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Successful ultrarapid cryopreservation of wild Iberian ibex (*Capra pyrenaica*) spermatozoa

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ABSTRACT

A method for cryopreserving wild ibex sperm at high cooling rates was developed. To design a freezing solution based on Tris, citric acid, and glucose (TCG), two preliminary experiments were performed using glycerol (GLY) and dimethyl sulfoxide (DMSO) at different concentrations (5%, 10%, 20%). The 10% GLY + 10% DMSO combination reduced ($P < 0.05$) frozen-thawed sperm motility, which reached a minimum when 20% GLY + 20% DMSO was used. In the second experiment, sperm tolerance to three sucrose concentrations was evaluated (100-mM sucrose, 300-mM sucrose, 500-mM sucrose). Frozen-thawed sperm motility and sperm viability decreased ($P < 0.05$) at concentrations above 300 mM. The ultrarapid cooling procedure finally used involved a TCG egg yolk (ey)–based extender with 100-mM sucrose, either alone or with 5% GLY with or without BSA. Two warming procedures (37 °C vs. 60 °C) were also evaluated. The TCG ey with 100-mM sucrose but without GLY/BSA returned the best sperm quality variables. Slow warming at 37 °C strongly affected ($P < 0.05$) sperm motility and viability in all groups. Sperm selection by density gradient centrifugation produced no motile sperm when slow warming was performed. In contrast, when fast warming was used, sperm selection increased ($P < 0.05$) percentage of motility, viability, and the percentage of sperms with intact acrosomes. Heterologous *in vivo* fertilization involving domestic goats was performed to evaluate the *in vivo* fertilization capacity of the ultrarapidly cooled cryopreserved sperm (in TCG-ey + 100 mM sucrose), with warming undertaken at 60 °C. Inseminations of domestic goats resulted in three pregnancies (3 of 16, 18.7% fertility). In conclusion, ibex spermatozoa are strongly sensitive to high concentrations of permeable cryoprotectants and sucrose. However, the combination of ultrarapid cooling, using TCG-ey + 100-mM sucrose, and fast warming at 60 °C, followed by sperm selection by density gradient centrifugation to collect the motile sperm, has a positive effect on sperm viability.

1. Introduction

Freezing at ultrarapid cooling rates and vitrification (in which living cells undergo glass-like solidification) are cryopreservation methods that can successfully preserve the embryos, oocytes [1–7], and even the sperm [8–14] of certain species. Equilibrium vitrification, which is used for embryo preservation [9,15], requires high concentrations of cryoprotectant; this raises the viscosity of the milieu and prevents ice formation during cooling, as well as during warming. However, high concentrations of cryoprotectant are harmful to the cytoskeletons of oocytes, and especially to those of sperm cells. For sperm [16]—at least in the species studied so far—kinetic vitrification would appear to

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be a better alternative [8,16–18]. This consists of the ultrafast cooling (by tens of thousands °C/min) of small volume samples without any permeable cryoprotectant [19]. Such high cooling rates prevent ice formation inside the cells. As a result, the entire cell suspension vitrifies [20]; no other vitrifying agents are needed [8,19]. Some nonpermeable additives with cryoprotective activity, such as human serum albumin and sucrose, have been successfully used [21] in the kinetic vitrification of human [8,22] and dog sperm [18]. In patients with severe oligozoospermia, vitrification provides a means of freezing sperm without the use of a potentially toxic permeable cryoprotectant [23].

Whether vitrification occurs is dictated by the interaction between the cooling rate, the viscosity of the solution, and the volume [24]. It has been suggested that both the intracellular milieu and the extracellular environment of the sperm cells must become vitrified [19] for the term "vitrification" to be applicable. However, both vitrifying and ice-forming regions may exist within the same solution, and it can become difficult to differentiate between vitrification and ultrarapid freezing [24]. In addition, debate surrounds whether the terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos is applicable to vitrification in sperm cells. Some authors have even referred to the possibility of vitrifying large volumes of human sperm without the use of a permeable cryoprotectant [25,26].

