Short communication

Superovulation, *in vivo* embryo recovery and cryopreservation for Aoudad (*Ammotragus lervia*) females using osmotic pumps and vitrification: a preliminary experience and its implications for conservation.

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Abstract

The aoudad *(Ammotragus lervia) is a wild ruminant considered the living ancestor of domestic sheep and goats. The original distribution of aoudads includes several countries in the North of Africa, but it has* been introduced, for trophy-hunting purposes, into other countries (USA, Mexico and Spain). The species was declared vulnerable in the 2012 IUCN's Red List and is also included in the CITES II Appendix. Surprisingly, little is known about its conservation status or the reproductive biology of the natural populations. There are some reports of the application of basic assisted-reproduction techniques in captive aoudads. In this preliminary study, we explored the feasibility of implementing assisted reproduction procedures in captive aoudad females using non-traditional techniques for *in vivo* embryo production. This approach was used to obtain the best results using the minimum possible number of animals. Three aoudad females were synchronized using a domestic sheep protocol and subcutaneous osmotic pumps for the delivery of follicle-stimulating hormone. A mini-surgical approach combined with laparoscopy was performed to obtain *in vivo*-produced embryos. All females had an ovulatory response of more than three *corporea lutea*, but only five good quality morulae were obtained from one female. Those were cryopreserved by vitrification using a Cryotop®. In conclusion, our approach was successful in obtaining *in vivo* embryos using a limited number of females. Further studies are necessary to optimize the fertilization rate and clarify the effect of our protocol on embryo implantation and the production of offspring.

Keywords: Aoudad; Cryotop®; osmotic pumps; superovulation; vitrification, wild ruminant.

Resumen

Ammotragus lervia, aoudad, es un rumiante silvestre considerando el ancestro de los borregos y cabras domésticas. La distribución original de los aoudad incluye varios países en el Norte de África, pero ha sido introducido, con propósitos de cacería en otros países (EUA, México y España). La especie se declara como vulnerable en la Red List 2012 de la IUCN y también se incluye en el Apéndice II del CITES. Sorprendemente, poco se sabe del estado de conservación o de la biología reproductiva de las poblaciones naturales. Existen algunos reportes de la aplicación de técnicas básicas de reproducción asistida en hembras de aoudad captivas. En este estudio preliminar, exploramos la factibilidad de implementar procedimientos de reproducción asistida en hembras de aoudad captivas usando técnicas no tradicionales para la producción de embriones *in vivo*. Este enfoque fue utilizado para obtener los mejores resultados utilizando el menor número posible de animales. Tres hembras de aoudad fueron sincronizadas usando un protocolo de borregas domésticas y bombas subcutáneas para la administración de la hormona folículo estimulante. Un abordaje mini-quirúrgico combinado con laparoscopia fue realizado para obtener los embriones producidos *in vivo.* Todas las hembras tuvieron una respuesta ovulatoria de más de tres cuerpos lúteos, pero solo cinco mórulas de buena calidad se obtuvieron de una hembra. Estos embriones fueron criopreservados por vitrificación usando un Cryotop®. En conclusión, nuestro abordaje fue exitoso en obtener embriones *in vivo* usando un número limitado de hembras. Se requieren estudios subsecuentes para optimizar la tasa de fertilización y clarificar el efecto de nuestro protocolo en la implantación y la producción de crías.

Palabras clave: Aoudad; bombas osmóticas; Cryotop®; rumiante silvestre, superovulación; vitrificación.

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Introduction

Ammotragus lervia, also called the Barbary sheep or aoudad (an anglicization of its Tunisian name: *udad*), is an interesting ruminant from a taxonomical point of view because it is the sole member of the *Ammotragus* genus. Its evolutionary origin is controversial, but aoudads are considered the living ancestor of domestic sheep and goats (reviewed in [1,2]).

The aoudad has 6 subspecies described: *A. l. lervia, A. l. ornatus, A. l. sahariensis, A. l. blainei, A. l. angusi and A. l. fassini*. The original living range of aoudads includes the countries of North Africa: Algeria, Chad, Egypt, Libyan Arab Jamahiriya, Mali, Mauritania, Morocco, Niger, Sudan and Tunisia [2,3]. In addition, the aoudad has been introduced into other countries for trophy-hunting purposes, and there are currently introduced populations living in the USA (Texas, New Mexico and California), Mexico (Northern states) and Spain (Canary Islands and other provinces). These populations have a large number of individuals and are free-ranging, competing with the native mammals for resources [3].

