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

Stănescu (Pascal) M.[1], **Bîrţoiu, I.[1]**, **Deleuze, S.[2]**

[1] *Faculty of Veterinary Medicine of Bucharest, Romania*
manuelastanescu@hotmail.com
[2] *Faculty of Veterinary Medicine, Liège, Belgium*

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-

Forsberg, 2000; Pena et al., 2003). 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 accesory 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 (T_0, T_1, T_2).
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).

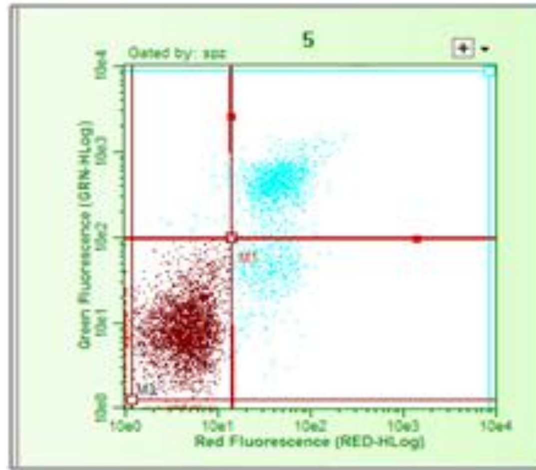

Figure 1. Histogram of a dual stained sample PI-PNA: low-left area – live-acrosome intact; low-right area – live-acrosome reacted; upper-left area – dead-acrosome intact; upper-right – dead-acrosome reacted.

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).

Treatment	Time	Total motility	Progressive motility	Live - AI	Live - AR	Dead - AI	Dead - AR
TFG- EY	T0	75,37 ± 9,13	71,34 ± 10,03	73,04 ± 10,03	9,76 ± 3,35	3,63 ± 1,92	13,56 ± 3,94
	T1	46,64 ± 19,56	42,51 ± 18,66	63,03 ± 5,37	12,71 ± 6,62	5,44 ± 3,63	18,99 ± 7,81
	T2	29,67 ± 19,38	28,13 ± 17,24	57,94 ± 5,45	12,88 ± 4,97	4,88 ± 2,78	24,30 ± 4,76
TFG-EY + PF	T0	63,08 ± 23,80	60,06 ± 23,80	64,21 ± 19,67	11,93 ± 7,95	6,70 ± 3,70	17,17 ± 13,69
	T1	34,93 ± 23,26	31,60 ± 22,85	51,23 ± 22,29	16,69 ± 9,62	8,36 ± 5,82	23,71 ± 17,33
	T2	23,96 ± 21,46	22,23 ± 20,91	37,03 ± 18,79	16,75 ± 9,47	10,30 ± 7,94	35,37 ± 18,00

Figure 2. Motility, viability and acrosome status data for the two groups of the experiment presented as mean±standard deviation.

The addition of prostatic fluid (PF) significantly reduced the total and progressive motility at T_0, T_1 and T_2. 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_0 and T_1. 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.

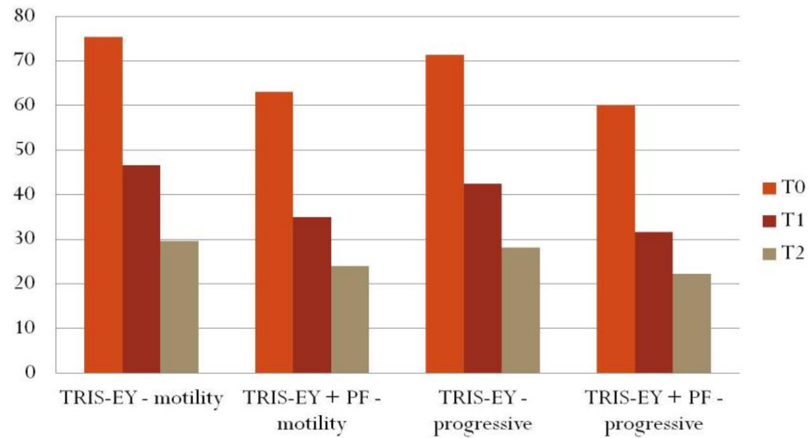

Figure 3. Comparison of total and progressive motility values

The addition of prostatic fluid significantly increased the percentage of reacted acrosomes at T_0, T_1 and T_2 ($P < 0.05$). 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.

