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Influence of cooling rates and addition of Equex pasta on cooled and frozen-thawed semen of generic gray (*Canis lupus*) and Mexican gray wolves (*C. l. baileyi*)

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Abstract

A current priority for the preservation of the endangered Mexican gray wolf (*Canis lupus baileyi*) is the development of a sperm-based genome resource bank for subsequent use in artificial insemination. To optimize the quality of cryopreserved sperm, the procedures involved in processing semen before and during freezing need to be improved. The aim of this study were to examine the effects of: (i) different cooling periods before freezing and (ii) addition of Equex pasta (Minitüb, Tübingen, Germany) on the characteristics of sperm from the generic gray wolf and the Mexican gray wolf after cooling and cryopreservation. For Mexican wolf sperm, cooling for 0.5 and 1.0 h had a less detrimental effect on cell morphology than cooling for 2.5 h, whereas the slower cooling rate (2.5 h) had a less detrimental effect on functional parameters and seemed to cause less damage to plasma membrane and acrosome integrity than 0.5 and 1.0 h. For the generic gray wolf, cooling semen for 2.5 h had less detrimental effect on plasma membrane integrity and viability; together with the 0.5 h cooling time, it yielded the highest percentages of intact acrosomes. As previously shown in the domestic dog, Equex pasta had no beneficial effect on sperm characteristics in either wolf species.

Keywords: Generic gray wolf (*Canis lupus*); Mexican gray wolf (*Canis lupus baileyi*); Semen cryopreservation; Cooling period; Equex pasta

1. Introduction

Recovery of the Mexican gray wolf (*Canis lupus baileyi*), a subspecies of the gray wolf (*Canis lupus*), depends on careful genetic management of the captive population. Because of their monogamous mating system, transfer of gametes using cryopreservation and artificial insemination is preferable to breaking pair-bonds and transfer of animals. As there are only few Mexican wolves available for evaluating the requisite technology, such as sperm cryopreservation, it has been necessary to use the generic gray wolf as a model and to base the techniques on those developed for the domestic dog, the wolf's closest relative. As cooling is one of the critical steps in semen freezing, the effect of various cooling rates on semen quality was compared in both generic gray and Mexican wolves.

Sperm of several species require a pause of several hours during cooling, before freezing, to develop maximal resistance to the effects of freezing. In the first studies regarding cooling and equilibration of canine sperm [1], it was stated that none of the temperature or time factors significantly affected the revival rate. However, there have been a number of studies investigating the effects of slow and rapid

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cooling of canine semen [2,3]. Some studies [4,5] showed that the time taken during the cooling process is an important factor. However, most studies have not fully evaluated cooling rates or equilibration time before freezing and have used arbitrary values [6].

Equex STM paste (Nova Chemical Sales, Scituate, USA) was beneficial in dog semen cryopreservation; it resulted in higher post-thaw survival, thermoresistance, increased longevity and a higher proportion of sperm with an intact plasma membrane [7,8]. In contrast, Equex pasta (Minitüb, Tübingen, Germany) did not have the beneficial effect of Equex STM paste on domestic dog semen [9]. The aim of the present study was to investigate the effects of various cooling rates on semen quality of both the generic gray and Mexican wolf. Furthermore, the study was designed to explore if sodium dodecyl sulphate (Equex pasta) had a beneficial effect on wolf sperm.

2. Materials and methods

A total of 32 ejaculates (one ejaculate/week) from four adult Mexican gray wolves (3 year of age) and 13 ejaculates (two ejaculates with a 2 day interval) of seven generic gray wolves (3–6 year of age) was collected by electroejaculation during the breeding season (January–March) at the Wild Canid Research and Survival Center, Eureka, MO, USA and the Wildlife Science Center, Forest Lake, MN, USA, respectively.