The aoudad is declared to be vulnerable in the 2012 Red List of the International Union of Conservation of Nature (IUCN) [3], and it is also included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [4]. However, with regard to the natural populations, little is known of their biology [3]. Indeed, virtually all the information for this species comes from *ex-situ* populations, and unfortunately the good adaptation and reproduction of introduced and captive populations give a false sense of security regarding its conservation. Although *A. l. ornatus* had been considered extinct, an article in the last decade reported its presence in Egypt [5].

The aoudad has 58 chromosomes, an intermediate number between the chromosome complement of sheep (n=54) and goats (n=60) [1], making the aoudad an interesting species from a genetic and reproductive point of view. Although there is little information about its basic reproductive biology and relationship with other members of the subfamily *Caprinae*, there are some reports on the application of basic reproductive techniques in this species, including sperm analysis in captive males [6,7], sperm freezing [8,9,10], the production of offspring using estrus synchronization and intrauterine artificial insemination [10] and, recently, the description of estrus cycle in aoudad females using fecal steroids [11].

In this study we explored the feasibility of assisted reproduction in captive aoudad females using non-traditional techniques, in order to obtain the best results using the minimum possible number of animals (5 females were available). Our main goals in this preliminary study were to synchronize the females using a domestic sheep protocol with osmotic pumps for the delivery of hormones, to obtain *in vivo*-

produced embryos using a mini-surgical approach, and to vitrificate these embryos using a Cryotop® device for future embryo transfers.

Methods

Animals

The present research was performed at the Zoological Park of Leon (www.zooleon.org) located in Guanajuato, Mexico (coordinates: 21°18´ N, 101°65´W) between months of June and December. The animals proposed for this study were 5 females of 2 years and 1 male of 3.5 years, which inhabited the Safari area of the park (a large exhibit with semi-free conditions) (Figure 1). The animals were fed mixed grass and water *ad libitum*. The training consisted of the habituation to human presence with touching and physical restraining. After this training period, an ultrasound scan (Aquila Pro Vet®, Esaote-Pie Medical) was performed for pregnancy diagnosis, and the non-pregnant females were relocated to a smaller enclosure where they were maintained for the study.

All of the procedures were conducted according the Mexican laws for wildlife protection (NOM-059-ECOL-2001) and the Mexican regulations for animal research (NOM-062-ZOO-1999).

Synchronization of estrus and superovulation

Estrus synchronization consisted of the insertion of intravaginal sponges impregnated with 20 mg of micronized chronogestone (Chronogest®, Intervet) for 14 days, with a replacement at day 7, and simultaneous i.m. administration of 15 mg of dinoprost (Lutalyse®, Pfizer Animal Health).

For the superovulation procedure, the females were sedated at day 11 with a combination of xylazine (Rompun®, Bayer Mexico) and ketamine (Inoketam®, Virbac Mexico) administered intramuscularly at doses of 0.3 mg/kg and 10 mg/kg respectively. Two micro-osmotic pumps (model 1003D, Alzet®) containing 200 mg of porcine FSH (Folltropin-V®, Bioniche Animal Health) were applied subcutaneously, and 60 mg of FSH was intramuscularly injected into each female. The intravaginal sponges were removed at day 14, and 100 µg of GnRH (Fertagyl®, Intervet) was administered at day 15. A ram (Figure 1) was then introduced 36 hours after the removal of the sponges, and visual monitoring during daylight was performed for the signs and behavior of estrus in the females and mating-associated behavior in the male.

Embryo collection

The embryo collection was performed at seven days after the removal of the sponges. The three females were anesthetized using 4 mg/kg of a combination of tiletamine and zolazepam (Zoletil®, Virbac Mexico). Prior to the embryo collection, ovarian status was evaluated by endoscopy to determine whether surgery was necessary. The mini-laparotomy for the embryo collection was performed according to the technique described by Ramon-Ugalde *et al*. [12]. The flushing medium used was TCM-199 with Hank's Salts containing L-glutamine, HEPES 25 mM (Gibco®, cat. 12350), 50 µg/ml gentamicin and 15% (v/v) fetal bovine serum (FBS).

