motility. No statistically significant differences were found for the total motility parameter in any of the groups tested (Table 1; $p>0.05$).

Determination of the percentage of live cells producing ROS

Our data showed an apparent increase in the percentage of live sperm producing ROS when TFY2-C was used compared to TFY1-C at 24, 48, 72 and 96 h. In addition, a tendency for ROS production alleviation was observed when TFY2 was supplemented with NAC either at 1 mM or 2.5 mM at any time point studied (Table 1), although the values obtained did not vary significantly. Nevertheless, statistically significant differences in the percentage of live cells producing ROS were not found between groups disregarding the length of the epididymal storage, freezing medium used and/or NAC addition ($p>0.05$; Table 1).

Determination of sperm with high mitochondrial membrane potential

Our data showed that storage time of the epididymis, freezing media or NAC addition did not significantly affect the percentage of sperm with high mitochondrial membrane potential ($p>0.05$; Table 1) which ranged between 33.5% ± 1.0 for TFY2-2.5 mM at 72 h (mean ± SEM; minimum value) to 52.2% ± 1.81 for TFY1-C at 48 h (mean ± SEM; maximum value).

Determination of the sperm viability and acrosomal integrity

Our data showed that sperm viability and acrosomal intactness was maintained despite prolonged epididymal cooled storage, varying freezing medium or NAC addition. No statistically significant differences were observed between groups (Table 2; $p>0.05$).

Determination of sperm chromatin status

The percentage of sperm showing DNA fragmentation (%DFI) or high DNA stainability (%HDS) did not significantly vary despite NAC addition, freezing extender used or prolonged refrigerated epididymal storage (Table 2; $p>0.05$); DFI was below 2.2% and HDS remained below 5.7% for all cases, indicating good chromatin status.

Table 1. Total and progressive motility, percentage of living cells producing ROS and percentage of sperm depicting high mitocondrial membrane potential of thawed Lidia bull sperm from epididymis stored at 4°C at different time points prior cryopreservation.

Storage time 4ºC	Freezing medium[1]	n	TM (%)[2]	PM (%)[3]	% Living cells ROS+[4]	hMMP(%)[5]
24 hours	TFY1-C	4	33.1 ± 1.4	16.2 ± 0.6[a]	16.5 ± 0.9	40.6 ± 1.3
	TFY1-1 mM	4	29.3 ± 3.8	12.6 ± 1.9	16.2 ± 1.4	37.0 ± 4.8
	TFY1-2.5 mM	4	29.1 ± 0.8	12.9 ± 0.9	16.0 ± 1.4	34.7 ± 2.0
	TFY2-C	4	26.1 ± 2.8	10.5 ± 1.8	28.4 ± 2.8	34.4 ± 2.3
	TFY2-1 mM	4	34.6 ± 1.6	18.3 ± 2.6	19.9 ± 2.5	37.0 ± 3.9
	TFY2-2.5 mM	4	33.4 ± 2.1	17.0 ± 2.8	18.2 ± 1.8	34.4 ± 2.6
48 hours	TFY1-C	5	21.7 ±1.6	6.4 ± 1.0	11.4 ± 1.7	52.2 ± 1.8
	TFY1-1 mM	5	21.1 ± 2.1	5.9 ± 0.6	11.9 ± 1.1	45.0 ± 2.3
	TFY1-2.5 mM	5	19.6 ± 0.9	6.7 ± 0.7	13.6 ± 1.3	44.4 ± 2.4
	TFY2-C	5	19.5 ± 1.1	5.8 ± 0.7	17.4 ± 1.2	43.0 ± 2.6
	TFY2-1 mM	5	19.6 ± 1.5	5.1 ± 0.8	10.2 ± 3.3	38.6 ± 3.3
	TFY2-2.5 mM	5	19.8 ± 0.8	5.1 ± 0.9	14.0 ± 3.0	40.5 ± 1.5
72 hours	TFY1-C	5	21.0 ± 1.4	7.3 ± 1.0	21.9 ± 3.5	38.6 ± 0.8
	TFY1-1 mM	5	23.5 ± 1.8	7.1 ± 0.3	16.2 ± 1.7	40.9 ± 1.8
	TFY1-2.5 mM	5	25.4 ± 1.5	9.1 ± 0.9	18.6 ± 1.4	40.4 ± 1.2
	TFY2-C	5	24.3 ± 1.5	7.3 ± 0.8	29.1 ± 2.5	34.5 ± 1.5
	TFY2-1mM	5	24.8 ± 2.2	7.1 ± 0.6	24.0 ± 2.1	34.7 ± 1.0
	TFY2-2.5 mM	5	24.3 ± 0.5	6.4 ± 0.6	18.7 ± 3.0	33.5 ± 1.0
96 hours	TFY1-C	3	12.1 ± 0.4	2.8 ± 0.4[b]	24.0 ± 1.3	42.8 ± 1.0
	TFY1-1 mM	3	15.1 ± 1.8	2.2 ± 0.5	24.9 ± 4.5	40.9 ± 1.9
	TFY1-2.5 mM	3	16.2 ± 2.8	3.5 ± 0.5	22.8 ± 3.4	46.8 ± 1.8
	TFY2-C	3	15.3 ± 2.2	2.5 ± 0.5	34.9 ± 3.4	41.5 ± 0.7
	TFY2- 1 mM	3	17.2 ± 2.1	4.0 ± 0.7	30.6 ± 2.9	38.7 ± 0.6
	TFY2-2.5 mM	3	19.2 ± 1.1	4.1 ± 0.4	27.8 ± 5.1	40.0 ± 2.3

