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SHORT COMMUNICATION

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The percentage of egg yolk in the freezing media affects mouflon (*Ovis musimon*) epididymal sperm cryosurvival

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Abstract

Sperm cryopreservation protocols are not well defined in many wild species such as the mouflon. The aim was to study the effect of different concentrations of EY on mouflon epididymal sperm cryosurvival. Samples were collected by the flushing method and cryopreserved by the conventional freezing technique in straws using a TEST (TES, Tris, glucose) 5% v/v glycerol medium containing either 6% v/v (n=16) or 12% v/v (n=13) clarified EY. The membrane integrity, acrosome integrity, motility, and morphological abnormalities were assessed in fresh and frozen/thawed samples. Fresh sperm quality parameters did not differ between groups except for the acrosome integrity that was lower in the TEST-6% EY than in the TEST-12% EY group (88.9 ± 2.1% *vs* 94.7 ± 0.8%). Membrane integrity (31.6 ± 4.6% *vs* 11.6 ± 4.5%), total motility (32.8 ± 4.6% *vs* 17.2 ± 5.6%), progressive motility (13.3 ± 2.7% *vs* 6.1 ± 2.9) were higher in frozen-thawed sperm with TEST-6% EY than with TEST-12% EY ($p<0.05$). Other motility parameters such as curvilinear velocity, straight-line velocity, average path velocity and amplitude of lateral head displacement were also higher ($p<0.05$) in frozen-thawed sperm with TEST-6% EY. Frozen-thawed acrosome integrity (85.1 ± 3.3% *vs* 91.9 ± 2.3) and morphological abnormalities (34.0 ± 3.7% *vs* 29.1 ± 3.6%) did not differ between extenders. In conclusion, high EY concentration had detrimental effects on post-thaw quality parameters, therefore the use of TEST based extender containing 6% EY is recommended for the cryopreservation of mouflon epididymal sperm.

Additional keywords: cryobank; hunting resource; reproductive technologies; wild sheep.

Abbreviations used: CASA (computer-assisted sperm analysis system); E/N (eosin-nigrosin staining); EY (egg yolk); HOST (hypo-osmotic swelling test); NAR (normal acrosome ridge); PI (propidium iodide); PNA-FITC (fluorescein isothiocyanate-conjugated peanut agglutinin).

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Introduction

The mouflon (*Ovis musimon*), a wild ancestor of domestic sheep, is widely distributed in Europe and has an important hunting interest. The recovery of epididymal sperm samples from death animals followed by its cryopreservation is an important source of genetic material that can be preserved in genetic resource banks for conservation purposes, but also for improving the stock on hunting reserves. Nevertheless, sperm cryopreservation protocols are not well defined yet in many wild species such as the mouflon. The egg yolk (EY) is a common component of semen extenders of several species. It has been suggested that the main protective effect of EY from freezing damage is related with the interaction of the low-density lipoproteins (LDL) of the EY with some seminal plasma proteins such as the Binder of Sperm proteins (Manjunath, 2012). Also, the presence of EY in semen extenders not only prevents the lipid efflux from the sperm plasma membrane, but stimulates cholesterol and choline phospholipids gain (Bergeron

et al., 2004). The use of EY in ram sperm diluents prevents ultrastructural changes such as the swelling of the acrosome during the cooling (Jones & Martin, 1973). Moreover, the EY protects spermatozoa during chilling and freezing by its attachment to the sperm plasma membrane by coating the cell membrane (Watson, 1975). This cryoprotective action is due to the specific binding of lipoproteins present in the EY to the sperm membrane (Watson, 1981; Cookson *et al.*, 1984). High concentrations of EY are successfully used in semen extenders from domestic sheep (Álvarez *et al.*, 2012). In contrast, low EY concentrations are usually used as additives in extenders from wild small ruminant species, such as ibexes (Santiago-Moreno *et al.*, 2006) or ejaculated sperm from mouflon (Pradiee *et al.*, 2017). It is hypothesised that mouflon epididymal sperm might require higher EY concentrations like domestic phylogenetic descendants. The objective of the present work was to study the effect of different concentrations of EY (6% and 12% v/v) on mouflon epididymal sperm cryosurvival.

