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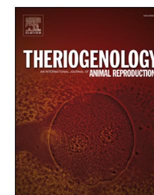
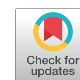


Red wolf (*Canis rufus*) sperm quality and quantity is affected by semen collection method, extender components, and post-thaw holding temperature



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ABSTRACT

Cryopreserving genetic resources is becoming increasingly important for species management. In the zoo-based red wolf (*Canis rufus*) population, inbreeding continues to increase in the absence of new founders. Through banking sperm, we preserve genetic diversity and create the ability to decrease inbreeding accumulation in the future. The quality and quantity of banked sperm can be affected by cryopreservation media and semen collection methods. This study's objectives were to further optimize semen extender used for red wolf sperm cryopreservation, investigate effects of post-thaw holding temperature, and to determine if urethral catheterization is an effective method for semen collection in this species. Semen collection via electroejaculation (EE) was performed on 39 adult red wolf males (ages 1 to 11) from 15 institutions. Urethral catheterization (UC) was attempted on a subset ($n = 14$) of those males, prior to EE. Thirteen different semen extenders were used for cryopreservation, which varied in osmolarity (HI or NORM), sugar source (glucose, fructose, or a combination), and cryoprotectant (glycerol or DMSO). Significant decreases in percent motility, forward progressive status (FPS), and acrosomal integrity were observed over time across all extenders ($P < 0.0001$). Among the extender components examined, post-thaw sperm motility and FPS were lower in DMSO versus glycerol based treatments ($P < 0.005$). Therefore, DMSO should be considered unsuitable as a cryoprotectant when freezing red wolf sperm. Effects of osmolarity and sugar source were minimal and temporally variable, however notably, a higher percentage of morphologically normal sperm were observed in the fructose-based extenders compared to glucose-based extenders post-thaw ($P < 0.05$). Additionally, post-thaw sperm motility and FPS declined more rapidly in samples maintained at 37 °C compared to samples held at room temperature ($P < 0.05$). Greater volumes of semen were collected using EE compared to UC ($P = 0.041$), and sperm samples collected using EE also had greater motility and FPS ($P < 0.05$). Additionally, though no gross morphological differences were observed, there were fewer sperm with intact acrosomes in the samples collected via UC ($P = 0.0443$). Thus, UC should not be considered sufficient for semen collection in red wolves when the desired fate of sperm is cryopreservation and/or AI. However, UC does provide an opportunity for a basic reproductive evaluation of a red wolf male.

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1. Introduction

Reproductive studies in the red wolf focusing on semen collection, processing and cryopreservation have been ongoing for almost two decades [1–5]. These techniques have the potential to assist species management by: reducing loss of genetic diversity through use of banked sperm for artificial insemination (AI) (including posthumous male reproduction), providing a genetic reservoir to safeguard against natural disasters or epizootics, and transporting frozen semen to allow males to simultaneously reproduce at multiple locations. Semen collection and cryopreservation for these purposes, as well as the maintenance of "the red wolf into perpetuity" was identified as a priority in the 1989 Recovery Plan for the red wolf by the U.S. Fish and Wildlife Service [6].

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The optimal components of cryopreservation media for freezing sperm (semen extender) differ between species [7], therefore experiments with different types of media are necessary to determine what is best to use. Previous research on red wolf sperm cryopreservation has investigated the effects of osmolarity [4], filtered vs. unfiltered egg yolk [4], holding temperature [2], cooling method [2], as well as age [4,5] and inbreeding [4,5] on the quality of cryopreserved red wolf sperm. The major implications for red wolf sperm cryopreservation based on this work have been the suggested use of extender with higher osmolarity (350 mOsm vs. 305 mOsm) for increased post-thaw motility [4], and filtered egg yolk to increase post-thaw acrosomal integrity [4]. It is also suggested to collect semen before males reach an advanced age, as increasing age has been shown to be associated with a decrease in sperm motility, forward progressive status, percent morphologically normal sperm, sperm cell concentration, and total cell counts after age six, with the effect more pronounced with higher individual levels of inbreeding [5]. Overall, the quality of post-thaw red wolf sperm is inferior to other canid species [1–3], thus further investigations are warranted to seek improvement upon current methods.

Possible modifications to the current red wolf semen extender (a TRIS-glucose-glycerol media) include changing the energy source (sugar) to fructose or a glucose/fructose combination, as well as using a different cryoprotectant, dimethyl sulfoxide (DMSO), in lieu of glycerol. Coyote sperm frozen in a TRIS-fructose extender has been shown to result in numerically (though not statistically significant) higher percent motility compared to TRIS-glucose extender [8]. In the domestic dog, the addition of fructose to egg-yolk Tris extender results in a slower decline in percent motility of chilled semen [9]. In maned wolves, the use of DMSO shows improved post-thaw sperm motility and sperm cell plasma membrane integrity without any significant change in acrosomal integrity compared to glycerol [10]. The effect of using a TRIS-fructose or DMSO based extender for red wolf sperm has previously not been evaluated.