Embryo Vitrification

The vitrification was performed according to a method described for sheep by Kelly *et al*. [13] with minor modifications. First, the collected embryos were equilibrated at room temperature in a solution of 7.5% ethylene glycol (EG) and dimethyl sulfoxide (DMSO) in TCM-199 with Hank's Salts containing L-glutamine, HEPES 25 mM (Gibco®, cat. 12350), 50 µg/ml gentamicin and 20% (v/v) FBS. The embryos were transferred to a vitrification solution (15% EG-DMSO-0.5 M sucrose) and maintained for 3 min to ensure

the collapse and re-expansion of the embryos. Lastly, the embryos were loaded in a Cryotop®, frozen with liquid nitrogen (LN2), and stored in a tank with LN2.

Results

The number of females used was dependent on the ultrasonographic pregnancy diagnosis performed during the training. All of the five females responded well to the handling training, and it was possible to maintain a relatively low level of stress. Of the five female aoudads available at the Zoo, two were pregnant. We therefore performed the superovulation treatment using three females.

It was possible to administer the FSH through the osmotic pumps with a combination of sedation while maintaining good results in the superovulation response. Due to heavy rain and fog during the day-light hours, it was difficult to determine in detail and with confidence the estrus and male mating behavior. However, it was possible to observe that the male aoudad attempted to mate with female number 3. Table 1 shows the superovulatory response examined by laparoscopy in the treated females, the number of follicles (F), the *corporea lutea* (LC) and the embryos obtained in each flushed female.

Table 1. Superovulatory response in aoudad females.

	Right Ovary	Left Ovary			
Female	**# F (mm)**	**# CL**	**# F (mm)**	**# CL**	**# embryos**
1	4 (3) 2 (2) 2 (1)	0	0	4	0
2	1 (5) 1 (2)	3	1 (>5)	6	1 degenerate oocyte
3	0	16	0	11	5 morulae

F: Follicles; CL: *Corporea lutea*

Figure 2 shows an embryo obtained prior to vitrification. The behavior of the embryos during the vitrification was similar to that of human embryos. After one minute in the vitrification solution, the embryo suffered a shrinking caused by dehydration, which was similar to what we observed in other mammalian embryos (mouse and human). The 5 embryos were loaded without complication, leaving a minimal volume of solution (approximately 1 µl per embryo).

Discussion

The female reproductive physiology of aoudads has been poorly studied. To date, information is limited to reports of progesterone levels and sources during pregnancy [14], the pregnancy levels of progesterone and estradiol compared with domestic goat and sheep [15], and the relationship of inbreeding to reproductive success in captive females [16]. Consequently, it was difficult to design an optimal multiple ovulation and embryo transfer (MOET) scheme based on the published data for the aoudad female reproductive cycle. It was very recently described that the estrus cycle of captive aoudad, using fecal steroids, is around 23 days (range 16-32 days), being more similar to *Capra* genus [11].

We utilized an estrus synchronization scheme, similar to that for domestic sheep females, which had been successful for aoudad insemination [8]. However, the traditional procedures for superovulation in domestic ruminants are complicated and difficult to perform in wild females because the procedure requires many physical contact and repetitive procedures, i.e., the administration of FSH two times per

day. Thus, we choose to deliver FSH using osmotic pumps to minimize such manipulations. Previously, osmotic pumps were used with success for the administration of FSH to Spanish ibex females [17], and we considered that it was the best approach for wild ruminant females and most likely represents a more "physiological" delivery method, with the continuous administration of FSH rather than blood level spikes.

The reason for using only one male aoudad in this work was because the Leon Zoological Park only had two males available for this study and one of them is a young male with an unproven fertility. The detection of estrus can determined using a vasectomized male or a heterospecific male (buck or ram); however, the feasibility of using a male of another species for the detection of estrus in wild goats has not been established. In addition, estrus detection in wild females can generate stress just before the moment of ovulation., We therefore decided to use a natural approach, only introducing the aoudad male with proven fertility, and to monitor the copulation behavior.

