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Research Article/Artículo de Investigación

N-ACETYLCYSTEINE DOES NOT IMPROVE SPERM MOTILITY OF LIDIA BULL AFTER PROLONGED EPIDIDYMAL STORAGE

LA N-ACETILCISTEINA NO MEJORA LA MOTILIDAD ESPERMATICA EN TOROS DE LIDIA LUEGO DEL ALMACENAMIENTO EPIDIDIMARIO PROLONGADO

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ABSTRACT

The Lidia bovine breed is considered a hallmark of Spanish cattle industry. Assisted reproductive techniques like cryopreservation of epididymal spermatozoa could be considered as an important tool to obtain more offspring and store its genetics. As these bulls are not selected by their reproductive performance or sperm freezability, the quality of their ejaculates is poor and addition of antioxidants prior cryopreservation could exert beneficial effects on the post-thaw sperm quality. The aim of this study was to evaluate the effect of the supplementing a tris-fructose-egg yolk based freezing extender with 1 mM and 2.5 mM of N-acetylcysteine to sperm recovered from epididymis stored at 4ºC for 24, 48, 72 or 96 hours prior cryopreservation. Motility values and sperm kinematic parameters were compared against control (epididymis stored for 24 hours and no antioxidant addition). Our results showed that N-acetylcysteine addition did not improve sperm motility parameters at any of the time points or dosages tested. In addition, storage of bullfight epididymis up to 96 hours did not significantly affect sperm kinematic parameters or total and progressive motility.

Keywords: Lidia breed; Epididymal sperm; Cryopreservation; N-acetylcysteine.
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RESUMEN

La raza de Lidia es considerada una insignia de la industria ganadera española. Las tecnologías de reproducción asistida como la criopreservación de espermatozoides epididimarios podría ser considerara como una herramienta importante para obtener más crías y conservar su genética. Ya que estos toros no son seleccionados por su desempeño reproductivo o congelabilidad espermática, la calidad de sus eyaculados es pobre y la adición de antioxidantes antes de la criopreservación podría tener efectos beneficiosos sobre la calidad del semen descongelado. El propósito de este estudio fue evaluar el efecto de la suplementación del medio a base de tris-fructosa y yema de huevo con 1 mM y 2,5 mM de N-acetilcisteína a espermatozoides recuperados de epidídimos almacenados a 4°C por 24, 48, 72 o 96 horas antes de la criopreservación. Los valores de movilidad y los parámetros de cinética espermática fueron comparados con el control (epidídimos almacenados por 24 horas y sin la adición de antioxidantes). Nuestros resultados muestran que la adición de N-acetilcisteína no mejora los parámetros de motilidad espermática en ninguno de los momentos o dosis evaluadas. Además, el almacenamiento de epidídimos refrigerados hasta 96 horas no afecta significativamente los parámetros de cinética espermática o la movilidad total o progresiva.

Palabras clave: Raza de Lidia; Espermatozoides epididimarios; Criopreservación; N-acetilcisteína.
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INTRODUCTION

The Lidia bovine breed is an Iberian breed considered a hallmark of Spanish cattle industry. As a general rule, Lidia bulls are selected based upon temperament and aggressiveness disregarding their reproductive performance (Jiménez et al., 2007). The descendants are not allowed to sire, as they are not tested until they reach the fighting ring and this fact has lead to an increased in inbreeding (Canon et al., 2008). Assisted reproductive techniques (ARTs) could be considered as an important tool to obtain more offspring from certain maternal lineages or from a particular bull killed during the bullfight (Katska-Ksiazkiewicz et al., 2006).

Cryopreservation of epididymal spermatozoa allows for an efficient use of the genetic material as it can be successfully cryopreserved and used for in vitro embryo production or artificial insemination (Martins et al., 2007). Usually, assisted reproductive facilities are far from bullrings and thus, transport and/or storage of the epididymis is necessary prior sperm harvesting. It has to be noted that even when epididymal spermatozoa are known to be moderately protected by a variety of antioxidant enzymes (Chen et al., 2003), seminal plasma is recognized as their predominant source of antioxidant defenses (Vernet et al., 2004). Cryopreservation exerts deleterious effects on sperm cells impairing their fertility due to the thermic, osmotic and oxidative stresses triggered during the freezing and thawing cycles (Amidi et al., 2016). In fact, these processes have been demonstrated to negatively impact plasma membrane, acrosome and mitochondrial integrity (Januskauskas et al., 2003). Sperm cryopreservation has also been demonstrated to generate reactive oxygen species (ROS) which rapidly overwhelms the antioxidant defenses of the spermatozoa (Bilodeau et al., 2000). Accordingly, sperm removal from the epididymal tail followed by cryopreservation detrimentally affects the oxidant/antioxidant balance (Silva and Guerra, 2011).