Another factor that can affect sperm quality is the method of collection. Semen collection in red wolves has traditionally been done using an electroejaculation (EE) procedure [1–5]. In other carnivore species, including domestic cat [11,12], lion [13], and polar bear [14], a less invasive method of semen collection has been developed: urethral catheterization (UC) following alpha-2 agonist drug administration (medetomidine or dexmedetomindine). In domestic cat, sperm collected via UC has lower volume, higher concentration, and lower pH compared to sperm collected via EE [11,12] and the post-thaw characteristics of sperm, including percent motility, forward progressive status, viability, percent of sperm with intact acrosome, and percent morphologically normal sperm, did not differ between semen collection methods, fresh or post-thaw [12].

This study's objectives were to: (1) further optimize the semen extender media used for sperm cryopreservation, including sugar source (glucose only, fructose only, vs. a combination) and cryoprotectant (glycerol vs. DMSO), (2) evaluate post-thaw holding temperature on motility and forward progression of cryopreserved sperm, and (3) determine if urethral catheterization following alpha-2 agonist drug administration can be used as an effective method for semen collection in the red wolf.

2. Material and methods

2.1. Animals

The study was conducted over three years. Adult red wolf males (n = 39, ages 1 to 11) from 15 institutions in the USA were sampled during the breeding season, January to March, in 2015–2017 (Supplementary Table 1). Animals were housed singly, in breeding/companion pairs, with multiple male conspecifics, or in family units (packs). All institutions adhered to the husbandry guidelines put forth by the Red Wolf Species Survival Plan® (SSP), including housing animals in pens containing natural substrate, foliage, and sheltered dens with bedding material. Animals were exposed to natural photoperiod across a wide geographic scale and fed diets ranging from whole carcass to a commercially available dry dog food. All animals were provided water ad libitum. Inbreeding coefficients for each animal were calculated in PMx software [15] using data from the red wolf studbook [16]. Inbreeding coefficients ranged from 0.0623 to 0.0896.

2.2. Semen collection

Semen collection methods were approved by the Animal Welfare Committees at Point Defiance Zoo & Aquarium and Roger Williams Park Zoo, and the North Carolina Zoo Institutional Animal Care and Use Committee. Males were fasted one day prior to anesthesia. On the day of semen collection, each male was captured, transferred to a crate for holding, and transported to an indoor collection site. Wolves were anaesthetized using multiple drug protocols, depending on the institution. The majority of procedures were carried out using butorphanol (0.4 mg/kg im) with medetomidine (0.04 mg/kg im) or dexmedetomidine (0.02 mg/kg im), sometimes also including midazolam, maintained with isoflurane as needed. Alternative drug protocols included the use of an injection of Telazol (tiletamine hydrochloride and zolezapam), ketamine hydrochlorine with zylazine, or ketamine hydrochlorine with midazolam and butorphanol. Before semen collection, the length and width of each testis was recorded using calipers. Testis volume was estimated using the ellipsoid equation: $W^2 \times L \times 0.5236$ [17].

In the 2016 and 2017 breeding seasons, prior to EE, semen collection was attempted via UC on animals receiving a drug protocol utilizing an alpha-2 agonist (medetomidine or dexmedetomidine). Approximately 20–30 min after drug administration, a 3.5 fr x 22 in polypropylene catheter was advanced through the penis until reaching the prostate (visualized via rectal ultrasound) and left in place for 30–60 s to allow collection of sperm via capillary action. The catheter was then removed and contents were ejected, using a syringe, into a pre-warmed sterile tube. The average distance of the insertion of the catheter was 26.3 ± 7.0 cm. The semen sample was evaluated as described in 2.4 before the addition of extender. Samples without any motile sperm present were discarded.

The penis was re-catheterized with another 3.5 fr x 22 in polypropylene catheter to drain the bladder of urine. The bladder was flushed with sterile saline to remove any residual urine. Electroejaculation was then performed using a P.T. Electronic Model 302 ejaculator and no. 4 (1.6 cm diameter) lubricated rectal probe (P.T. Electronics, Boring, OR, USA). A set of one to three series of stimulations with voltage ranging from three to eight were used to achieve ejaculation, with each series consisting of multiple (25–60) on–off stimuli and a 5–10 min rest between series. The voltages that elicited ejaculation varied among individuals. Semen from each series was collected into plastic containers. There were no attempts to fractionate the ejaculates. Ejaculates without any significant urine contamination were pooled and the total volume recorded. The "Fresh" semen sample was evaluated as described in 2.4. Ejaculates were split into equal aliquots (up to eight) before addition of different extenders depending on volume and concentration of the sample. A balanced, randomized incomplete block design was used to assign the extenders outlined in 2.3. Samples

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