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Slow and ultra-rapid freezing protocols for cryopreserving mouflon (*Ovis musimon*) and fallow deer (*Dama dama*) epididymal sperm

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ABSTRACT

This study examines the effectiveness of two methods for cryopreserving post-mortem epididymal sperm - conventional slow freezing employing a short equilibration time with glycerol, and ultra-rapid freezing - from the wild ruminant species *Ovis musimon* (mouflon) and *Dama dama* (fallow deer). A Tris-citric acid-glucose (TCG) + 12% egg yolk-based medium was used for the conventional slow freezing of the fallow deer sperm, whereas a Tes-Tris-glucose (TEST) + 6% egg yolk-based medium was used for the mouflon sperm. Glycerol was added to a final concentration of 5% to both media. The same diluents were used for ultra-rapid freezing but replacing the glycerol with 100 mM of sucrose. Sperm variables (motility, viability, acrosome integrity, membrane integrity, and morphological abnormalities) were analyzed before and after cryopreservation. Although values were generally better after the thawing of the conventionally cryopreserved sperm, total sperm motility (38.40 ± 4.44% in mouflon and 31.25 ± 3.37% in fallow deer) and total live sperm (47.19 ± 5.18% in mouflon and 43.13 ± 2.43% in fallow deer) were acceptable for the ultra-rapidly cooled sperm. Independent of the cryopreservation method, membrane integrity, acrosome integrity and the percentages of dead sperm and sperms with a damaged acrosome were better for the cryopreserved mouflon sperm than the fallow deer sperm ($P < 0.05$). Despite exerting a more harmful effect on sperm variables than conventional freezing, ultra-rapid freezing may be a useful alternative for the cryopreservation of these species' epididymal sperm in the field, as this simple technique does not require sophisticated equipment and expertise.

1. Introduction

The development of new techniques for sperm cryopreservation (e.g. species-specific customization of freezing extenders, freezing at ultra-rapid cooling rates, directional freezing technique) has allowed progress to be made in the banking of genetic resources and improved the conservation of endangered wild species (Sánchez et al., 2012; Prieto et al., 2014). The widespread use of cryopreservation is, however, limited by the difficulties involved in collecting sperm and freezing it under field conditions. In wild ruminant species such as the European mouflon (*Ovis musimon*) and fallow deer (*Dama dama*), sperm cryopreservation is of great interest not only for conservation purposes, but also for improving the stock on hunting reserves. The methods of assisted reproduction commonly

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used in domestic animals (e.g. artificial insemination with frozen-thawed sperm) are currently being applied to game species, allowing the optimization of wild game management, by means of selection of animals with high genetic value, and improving the quality of the trophies. Moreover, genome resource banking may be the only way to guarantee the continued survival of certain species, subspecies or ecotypes (Santiago-Moreno and López-Sebastián, 2010).

Viable epididymal sperm can be collected and cryopreserved from dead and hunter-killed males of several wild species (Soler et al., 2003; Pérez-Garnelo et al., 2004; Santiago-Moreno et al., 2006a). In Iberian ibexes (*Capra pyrenaica*), the cryopreservation of such sperm can be field-adapted through the use of a short equilibration time and a permeant cryoprotectant (Pradiee et al., 2014). Ultra-rapid cooling might also be used in the field since it requires less equipment than conventional freezing, and is much faster, simpler and cheaper (Isachenko, 2003). It was first used successfully for cryopreserving human (Isachenko et al., 2011) and fish (Merino et al., 2012) sperm, and more recently it has been used to preserve ejaculated Iberian ibex (Pradiee et al., 2015) and European mouflon (Pradiee et al., 2017) sperm. The key to success when using this technique with wild ruminant sperm is to add only moderate concentrations of sucrose (about 100 mM) to the extender, and to use rapid thawing rates when later thawing the sperm (Pradiee et al., 2015, 2017). Both shortening the prefreezing equilibration time with glycerol and ultra-rapid cooling rates have been used with ejaculated mouflon sperm, but with only very moderate success (Pradiee et al., 2016, 2017). Epididymal sperm from some studied species, such as sheep (García-Álvarez et al., 2009) and ibex (Pradiee et al., 2014, 2016), were found to be more cryoresistant than sperm obtained by electro-ejaculation. This advantage was also seen when comparing epididymal ram sperm with normally ejaculated sperm (Woelders et al. 2012). Therefore, ultra-rapid freezing of epididymal mouflon sperm might give good results.

