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Cryopreservation of captive roe deer (*Capreolus capreolus*) semen

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ABSTRACT

To address the need to preserve current genetic diversity before it is lost forever; further studies to adapt assisted reproductive technologies to various endangered species are needed, among other things. Roe deer (*Capreolus capreolus*), an over abundant wild deer, can serve as model species to develop or improve sperm cryopreservation of threatened or endangered deer species. The aim of this study was to compare the ability of three diluents (Berliner Cryomedium [BC]; Tris, citric acid, glucose [TCG]; TES, Tris, glucose) to support chilling, cryopreservation (with 5% glycerol; G) and postthaw incubation (at 22 °C and 37 °C) of roe deer spermatozoa collected by electroejaculation. Berliner Cryomedium was the diluent that better preserved roe deer spermatozoa during refrigeration, able to maintain motility for at least 14 days, longer than the other extenders. BC + G was the extender of choice for cryopreservation, showing higher viability compared with TCG + G (66.7 ± 3.4 vs. 54.5 ± 6.5; $P < 0.05$) and higher level of acrosome integrity compared with TES, Tris, glucose + G (79.4 ± 3.4 vs. 67.9 ± 5.0; $P < 0.05$). Maintaining the samples at 22 °C after thawing presented higher values in various parameters compared with 37 °C. The knowledge gained through this study can potentially act as a preliminary step toward development of new protocols to help increase the reproductive success of biologically similar, yet endangered, wild species.

1. Introduction

Accelerated loss of global biodiversity and an increase in the number of endangered and threatened species are matters of great concern. The increased inbreeding witnessed in many animal populations is a cause for further concern. Although efforts should be invested into reversing the processes that led to the current dire state, supportive technologies must be developed to preserve current genetic diversity, at least *in vitro* [1]. Sperm cryopreservation, one such conservation tool, has been of interest to both captive- and wild-populations managers.

The biology and reproductive pattern in mammals is well characterized for only a small fraction of species, the vast majority of which are domestic and laboratory species and species of commercial or scientific interest. This is considered a big obstacle when attempting to apply assisted reproductive techniques such as sperm cryopreservation, estrus synchronization, or artificial insemination to rare or endangered species [2–4]. The use of model species, such as domestic animal or more accessible wild species, coupled with an increase in the understanding of basic processes in cryobiology, may help in developing protocols for preservation of spermatozoa from endangered wild species [1,5–7]. Technology could be transferred from well-studied species to related, but less studied, species, possibly leading to improvement in their sperm preservation. However, even when it is

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possible to develop reproductive protocols based on such model species, it is imperative to eventually experiment in the target species for final adaptation [8,9,12–14]. Species differ in many traits related to their reproductive biology, one of which is the wide variation in sperm membrane lipid and protein composition and its permeability to water and solutes. Such differences, no doubt, contribute to differences in survival of spermatozoa through the chilling, freezing, and thawing processes [15]. Owing to this phenomenon, there is a need to customize the freezing protocols for each species individually.

Important steps during semen cryopreservation include the exposure to cryoprotectants, cooling, freezing, and thawing [8,9,12–14]. Spermatozoa of many species are very sensitive to rapid reduction in temperature from 25°C to 5 °C [16]. Fast chilling may result in chilling injury that is associated with the lipid phase transition of biological membranes [17]. The presence and amount of glycerol, or other cryoprotectants, as well as the presence or absence of sugars and lipids in the cryodiluent, may all be important [18–25], helping protect the spermatozoa from chilling injury [10–26]. Since the discovery of its cryoprotective properties, glycerol has become the most effective cryoprotectant for ruminant spermatozoa [9]. The beneficial effects of sugars in the diluent on postthaw viability of mammalian spermatozoa have also been vastly described [27–30]. Sugars provide energy substrate for the spermatozoa during incubation, they help maintain the osmotic pressure of the diluent, and they act as cryoprotectants [9]. In many studies, glucose, fructose, and/or lactose were included in TEST extenders for freezing ungulate spermatozoa [18,19,30–33].