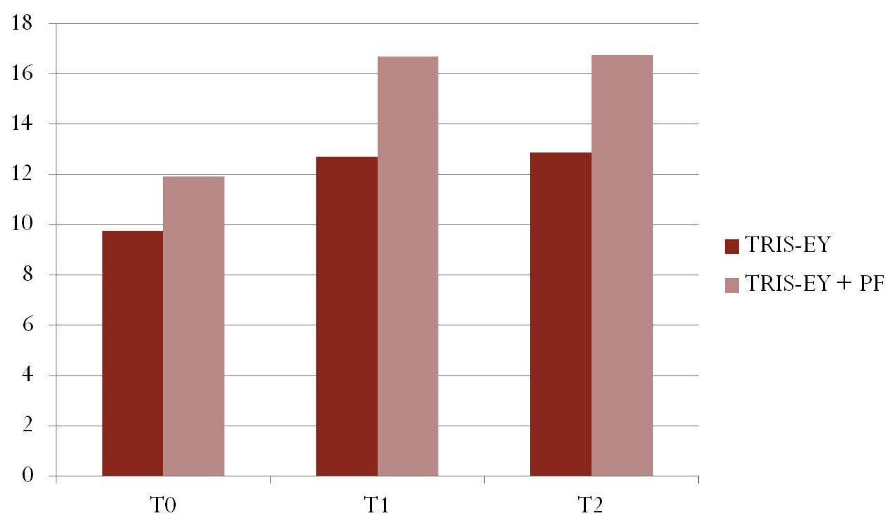

Figure 4. Acrosome reaction for live spermatozoa according to time dynamics

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.

ACKNOWLEDGEMENTS

This work was supported by the POSDRU project 88/1.5/S/52614 „Doctoral scolarships for high quality training for young researchers in the field of agronomy and veterinary medicine”.

REFERENCES

Eilts B.E., 2005. Theoretical aspects of canine cryopreserved semen evaluation. Theriogenology 64, 685-691.
England G.C.W., Allen W.E., 1992. Factors affecting the viability of canine spermatozoa. II. Effects of seminal plasma and blood. Theriogenology 37, 373-381.
Gunzel-Apel A.R., Ekrod B., 1991. Influences of seminal plasma and extender on sperm motility, ATP-concentration, and the activity of acid and alkaline phosphatases of Beagle dog semen. Reprod Dom Anim 26, 31-41.
Holt W.V., 2000. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology 53, 47-58.
Hori T., Hagiuda K., Kawakami E., Tsutsui T., 2005. Unilateral intrauterine insemination with prostatic fluid-sensitized frozen caudal epididymal sperm in beagle dogs. Theriogenology 63, 1573-1583.
Koderle M., Aurich C., Schafer-Somi S., 2009. The influence of cryopreservation and seminal plasma on the chromatin structure of dog spermatozoa. Theriogenology 72, 1215-1220.
Kutzler M.A., 2005. Semen collection in the dog. Theriogenology 64, 747-754.
Linde-Forsberg, C., Strom Holst, B., Govette, G., 1999. Comparison of fertility data from vaginal vs intrauterine insemination of frozen-thawed dog semen: a retrospective study. Theriogenology 52, 11-23.
Nothling J.O., Gerber D., Colenbrander B., Dijkstra M., Bakker T., De Cramer K., 2007. The effect of homologous prostatic fluid on motility and morphology of dog epididymal spermatozoa extended and frozen in Biladyl with Equex STM paste or Andromed. Theriogenology 67, 264-275.
Nothling J.O., Gerstenberg C., Volkmann D.H., 1997. Semen quality after thawing: correlation with fertility and fresh semen quality in dogs. Journal of reproduction and fertility. Supplement 51, 109-116.
Nothling J.O., Shuttleworth R., 2005. The effect of straw size, freezing rate and thawing rate upon post-thaw quality of dog semen. Theriogenology 63, 1469-1480.
Nothling J.O., Shuttleworth R., de Haas K., Thompson, P.N., 2005. Homologous prostatic fluid added to frozen-thawed dog spermatozoa prior to intravaginal insemination of bitches resulted in better fertility than albumin-free TALP. Theriogenology 64, 975-991.
Nothling J.O., Volkmann D.H., 1993. Effect of addition of autologous prostatic fluid on the fertility of frozen-thawed dog semen after intravaginal insemination. Journal of reproduction and fertility. Supplement 47, 329-333.
Oettlé E.E., 1986. Changes in acrosome morphology during cooling and freezing of dog semen. Anim Repro Sci 12, 145-150.
Okano T., Murase T., Asano M., Tsubota T., 2004. Effects of final dilution rate, sperm concentration and times for cooling and glycerol equilibration on post-thaw characteristics of canine spermatozoa. The Journal of veterinary medical science / the Japanese Society of Veterinary Science 66, 1359-1364.
Pena A., Linde-Forsberg C., 2000. Effects of Equex, one- or two-step dilution, and two freezing and thawing rates on post-thaw survival of dog spermatozoa. Theriogenology 54, 859-875.