The Mexican gray wolves were maintained outdoors in pens of 312 m^2 (three wolves) and of 215 m^2 (two wolves) near St. Louis, MO, in groups of two or three animals, and fed a commercial dry chow diet (Mazuri Exotic Canine Diet, PMI Nutrition International, St. Louis, MO, USA; 1 kg/wolf/day). The generic gray wolves were maintained in large outdoor pens near Minneapolis, MN, USA, either in family packs or in sibling groups of three to eight animals. They were fed carcasses of white-tailed deer. In both locations, water was provided ad libitum, and the wolves had valid vaccination status.

For electroejaculation, the wolves were anesthetized with ketamine (Ketaset®; Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO, USA; 4.2 mg/kg body weight, i.m.) and xylazine (Rompun®; Bayer Corporation, Shawnee Mission, Kansas, USA; 2.3 mg/kg body weight, i.m.) alone (generic gray wolves) or in conjunction with isofluorane (1–3%; Isoflo®) (Mexican gray wolves). Semen was collected by electroejaculation using a Seager model ejaculator and a Platz no. 6 rectal probe, based on methods described by Platz and Seager [10]. Semen of generic gray wolves were collected at two collection days, semen of the Mexican gray wolf was collected on seven collection days during the 2001 breeding season. Following a successful ejaculation series, one drop of semen was subjectively examined for motility under a light microscope and samples of similar quality from each animal were pooled; color, volume, concentration and total sperm number were recorded and the sample was centrifuged at 1390 × *g* for 10 min at room temperature. Ejaculate characteristics for generic and Mexican gray wolves included volume (7.0 ± 1.0 and 4.2 ± 0.5 mL), concentration ($271.7 \pm 59.4 \times 10^6$ and $181.4 \pm 26.1 \times 10^6$ sperm/mL) and total sperm count ($1597.4 \pm 390.4 \times 10^6$ and $756.2 \pm 153.9 \times 10^6$ sperm). After dilution of the sperm pellet with a TRIS–egg yolk (20%)-extender with 4% glycerol (modified from Ref. [2]; 1 L contains 9.008 g Dextrose (Glucose), 24.228 g TRIS (Tris[hydroxymethyl]aminomethane), 11.478 g citric acid (anhydrous), 10,000 IU/mL penicillin and 10,000 μg/mL streptomycine (pH 7.45, 329 mOsM)) to obtain a concentration of 40×10^6 sperm/mL (Mexican and generic gray wolf) and 100×10^6 sperm/mL (generic gray wolf, in case of a highly concentrated semen sample), the extended semen was divided in three aliquots into 15 mL tubes. Volumes of the samples for generic and Mexican gray wolves were 5.1 ± 0.5 and 4.2 ± 0.4 mL, respectively and were cooled from 16 to 18 °C to 4 °C over 0.5, 1 or 2.5 h. The 0.5 h cooling time was achieved by placing the extended samples directly in the refrigerator at 4 °C, to achieve a cooling rate of 0.4–0.5 °C/min (final temperatures for generic and Mexican gray wolf semen samples were 5.5 ± 0.6 and 3.7 ± 0.5 °C, respectively). The 1 h cooling time was achieved by placing the tube of extended semen in a 500 mL plastic beaker (9 cm in diameter), containing 250 mL of water as 16–18 °C, to achieve a cooling rate of 0.1–0.2 °C/min (final temperatures of generic and Mexican gray wolf semen samples were 9.5 ± 0.7 and 7.5 ± 0.5 °C). The 2.5 h cooling time was achieved by submersing the tube with extended semen in a 250 mL glass beaker (diameter, 6.5 cm), containing 250 mL of water at 16–18 °C, to achieve a cooling rate of 0.08–0.1 °C/min (final temperature for generic and Mexican gray wolf semen was 5.2 ± 0.4 and 3.2 ± 0.2 °C). After completion of cooling of the three different semen portions, the cooled portions were divided in two split-samples and 1% Equex pasta (Minitüb, Tübingen, Germany) was added to one of the split-samples. Semen was frozen in pellets (30 μL) on dry ice for 1 min, and then transferred into liquid nitrogen for storage. Each pellet was thawed in 1 mL TRIS-extender (no glycerol or egg yolk) [2] at 37 °C until dissolution of the pellet.