Material and methods

Epididymal sperm samples were collected from 29 mouflons legally hunted in their natural habitat in the Andalusian hunting reserve of Cazorla and Segura (Jaén, Spain). Sperm collection and cryopreservation were performed in January (at the end of the rutting season) through two different hunting seasons in two consecutive years. Epididymal sperm was collected within 12 h postmortem. Sperm samples were flushed out with 1 mL of medium containing Tris (95.8 mM), TES (210.6 mM), glucose (10.1 mM) and either 6% v/v (n=16) or 12% v/v (n=13) of clarified EY (prepared by centrifugation and filtration). Chemicals were purchased from Panreac Química S.A. (Barcelona, Spain) and Sigma Chemical Co. (St. Louis, Mo, USA). The osmolality was 320 mOsm/kg and the pH 6.8. After 1 hour of equilibration at 5ºC, glycerol was added to a final concentration of 5% (v/v) and sperm concentration was adjusted to $800 \cdot 10^6$ sperm/mL. Sperm samples with glycerol were maintained another 15 min at 5ºC, loaded into 0.25 mL French straws and frozen by placing them into nitrogen vapor for 10 min before plunging into liquid nitrogen. The following fresh sperm quality parameters were evaluated immediately after collection at the "field" laboratory located next to the hunting area, as previously described (Santiago-Moreno *et al.*, 2006): sperm concentration, subjective motility, plasma membrane integrity, morphological abnormalities and the acrosome integrity. Sperm concentration was calculated with a Neubauer Chamber (Marienfeld, Lauda-Königshofen, Germany) and subjective motility was assessed using a phase contrast microscope. Plasma membrane integrity was assessed using the hypo-osmotic swelling test (HOST) and the eosin-nigrosin staining technique (E/N). Morphological abnormalities and the percentage of spermatozoa with intact acrosomes (NAR: normal acrosome ridge) were evaluated in glutaraldehyde fixed samples using phase-contrast microscopy. Sperm samples were thawed at the INIA laboratory in a water bath at 37ºC for 30 seconds, and the same sperm quality variables were assessed as for fresh samples. In addition, due to equipment availability, sperm motility parameters were assessed by a computer-assisted sperm analysis system (CASA) (Santiago-Moreno *et al.*, 2013) and membrane integrity and acrosome integrity were evaluated by fluorescence microscopy using the fluorochrome combination of propidium iodide (PI) and fluorescein isothiocyanate-conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC) (Soler *et al.*, 2005). The statistical analysis was performed using Statistica v.12.0 software (StatSoft, Tulsa, OK, USA). Data did not follow a normal distribution therefore were analyzed by the non-parametric Mann-Whitney U-test for unmatched samples. Statistical results were confirmed also by ANOVA analysis of the transformed data.

Results and discussion

Fresh sperm quality parameters did not differ between groups (Table 1) except for the percentage of NAR that was higher in fresh samples cryopreserved with 12% EY ($p<0.05$). Results in Table 2 show that membrane integrity assessed by fluorescence and by the E/N technique was higher ($p<0.05$) in frozen-thawed samples cryopreserved with 6% EY than 12% EY. Total motility, progressive motility, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP) and amplitude of lateral head

Table 1. Fresh sperm quality parameters of mouflon epididymal spermatozoa (mean ± S.E).

	TEST-6% EY	TEST-12% EY	*p* value
Motility (%)	74.4 ± 3.8	72.5 ± 6.6	0.436
Morphological abnormalities (%)	12.7 ± 2.5	10.4 ± 1.4	0.774
HOST (%)	85.0 ± 2.9	84.2 ± 3.3	0.964
NAR (%)	88.9 ± 2.1	94.7 ± 0.8	0.011
E/N (%)	83.2 ± 1.5	83.7 ± 1.8	0.982

EY: egg yolk. HOST: hypo-osmotic swelling test. NAR: normal acrosome ridge. E/N: eosin-nigrosin.

Table 2. Frozen-thawed sperm quality parameters of mouflon epididymal spermatozoa (mean ± S.E).