Pena A.I., Lopez-Lugilde L., Barrio M., Becerra J.J., Quintela L.A., Herradon, P.G., 2003. Studies on the intracellular Ca2+ concentration of thawed dog spermatozoa: influence of Equex from different sources, two thawing diluents and post-thaw incubation in capacitating conditions. Reproduction in domestic animals = Zuchthygiene 38, 27-35.

Rijsselaere T., Van Soom A., Maes D., de Kruif A., 2002. Effect of centrifugation on in vitro survival of fresh diluted canine spermatozoa. Theriogenology 57, 1669-1681.

Rijsselaere T., Van Soom A., Maes D., de Kruif A., 2003. Effect of technical settings on canine semen motility parameters measured by the Hamilton-Thorne analyzer. Theriogenology 60, 1553-1568.

Rijsselaere T., Van Soom A., Tanghe S., Coryn M., Maes D., de Kruif A., 2005. New techniques for the assessment of canine semen quality: a review. Theriogenology 64, 706-719.

Root Kustritz M.V., Olson P.N., Johnston S.D., Root T.K., 1998. The effects of stains and investigators on assessment of morphology of canine spermatozoa. Journal of the American Animal Hospital Association 34, 348-352.

Rota A., Milani C., Romagnoli S., 2007. Effect of post-thaw dilution with autologous prostatic fluid on dog semen motility and sperm acrosome status. Theriogenology 67, 520-525.

Rota A., Pena A.I., Linde-Forsberg C., Rodriguez-Martinez H., 1999. In vitro capacitation of fresh, chilled and frozen-thawed dog spermatozoa assessed by the chloretetracycline assay and changes in motility patterns. Anim Reprod Sci 57, 199-215.

Rota A., Strom B., Linde-Forsberg C., 1995a. Effects of seminal plasma and three extenders on canine semen stored at 4 degrees C. Theriogenology 44, 885-900.

Rota A., Strom B., Linde-Forsberg C., 1995b. Effects of seminal plasma and three extenders on canine semen stored at 4°C. Theriogenology 44, 885-900.

Schafer-Somi S., Kluger S., Knapp E., Klein D., Aurich C., 2006. Effects of semen extender and semen processing on motility and viability of frozen-thawed dog spermatozoa. Theriogenology 66, 173-182.

Silva A.R., Cardoso Rde C., Silva L.D., Chirinea V.H., Lopes M.D., Souza F.F., 2006. Prognostic value of canine frozen-thawed semen parameters on in vitro sperm-oocyte interactions. Theriogenology 66, 456-462.

Silva L.D., Verstegen J.P., 1995. Comparisons between three different extenders for canine intrauterine insemination with frozen-thawed spermatozoa. Theriogenology 44, 571-579.

Sirivaidyapong S., Bevers M.M., Colenbrander B., 1999. Acrosome reaction in dog sperm is induced by a membrane-localized progesterone receptor. Journal of andrology 20, 537-544.

Sirivaidyapong S., Cheng F.P., Marks A., Voorhout W.F., Bevers M.M., Colenbrander B., 2000. Effect of sperm diluents on the acrosome reaction in canine sperm. Theriogenology 53, 789-802.

Sirivaidyapong S., Ursem P., Bevers M.M., Colenbrander B., 2001. Effect of prostatic fluid on motility, viability and acrosome integrity of chilled and frozen-thawed dog spermatozoa. Journal of reproduction and fertility. Supplement 57, 383-386.

Strom B., Rota A., Linde-Forsberg C., 1997. In vitro characteristics of canine spermatozoa subjected to two methods of cryopreservation. Theriogenology 48, 247-256.

Thomassen R., Sanson G., Krogenaes A., Fougner J.A., Berg K.A., Farstad W., 2006. Artificial insemination with frozen semen in dogs: a retrospective study of 10 years using a non-surgical approach. Theriogenology 66, 1645-1650.
Thurston L.M., Watson P.F., Holt W.V., 1999. Sources of variation in the morphological characteristics of sperm subpopulations assessed objectively by a novel automated sperm morphology analysis system. Journal of reproduction and fertility 117, 271-280.
Yamashiro H., Narita K., Sugimura S., Sugawara A., Hoshino Y., Sakurai M., Yokoo M., Konno T., Yoshida M., Sato E., 2009. Influence of the prostatic fluid from the first and third fractions of the ejaculates on the cryosurvival of poodle dog sperm. American Journal of Animal Veterinary Science 4, 14-20.