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Use of the powdered coconut water (ACP-106®) for cryopreservation of canine spermatozoa

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Abstract

The purpose of the present study was to test powdered coconut water (ACP-106®) as an alternative extender for cryopreserved canine semen. Ejaculates from six dogs were collected by manual stimulation and evaluated grossly and microscopically. Semen was divided into two aliquots to be extended with either natural coconut water (NCW) or powdered coconut water (ACP-106®). Semen was initially extended with A-fraction (27 °C) containing 20% egg yolk at a ratio of 1:0.5 (semen:extender). After dilution, the semen was cooled for 40 min in a thermal box (15 °C) and for 30 min in a refrigerator (4 °C). The other half of the extender (B-fraction) containing 20% egg yolk and 12% glycerol was added to both aliquots in a ratio 1:1, so that, the final concentration of glycerol in the extender was 6%. Ejaculates were frozen in 0.25 ml straws and stored in liquid nitrogen. After one week, straws were thawed at 37 °C for 1 min, and the semen quality was evaluated. Both extenders were efficient in conserving sperm motility, vigor, and morphology after the freezing and thawing procedure. Results showed that ACP-106® can be used as an extender for cryopreservation of canine semen. However, only insemination trials will prove the fertilization potential of the frozen-thawed semen.

Keywords: canine, semen, freezing, coconut water, ACP®.

Introduction

The effects of an ideal semen extender for the cryopreservation of canine semen would be to minimize damage resulting from the freezing and thawing procedures and to maximize recovery of motile and viable spermatozoa. Many scientific studies have been carried out to improve the quality of frozen-thawed canine semen. For this purpose, different cryoprotective agents, freezing and thawing protocols, and various extenders containing PIPES, TES, and TRIS, for example, have been used; the latter is the most frequently used diluent for canine semen preservation (England, 1993; Silva *et al*., 2002; 2003).

Other authors have tried to develop alternative extenders that were non-toxic, isotonic, buffering, low cost, practical, and effective. An extender based on sucrose and lactose was used for canine semen cryopreservation, but the results were not favorable (33% motile spermatozoa). Tris is one of most used extenders for cryopreservation of canine semen and has been tested by several authors (Ström et al., 1997; Rota et al., 1997; Peña et al., 2003; Álamo et al., 2005). Ström et al. (1997) compared the most used extenders in Scandinavia, Tris and CLONE, and obtained approximately 70% sperm motility. Recently, it has been shown that fresh coconut water is an effective extender for freezing canine semen (Cardoso *et al*., 2003). However, the use of this extender had some disadvantages such as the inability to store the coconut water for long periods and limited availability of fruits in some regions of the world. Furthermore, the biochemical constitution of one coconut can be very different from another, and this can directly affect the ability of the extender to preserve spermatozoa. Thus, studies were conducted to develop a powdered coconut water (ACP®), which has already been tested for use with goat (Salgueiro *et al*., 2002), stallion (Sampaio Neto *et al*., 2002), and canine semen (Cardoso *et al*., 2004).

After dissolution, the biochemical characteristics of ACP® are very similar to those of fresh coconut water. The powder can be easily stored and readily sent to regions where fresh coconuts are not available. In addition, the composition of this extender is standardized, since it is obtained from fruits of the same plantation. ACP® has been approved for different animal species including dogs (ACP-106®). This study was conducted to compare powdered coconut water (ACP®) with fresh coconut extender for use in freezing canine semen.

Materials and Methods

Animals

Six proven stud dogs from private kennels, 1 to 6 years of age, were selected for this experiment: one Brazilian Mastiff, one Doberman, one American Staffordshire Terrier, one Rottweiler, and two Boxers. The animals were kept in individual cages and fed dry food once daily and had free access to water.