The addition of antioxidants to the freezing media has been demonstrated to reduce the negative effects induced by ROS in spermatozoa (Aitken, 1995; Bilodeau et al., 2000; Yoshimoto et al., 2008; Taylor et al., 2009; Gadea et al., 2011) and thus, antioxidants may have beneficial effects on the function of epididymal spermatozoa submitted to cryopreservation. N-acetylcysteine (NAC) is a potent free radical scavenger that can be considered as a supplement to alleviate Glutathione (GSH) depletion and free radical formation during oxidative stress as previously reported (Wu et al., 2006). Accordingly, our study aimed to evaluate the effect of NAC (1 mM and 2.5 mM) addition to Tris-fructose-egg-yolk (TEY) freezing medium with 7% glycerol (v/v) on kinematics of epididymal Lidia bull sperm cooled at 4ºC for 24, 48, 72 or 96 hours prior cryopreservation.

MATERIALS AND METHODS

Reagents

All the reagents used were purchased from Sigma-Aldrich (Barcelona, Spain) unless otherwise stated.

Media

The base medium was composed of Tris-fructose-citrate (TF) (Tris 250 mM, citric acid 86.9 mM, fructose 36 mM, 1 mg/ml penicillin and 0.5 mg/ml gentamicin in sterile Milli-Q water) as previously described (Van Wagtendonk-De Leeuw et al., 2000; Chaudhari, et al., 2015). Base freezing medium was composed of Tris-fructose added with 20% (v/v) of egg yolk (Van Wagtendonk-De Leeuw, et al., 2000), and 7% glycerol (TEY). Treatment groups were added with 1 mM NAC (TEY-1) and 2.5 mM NAC (TEY-2.5); non-NAC added groups were considered as controls and are referred as TEY-C.

Sperm harvesting and processing

Spermatozoa were collected from the epididymis of 18 Lidia Spanish bulls aged 3-4 years. Testes with attached epididymis were obtained post-mortem at the bullring. Immediately after removal, the testis were placed into plastic bags in an isothermal box at 4ºC and shipped to the laboratory overnight. Once arrived to the laboratory the epididymis were separated from the testis, as previously described (Yu and Leibo, 2002). Epididymis were stored in the fridge (4ºC) and processed at 4 different time points: a) 24 hours (n = 5), b) 48 hours (n = 5), c) 72 hours (n = 5) and d) 96 hours (n = 3). Connective tissue was carefully dissected, and the cauda epididymis was straightened to allow for flushing medium 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 TF medium pre-warmed at 37ºC (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 minutes at room temperature, the supernatants were discarded and the pellets were diluted in 1.5-2 ml of TF at room temperature (22-25ºC) and centrifuged again. The supernatant was discarded once more and the remaining pellets were resuspended in 600-800 µl of TF. Sperm concentration was determined using a Neubauer chamber and freezing medium was slowly added to reach a final concentration of 100×10^6 spz/ml. 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

placed in the fridge at 4°C for 2 hours; then, the straws were placed 4 cm above liquid nitrogen vapors for 20 minutes, seeded and subsequently plunged into liquid nitrogen as previously described (Chaveiro et al., 2006). The straws were stored for at least 1 month prior thawing and subsequent analysis. Thawing was achieved by immersing the straws for 1 minute in a water bath set at 37°C. After thawing 50 µl of each sample were resuspended with an equal volume of TF and subjected to a short spin (MiniSpin®, eppendorf) for 10 seconds. The supernatant was removed and the remaining pellet was resuspended in 90 µl of pre-warmed TF.

