

Coatis (*Nasua nasua*) semen cryopreservation

Criopreservação do sêmen de quatis (*Nasua nasua*)

Regina Celia Rodrigues da PAZ¹; Heid Belle dos Santos AVILA¹

¹ Universidade Federal de Mato Grosso,
Faculdade de Agronomia, Medicina Veterinária e Zootecnia, Cuiabá – MT, Brazil

Abstract

Carnivore semen cryopreservation procedures started with semen washing and centrifuging in culture media for seminal plasma removal and microorganisms elimination. The objective of this study was to perform coatis semen cryopreservation comparing the effects between two extenders Ham's F-10 and M199 for washing and centrifugation before cryopreservation using Dilutris medium. Semen samples (n = 36) were collected by electroejaculation from six adult male coatis (*Nasua nasua*) between May and October of 2008 at the Universidade Federal de Mato Grosso Zoo. Sperm total motility (%), progressive sperm motility (0-5), plasma membrane integrity spermatozoa rates (%), and acrosome integrity (%) were analyzed. These fresh semen samples were divided in two fractions, diluted in 1 ml of Ham's F-10 (Ham's F-10, Nutricel S.A., Brazil) or M199 (M199, Nutricel S.A., Brazil) and centrifuged at 300 g for 10 min. The supernatant was discarded and pellets resuspended in 1 ml of Dilutris (Dilutris, Minitube®, Brazil), stored at 5°C for 3 hours, transferred to 0.25 ml straws, placed in liquid nitrogen vapor for 20 min, and immersed in liquid nitrogen. The means/SD for fresh semen and cryopreserved semen using Ham's F-10/Dilutris and M199/Dilutris were, respectively: 84.28 ± 11.57, 45.38 ± 27.26, and 44.61 ± 25.03 for total motility; 3.64 ± 1.44, 2.15 ± 1.14, and 2.07 ± 1.03 for progressive sperm motility; 92.76 ± 3.46, 84.69 ± 15.77, and 89.76 ± 13.97 for live spermatozoa rate; and 94.76 ± 2.89, 92.35 ± 4.73, and 90.58 ± 7.17 for acrosome integrity. No significant difference (P < 0.05) were observed between the values obtained from the Ham's F-10/Dilutris or M199/Dilutris treatments. Both treatments demonstrated to be suitable for freezing semen from this species.

Keywords: Procyonidae. Carnivore. Semen. Cryopreservation. Reproduction.

Resumo

Os procedimentos de criopreservação do sêmen em carnívoros devem ser iniciados após a lavagem e centrifugação em meios de cultura para retirada do plasma seminal e eliminação de microrganismos. O objetivo deste estudo foi realizar a criopreservação do sêmen de quatis comparando os efeitos entre dois extensores Ham's F-10 e M199 para lavagem e centrifugação do sêmen antes do congelamento com o meio Dilutris. Amostras de sêmen (n = 36) foram coletadas por eletroejaculação em seis quatis (*Nasua nasua*) machos adultos entre maio e outubro de 2008, no Zoológico da Universidade Federal de Mato Grosso. Motilidade total (%), motilidade espermática progressiva (0-5), integridade da membrana plasmática (%) e integridade do acrossoma (%) foram analisados. Amostras de sêmen a fresco foram divididas em duas frações, diluídas em 1 mL de Ham's F-10 (Ham's F-10, Nutricel S.A., Brasil) ou M199 (M199, Nutricel S.A., Brasil) e centrifugado a 300 g por 10 min. O sobrenadante foi descartado e pellets ressuscitados em 1 mL de Dilutris (Dilutris, Minitube®, Brasil), armazenados a 5°C durante 3 horas, transferidos para palhetas de 0,25 mL, colocadas em vapor de nitrogênio líquido por 20 min e imersos em nitrogênio líquido. Os resultados para sêmen a fresco e sêmen congelado utilizando Ham's F-10/Dilutris e M199/Dilutris foram, respectivamente: 84,28 ± 11,57, 45,38 ± 27,26 e 44,61 ± 25,03 para motilidade total; 3,64 ± 1,44, 2,15 ± 1,14 e 2,07 ± 1,03 para a motilidade progressiva; 92,76 ± 3,46, 84,69 ± 15,77 e 89,76 ± 13,97 para integridade da membrana plasmática; e 94,76 ± 2,89, 92,35 ± 4,73 e 90,58 ± 7,17 para integridade do acrossoma. Não houve diferença significativa (P < 0,05) entre os valores obtidos nos tratamentos Ham's F-10/Dilutris ou M199/Dilutris. Ambos os tratamentos demonstraram ser adequados para a congelamento de sêmen desta espécie.