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Semen collection and evaluation

Each dog was submitted to two semen collections by manual stimulation. Ejaculates were collected into a sterile glass tube connected to a funnel, and fractions were separated by color modification (Johnston *et al*., 2001). The sperm-rich fraction of each ejaculate was macroscopically evaluated, and the volume was measured. Sperm motility (percentage of motile spermatozoa) and vigor (sperm motility status or quality of motility), scored on a scale from 0 (without movement) to 5 (fast progressive movement), were evaluated using a light microscope (100x) according to the procedures of Johnston *et al*. (2001). Sperm morphology was evaluated microscopically (1000x) from 200 cells per slide after staining cells with eosin-nigrosin (Johnston *et al*. 2001). Sperm concentration was determined with a Neubauer counting chamber (Johnston *et al*., 2001). Only samples with a volume ≥ 0.6 ml, concentration ≥ 200 x 10^6 spermatozoa/ml, sperm motility ≥ 80%, and vigor ≥ 4 were used in the study.

Semen dilution

Semen samples were submitted to a volume:volume extension based on a proportion of one part semen to one part extender (1:1). Semen samples were divided into two aliquots, and each was diluted with one of the two extenders to be tested. The first extender was a solution based on fresh coconut water (NCW – Cardoso *et al*., 2003) consisting of 50% coconut water, 25% anhydrous monosodium citrate solution (5%), and 25% ultra-pure water (pH 6.6, 300-310 mOsm/L). The second extender was powdered coconut water (ACP-106®, ACP Biotecnologia®, Fortaleza-Ceará, Brazil) obtained by an atomization process in a spray dryer (Salgueiro *et al*., 2002). The extender was prepared according to the manufacturer's recommendation. Powdered coconut water was dissolved in ultra-pure water (294 mOsm/L and pH 6.6).

Semen freezing

After the initial semen analysis, the A-fraction of both extenders, which contained 20% egg yolk, was added to both aliquots at a ratio of 1:0.5 (semen:extender, 27 °C). The semen was stored in a sealed tube, and a cooling period of 40 min at 15 °C in a thermal box standing in a beaker full of water was allowed. The semen was then transferred to a refrigerator for equilibration (30 min at 4 °C). After equilibration, the B-fraction of the extender, which contained 20% egg yolk and 12% glycerol, was divided into three aliquots that were added to the sample at 5 min intervals. The prediluted semen:extender ratio was 1:1, and the final glycerol concentration in the extender was 6%. Immediately after the final dilution, 0.25 ml plastic straws were filled with samples, placed on a grate 5 cm above the surface of liquid nitrogen for 5 min, and then plunged into the liquid nitrogen (Fig.1). After one week, samples were thawed in a water bath for 1 min at 37 °C, and the quality was evaluated. Twenty-four straws were obtained for each dilution method, that is, two straws per replicate (n = 12).

Statistical analyses

The results are expressed as means ± standard deviations and were analyzed using the Statview 5.0 software (SAS Institute Inc., Cary, NC, USA). Differences among the semen characteristics of individual dogs were analyzed using the Kruskal-Wallis test. Sperm motility (%) and morphology (%) were transformed to arcsine because these data were not normally distributed. The effects of extenders on sperm motility and morphology as well as the effects of the cryopreservation process on motility were evaluated by the Student *t*-test. The same effects on vigor were analyzed by the Mann-Whitney test. Differences were considered significant at $P < 0.05$.

Results

Fresh canine semen has a white milky appearance. The volume of the sperm-rich fraction was 1.2 ± 0.5 ml with a sperm concentration of 1.6 ± 0.7x 10^9 spermatozoa/ml. Sperm motility was 95.3 ± 0.9%, and vigor was 5.0 ± 0.0. The percentage of morphologically normal sperm was 77.5 ± 5.1%. Statistical analysis demonstrated that the population of dogs was homogeneous because no differences were detected among the samples ($P > 0.05$).

Figures 2 and 3 show similar patterns of sperm motility and vigor between NCW and ACP®-106 during each of the evaluation stages. After thawing, a reduction in the semen parameters was seen for both treatments with significant differences at each evaluation stage except for the initial dilution. Table 1 shows the evaluation of sperm morphology prior to and after freezing and thawing. A significant reduction in the proportion of morphologically normal spermatozoa was seen after thawing for both extenders, but there was no difference between groups. Tail defects were the most frequently observed damage that occurred ($P < 0.05$).