Unfortunately, vitrification and other cryopreservation methods that use high freezing rates have failed in all other species in which they have been tried (see papers on failures in the kangaroo [27], Rhesus monkey [28], rabbit [29], rodents, polar bear, and raptors [19]). However, in humans and dogs at least, it has been shown quick and simple to perform, to require no expensive cryobiological equipment or special cooling procedures, and to be within the capabilities of any reasonably equipped laboratory [8]. It also renders the use of permeable cryoprotectants superfluous and is much faster, simpler, and cost-effective [30].

In wild ungulates, sperm cryopreservation very often has to be performed in field laboratories, with all the inconveniences this entails. Vitrification and freezing at high cooling rates might, therefore, be extremely useful when working with such species in wildlife parks, zoological gardens or on game reserves, and so forth, which generally lack the required sophisticated equipment and human expertise—if, of course, they can be made to work.

No studies have been performed on the cryopreservation at high cooling rates of wild ruminant ungulate sperm. Using the Iberian ibex (*Capra pyrenaica*) as a model, the present work reports the development of a rapid and efficient cryopreservation method. Ibexes have been shown a useful animal model in which to develop reproductive techniques that can then be used with other wild mountain ruminants [31]. The tolerance of these animals' sperm to different concentrations of cryoprotectant was first assessed to determine whether equilibrium vitrification might be effectively used in this species. Sperm tolerance to different concentrations of freezing solution sucrose (acting as an osmoprotectant) was also evaluated. A cryoprotectant-free methodology based on a high cooling rate, plus different warming protocols, was then established according to the results of the previous experiments. Heterologous *in vitro* fertilization experiments showed sperm frozen at high cooling rates to have fertilizing capacity.

2. Materials and methods

2.1. Animals and sperm collection

Nine adult (4–9 years of age) Iberian ibex were housed in captivity at the animal reproduction department of the INIA (40°N, Madrid, Spain). All had been born at the INIA facilities. They were kept in a 250-m^2 enclosure with partial roof cover. All animals were fed a balanced diet based on Visan K-59 (Visan Ind. Zoot., S.A., Arganda, Madrid, Spain), supplemented with barley grain, barley straw, and dry alfalfa. Free access was provided to water and vitamin/mineral blocks. To alleviate stress during experimental procedures, animals were accustomed to handling in a small restraining stall (2 m^2) in which anesthesia was administered. During all manipulations, the eyes were covered with a mask to further reduce stress. All handling procedures were approved by the INIA Ethics Committee (reference number CEEA 2011/017) and were performed in accordance with the Spanish Policy for Animal Protection RD1201/2005, which conforms to European Union Directive 86/609 regarding the protection of animals used in scientific experiments. The INIA Ethics Committee approved the entire study.

The animals were anesthetized using 50-μg/kg intravenous detomidine (Domosedan; Pfizer Inc., Amboise Cedex, France), 0.5-mg/kg ketamine hydrochloride (Imalgene 1000; Rhône Mérieux, Lyon, France), and 0.5-mg/kg tiletamine-zolazepam (Zoletil 100; Virbac España S.A., Barcelona, Spain). Anesthesia was maintained with 1.5% isoflurane (Isobavet; Intervet/Schering Plough Animal Health, Madrid, Spain) in oxygen (flow rate 2.5 L/min) administered *via* an endotracheal tube. A pulse oximeter was used to monitor the condition of the animals. All 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 that 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 (TCG; 345 mOsm, pH 6.8) [32]. Sperm was obtained by transrectal ultrasound-guided massage of the accessory sex glands combined with electroejaculation, as previously described [33]. The use of transrectal ultrasound-guided massage of the accessory sex glands decreases or even avoids the use of electrical pulses; and thus, it provides practical advantages in animal welfare. Ultrasound was performed using real-time transrectal ultrasonography using a 7.5-MHz linear array probe (Prosound 2, Aloka Co., Ltd, Tokyo, Japan). Zero to four electrical pulses were required to induce ejaculation with this technique. The electroejaculator used was a Lane Pulsator IIIZ model (Lane Manufacturing Inc., Denver, CO, USA) consisting of a rectal probe 2.5 cm in diameter and 20.5 cm in length.