Roe deer (*Capreolus capreolus*) is a relatively small species of deer well adapted to the cold environment of central and northern Europe. It is a highly successful deer species and its conservation status on the IUCN Red List of Threatened Species is least concern [34]. Roe deer is a seasonal breeder. The apex of the reproductive season is between mid-July and mid-August. Mature males show cyclic changes between totally arrested and highly activated spermatogenesis, making this specie ideal for studying mechanisms related to spermatogenesis. Has been shown in several studies [35,36], this testicular activity in connection with plasma concentrations of hormone and their interaction with antler growth. Cellular composition changes in roe deer gonads were also shown to depend on the season, showing lack of spermatogenesis during the winter months [37–39].

Roe deer can thus act as a model species for other, threatened or endangered deer species. Besides, the important improvement in the knowledge of reproductive biology in different deer species, wild ruminants are very popular species. The interest in deer farming has increased in Europe and elsewhere as a mean to provide game reserves with good quality animals. Deer species are also very important for society being associated with hunting and eco-tourism activities. Hunting has a great economic relevance, constituting an important factor for the subsistence of many rural communities throughout Europe [40].

However, studies on cryopreservation of roe deer spermatozoa are lacking. The influence of different sugars in TEST diluent and the effects of cooling and freezing procedures on roe deer sperm cryosurvival were not yet studied. The aim of this study was to compare the ability of three diluents, used previously in other related species, to support chilling and cryopreserving roe deer spermatozoa collected by electroejaculation. The knowledge gained from this study can potentially be an important preliminary step toward developing new protocols to help increase the reproductive success of biologically similar, yet endangered, wild artiodactyl species.

2. Material and methods

2.1. Animals

This study is part of a long-term study that includes periodic assessment of body condition, and general and reproductive health of a large group of roe deer. All procedures were conducted within the guidelines for IUCN CITES (International Union for Conservation of Nature and Natural Resources, Convention on International Trade in Endangered Species) requirements and in accordance with the guidelines of the Internal Committee of Ethics and Animal Welfare of the Leibniz Institute for Zoo and Wildlife Research under approval number 2013 to 01 to 02.

Semen was collected from 14 male roe deer (*Capreolus capreolus*) (17–24 kg) aged 1 to 9 years, maintained at the Field Research Station of the Leibniz Institute for Zoo and Wildlife Research, located in Niederfinow, Germany. This flock consists of animals in small females-males mixed groups in enclosures of about 250 m^2. Nine males were proven breeders, four were bachelors, and the fertility status of the last one was unknown. All the animals were within their rutting season and exposed to the smell of the females at the time of collection and were therefore reproductively active.

Animals had access to natural vegetation and were additionally fed with pelleted compound feed for dairy calves (Märka KA-18–3, Märkische Kraftfutter GmbH, Guben, Germany) that provided a balanced diet. The chemical composition of the dairy calf pellets was 23% crude protein, 17% neutral detergent fiber, and 7% acid detergent fiber, all on a dry matter basis. This diet was supplemented with hay, straw, and dry alfalfa. Pellets, water, and mineral/vitamin blocks were available *ad libitum*.

2.2. Semen collection

The experiment was conducted during the roe deer rutting season. All semen collection procedures were conducted under general anesthesia. Animals were immobilized by intramuscular administration of xylazine hydrochloride (2 mg/kg body mass (bm); Rompun 10%, Bayer AG, Leverkusen, Germany) in combination with ketamine hydrochloride (4 mg/kg bm; Ketamine 10%, Essex Pharma GmbH, Munich, Germany) using a blowpipe. Anesthesia was maintained with isoflurane (1.0–2.5 vol %; Forane, Abbott Laboratories GmbH, Hanover, Germany) delivered with oxygen at a flow rate of 1.5 to 2.0 L/min via endotracheal tube. Anesthesia was reversed with atipamezol hydrochloride (0.2 mg/kg bm, Antisedan, Pfizer Pharma GmbH, Karlsruhe, Germany).