Palavras-chave: Procyonidae. Carnívoros. Sêmen. Criopreservação. Reprodução.

Introduction

Coatis are carnivores from the Procyonidae family with geographical distribution from southern Canada to northern Argentina (GUZMAN-LENIS, 2004). Despite their wide geographical distribution and abundance, the majority of studies on this species present data

Correspondence to:

Regina Celia Rodrigues da Paz
Av. Fernando Correa da Costa, 2367
CEP 78060-900, Cuiabá, MT, Brazil
e-mail: reginacrpaz@gmail.com

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focused on behavior and ecology (BEISIEGEL, 2001; INDRUSIAK; EIZIRIK, 2003; LABATE; NUNES; GOMES, 2001; SMYTHE, 1970; VALENZUELA, 1998). Few studies report on the characteristics of the species' reproductive physiology (BARROS et al., 2009; LIMA et al., 2009, PAZ et al., 2012a).

Significant correlations between seasons and testosterone concentrations, testicular volume, and sperm concentration in coatis from Pantanal/MT (western Brazil) have been reported (PAZ et al., 2012a). The distinct seasonal changes observed in these parameters indicated the highest values occurring during the dry season (winter) and coinciding with the breeding season for coatis in this geographic location. However, no seasonal differences in the production of both, functionally intact and morphologically normal sperm have been reported (PAZ et al., 2012b). According to Beisiegel (2001), the birthing period in *Nasua nasua* occurs between October and November in southeastern Brazil. In western Brazil, the breeding season for coatis occurs between July and August and birthing between September and October coinciding with the onset of the rainy season. The reproductive seasonality of this species did not seem to be correlated to photoperiod; however, it was correlated to the availability of resources.

Destruction of tropical environments and reduction in natural habitats for Brazilian species caused by urbanization, agricultural expansion, illegal hunting, and wild animal trade reinforce the importance of the development of reproductive biotechnologies to prevent the extinction of many species.

The establishment of a semen bank would help in maintaining genetic diversity and promoting the viability of isolated populations by enabling the introduction of different genetic material and facilitating the development of other assisted reproductive technologies (WILDT, 1989).

Regardless of the fact that coatis are not listed as endangered species at Instituto Chico Mendes de Conservação da Biodiversidade Red List (ICMBIO,

2013) and listed as "Least Concern" at International Union for Conservation of Nature Red List of Threatened Species (IUCN, 2010), information obtained about this species could be applied to conservation programs for other species from the Procyonidae family such as the white-nosed coati (*Nasua narica*), Southern-Brazilian coati (*Nasua nasua solitaria*), jupará (*Potos flavus*), or other endangered carnivores.

Semen cryopreservation procedures in carnivores started with semen washing and centrifuging in culture media. This procedure is essential for seminal plasma removal and microorganism elimination (PAZ, 2013). The processing methods of semen without centrifugation and seminal plasma removal led to the development of pyometra in 40% of domestic cats inseminated despite the extender containing antibiotics (HOWARD, 1999).

Many culture media were predominantly formulated from animal products and combinations of vitamins and amino acids. Medium 199 is widely used in virology, vaccine production and in vitro cultivation of primary explants tissues. Ham's F-10 has been shown to support the growth of human diploid cells, white blood cells for chromosomal analysis, primary explants of rat, rabbit and chicken tissues. HEPES, an organic chemical buffering widely used in cell culture, is added to various commercial culture media because of its ability to stabilize pH (ZIGLER JUNIOR et al., 1985).

Tris(hydroxymethyl)aminomethane is a common organic buffer used in extenders for spermatozoa preservation due to its buffering capacity, diuretic, and osmotic activity, and low toxicity at high concentrations (NIASARI-NASLAJI et al., 2006). Egg yolk protects cell membranes against cold shock, and it prevents or restores the loss of phospholipids from the membrane (FARSTAD, 2009). Glycerol has been one of the most widely applied cryoprotectant additives for mammalian spermatozoa; despite its benefits, glycerol can induce chemical and osmotic toxic effects on spermatozoon (FAHY, 1986).

The most widely used dilution media for carnivore semen washing and centrifugation before cryopreservation are Ham's F10 and HEPES (HOWARD, 1999). The Dilutris is a commercial dilutant composed of tris-egg yolk-glycerol indicated for bovine, ovine and caprine semen cryopreservation.

