Animal Reproduction Science 162 (2015) 73–79

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Reduced glutathione addition improves both the kinematics and physiological quality of post-thawed red deer sperm

L. Anel-López [a], O. Garcia-Alvarez [a], A. Maroto-Morales [a], M. Iniesta-Cuerda [a], M. Ramón [b], A.J. Soler [a], M.R. Fernández-Santos [a], J.J. Garde [a,*]

[a] SaBio IREC (CSIC-UCLM-JCCM), Campus Universitario s.n., 02071 Albacete, Spain
[b] CERSYRA, Valdepeñas, Spain

ARTICLE INFO

Article history:
Received 6 July 2015
Received in revised form
22 September 2015
Accepted 25 September 2015
Available online 28 September 2015

Keywords:
Red deer
Sperm
Cryopreservation
Antioxidant
Reduced glutathione
Trolox

ABSTRACT

The potential protective effect of reduced glutathione (GSH) and trolox (TRX), an analogue of vitamin E, supplementation during *in vitro* culture (2 h, 39 °C) of electroejaculated frozen/thawed red deer sperm was investigated. Cryopreserved sperm were thawed and incubated with no additive (Control) and 1 mM or 5 mM of each antioxidant to find out whether these supplementations can maintain the sperm quality, considering the use of thawed samples for *in vitro* techniques such as *in vitro* fertilisation (IVF), sperm sex sorting or refreezing. The effect of GSH on sperm motility was positive compared to TRX which was negative ($P < 0.001$). After 2 h of incubation at 39 °C, use of GSH improved motility while TRX supplementation reduced sperm motility compared with Control samples without antioxidant. Use of TRX at both concentrations (1 and 5 mM; TRX1 and TRX5) resulted in lesser percentages of apoptotic sperm ($12.4 \pm 1.1\%$ and $11.7 \pm 0.9\%$) than GSH1, GSH5 ($15.2 \pm 1\%$ and $14.6 \pm 1.1\%$) and Control samples ($16.9 \pm 1.2\%$) ($P < 0.001$). Use of GSH at both concentrations (1 and 5 mM) resulted in greater mitochondrial activity as compared with findings for the Control, TRX1 and TRX5 groups. Results of this study indicate that GSH is a suitable supplement for electroejaculated red deer sperm. It would be necessary to conduct fertility trials (*in vivo* and *in vitro*), to assess whether GSH supplementation of thawed red deer sperm could improve fertility rates.

1. Introduction

In red deer, post-mortem collection has been considered as a very important germplasm resource because of the hunting activity in Spain during the past few decades (Garde et al., 2006). Studies using this sperm collection approach have provided for significant improvements by implementing different cooling and freezing rates, extender composition or antioxidant supplementation (Fernandez-Santos et al., 2006; Martinez-Pastor et al., 2006a; Martínez-Pastor et al., 2006b; Anel-López et al., 2012).

The production of red deer on farms is becoming an important source of economic resource in many countries. In this production situation, electroejaculation is the obvious and the most suitable choice for sperm collection (Asher et al., 2000). It allows performance of intensive breeding selection and, therefore, more rapid improvement of genetic quality than postmortem collection. For these reasons the improvement in specific cryopreservation protocols for use of ejaculated and electroejaculated sperm samples should be considered a priority.

Mammalian sperm are known to be especially susceptible to oxidative stress (Baker and Aitken, 2004). Oxidative

* Corresponding author.
E-mail address: Julian.garde@uclm.es (J.J. Garde).

http://dx.doi.org/10.1016/j.anireprosci.2015.09.012
0378-4320/

stress can be defined as the loss of the balance between reactive oxygen species (ROS) production and the ability of antioxidants to scavenge ROS. The susceptibility of sperm to oxidative damage arises as an important problem because this might lead to loss of motility, membrane integrity, fertilising capability and other physiological changes in sperm cells (Aitken, 1995; Aitken and Baker, 2004; Rath et al., 2009; Aitken et al., 2012). Previews studies have demonstrated the use of some antioxidants could enhance the viability of epididymal red deer sperm either in refrigerated storage (Fernández-Santos et al., 2009a), cryopreservation (Fernandez-Santos et al., 2007) or post-thawing incubation (Domínguez-Rebolledo et al., 2010). However, further investigations should be conducted because of the great variability of antioxidant effects. These effects may vary, not only depending on concentration (Domínguez-Rebolledo et al., 2010), but also from differences in sperm responses among species, time of application of antioxidants during sperm processing, and medium or temperature used for sperm storage. For example, in a previous study (Mara et al., 2005) refrigerating ram semen in presence of the antioxidant TEMPOL improved sperm quality and fertility. In contrast, use of TEMPOL reduced the motility of deer sperm during incubation at 39 °C, but it was able to protect DNA against oxidative stress (Mata-Campuzano et al., 2012).

