Theriogenology 79 (2013) 383–391

Contents lists available at SciVerse ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Cryopreservation of aoudad (*Ammotragus lervia sahariensis*) sperm obtained by transrectal ultrasound-guided massage of the accessory sex glands and electroejaculation

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ARTICLE INFO

Article history:
Received 23 July 2012
Received in revised form 9 October 2012
Accepted 16 October 2012

Keywords:
Aoudad
Electroejaculation
Extender
Freezing spermatozoa
Ultrasonography

ABSTRACT

This study examines (1) the effectiveness of transrectal, ultrasound-guided massage of the accessory sex glands (TUMASG) combined with electroejaculation for obtaining aoudad (*Ammotragus lervia sahariensis*) sperm samples for cryopreservation, and (2) the effectiveness of a Tris-citric acid–glucose-based medium (TCG; usually used for freezing ibex sperm) and a TES-Tris–glucose-based medium (TTG; typically used in the cryopreservation of mouflon sperm) as sperm extenders. After TUMASG, just one to three electrical pulses were required for ejaculation to occur in five of the six animals studied; one ejaculated after TUMASG alone. Transrectal, ultrasound-guided massage of the accessory sex glands would therefore appear to be useful in obtaining sperm samples from this species, requiring few subsequent electrical electroejaculation stimuli and sometimes none at all. After thawing, the membrane integrity (assessed by nigrosin-eosin staining) of sperm extended with TTG was greater than that of sperm extended with TCG ($P < 0.05$). The total percentage of sperm showing an intact acrosome, as assessed by fluorescein isothiocyanate-conjugated peanut (*Arachis hypogea*) agglutinin, was also higher in the TTG-extended sperm ($P < 0.05$), and the percentage of dead sperm with a damaged acrosome was lower ($P < 0.05$). No differences were seen between TCG and TTG in terms of apoptotic manifestations (DNA damage, caspase activity, mitochondrial membrane potential, and plasmalemma stability). Therefore, TTG appears to be a better extender than TCG for cryopreserving aoudad sperm.

1. Introduction

The aoudad (*Ammotragus lervia*), a wild mountain ungulate, was formerly widespread in the rugged, mountainous terrains of the deserts, semideserts, and even open forests of North Africa. Currently, however, it is listed as vulnerable in the 2011 International Union for Conservation of Nature Red List of Threatened Species, and it is considered to be facing a high risk of extinction in the wild [1], the victim of poaching and competition from domestic stock. Currently, the total population is of the order of 5000–10,000 individuals, but it is estimated that a decline in excess of 10% will occur over the coming 15 years [1]. Reproductive biotechnologies will likely be required if populations of aoudad are to be adequately maintained. Gamete cryopreservation and the development of a gene bank might therefore be of help in efforts to conserve the species [2].

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0093-691X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2012.10.011

Viable epididymal spermatozoa can be retrieved from dead wild ruminants and then frozen [3,4], but the availability of such postmortem sperm samples is low. Fortunately, live aoudads can be subjected to electroejaculation, allowing for the repeated collection of sperm from individual specimens. Electroejaculation has been successfully used with a wide range of wild species [5–11]. However, early studies in domestic caprines showed that semen collected by electrical stimulation is of lower quality than that collected using artificial vaginas [12]: the pH is higher and it contains a larger amount of seminal plasma [13]. The response to cryopreservation of electroejaculated samples thus collected might therefore be different to those obtained by other methods.

Transrectal massage of the accessory sex glands is an alternative to electrical stimulation, and has been successfully used in a number of species. In humans, vigorous prostatic massage has been described as a simple noninvasive method to recover sperm [14]. Transrectal massage of the ampulla of the vas deferens has been used for sperm recovery in bulls, but this usually also requires electroejaculation [15]. Schmitt and Hildebrandt [16] described a successful method of sperm collection for use in elephants, involving the manual stimulation of the accessory sex glands. Ultrasound scanning of the ampulla glands was found to be a useful for monitoring the ejaculatory response [17].