The objective of this study was to perform coatis semen cryopreservation comparing the effects between two extenders (Ham's F-10 and M199) for washing and centrifugation before cryopreservation using Dilutris medium.

Materials and Methods

The Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio-SISBIO/Brazil) and UFMT Bioethical Committee approved the procedures used in this study.

The study was conducted between May and October of 2008 at the Universidade Federal de Mato Grosso Zoo, Cuiabá, Brazil. The study period was chosen to obtain the best semen production, which is important for the process of semen cryopreservation. Thirty-six attempts of sample collection were made; semen samples were collected once a month from all animals with a 30-day minimum interval between each collection.

The male coatis (six, 2 to 10-year-old, 5-7 kg of body weight) were housed together in outdoor pens in the absence of females. The animals had ad libitum access to water and fruit, vegetables, and commercial dog food. Egg and fish, or red meat (beef) was offered once daily. Commercial vitamins and mineral supplements (Promater®/Vetnil, Brazil) were added to the food daily (5 ml/animal) during the experimental period.

Animals were anesthetized with Tiletamina-Zolazepan (Zoletil50®/Virbac, Brazil), 7mg/kg, i.m. and semen samples collected using electroejaculation techniques. The electroejaculator (Torjet 65C/Eletrovect®, Brazil) was used in conjunction with a rectal bipolar electrode with three longitudinal copper bands 8.8 cm long and 0.2 cm in diameter. The electrode

was 17 cm long and 1.8 cm in diameter (VIANA et al., 2007). The electrical stimuli were administered through three series (30:30:30) as follows: series 1 (10 stimuli at 200 mA, 10 at 250 mA, and 10 at 300 mA), series 2 (10 stimuli at 300 mA, 10 at 350 mA, and 10 at 400 mA), and series 3 (10 stimuli at 350 mA, 10 at 400 mA, and 10 at 450 mA) (VIANA et al., 2007).

The total volume of ejaculated semen was recorded immediately after collection using graduated tubes and an aliquot was placed on a microscope slide at 37°C, covered with a warm glass cover slip, and examined at 40X magnification. Total motility was assessed on a 0-100% scale and progressive sperm motility on a 0-5 scale.

One drop of semen was mixed with one drop of Eosin Y (Eosin Y, Merck®, Brazil) to determine the plasma membrane integrity rate (%); one drop of semen was mixed with one drop of rose Bengal / fast green stain (POPE; ZHANG; DRESSER, 1991) to determine the proportion of acrosome integrity (%). Smears were prepared for each type of staining and 200 sperm cells were assessed in an optical microscope at 1000X magnification.

The fresh semen samples were divided in two fractions, diluted in 1 ml of Ham's F-10 medium (Nutricel S.A., Brazil) or M199 (Nutricel S.A., Brazil) at 37°C and centrifuged at 300 g for 10 min. The supernatant was discarded and pellets resuspended in 1 ml of Dilutris (Minitube®, Brazil) at 37°C. The tubes were placed in containers with water at 37°C and kept in the refrigerator at 5°C for 3 hours. These materials were subsequently transferred to cooled 0.25 ml straws, which were sealed with polyvinyl alcohol, placed in 6 cm of liquid nitrogen vapor for 20 min, immersed in liquid nitrogen, and transferred to racks that were loaded into canes for liquid nitrogen storage. Semen straws were removed from liquid nitrogen, thawed immediately at 37°C in a water bath for 1 min and evaluated for total motility (%), progressive sperm motility (scale, 0-5), plasma membrane integrity (%), and acrosome integrity (%).

Values were reported as mean \pm SD and $P < 0.05$ was considered significant. The t-Test was used for total motility, progressive sperm motility, plasma membrane integrity rates, and acrosome integrity pre- and post-cryopreservation (Statistic Analyses Program. 99 2th Ed. Stat Soft Inc.).

Results and Discussion

The present study evaluated, for the first time, the cryopreservation of coatis semen, collected by electroejaculation, using two combinations of extenders. The semen collection technique used was highly efficient and 100% of the attempts produced ejaculate. The electroejaculation procedure was suitable for semen collection in this species; all collections were successful and performed without problems. The mean and SD obtained for the physical and morphological characteristics of fresh and cryopreserved semen are presented in table. 1.

Significant differences were observed for total motility between pre- and post-cryopreservation for both treatments. No significant differences were observed between progressive sperm motility, plasma membrane integrity rates, and acrosome integrity before and after cryopreservation or between treatments.