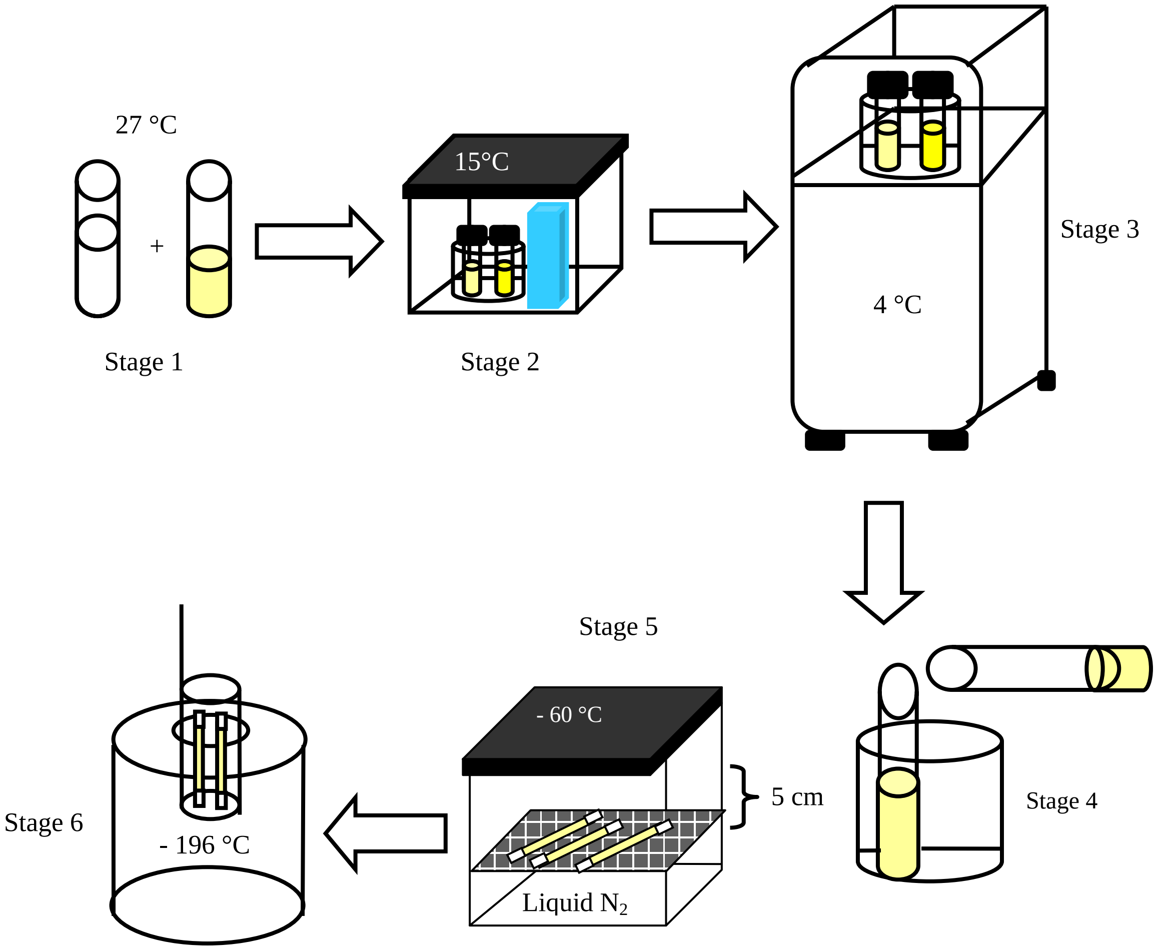
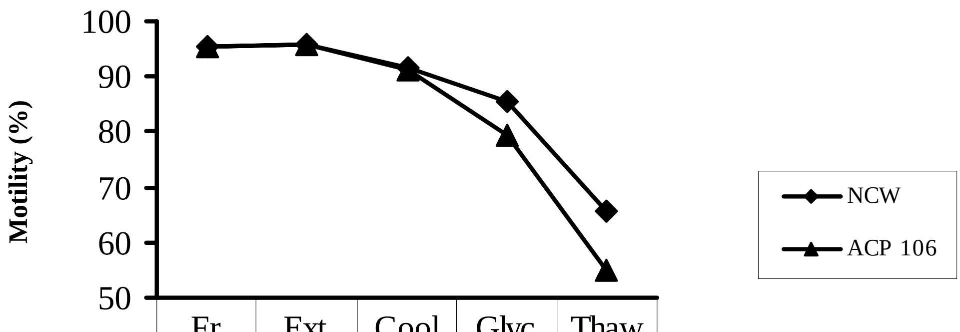