The aim of the present work was to examine the effect of two freezing protocols - conventional freezing with a short equilibration time with glycerol, and ultra-rapid freezing - on the quality of frozen/thawed epididymal sperm from mouflon and fallow deer. The literature contains no information on the use of short equilibration times or ultra-rapid freezing applied to ejaculated or epididymal sperm from fallow deer, or to mouflon epididymal sperm.

2. Materials and methods

2.1. Animals and sperm collection

Testes were collected during the rutting season (December of 2014 and February of 2015) from dead mature mouflons (n = 16) and fallow deer (n = 8). All animals had been legally hunted in their natural habitat in the Andalusian hunting reserve of Cazorla and Segura (Jaén, Spain), in accordance with the reserve's harvesting plan. The latter plan followed Spain's 'Harvest Regulation, Forest and Wild Animal Law 8/2003' issued by the Andalusian Regional Government, which adheres to European Union regulations.

2.2. Sperm collection

The sperm collected from the left and right epididymides was cryopreserved differently: classic slow freezing for the sperm from the left epididymis with a short equilibration time with glycerol (Pradiee et al., 2014), and ultra-rapid freezing for sperm collected from the right epididymis. Sperm collected from the left epididymis (for conventional freezing) of the fallow deer was collected by retrograde flushing with 1 ml of TCG (Tris (313.7 mM), citric acid (104.7 mM), glucose (30.3 mM)) + 12% egg yolk (vol/vol) at 5 °C (Santiago-Moreno et al., 2009), while the corresponding sperm from the mouflon was collected in the same way using TTG (Tes (210.6 mM), Tris (95.8 mM), glucose (10.1 mM) + 6% egg yolk (vol/vol)). Sperm collected from the right epididymis (for ultra-rapid cryopreservation) was flushed out using the same method and extenders for each species, but including additionally 100 mM of sucrose in the flushing solution. These flushing agents were prepared using reagent-grade chemicals purchased from Panreac Química S.A. (Barcelona, Spain) and the Sigma Chemical Co. (St. Louis, Missouri, USA).

2.3. Sperm assessment

Total sperm concentration in the collected samples was calculated before freezing using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany). Sperm motility variables, sperm morphology, acrosome and plasma membrane integrity were assessed for each sample both prior to freezing and after later thawing. The percentage of motile spermatozoa and the quality of motility were evaluated subjectively using a phase contrast microscope (Zeiss, Oberkochen, Germany). The quality of sperm movement was scored on a scale from 0 (lowest) to 5 (highest). Sperm motility was also evaluated objectively using a computer-aided sperm analysis system (CASA) (SCA, Barcelona, Spain) coupled to a Nikon Eclipse model 50i phase contrast microscope with negative contrast capability. For this, sperm samples were diluted with the same extender used for flushing the sperm from the epididymis and 3 µL drops were placed on a Leja eight-chamber slide (Leja Products B.V., Nieuw Vennep, The Netherlands). A minimum of three fields and 500 sperm tracks were assessed to determine the percentage of immotile sperm, sperm showing non-progressive motility, and sperm showing progressive motility.

Plasma membrane integrity was assessed by nigrosin-eosin staining (Campbell et al., 1956) and the hypo-osmotic swelling test (Jeyendran et al., 1984). The percentages of spermatozoa with a normal acrosomal ridge (NAR; Pursel and Johnson, 1974; Pintado and Pérez-Llano, 1992) and morphological abnormalities (Frank 1950) were assessed by examination of glutaraldehyde-fixed samples under a phase-contrast microscope. Sperm cell morphology was categorized as either normal or as showing coiled tails, bent tails, loose normal heads, broken necks, abnormal heads, mid-piece defects, or proximal or distal cytoplasmic droplets. Spermatozoa with cytoplasmic droplets were considered morphologically normal since these are commonly seen in epididymal sperm cells. Sperm