[1] Cryopreservation media were supplemented with varying doses of NAC (0, 1 and 2.5 mM). TFY-1: TFY with 7% glycerol, TFY-2: TFY with 3.5% glycerol and 3.5% dimethylformamide (v/v). [2] TM: total motility. [3] PM: progressively motility. [4] % Living cells ROS+: percentage of live cells producing ROS. [5]hMMP: percentage of high mitochondrial membrane potential. [a,b] Values between TFY1-C at 24 h and TFY1-C at 96 h differ statistically (p <0.05). All groups were compared between them.

Discussion

Sperm recovery from the cauda epididymis for subsequent cryopreservation is the most practical method to obtain and store semen from Lidia bulls due to their risky field handling. However, glycerol concentrations currently added to bovine freezing extenders vary from 3% to 7% depending on the research laboratory (Chaveiro *et al.*, 2006; Fernández-Santos *et al.*, 2009a), and marked differences in sperm tolerance to glycerol has been demonstrated between breeds of the same species (*i.e.* goats and turkeys) (Kulaksiz *et al.*, 2013; Long *et al.*, 2014). Moreover, glycerol and DMF have also been shown to detrimentally affect sperm motility, viability and mitochondrial status of bull and stallion semen when used as single CPA (Alvarenga *et al.*, 2005; Forero *et al.*, 2012). Therefore, in our study the alternative cryoprotectant DMF combined with glycerol was tested, as this CPA mixture has yielded promising results in the equine species (Álvarez *et al.*, 2014). Our data demonstrate that total and progressive sperm motility, viability, mitochondrial membrane potential, ROS production, DNA status and acrosomal intactness of Lidia bull sperm post-thaw are well maintained in TFY medium added either with 7% glycerol or the combination of 3.5% glycerol and 3.5% DMF for

up to 96 h post-mortem (Tables 1 and 2; $p>0.05$). Coinciding with previous observations in boar, our work demonstrates that, in Lidia bull, the combination of DMF and glycerol is not better than glycerol alone for epididymal sperm cryopreservation (Malo *et al.*, 2012). However, the combination of DMF and glycerol seems to effectively decrease the toxic effect of the latter, as DMF alone at 3% or 5% (v/v) significantly impairs motility, mitochondrial function and acrosomal intactness of bull sperm (Forero *et al.*, 2012). However, it has to be mentioned that the percentage of live sperm producing ROS was higher and the hMMP was lower when the combination of 3.5% glycerol and 3.5% DMF was used compared to glycerol alone (Table 1). Thus, as the use of a single CPA agent facilitates the preparation of freezing extenders, glycerol seems to be the cryoagent of election for Lidia bull sperm.

Even when total motility decreased during prolonged storage and a similar pattern was followed by progressive motility (Table 1), no statistically significant differences were found between treatments except for PM of sperm frozen in TFY1-C at 24 h and 96 h (Table 1). This lack of significance can be explained due to the high individual variability existing between bulls and their sperm tolerance for the cooling and freezing-thawing cycles (Posado *et al.*, 2008). A recent report by Nichi *et al.* (2016) established that total motility of post-thaw epididymal sperm from Charolais, Limousin and Belgian Blue bovine breeds after 2-3 h of refrigerated storage was 16.2% ± 4.3, which is comparable to the results obtained in the present study after 96 h (Table 1), highlight the fact that Lidia bull tolerance

Table 2. Simultaneous determination of viability, live acrosome-intact sperm and DNA fragmentation of thawed Lidia bull sperm from epididymis stored at 4°C at different time points prior cryopreservation.

