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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Received: October 13, 2005
Accepted: April 18, 2006
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 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 thermal 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).

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®).
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®).

<table>
<thead>
<tr>
<th>Sperm morphology (%)</th>
<th>Fresh Semen</th>
<th>NCW</th>
<th>ACP® 106</th>
</tr>
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<tbody>
<tr>
<td>Normal sperm</td>
<td>77.5 ± 5.1a</td>
<td>71.4 ± 6.4b</td>
<td>70.7 ± 9.0b</td>
</tr>
<tr>
<td>Head abnormalities</td>
<td>13.5 ± 5.3a</td>
<td>10.7 ± 5.1a</td>
<td>9.0 ± 5.5a</td>
</tr>
<tr>
<td>Midpiece abnormalities</td>
<td>1.9 ± 1.4a</td>
<td>2.8 ± 1.6a</td>
<td>2.4 ± 1.9a</td>
</tr>
<tr>
<td>Tail abnormalities</td>
<td>8.9 ± 4.7a</td>
<td>15.2 ± 3.8a</td>
<td>17.9 ± 5.7a</td>
</tr>
</tbody>
</table>

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. Oettlé (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 use as an extender. The product was standardized for a powder of coconut water. After spray drying, coconut water extender with egg yolk and three different glycerol concentrations. Theriogenology, 59:743-751.