Fig. 1. (A) Aoudad group at "Safari" (semi-free enclosure) of Leon Zoological Park. (B) Male used in the experiment.

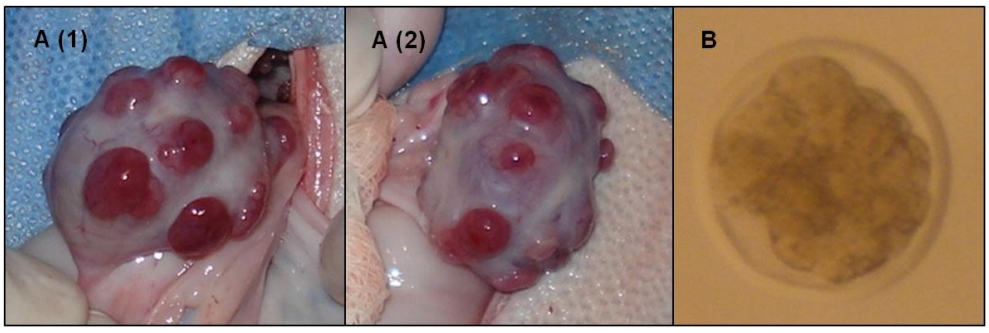

Fig. 2. (A) An ovary at day of surgery (seventh day after sponge removal) showing the superovulatory response to FSH in female number 3. (1) Right ovary. (2) Left ovary. (B) Morula from auodad before vitrification.

We had a low embryo recovery rate in comparison with the same procedure in domestic ewes [12], which can be attributed to the low level of fertilization *in vivo* rather than a failure of the technique, because our procedure has an embryo recovery of more than 85% in domestic ewes when it is performed by the same surgeon [12]. Such sub-optimal fertilization can occur in domestic ewes due to changes in the cervical mucus that generate a deficient sperm transport; this difficulty can be avoided using intrauterine insemination [18]. Consequently it might be better to fertilize aoudads using intrauterine insemination, although this would require another anesthetic procedure during the ovulation period. One possible approach to circumvent this problem without using extra-manipulation can be to use another male for additional mating during the fertilization window (24-40 hrs post GnRH administration).

Although vitrification is changing the success of cryopreservation in some species, particularly in humans, it is not traditionally used for domestic animal embryos. However, there are reports of good pregnancy rates of *in vivo*-vitrificated domestic sheep embryos [19]. One of the main limitations of this method is the high cost of the commercial vitrification devices (as, Cryotop®, Cryolock® and Cryoleaf®), so only a few works have reported the use of these commercial devices in domestic animal embryos, mainly in bovines [20], and only one article has reported its use in wildlife species oocytes: the Mexican gray wolf [21]. To our knowledge, this is the first report of the use of Cryotop® in wild ruminant embryos. More studies are necessary to address the success of these devices with regard to the pregnancy rates and cost-benefits.

The next step in the development a MOET scheme for aoudad is embryo transfer. Although it is possible to use *ex-situ* populations of aoudads as the recipients, to maximize the conservation goals, it is necessary to attempt to use hybrids or domestic surrogate mothers. In this respect, there are reports of aoudad hybridization attempts with goats and rams [22,23,24]. In these reports, it was possible to obtain live hybrid offspring from an aoudad male and female goats using both natural mating [22,23] and artificial insemination [24]. The ewe inseminated with aoudad semen produced embryos, but no pregnancies resulted when the embryos were transferred to ewes or goats [24].

Inter-species pregnancies have been published for some species of wild ungulates, such as Spanish ibex [14], Texas red sheep [21] and mouflons [22]. However, no report has been published for inter-species embryo transfer of ungulates species with different numbers of chromosomes in embryo and receptor, which is the first obstacle to overcome for aoudad inter-species pregnancies to sheep or goats. In future reproductive seasons, we will attempt to obtain additional embryos, with the final goal of transferring the vitrified embryos using goats or aoudad-goat hybrids as the recipients.

In conclusion, our protocol using osmotic pumps and natural mating is appropriate to obtain *in vivo* embryos. However, refinements are required to maintain a high embryo recovery rate. The vitrification procedure using Cryotop® can be applied to wild ruminants using conventional procedures. Future studies are necessary to explore their success in pregnancy rates and offspring production.