Storage time 4ºC	Freezing medium[1]	n	PNA+/PI-[2]	Viability[3]	TDFI (%)[4]	HDS (%)[5]
24 hours	TFY1-C	4	3.1 ± 4.0	50.8 ± 1.9	0.8 ± 0.1	4.5 ± 02
	TFY1-1 mM	4	3.9 ± 5.0	44.5 ± 3.5	0.9 ± 0.1	5.1 ± 0.2
	TFY1-2.5 mM	4	3.5 ± 0.5	45.4 ± 3.0	1.0 ± 0.3	4.1 ± 0.4
	TFY2-C	4	6.1 ± 1.1	52.6 ± 1.6	0.8 ± 0.1	4.3 ± 0.2
	TFY2-1 mM	4	4.3 ± 0.3	56.3 ± 1.7	1.2 ± 0.3	5.1 ± 0.2
	TFY2-2.5 mM	4	3.2 ± 0.5	53.3 ± 3.1	0.6 ± 0.1	5.1 ± 0.4
48 hours	TFY1-C	5	3.0 ± 0.1	52.5 ± 2.9	1.5 ± 0.2	4.9 ± 0.2
	TFY1-1 mM	5	2.9 ± 0.2	47.9 ± 2.8	1.4 ± 0.1	5.6 ± 0.5
	TFY1-2.5 mM	5	2.9 ± 0.4	46.2 ± 1.9	1.6 ± 0.2	5.5 ± 0.2
	TFY2-C	5	3.4 ± 0.1	49.1 ± 2.6	1.0 ± 0.1	5.6 ± 0.2
	TFY2-1 mM	5	3.9 ± 0.9	45.1 ± 1.5	1.4 ± 0.1	5.1 ± 0.4
	TFY2-2.5 mM	5	2.5 ± 0.5	46.0 ± 2.4	1.5 ± 0.1	5.6 ± 0.2
72 hours	TFY1-C	5	2.0 ± 0.2	51.6 ± 3.1	1.5 ± 0.1	3.4 ± 0.2
	TFY1-1 mM	5	1.8 ± 0.3	49.3 ± 2.0	2.1 ± 0.3	3.3 ± 0.2
	TFY1-2.5 mM	5	1.4 ± 0.2	53.2 ± 2.0	1.7 ± 0.0	3.3 ± 0.2
	TFY2-C	5	2.3 ± 0.3	56.6 ± 1.1	1.6 ± 0.2	3.4 ± 0.1
	TFY2-1 mM	5	2.6 ± 0.3	55.8 ± 0.7	1.5 ± 0.2	3.3 ± 0.2
	TFY2-2.5 mM	5	1.8 ± 0.2	58.9 ± 2.0	1.7 ± 0.1	3.6 ± 0.2
96 hours	TFY1-C	3	10.2 ± 1.5	52.4 ± 0.8	1.4 ± 0.1	3.0 ± 0.1
	TFY1-1 mM	3	11.4 ± 1.2	53.6 ± 1.4	0.9 ± 0.1	2.9 ± 0.4
	TFY1-2.5 mM	3	8.5 ± 1.9	55.7 ± 1.4	1.4 ± 0.3	3.0 ± 0.4
	TFY2-C	3	13.9 ± 0.4	56.1 ± 1.8	1.1 ± 0.1	2.7 ± 0.2
	TFY2-1 mM	3	12.1 ± 1.1	57.7 ± 1.9	1.1 ± 0.3	3.2 ± 0.5
	TFY2-2.5 mM	3	9.9 ± 2.5	59.7 ± 1.6	1.3 ± 0.2	3.5 ± 0.8

[1] Cryopreservation media were supplemented with varying doses of NAC (0, 1 and 2.5 mM). TFY-1: TFY with 7% glycerol, TFY-2: TFY with 3.5% glycerol and 3.5% dimethylformamide (v/v). [2] PI-/PNA+: live sperm with intact acrosome. [3]Viability: sperm viability was extracted from the sum the PI-quadrants. [4]TDFI (%): percentage of total DNA fragmentation index. [5]HDS (%): percentage of immature sperm with intact DNA. All groups were compared between them. Values do not differ statistically ($p>0.05$).

for prolonged epididymal cooled storage is substantial as previously shown in other species of ruminants (Fernández-Santos *et al.*, 2009b).