The values determined for fresh semen about sperm total motility ($84.28 \pm 11.57\%$) and progressive sperm motility (3.64 ± 1.44) in the present study were higher than the values reported by Viana et al., 2007 ($22.5 \pm 38.62\%$ and 1.5 ± 2.38 , respectively), Barros et al., 2009 ($66.1 \pm 30.1\%$ and 3.1 ± 1.7 , respectively), Lima et al., 2009 (68% and 3.2 , respectively) and Paz et al., 2012 ($44.8 \pm 8.3\%$ and 2.1 ± 0.38 , respectively).

Poor semen quality is a limiting factor for using biotechnology of reproduction in most wild animals in captivity. Inadequate environment and nutrition deficiencies are two specific factors that interfere with such reproduction performance (MELLEN, 1991).

Optimum nutrition and dietary husbandry are fundamental to semen quality (PAZ et al., 2006). The high quality semen observed in this study could be explained by the vitamin and mineral supplementation (Promater®, Vetnil, Brazil) offered to the animals before (16 months) and during the experiment and because the collections were focused in the specific season period where the animals are in their best reproductive performance.

Coatis have been classified in previous studies as seasonal breeders. According to Beisiegel (2001), the breeding season occurs between August and September and births result between October and November in Brazil. The study period in the current study was chosen to obtain the best semen production, which is important for the process of semen cryopreservation.

Since the early developments of synthetic solutions for cryopreservation it has been accepted that some intracellular solutions provide the best cryoprotection during hypothermic storage. A general consensus of the most important characteristics in the design of hypothermic storage solutions is acidity control to preventing intracellular acidosis, recognized as critical properties of an effective preservation solution. Control of pH and buffering capacity are therefore an important consideration in comparing the respective merits of hypothermic preservation solutions. These

Table 1 – Semen characteristics (Mean \pm SD) in fresh and cryopreserved semen (Ham's F-10/Dilutris or M199/Dilutris) collected from coatis by electroejaculation – Cuiaba/MT, Brazil – 2008

End point	Fresh semen	Ham's F-10/Dilutris	M199/Dilutris
Total motility (%)	84.28 ± 11.57^a	49.28 ± 29.99^b	44.61 ± 25.03^b
Progressive sperm motility (0-5)	3.64 ± 1.44^a	2.15 ± 1.14^a	2.07 ± 1.03^a
Plasma membrane integrity (%)	92.76 ± 3.46^a	84.69 ± 15.77^a	89.76 ± 13.97^a
Acrosome integrity (%)	94.76 ± 2.89^a	92.35 ± 4.73^a	90.58 ± 7.17^a

^{a,b} Different letters in the same line represents significant differences ($P < 0.05$)

data are necessary for evaluation of the relative merits of cold storage solutions in terms of their abilities to combat acidosis during low temperature preservation applications (BAICU; TAYLOR, 2002).

Glycerol has been one of the most widely applied cryoprotectant additives for mammalian spermatozoa and it has been used successfully to freeze giant panda (SPLINDER et al., 2004) and Japanese black bear spermatozoa (OKANO et al., 2006). Giant panda spermatozoa were cryopreserved adding glycerol at room temperature, immediately before slow cooling (SPLINDER et al., 2004). In other species, many studies have been carried out on the protocol of addition of glycerol, some concluding that glycerol should be added after cooling (4-5°C), for example in sheep (GIL et al., 2003), pig (ALMLID; JOHNSON, 1988) and dog (PEÑA et al., 1998). Although glycerol always has been the accepted cryoprotectant for freezing semen, the level of glycerol added to freezing media is essentially limited by its toxicity (MALO et al., 2012). The use of amides as penetrating cryoprotectant in freezing extender for boar semen resulted in higher motility than glycerol (BIANCHI et al., 2008).

Assisted reproduction techniques in wild animals, such as artificial insemination (AI) and *in vitro* fertilization (IVF), have been considered the solution to sexual incompatibility problems and introduction

of genetic material in isolated populations. These findings are of considerable importance for conservation biology and to the creation of a gamete bank. Such a bank could represent an asset toward increasing the opportunities to warrant the survival of a number of present and future endangered carnivores. The post-thawed quality of spermatozoa observed in this study demonstrated the feasibility of the methods used in producing appropriate sperm samples for the formation of a carnivore gamete bank in the future. The results from this study suggest that populations of Procyonidae species may be intensively managed by using cryopreserved semen for assisted reproductive procedures such as AI and IVF.

Conclusion

Both treatments demonstrated suitability for freezing semen from this species. No significant differences were observed between the Ham's F-10/Dilutris or M199/Dilutris treatments.

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