Motility Assay

All samples were examined using a CASA system (ISAS®, Proiser R+D, Paterna, Valencia, Spain). Two microliters of each sample were placed in a pre-warmed counting chamber (Leja®, Nieuw-Vennep, The Netherlands). Sperm motility was assessed with a microscope (Nikon Eclipse 50i) equipped with a 10x negative-phase contrast objective and a heated stage at 38°C. Analysis was based on the examination of 25 consecutive digitalized images and at least 200 spermatozoa per sample were analyzed. After acquiring at least 3 representative fields, the following sperm motility descriptors were recorded: total motility (TM) and progressive motility (PM), VCL (curvilinear velocity in µm/sec), VSL (straight-line velocity in µm/sec), VAP (average path velocity in µm/sec), LIN (linearity coefficient in %), STR (straightness coefficient in %), ALH (amplitude of lateral head displacement in µm) and BFC (beat cross frequency in Hz).

Statistical analysis

Data were tested for normality using a Shapiro–Wilk test; results are reported 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 against the control (TEY-C at 24 hours) were found, a Dunn's post-hoc test was used. 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 motility values did not show statistically significant differences among groups (Table 1; $p > 0.05$). Conversely, for progressive motility, statistically significant differences were found between TEY-C at 24 h (14.22% ± 3.51; mean ± SEM), and TEY-C and TEY-1 at 96 hours (2.8% ± 0.95 and 2.23% ± 0.96; mean ± SEM; respectively). No statistically significant differences were found in the velocity parameters (VCL, VSL, VAP, LIN and ALH) among control (TEY-C) and treatment groups (TEY-1 and TEY-2.5) at any storage time studied (Tables 2 and 3; $p > 0.05$). Conversely, significant differences among control (TEY-C) and TEY-1 (68.70% ± 2.48 vs. 52.47% ± 2.60; mean ± SEM) and TEY-2.5 (68.70% ± 2.48 vs. 57.27% ± 1.93; mean ± SEM) at 96 hours were found for STR. BCF differed only among control (TEY-C) and TEY-1 after 96 hours ($p < 0.05$; Table 3).

Table 1. Total and progressive motility of frozen-thawed Lidia bull epidydimal sperm

Storage time(4ºC)	Freezing medium	n	TM (%)	PM (%)
24 hours	TEY-C	5	30.22 ± 5.96	14.22 ± 3.51
	TEY-1	5	26.54 ± 5.21	11.44 ± 2.28
	TEY-2.5	5	25.08 ± 6.19	11.36 ± 2.92
48 hours	TEY-C	5	21.72 ± 5.10	6.44 ± 2.51
	TEY-1	5	21.18 ± 5.4	5.98 ± 1.76
	TEY-2.5	5	19.66 ± 4.9	6.78 ± 2.18
72 hours	TEY-C	5	21.08 ± 2.87	7.36 ± 2.22
	TEY-1	5	23.52 ± 1.88	7.12 ± 0.89
	TEY-2.5	5	25.46 ± 2.67	9.16 ± 2.03
96 hours	TEY-C	3	12.16 ± 3.10	2.8 ± 0.95*
	TEY-1	3	15.1 ± 5.25	2.23 ± 0.96*
	TEY-2.5	3	16.26 ± 4.60	3.56 ± 0.35

Total and progressive motility of thawed Lidia bull sperm from epididymis stored at 4 ºC at different time points prior cryopreservation. TEY-C is a TF based medium added with 20% egg yolk, 7% glycerol (v/v) used as control; TEY-1 is a TEY-C medium added with 1 mM NAC and TEY-2.5 is a TEY-C medium added with 2.5 mM NAC. All data were compared against TEY-C 24h using an ANOVA and are represented as mean ± SEM; values marked with * differ statistically $p < 0.05$.