The genus *Ammotragus* is understood as an intermediate between *Ovis* and *Capra*, with its members sharing characteristics of both sheep and goats [18]. The sexual cycle in the female, which lasts 23 days [19], however, appears to be most similar to that of goats and ibexes. Unfortunately, information regarding the handling and cryopreservation of sperm in the aoudad is scarce [20,21], and success could depend on whether it is treated like that of sheep or goats [21]. In caprines, the secretion of the accessory bulbourethral gland shows phospholipase activity; lipids in the egg yolk used in the extender are therefore converted to fatty acids and lysophospholipids—compounds that might be toxic to spermatozoa [22–24]. Thus, washing sperm is recommended in domestic and wild caprines [25]. In contrast, the bulbourethral glands of ovines show no phospholipase activity, so no such washing is needed. In addition, the types of buffering additive used and the interaction of these with the sugars [26,27] and egg yolk [28] in the extender might affect sperm viability after thawing, and these interactions can be species-dependent. For example, in the ibex (*Capra pyrenaica*), a wild caprine from the Mediterranean area, the use of a Tris-citric acid-yolk-based medium for freezing spermatozoa affords better postthawing results than a medium containing egg yolk and the two buffers TES and Tris [28]. In mouflon (*Ovis musimon*), however, TES-Tris-glucose-based extenders have been successfully used for sperm cryopreservation [29]. TES, a zwitterion, aids in the cellular dehydration process by creating an osmotic force [30]; this helps to increase the stability of the plasma membrane and to neutralize any acids generated during storage [31]. Similarly, TES, and indeed other zwitterion buffers such as HEPES and PIPES, appear to be better than Tris-citrate-based media for cryopreserving ram (*Ovis aries*) semen [30]. Because the aoudad shares affinities with sheep and goats, the use of caprine and ovine extenders needs to be tested [21].

With the aim of optimizing biological resources for cryopreservation in germplasm banks, this study assesses the effectiveness of transrectal ultrasound-guided massage of the accessory sex glands (TUMASG) combined with electroejaculation for the collection of aoudad sperm samples. In addition, a Tris-citric acid-glucose-based (caprine) and a TES-Tris-glucose-based (ovine) extender were tested to see which provided the best cryopreservation results.

2. Materials and methods

2.1. Animals

The study animals were six male Saharan aoudad (*Ammotragus lervia sahariensis*) aged 3 to 9 years, all maintained at the Cordoba Zoological Gardens in southern Spain. The mean body weight of these animals was 80 kg (range, 46 to 110 kg). These animals, plus four female Saharan aoudad, were kept together throughout the year. The entire flock (N = 10) was derived from a single pair brought to Cordoba from Libya in 1967; its members therefore have a high degree of consanguinity. All animals were fed with dry alfalfa and barley straw, supplemented with maize. Free access was provided to water and vitamin/mineral blocks. The holding facility consisted of an outdoor enclosure (500 m^2) with areas of sand and rocks, plus an indoor stall (75 m^2) with a restraining-isolating area. All handling procedures were performed in accordance with the Spanish Policy for Animal Protection RD1201/2005, which conforms to the European Union Directive 86/609 regarding the protection of animals used in scientific experiments.

2.2. Transrectal, ultrasound-guided massage of the accessory sex glands and electroejaculation for sperm recovery

Sperm was collected in early February, at the end of the rutting season [19]. Food and water were withheld for 24 hours before the male animals were anesthetized using a projectile dart (TeleDart, GMBH & CO., KG, Westheim, Germany) providing intramuscular detomidine (138 μg/kg; Domosedan, Pfizer Inc., Amboise, France), ketamine hydrochloride (1.3 mg/kg; Imalgene-1000, Rhône Mérieux, Lyon, France), and tiletamine-zolazepam (1.3 mg/kg; Zoletil-100, Virbac España S.A., Barcelona, Spain). The mean anesthetic induction time was 5 minutes. Anesthesia was maintained with isoflurane (Isobavet, Intervet Schering Plough Animal Health, Madrid, Spain) [32]. Animals were placed in the lateral recumbent position. After clipping the hair around the penis and cleaning the surrounding area, the penis was manually made to protrude; it was maintained protruded by holding it with the help of gauze just caudal to the glans. This guaranteed the urethra did not collapse during ejaculation. The protruded penis was then cleaned with a sterile gauze wetted in a sperm-washing solution composed of Tris, citric acid, and glucose (345 mOsm, pH 6.8) [20].