INFLUENCE OF AUTOLOGOUS PROSTATIC FLUID ADDED TO FROZEN-THAWED DOG SEMEN

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Abstract

The fertilizing capacity of dog spermatozoa depends on many factors, like: motility, plasmatic membrane integrity (viability), acrosome integrity. The role of the prostatic fluid in the fertilization process is still controversial. The aim of our study was to evaluate the effect of post-thaw dilution with autologous prostatic fluid on viability, motility and acrosome status of cryopreserved dog spermatozoa. Semen was collected from 6 Beagle dogs. The sperm rich fraction was frozen with a standard extender for dog semen containing Tris, fructose, glycerol and egg yolk (TFG-EY). For each dog, six straws were thawed: three straws were diluted 1:2 with autologous prostatic fluid, while the others were not diluted at all. Motility (CASA), viability and acrosome status (flow cytometry), morphology (Diff-Quick stain) were assessed at 5 minutes, 1 hour and 2 hours post-thaw (T0, T1, T2). There were no significant differences regarding the morphology of fresh and frozen semen. The addition of prostatic fluid significantly reduced the total and progressive motility and increased the percentage of reacted acrosomes at T0, T1 and T2 (P < 0.05). Although the addition of prostatic fluid did not affect the viability and the morphology of frozen-thawed semen, it reduced the motility and increased the percentage of acrosome reactions.

Key words: cryopreservation, dog semen, prostatic fluid

INTRODUCTION

Although there are many protocols developed for the cryopreservation of canine semen, fertilizing results still vary (Linde-Forsberg et al., 1999; Thomassen et al., 2006). There are a many known factors that influence these results: technique of semen collection, extender and the final concentration of spermatozoa (Okano et al., 2004; Pena and Linde-Forsberg, 2000), semen processing (Nothling and Shuttleworth, 2005; Rijsselaere et al., 2002), the combination of extender and cooling rate during the freezing procedure (Pena and Linde-Forsberg, 2000; Schafer-Somi et al., 2006; Silva and Verstegen, 1995; Sirivaidyapong et al., 2000), the thawing technique (Pena and Linde-Forsberg, 2000; Strom et al., 1997) and the use of a thawing medium (Oettlé, 1986; Okano et al., 2004; Pena and Linde-
Individual factors that make individual dogs or individual ejaculates more resistant to freezing and thawing damage of spermatozoa are also important (Holt, 2000; Pena et al., 2003; Thurston et al., 1999).

The prostate is the only accessory gland of the genital system in the dog. Consequently, the prostatic fluid is the main component of seminal plasma. The role of the prostatic fluid in the fertilization process is still controversial. In vivo experiments showed that prostatic fluid increased fertility of frozen-thawed semen: improved conception rate after intrauterine insemination (Hori et al., 2005), and yielded higher fertility rates after intravaginal insemination when prostatic fluid was added to frozen-thawed semen (Nothling et al., 2005; Nothling and Volkmann, 1993). In vitro studies showed a negative effect of prostatic fluid on sperm incubated at 37°C (England and Allen, 1992). Prostatic fluid also has a detrimental effect on semen preservation if it is added to refrigerated semen (Rota et al., 1995b), refrigerated semen followed by freezing (Sirivaidyapong et al., 2001) and when it is added after thawing (Rota et al., 2007; Yamashiro et al., 2009). Conversely, other in vitro studies demonstrated no effect on frozen-thawed sperm (Koderle et al., 2009) or a positive effect of prostatic fluid added to frozen-thawed semen on fertility (Nothling et al., 2005; Nothling and Volkmann, 1993).

Spermatozoa undergo capacitation and acrosome reaction inside the female genital tract. One of the functions of dog prostatic fluid is to coat sperm membranes, thus masking progesterone receptors and delaying capacitation (Sirivaidyapong et al., 1999). The freezing–thawing process induces destabilization of sperm membranes, which is similar to capacitation (Rota et al., 1999).

The aim of our study was to evaluate the effect of post-thaw dilution with autologous prostatic fluid on viability, motility and acrosome status of cryopreserved dog spermatozoa.

**MATERIALS AND METHODS**

Semen was collected from 6 Beagle dogs (aged 4 to 7 years) by digital manipulation into pre-warmed tubes (+37°C) and separated into the three different fractions (Kutzler, 2005). Each sperm rich fraction was assessed immediately after collection and the following parameters were determined: volume, motility (computer assisted sperm analyzer, CASA, IVOS, Hamilton Thorne, USA), concentration (Toma cell) and morphology (Diff-
Quick stain, 200 sperm cells under 100 magnification). Only good quality ejaculates were frozen.

The sperm reach fraction was diluted with a standard extender for dog semen containing Tris, fructose, glycerol and egg yolk (TFG-EY), cooled at 5°C for 3 hours and the straws were placed at 7 cm above liquid nitrogen for 10 minutes before the transfer into liquid nitrogen.