Table 2. Sperm velocity parameters of frozen-thawed Lidia bull epidydimal sperm

Storage time (4ºC)	Freezing medium	n	VCL (μm s^{-1})	VSL (μm s^{-1})	VAP (μm s^{-1})
24 hours	TEY-C	5	82.45 ± 4.48	27.78 ± 2.07	40.29 ± 2.17
	TEY-1	5	86.47 ± 5.2	27.43 ± 2.01	40.17 ± 2.51
	TEY-2.5	5	74.79 ± 12.9	23.21 ± 4.35	34.19 ± 6.22
48 hours	TEY-C	5	81.22 ± 5.12	21.34 ± 2.71	34.21 ± 3.57
	TEY-1	5	82.16 ± 1.80	20.41 ± 1.31	33.81 ± 1.92
	TEY-2.5	5	82.31 ± 3.14	22.62 ± 2.23	35.32 ± 2.49
72 hours	TEY-C	5	80.53 ± 4.64	22.36 ± 2.89	34.70 ± 2.65
	TEY-1	5	80.47 ± 2.99	22.37 ± 2.90	35.76 ± 1.73
	TEY-2.5	5	86.55 ± 5.76	22.50 ± 1.82	41.63 ± 4.15
96 hours	TEY-C	3	69.35 ± 2.49	27.76 ± 3.59	31.54 ± 2.18
	TEY-1	3	64.59 ± 8.65	19.02 ± 1.07	30.83 ± 6.49
	TEY-2.5	3	64.43 ± 8.65	15.88 ± 2.59	28.12 ± 4.53

Sperm velocity parameters of frozen-thawed Lidia bull sperm recovered from epididymis stored at 4 ºC at different time points prior cryopreservation. TEY-C is a TF based medium added with 20% egg yolk, 7% glycerol (v/v) used as control; TEY-1 is a TEY-C medium added with 1 mM NAC and TEY-2.5 is a TEY-C medium added with 2.5 mM NAC. All data were compared against TEY-C 24 h using an ANOVA and represent the mean ± SEM; no statistically significant differences were detected among groups $p > 0.05$.

Table 3. Sperm kinematic parameters of frozen-thawed Lidia bull epididymal sperm

Storage time(4ºC)	Freezing medium	n	LIN (%)	STR (%)	ALH (μm)	BCF (Hz)
24 hours	TEY-C	5	33.94 ± 2.78	68.70 ± 2.48	3.47 ± 0.20	10.52 ± 0.29
	TEY-1	5	31.94 ± 2.33	68.22 ± 2.30	3.74 ± 0.23	10.37 ± 0.55
	TEY-2.5	5	30.85 ± 1.49	67.36 ± 2.03	3.17 ± 0.52	9.33 ± 1.43
48 hours	TEY-C	5	26.03 ± 2.23	62.01 ± 2.19	3.57 ± 0.52	8.85 ± 1.23
	TEY-1	5	24.78 ± 1.22	60.39 ± 1.70	4.34 ± 0.33	8.63 ± 1.21
	TEY-2.5	5	27.37 ± 2.11	63.67 ± 2.73	3.72 ± 0.17	8.46 ± 0.82
72 hours	TEY-C	5	27.81 ± 2.99	63.98 ± 4.30	4.04 ± 0.32	9.54 ± 0.65
	TEY-1	5	27.82 ± 1.36	62.59 ± 2.24	3.92 ± 0.33	9.25 ± 0.78
	TEY-2.5	5	27.82 ± 1.56	65.96 ± 2.16	4.29 ± 0.30	10.60 ± 0.62
96 hours	TEY-C	3	31.64 ± 2.22	60.65 ± 3.32	3.42 ± 0.6	8.13 ± 1.59
	TEY-1	3	27.47 ± 1.47	52.47 ± 2.60*	2.76 ± 1.12	4.81 ± 1.35*
	TEY-2.5	3	24.40 ± 1.05	57.27 ± 1.93*	2.44 ± 0.28	6.70 ± 1.93

Sperm kinematics of frozen-thawed Lidia bull sperm recovered from epididymis stored at 4ºC at different time points prior cryopreservation. TEY-C is a TF based medium added with 20% egg yolk, 7% glycerol (v/v) used as control; TEY-1 is a TEY-C medium added with 1 mM NAC and TEY-2.5 is a TEY-C medium added with 2.5 mM NAC. All data were compared against TEY-C 24 h using an ANOVA; values marked with * differ statistically $p < 0.05$.