Implications for Conservation

This study is the first to obtain and cryopreserve *in vivo* embryos from aoudad. Considering the uncertainty of the conservation status of the population of aoudad subspecies *in situ* [3,4] and the possible need for intensive actions for the recovery of the natural populations in the near future, this study established the basis of a successful protocol that can help in intensive actions for its conservation.

Although the use of assisted reproductive technologies is a controversial issue in wildlife conservation, there are successful examples of partnerships between reproductive biologists and field conservationists to preserve non-domestic ruminants extinct in wild. The Saharawi Dorcas Gazelle (*Gazella dorcas neglecta*) is maintained and reproduced *ex situ* in zoological Parks in Europe to provide animals for reintroduction in Senegal. Animals are initially kept in enclosures at Guembeul Fauna Reserve and North Ferlo Fauna Reserve, to be later released to the wild [27]. The Estación Experimental de Zonas Aridas in Spain, in collaboration with other European institutions, maintains a population of Mohor gazelle (*Gazella dama mhorr*) and has obtained offspring from artificial insemination [28]. In the near future the program for Dorcas and Mohor gazelles will be enhanced by assisted reproduction techniques [28]. The Scimitar-horned Oryx (*Oryx dammah*) is another successful example; this species is extinct in the wild,

but captive herds are kept in fenced protected areas in Tunisia, Senegal and Morocco as part of long-term reintroduction programs, while the National Zoo in Washington has developed an artificial insemination procedure to preserve its population of Scimitar-horned Oryx and to avoid inbreeding [29]. Reintroduction of this species is currently planned in Niger [30]. All of these examples highlight the importance of intensive management and assisted reproduction techniques in the international conservation efforts for wild ungulates.

Therefore, we propose that aoudad should be part of similar management plans that include several components: a) an urgent survey of *in situ* populations in Africa, b) management at enclosure facilities in their original ranges, and, c) the use of semen/embryo related techniques, i.e., artificial insemination and superovulation and embryo recovery in females of subspecies of aoudad (*A. l. ornata, A. l. blainei, A. l. fassini, A. l. angusi*), using as embryo recipients the "generic" aoudad maintained in Zoos or introduced out of its original range. This approach is currently feasible, due to the existence of captive aoudad populations for conservation purposes, such as the one in the Estación Experimental de Zonas Áridas in Spain. This Institution keeps a herd of 50 individuals of *Ammotragus lervia sahariensis*, originated from 2 males and 1 female imported from Occidental Sahara to Spain in 1975 [11]. Unfortunately, this population has a high level of inbreeding; which can produce adverse consequences in reproductive fitness of females [13] and males [9]. Another population of 38 individuals of Kordofan Aoudad (*A. lervia blainei*) is managed in USA, under the auspices of the American Association of Zoo and Aquariums (AZA). This population is maintained in 3 institutions through the Kourdofan Aoudad Species Survival Plan® (SSP) and is considered on red Status due to the absence of physical space and infrastructure, the need for new genetics among the population, and the need to confirm the subspecies origin of animals in non-AZA institutions.

Using assisted reproduction techniques, the genetic pool can be refreshed without needing to extract new individuals from natural populations (the most desirable approach to avoid inbreeding). This can be achieved by obtaining semen from *A.l. sahariensis* males in Sahara of *A. l. blainei* males from Sudan, for artificial insemination as previously reported [6,7,9]. Alternatively, semen can be obtained from the epididymis of hunted males in its natural range (very successful in Spanish ibex [31]), or embryos from captive *A.l. saharensis* or *A.l. blanei* can be obtained and cryopreserved using the techniques described in the present report.

Management and surveillance by local authorities and IUCN are needed to optimize the global effects of assisted reproduction techniques for conservation purposes. Additionally, all types of assisted reproductive techniques should be incorporated Species Survival Plans®in larger associations of zoos (American Association of Zoo and Aquarium: AZA and European Association of Zoos and Aquaria: EAZA), to maintain the genetic pool of captive populations at a high level.

This integral path can facilitate the management and reintroduction of subspecies of aoudad to their original distribution areas and limit inbreeding. Finally, application and development of the proposed approach can add some conservation value to the populations of introduced aoudads in many places where they compete with local fauna.

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