TRX is an analogue of vitamin E with high capacity to capture free radicals (Mickle and Weisel, 1993), and usually it is used as the standard to assess antioxidant capacity of other molecules (Lipovac, 2000; Ronald et al., 2005). The supplementation of extender with TRX improved sperm motility and mitochondrial membrane integrity during post-thaw incubation of ejaculated boar sperm after thawing (Peña et al., 2003). Furthermore, use of TRX reduced intracellular reactive oxygen species, lipid peroxidation, and preserved membrane integrity of red deer epididymal sperm during post-thaw incubation, either with or without induced oxidative stress (Martínez-Pastor et al., 2008; Martinez-Pastor et al., 2009) in epididymal samples. TRX also protected motility and viability and abolished DNA damage in samples exposed to oxidative stress after thawing and washing (Domínguez-Rebolledo et al., 2009).

Reduced glutathione (GSH) is a tripeptid distributed within living cells. It has an important role in cell protection from the noxious effect of oxidative stress, directly and as a cofactor of GSH peroxidase (Atmaca, 2004). This enzyme acts on GSH to reduce hydrogen peroxide to H_2O and lipoperoxides to alkyl alcohols. The addition of GSH to cryopreservation extender has had variable results in several species (Anel-López et al., 2012; Câmara et al., 2011). Thus, the supplementation of GSH and TRX to extenders for epididymal red deer sperm was beneficial, although its effect on ejaculated sperm is still unknown.

The main aim of the present study was to assess the effect in electroejaculated frozen-thawed sperm samples of two different antioxidants (GSH and TRX) at two different concentrations (1 and 5 mM) after an incubation of 2 h at 39 °C. Attempts were also made to ascertain whether these supplementations can maintain sperm quality, considering the use of thawed samples for *in vitro* techniques such as IVF, sperm sex sorting or refreezing.

2. Materials and methods

2.1. Reagents and media

Flow cytometric equipment, software and consumables were purchased form Beckman Coulter (Fullerton, CA, USA). Fluorescence probes YO-PRO-1 and Mitotracker Deep Red were purchased from Invitrogen (Barcelona, Spain), propidium iodide (PI) and Peanut Agglutinin-Fluorescein isothiocyanate (PNA-FITC) were acquired from Sigma (Madrid, Spain) and acridine orange (chromatographically purified) was purchased from Polysciences (Warrington, PA, USA). Stock solutions of the fluorescence probes were: PI: 1.5 mM; PNA-FITC: 0.2 mg/mL; YO-PRO-1: 50 μM; Mitotracker Deep Red: 1 mM. All fluorescent stocks were prepared in DMSO, except for PI and PNA-FITC, which were prepared in distilled water and kept at −20 °C in the dark until needed. The stock solution of acridine orange was prepared in distilled water at 1 mg/mL and kept in the dark at 5 °C. The stock solutions of the antioxidants were prepared at 100 mM in DMSO (TRX) or in water (reduced GSH) and stored at −20 °C.

The work medium for cytometry assessment was bovine gamete medium (BGM-3) composed of 87 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2$, 0.4 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 40 mM HEPES, 21.6 mM sodium lactate, 1 mM sodium pyruvate, 50 μg/mL kanamycin, 10 μg/mL phenol red and 6 mg/mL BSA (pH 7.5). Solutions for SCSA® (Sperm Chromatin Structure Assay) were prepared following Evenson et al., 2000 (Evenson and Jost, 2000): TNE buffer (0.01 M Tris–HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4), acid-detergent solution (0.17% Triton X-100, 0.15 M NaCl, 0.08 N HCl, pH 1.4) and acridine orange solution (0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, pH 6.0; acridine orange was added from the stock up to 6 μg/mL). These solutions were kept at 5 °C in darkened conditions.

2.2. Ejaculate collection and cryopreservation

Samples were obtained from nine mature stags during breeding season (mid-September). Animals were housed in a semi-free ranging environment at Las Lomas Farm (Medianilla S.L., Cadiz, Spain). Animal handling and electroejaculation were performed in accordance with Spanish Animal Protection Regulation RD53/2013 which conforms to European Union Regulation 2010/63/UE. The electroejaculation procedure was conducted as described by (Martínez et al., 2008). Males were anesthetised with Xylacine (0.75 mg/Kg) (Rom-pun® 2%; Bayer AG, Leverkusen, Germany). The rectum was cleared of faeces and the prepucial area was shaved and washed with physiological saline solution. A three-electrode probe connected to a power source that allowed voltage and amperage control was used (P.T. Electronics, Boring, OR, USA). Probe diameter, probe length and electrode length were 3.2, 35.0 and 6.6 cm, respectively. The electroejaculation regimen consisted of consecutive series of 5 pulses of similar voltage and separated by 5 s. The initial voltage was 1 V that was increased in each series up to a maximum of 5 V. Semen was collected in fractions in graduated glass tubes at 37 °C. Sperm concentration was assessed using a hemocytometer