DISCUSSION

This study evaluated the effects of different dosages of NAC (1 mM and 2.5 mM NAC) on thawed Lidia bull sperm motility parameters after epididymal storage at 4ºC (24 to 96 hours). Our results show that total and progressive motility did not vary in thawed epididymal Lidia bull sperm despite prolonged epididymis storage at 4ºC. Furthermore, NAC addition at none of the dosages used exerted any significant effect in total or progressive motility (Table 1). Coinciding with our results, a high variability between bulls has been reported after 24 hours of refrigerated storage prior sperm freezing in Lidia breed (Posado et al., 2008). This variability observed between bulls explains why despite the apparent vivid differences in the total and progressive motility after epididymal refrigeration for 24 and 96 hours at 4ºC (30.22-12.16%

and 2.23-14.22%; respectively) no significant differences were found. Furthermore, although the total motility values obtained may appear to be low, epididymal sperm have been demonstrated to yield lower total and progressive motility, and lower straightness and linearity than freshly ejaculated bovine sperm (Goovaerts et al., 2006). However, it has to be noted that reference motility parameters are not established yet in the Lidia breed for epididymal or ejaculated sperm and ours are among the first results published.

Regarding the advanced sperm kinematic parameters, no changes were detected in VCL, VSL, VAP, ALH and LIN despite NAC addition or prolonged epididymal storage (Tables 2 and 3) and only STR and BCF experienced statistically significant alterations after 96 hours in the NAC supplemented groups. Individual velocities have been shown to predict the fertilizing potential of frozen-thawed semen in many species (Byrd et al., 1990; Fetterolf and Rogers, 1990; Adoyo et al., 1995) including the bull (Nagy et al., 2015). In this sense, it has been suggested that VAP may be the most useful parameter with clinical relevance to predict fertility in bulls (Nagy et al., 2015). Hence, our results suggest that prolonged storage of Lidia bull epididymis up to 96 hours may not significantly impair the fertility of the retrieved sperm and thus, processing of Lidia bull sperm epididymis can be done after epididymal storage for up to 96 hours at 4ºC.

On the other hand, prolonged storage is critical due to the accumulation of reactive oxygen species (ROS), which leads to the so called 'oxidative stress' (Nichi et al., 2007). Epididymal samples are particularly susceptible to attack by ROS, as they are not exposed to the complex secretions of the accessory sex glands (seminal plasma), which are recognized as the prime source of anti-oxidant protection (Chen et al., 2003). Antioxidant addition to sperm freezing media is backed by many authors (Bilodeau et al., 2000; Gadea et al., 2005; Yoshimoto et al., 2008; Taylor et al., 2009; Gadea et al., 2011; Olfati Karaji et al., 2014; Sapanidou et al., 2014; Mata-Campuzano et al., 2015), although its use is controversial (Seifi-Jamadi et al., 2016), as high antioxidant concentrations can also exert deleterious effects such as apoptosis due to cell pro-oxidation (Aisen et al., 2005; Atessahin et al., 2008; Kang et al., 2016). A number of studies have demonstrated the positive effect of ROS scavenger addition (such as superoxide dismutase, catalase, cytochrome C or Vitamin E among others) to ejaculated semen of ruminants for preservation at either 15 or 5ºC (Maxwell and Stojanov, 1996; Upreti et al., 1997; Upreti et al., 1998). NAC scavenging properties have been previously studied on fresh human spermatozoa incubated at room temperature (Oeda et al., 1997), and it has also been demonstrated a beneficial effect of NAC on refrigerated and cryopreserved canine and bovine spermatozoa motility and viability (Bilodeau et al., 2001; Michael et al., 2010; Pérez et al., 2015). However, our results are in agreement with previous research in different species in which it was concluded that antioxidant addition does not improve sperm motility parameters after 96 hours of refrigerated storage (Ball et al., 2001; Fernández-Santos et al., 2009) (horse and deer respectively). Our data and the previously mentioned works suggest that thermal and osmotic shocks (or others insults rather than ROS) are the main damages impairing sperm post-thaw quality and that these insults cannot be prevented by antioxidants. NAC addition to TEY medium at either 1 or 2.5 mM prior freezing does not induce any beneficial or detrimental effect on Lidia bull sperm after epididymal cooled storage. Furthermore, it was observed a high variability between males to withstand the sperm freezing and thawing processes.

CONCLUSIONS

In conclusion, in our study the possible beneficial effect of NAC in the freezing semen extender of Lidia bulls could not be demonstrated. Additionally, storage of bullfight epididymis up to 96 hours after animal slaughter, does not significantly affect total motility and progressive motility or sperm kinematic parameters. Due to the scant data available in Lidia bull sperm, more effort has to be put in establishing accurate protocols that maintain good quality of frozen epididymal sperm after the bullfight and to ensure its fertility.

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