The third fraction of each ejaculate (prostatic fluid - PF) was collected separately, centrifuged at 1118 x g for 10 minutes and the supernatant was frozen at -18°C until use.

For each dog six straws were thawed: three straws were diluted 1:2 with autologous prostatic fluid, while the others were not diluted at all. Motility (CASA, IVOS, Hamilton Thorne, USA) (Rijsselaere et al., 2003), viability and acrosome status (flow cytometry) as described below, morphology (Diff-Quick stain) (Root Kustritz et al., 1998) were assessed at 5 minutes, 1 hour and 2 hours post-thaw (T₀, T₁, T₂).

Each semen sample was differentially stained with a combination of PI and PNA (both fluorophores purchased from IMV Technologies, L’Aigle, France). Two μl of PI were added to 2 μl of semen diluted in 191 μl of Easy Buffer® (IMV Technologies, L’Aigle, France) and incubated at 37°C for 5 minutes. Five μl of PNA were added to the mix and incubated at 37°C for 5 more minutes.

Flow cytometric analysis was performed using Guava EasyCyte® flow cytometer (IMV Technologies, L’Aigle, France). Each analysis consisted of a minimum of 5000 events and 500 spermatozoa, which were quantified simultaneously for green and red fluorescence. The side and forward scatter light scatter parameters were gated so that only those cells possessing the light scatter characteristics of spermatozoa were analyzed for fluorescence. Green (PNA) and red (PI) fluorescence were collected at 548 nm. Data were analyzed using the Data Acquisition and Analysis Software (Guava Technologies Inc.®, Hayward, USA).

The double stain allowed the identification of four sperm population: live and intact acrosome spermatozoa (L-AI), live and acrosome reacted spermatozoa (L-AR), dead with intact acrosome spermatozoa (D-AI) and dead with acrosome reacted spermatozoa (D-AR) (figure 1).
RESULTS AND DISCUSSIONS

The experiment was replicated three times using two ejaculates from each of the six dogs. Statistical analyses were performed with IBM SPSS software (ver. 19 for Windows; IBM, New York, USA). The results are presented as mean values and a P value < 0.05 was considered statistically significant. There were no significant differences regarding the morphology of fresh and frozen semen (with or without the addition of PF).
The addition of prostatic fluid (PF) significantly reduced the total and progressive motility at T<sub>0</sub>, T<sub>1</sub> and T<sub>2</sub>. This finding partially disagrees with the results of a previous study (Nothling et al., 2005) that found no difference among thawed dog semen samples diluted with PF, saline or albumin-free TALP for the first 150 minutes. In our study, thawed semen samples from the TFG-EY group were not diluted at all and samples were evaluated for only 120 minutes. Our data contradicts the results of another study (Rota et al., 2007) where total motility of PF diluted samples were higher at T<sub>0</sub> and T<sub>1</sub>. This study compared the PF diluted samples with Tris buffer diluted samples. The difference between our data and the two previous mentioned studies could be explained by the lack of diluter in the TFG-EY samples, so no external influence was exerted. Our results come in agreement to other studies where the addition of PF to ejaculated dog spermatozoa resulted in a more rapid decrease in the percentage of progressive motility compared to when no fluid (Gunzel-Apel and Ekrod, 1991), an egg yolk-Tris extender (Gunzel-Apel and Ekrod, 1991; Rota et al., 1995a) or minimal essential medium (England and Allen, 1992) were added to frozen-thawed ejaculated spermatozoa. In most of the studies that found a positive effect for the addition of PF used epididymal sperm (Nothling et al., 2007), not ejaculated spermatozoa that already had contact with PF like we did in this study.
The addition of PF significantly reduced viability at all times (T0, T1, T2). This partially agrees with the results of Rota et al. (2007) where PF did not prolong semen viability, without mentioning a decrease of this parameter. In this study the viability was estimated according to motility and no specific staining for viability was used.

The addition of prostatic fluid significantly increased the percentage of reacted acrosomes at T0, T1 and T2 (P < 0.05). This completely contradicts the results of another study (Rota et al., 2007) that found no influence of PF dilution upon acrosome status. The discrepancy could be explained by the method used to evaluate acrosome reaction: flow citometry in our study, Spermac stain for the other. Nöthling et al. (2005) suggested that PF might postpone the acrosome reaction based on the increased fertility obtained after intravaginal insemination, but without a specific method for quantifying this assumption.
As laboratory analyses of semen can give only partial indication of semen fertility (Eilts, 2005; Nothling et al., 1997; Rijsselaere et al., 2005; Silva et al., 2006), our results and their impact on fertility should be further confirmed by a similar experiment with in vivo trials.

CONCLUSIONS

Although the addition of prostatic fluid did not affect the viability and the morphology of frozen-thawed semen, it reduced the motility and increased the percentage of acrosome reactions.

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Figure 4. Acrosome reaction for live spermatozoa according to time dynamics
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