Figure 1. Experimental design. Stage 1: dilution at 27 °C with ACP® (A-fraction). Stage 2: cooling in thernal box for 40 min. Stage 3: equilibration period for 30 min. Stage 4: dilution at 4°C with ACP® (B-fraction). Stage 5: freezing on a grate above liquid nitrogen. Stage 6: storage in liquid nitrogen (– 196 °C).



	Fr	Ext	Cool	Glyc	Thaw
NCW	95.3	95.7	91.7	85.6	65.8
ACP 106	95.3	95.7	91.3	79.2	55

Evaluation stages

Figure 2. Sperm motility of fresh (Fr), extended (Ext), cooled (Cool), glycerol-supplemented (Glyc), and frozen-thawed (Thaw) semen extended with fresh (NCW) or powdered coconut water (ACP-106®).

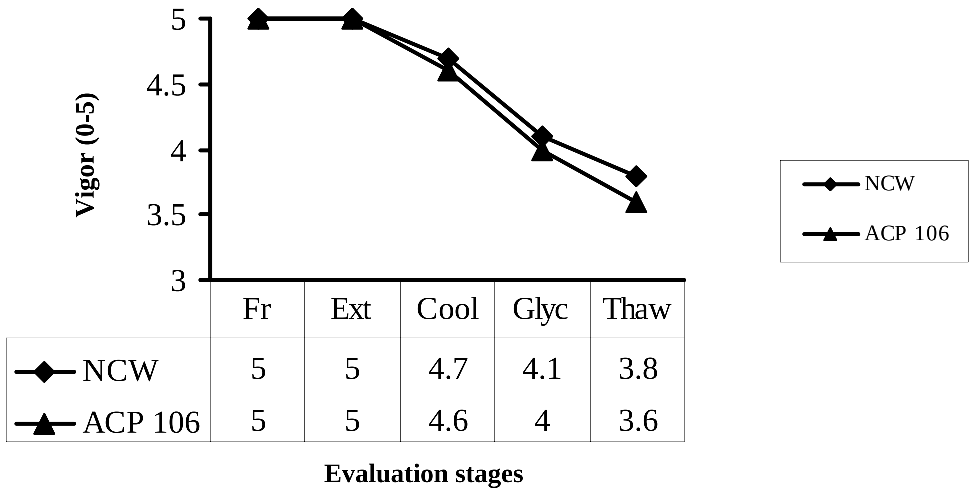


Figure 3. Sperm vigor of fresh (Fr), extended (Ext), cooled (Cool), glycerol-supplemented (Glyc), and frozen-thawed (thaw) semen extended with fresh (NCW) or powdered coconut water (ACP-106®).

Discussion

Several protocols have been developed for the cryopreservation of canine semen, and researchers are always trying to develop more practical and less-expensive methods. The fresh coconut water extender (Nunes, 1995) was proven to be effective for freezing canine semen (Cardoso *et al.*, 2003), but the solution could not be stored for more than two days (Cardoso *et al.*, unpublished data). Therefore, a powdered form of coconut water (ACP®) was developed and has already been tested with goat (Salgueiro *et al.*, 2002) and stallion semen (Sampaio Neto *et al.*, 2002). Other studies on cryopreservation of dog semen using ACP® (Cardoso *et al.*, 2004; Silva *et al.*, 2004) have also been published.

Table 1. Sperm morphology of canine fresh and frozen-thawed semen diluted in fresh coconut water (NCW) or powdered coconut water (ACP-106®).