	TEST-6% EY	TEST-12% EY	*p* value
Membrane integrity (PI)	31.6 ± 4.6	11.6 ± 4.5	0.002
Acrosome integrity (PNA)	85.1 ± 3.3	91.9 ± 2.3	0.147
Motility parameters (CASA)			
Total motility (%)	32.8 ± 4.6	17.2 ± 5.6	0.018
Progressive motility (%)	13.3 ± 2.7	6.1 ± 2.9	0.012
VCL (μm/s)	103.6 ± 9.5	70.6 ± 8.9	0.013
VSL (μm/s)	58.5 ± 5.9	38.0 ± 5.7	0.016
VAP (μm/s)	84.3 ± 8.1	55.9 ± 7.8	0.014
ALH (μm)	2.8 ± 0.2	1.9 ± 0.3	0.010
Morphological abnormalities (%)	34.0 ± 3.7	29.1 ± 3.6	0.496
HOST (%)	45.2 ± 4.5	37.8 ± 4.5	0.356
NAR (%)	60.6 ± 4.9	60.5 ± 4.7	0.877
E/N (%)	42.9 ± 4.5	20.9 ± 6.1	0.005

EY: egg yolk. PI: propidium iodide. PNA: peanut (*Arachis hypogaea*) agglutinin. CASA: computer-assisted sperm analysis. VCL: curvilinear velocity. VSL: straight-line velocity. VAP: average path velocity. ALH: amplitude of lateral head displacement. HOST: hypo-osmotic swelling test. NAR: normal acrosome ridge. E/N: eosin-nigrosin.

displacement (ALH) were also higher in frozen-thawed sperm with 6% EY than with 12% EY ($p<0.05$). The percentage of sperm with morphological abnormalities and with acrosome integrity did not differ between extenders. The membrane integrity assessed by the HOST did not differ between extenders although the presence of coiled tails could have interfered in the counting and interpretation of the results.

The addition of EY on epididymal sperm extenders has a protective effect from cold shock and freezing-thawing (Dong & VandeVoort, 2009; Jimenez *et al.*, 2013). However, different results have been reported regarding the optimum concentration of EY in freezing extenders. Previous studies in Iberian ibex reported higher fertility with epididymal sperm samples frozen in TCG (Tris, citric acid, glucose)-6% EY than for those extended with TCG-20% EY (Santiago-Moreno *et al.*, 2006). In agreement with these results, we obtained better freezability of mouflon epididymal sperm samples with freezing extender containing 6% EY than 12% EY. Nevertheless other studies obtained better results with high concentrations of EY. Fernández-Sántos *et al.* (2006) reported more vigorous post-thaw motility of red deer epididymal spermatozoa with 20% EY than with lower concentrations. Also, Álvarez *et al.* (2012) reported better post-thaw quality of ram spermatozoa with 20% EY both in epididymal and ejaculated sperm. The protective effect of EY is influenced by the sperm origin due to the fact that the phospholipid composition and the membrane properties differ between ejaculated and epididymal sperm (Scott *et al.*, 1967). Moreover, the interaction of the EY with the seminal plasma components seems to have a key role on its cryoprotective effect. Ferreira *et al.* (2014) reported higher post-thaw motility with 5% EY than with 10% EY in goat ejaculated sperm without seminal plasma while, with seminal plasma, the post-thaw motility was higher with 10% EY. The effect of EY concentration on ejaculated sperm cryosurvival differs between species and studies. Sperm cooling and freezability of ejaculated sperm was improved with high concentration of EY in domestic small ruminants (Cabrera *et al.*, 2005; Forouzanfar *et al.*, 2010; Rajabi-Toustani *et al.*, 2014; Şen *et al.*, 2015). On the contrary, increasing concentrations of EY resulted in detrimental effects on post-thaw ejaculated sperm parameters in other species such as gazelles (Holt *et al.*, 1996; Garde *et al.*, 2008). High proportions of EY increase the incidence of acrosomal damage in ram (Watson & Martin, 1975). Nonetheless, increasing EY concentration did not render any benefit on post-thaw sperm quality parameters in bucks (Ustuner *et al.*, 2009) and rhesus macaques (Dong & VandeVoort, 2009). It has been suggested that these differences could be related with the buffer system used (Watson, 1976; Garde *et al.*, 2008).

In conclusion, the optimum concentration of EY in sperm cryopreservation extenders should be adapted to the protocol, species and sperm origin. There is a maximum concentration of EY above which it has

detrimental effects on sperm freezability, therefore we recommend the use of a TEST-6% EY semen extender for mouflon epididymal sperm cryopreservation.

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