In addition, the percentage of viable sperm was well maintained in our setting disregarding the freezing medium used or storage time (over 44.5 %, see Table 2). These results coincide with previous reports in which viability after freezing-thawing decreased approximately to 50% in bovine sperm (Gürler *et al.*, 2016; Nichi *et al.*, 2016) and validates our freezing media and cooling/freezing protocols as suitable for routine Lidia bull epididymal storage and sperm cryopreservation.

Interestingly, despite the fact that total and progressive motility were lower after 96 h, the percentage of sperm with high mitochondrial membrane potential remained unchanged for all treatments (Table 1). Numerous studies have shown that the number of viable sperm with hMMP are significantly related to field fertility in buffalo (Minervini *et al.*, 2013), human (Marchetti *et al.*, 2002) and ram (Windsor, 1997), and thus, the fertility of Lidia bull epididymal sperm may be well preserved along the 96 h cooling period. In human and equine sperm, high mitochondrial potential and membrane integrity have also been positively correlated with sperm motility (Agnihotri *et al.*, 2016; Swegen *et al.*, 2016). However, in view of our results and previously published reports in bovine (Rocha *et al.*, 2006), this correlation needs to be more deeply studied in Lidia bulls.

Very few reports have been published in the post-thaw quality of Lidia bull breed, although Guijarro *et al.* (2014) described that in epididymal Lidia bull sperm cooled for 24 h, the percentage of PNA-/PI- subpopulation after thawing with Bioxcell® or Biladyl® was 58.0% ± 1.0 and 67.0% ± 1.0 respectively. This apparent divergence in sperm viability compared to the results obtained in the present work can be explained in part because sperm viability post-thaw depends on a wide variety of factors such as the pre-freezing sperm quality, cryopreservation medium and also on the individual male sperm freezability (Takahashi *et al.*, 2012). As previously stated, our study is amongst the first flow cytometry analysis of Lidia bull sperm, and therefore, there are not substantial reference values to compare with.

The last experiments of our work tried to determine if increased epididymal storage and sperm cryopreservation rendered a higher oxidative burst that could result in enhanced ROS production and DNA damage (Chatterjee & Gagnon, 2001). Theoretically, oxidative damage is increased in epididymal sperm, since they are not exposed to the accessory sex glands secretions (seminal plasma) (Chen *et al.*, 2003). Supporting this theory, Martínez-Pastor *et al.* (2006) demonstrated that epididymal sperm post-thaw quality can be substantially increased when seminal plasma is added to deer samples prior freezing. As Lidia bull seminal plasma cannot be easily obtained, antioxidant addition was tried to partially alleviate the oxidative burst. NAC antioxidant properties have been shown to exert a beneficial effect on fresh human sperm incubated at room temperature (Oeda *et al.*, 1997), and also on refrigerated and cryopreserved sperm of different species including cattle (Bilodeau *et al.*, 2001; Partyka *et al.*, 2013; Pérez *et al.*, 2015). Our results show that the percentage of live cells producing ROS were higher when the freezing extender included the combination of 3.5% glycerol and 3.5% DMF (Table 1), although the differences found were not significant. Also, we found a decrease in ROS production when NAC was added to TYF2. On the other hand, ROS imbalance and sperm cryopreservation have been shown to detrimentally affect the DNA intactness in bovine sperm (Gürler *et al.*, 2016). Our results did not show any significant increase in DNA damage as total DNA fragmentation remained under 2% and HDS was below 5.7 % for all treatments, coinciding with previous reports (Martínez-Pastor *et al.*, 2009). Interestingly, DFI below 10% have been related to high fertility status in humans and bulls (Evenson & Wixon, 2006; Waterhouse *et al.*, 2006), suggesting that epididymal Lidia bull sperm fertility is maintained despite prolonged storage.

In conclusion, our study demonstrates for the first time that Lidia bull epididymis can be refrigerated at 4ºC up to 96 h prior cryopreservation and that prolonged storage does not impair sperm quality. Furthermore, glycerol alone (7%) or the combination of glycerol and dimethylformamide (3.5% v/v each) added to TFY are equally safe choices as cryoprotective agents in Lidia bull sperm. N-acetylcysteine addition to sperm freezing extenders does not affect post-thaw sperm quality in Lidia bulls. More studies are needed to elucidate the *in vitro* and *in vivo* fertility of frozen Lidia bull epididymal sperm and to test if other antioxidants, CPAs or other combinations of dimethylformamide and glycerol help to increase its post-thaw quality.

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