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 4°C 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.

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 podrían ser considerada 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 a 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 evaluados. 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.
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-Książkiewicz 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 within 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 4°C 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 Wagendonk-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 Wagendonk-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 upon temperament and aggressiveness disregarding their reproductive performance (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 by 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 37°C (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 (22-25°C) 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

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

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 epididymal sperm

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

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

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

Sperm kinematics 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; 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 4°C.

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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REFERENCES


Chaudhari DV., Dhami AJ., Hadiya KK., Patel JA. 2015. Relative efficacy of egg yolk and soya milk-based extenders for cryopreservation (~196 °C) of buffalo semen. Veterinary Medicine 8:239-244.


Oeda T., Henkel R., Ohmori H., Schill WB. 1997. Scavenging effect of N-acetyl-L-
cysteine against reactive oxygen species in human semen: a possible therapeutic

antioxidant supplementation on microscopic and oxidative parameters of freeze—

protects cryopreserved bovine spermatozoa from reactive oxygen species without
compromising the in vitro developmental potential of intracytoplasmic sperm

Posado R., Hernández M., García J., Bartolomé D., Olmedo S., Rodríguez L., López-
Fernández C., Gosálvez J. 2008. DNA fragmentation in frozen semen samples of
fighting bulls. In: 24th Annual Meeting Association Europeene de
Transfert Embryonnaire, p. 220. Association Europeene de Transfert
Embryonnaire PAU, France, 12th -13th September 2008.

Sapanidou VG., Margaritis I., Siahos N., Arsenopoulos K., Dragatidou E., Taitzoglou
IA., Zervos IA., Theodoridis A., Tsantariotou MP. 2014. Antioxidant effect of a
polyphenol-rich grape pomace extract on motility, viability and lipid peroxidation
of thawed bovine spermatozoa. Journal of Biological Research-Thessaloniki

Quercetin Ameliorate Motility in Frozen-Thawed Turkmen Stallions Sperm.

Silva SV., Guerra MMP. 2011. Efeitos da criopreservação sobre as células
espermáticas e alternativas para redução das crioinjúrias. Revista Brasileira

Taylor K., Roberts P., Sanders K., Burton P. 2009. Effect of antioxidant
supplementation of cryopreservation medium on post-thaw integrity of human

Studies on aromatic amino acid oxidase activity in ram spermatozoa: role of

of ram spermatozoa during storage in a chemically-defined diluent containing

Van Wagendonk-De Leeuw AM., Haring RM., Kuul-Lansbergen LMTE., den Daas JHG.
2000. Fertility results using bovine semen cryopreserved with extenders based on
egg yolk and soy bean extract. Theriogenology 54:57-67.

Molecular and Cellular Endocrinology 216:31-39.

Wu W., Goldstein G., Adams C., Matthews RH., Ercal N. 2006. Separation and
quantification of N-acetyl-L-cysteine and N-acetyl-cysteine-amide by HPLC with

Yoshimoto T., Nakamura S., Yamauchi S., Muto N., Nakada T., Ashizawa K.,
Tatemoto H. 2008. Improvement of the post-thaw qualities of Okinawan native pig
spermatozoa frozen in an extender supplemented with ascorbic acid 2-O-α-
glucoside. Cryobiology 57:30-36.

Yu J., Leibo SP. 2002. Recovery of motile, membrane-intact spermatozoa from
canine epididymides stored for 8 days at 4 degrees C. Theriogenology 57:1179-
1190.