Sperm morphology (%)	Extenders		
	Fresh Semen	NCW	ACP® 106
Normal sperm	77.5 ± 5.1 [a]	71.4 ± 6.4 [b]	70.7 ± 9.0 [b]
Head abnormalities	13.5 ± 5.3 [a]	10.7 ± 5.1 [a]	9.0 ± 5.5 [a]
Midpiece abnormalities	1.9 ± 1.4 [a]	2.8 ± 1.6 [a]	2.4 ± 1.9 [a]
Tail abnormalities	8.9 ± 4.7 [a]	15.2 ± 3.8 [a]	17.9 ± 5.7 [a]

Values in the same row with different superscripts are different ($P < 0.05$).

A reduction in motility and vigor was seen from cooling up to thawing in both groups, except for vigor ($P>0.05$) when the stages of glycerol addition and thawing stages were compared. Silva *et al.* (2001) and Cardoso *et al.* (2002) reported a similar reduction in semen parameters during the freezing stages. Rota *et al.* (1997) found similar sperm motility (≅56%) using a Tris extender plus 20% egg yolk and 8% glycerol. However Peña and Linde-Forsberg (2000b) and Peña *et al.* (2003) obtained the greatest sperm motility using Tris plus Equex STM paste. The better results were probably caused by EQUEX, a detergent compound that has beneficial effects on the motility and integrity of the membranes after thawing (Peña and Linde-Forsberg, 2000a). Probably, an addition of a detergent to ACP-106® may increase the motility after thawing.

The post-thaw sperm motility and vigor observed for both extenders were within the optimal range for insemination (Concannon and Battista, 1989) and were similar to those reported in previous studies using coconut water extender (Cardoso *et al.*, 2003) and TRIS buffered extenders (Silva *et al.*, 2002; 2003). In addition, there was a decline on the percentage of normal spermatozoa after thawing semen for both extenders, but the frozen-thawed semen was considered to be of good quality (Johnston *et al.*, 2001) regarding the parameters observed here. Motility is one of the many important attributes of a fertile spermatozoon (Peña Martinez, 2004). However, motile sperm are not necessarily fertile because of acrosomal and membrane changes that may occur after cryopreservation and thawing that affect fertility, but not motility (Eilts, 2005).

The reduction in sperm motility and vigor observed after thawing in both groups might also have been caused by an increase in the number of sperm abnormalities, especially detached heads and coiled tails. Morton and Bruce (1989) have suggested that morphological abnormalities in the midpiece and tail may reduce post-thaw motility. More over, these authors reported that the number of morphologically abnormal spermatozoa is negatively correlated with sperm motility. Oéttle (1993) reported that normal morphology values below 60% adversely affected fertility rates. In the present work, the percentage of normal spermatozoa (Table 1) was higher than 60% (71.4%, ACP-106®; 70.7%, NCW).

Although there were no significant differences between ejaculates, interrelations between individual ejaculates and extenders were seen, and some ejaculates showed significantly decreased sperm motility after thawing. There was a significant difference between dogs regarding seminal quality after thawing. So it is important to use ejaculates from different dogs in a repeated measurement experimental design (Steel and Torrie, 1980).

A spray drier was used to produce the powdered coconut water. After spray drying, coconut water retains its functional properties and can still be used as an extender. The product was standardized for a pH of 6.6 and 300-310 mOsmol/L, which is ideal for canine semen (Johnston *et al*., 2001). However, ACP® has the disadvantage of not allowing complete dissolution of egg yolk in the solution, resulting in a higher content of debris that could possibly affect sperm motility (Hirai *et al*., 1997). This fact makes measurement of ACP®-diluted semen in CASA (Computer-aided semen analysis) systems difficult. This fact was observed in another work (unpublished data).

In conclusion, ACP®-106 can be successfully used in cryopreservation of canine semen as an alternative extender in locations where coconuts are not readily available. ACP® 106 is more practical for use in freezing canine semen when compared to NCW. In addition, further *in vitro* and especailly *in vivo* studies are needed to prove the efficiency of ACP®-106.

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