Effect of Post -Thaw Addition of Insemination Media and Prostatic Fluid on Dog Sperm Motility Parameters

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Abstract:

Improving sperm parameters with a role in fertilization and keeping them as long as possible at optimal levels is still a current requirement for frozen-thawed semen. The aim of the study was to evaluate the effect of the addition of an artificial insemination media (M) and prostatic fluid (PF), post thawing, on dog sperm motility parameters. The sperm rich fraction from nine dog ejaculates was cryopreserved. After thawing, three experimental variants were carried out: 1) semen diluted 1:1 with CaniPlus Enhance-media 2) with the PF and 3) semen without any addition. Samples were incubated at 37°C and sperm motility parameters were assessed after 10, 25, 40 and 50 minutes. Samples supplemented with the insemination media recorded the highest values of total motility, progressive motility and sperm velocity throughout incubation. Addition of PF had a negative effect on the total and progressive motility values and caused a slight increase in velocity.

Keywords: dog, frozen-thawed semen, insemination media, prostatic fluid, sperm motility

Introduction

Artificial insemination in dog has become a common demand in veterinary clinics. The possibilities of short and long-term preservation of dog spermatozoa generated a gradual development of international exchanges of genetic material in this species. The use of chilled or cryopreserved semen for insemination of the bitch involves a smaller volume of semen and lower quality sperm parameters compared to fresh semen. These are generally compensated by the use of uterine insemination techniques of semen. However, at least one unwanted aspect is associated with each uterine semen deposition techniques: it is difficult to master and cannot be used in all bitches (rigid catheter transcervical insemination); the equipment is not generally

available and its expense severely limits its widespread use (endoscopic transcervical insemination and laparoscopy); it is invasive and sedation or general anesthesia are needed (laparotomy and laparoscopy) (Nöthling and Volkmann, 1993). In addition, there are significant ethical and welfare concerns about the use of surgical insemination in bitches (England 2014), especially when non-invasive procedures are available (Thomassen, 2006). In contrast, vaginal insemination is a non-invasive technique, easy to perform, requires simple, cheap, readily available equipment, and does not require sedation or anesthesia of the bitch (Nöthling and Volkmann, 1993, Nöthling, 2007). On the other hand, vaginal insemination is only suitable when large quantities of good quality sperm are available, for example

when using fresh semen (England, 2014). In this context, vaginal deposition of semen may become the main insemination option, even for preserved semen (chilled or cryopreserved) if optimal solutions are found for some issues. One of them is identifying an insemination fluid to serve in both increasing semen volume and in improving sperm parameters and keeping them at optimal level as long as possible.

The effect of some substances addition to frozen-thawed semen was studied: caffeine, pentoxifylline, 2'-deoxyadenosine (Milani, 2010), albumin-free Tyrode's albumin lactate pyruvate (Nöthling, 2005), autologous prostatic fluid (Nöthling and Volkmann, 1993, Rota 2007, Stanescu Pascal, 2012), homologous prostatic fluid (Nöthling, 2005). Surprising and controversial results were obtained on the addition of prostatic fluid to frozen-thawed semen.

During copulation in dog, the third fraction (prostatic fluid) is ejaculated after the second fraction (sperm rich fraction) of semen. As the seminal fluid is delivered through the cervix into the uterus, this phenomenon tends to force the sperm-rich fraction into the uterus (Senger, 2003). In addition to transport into the uterus of spermatozoa deposited into the vagina, the third fraction has an important effect on regulating fertility and is recommended to be used as a flushing fluid in artificial insemination (England, 2014).

The aim of the study was to evaluate the effect of the addition of an artificial insemination media (M) and prostatic fluid (PF), post thawing, on dog sperm motility parameters.

Materials and methods

Semen collection and evaluation. Five male dogs of different pure breeds (Siberian Husky, American Staffordshire Terrier, English Springer Spaniel, Dogo Argentino and Bernese shepherd) aged 2 to 6 years, were used for semen collection. Nine ejaculates were collected by digital manipulation of penis, based on linear cranio-caudal massage of the prepuce, firm movements and compression beyond erectile bulbus, following the procedure described by Christiansen (1984). The second (sperm-rich) fraction and the third (post-sperm rich fraction / prostatic fluid) of the ejaculate were collected using glass containers, prewarmed at 37 ° C. Separate collecting of the two ejaculate fractions was attempted.

The evaluation of sperm-rich fraction quality immediately after collecting aimed to assess the following parameters: volume, concentration, total sperm count, total motility, progressive motility, path velocity and sperm morphology.

Concentration, total motility (TM), progressive motility (PM) and path velocity (VAP) were determined by computer-assisted assay with CASA system (IVOS, version 12,3 Hamilton Thorne Bioscience, USA) using Animal motility software and HTB-IVOS setting parameters recommended for dog semen. An aliquot from sperm-rich fraction was diluted with autologous prostatic fraction using the 1:5 dilution rate and gently mixed. A volume of 3 µl of the diluted sample was placed on the chamber of Leja slide (Leja Products B. V. Netherlands), and computer-assisted assay was performed.

Morphology was evaluated by examination of stained sperm using the bright field microscopy (Olympus BX51 microscope). Spermatozoa were stained with Spermac (Stain Enterprises, P.O. Box 152, Wellington, South Africa), according to the producers' staining procedure. About 200-215 sperm per sample were examined at 400x and 1000x magnification and classified in two categories: normal sperm and abnormal sperm. Immature spermatozoa, having a proximal or distal cytoplasmic droplet in the midpiece, were included in the abnormal sperm category. The percentage of normal spermatozoa was calculated for each semen sample.

Semen cryopreservation. Semen centrifugation was performed only to ejaculates that failed to separate collection of sperm-rich fraction and prostate fractions. The centrifugation parameters were 650 x g (2620 RPM) for 5 minutes.

The sperm-rich fractions were cryopreserved using CaniPlus Freeze (Minitub GmbH, Germany) extender. Semen was extended on a concentration of 100×10^6 spermatozoa ml^{-1} (50×10^6 spermatozoa / 0.5 ml straw). Straws of 0.5 ml volume were filled with extended semen and sealed with ultrasonic sealer. The semen was equilibrated at 4°C for 2 hours and then the straws were placed horizontally at 4 cm above the surface of liquid nitrogen for 10 min, followed by immersion in liquid nitrogen (-196 °C) and maintaining them in these conditions, in cryogenic container.

Prostatic fluid preservation. Post-sperm rich fraction (prostatic fraction) of each ejaculate was

Table 1. Mean values $\pm$ SD of sperm-rich fraction parameters, immediately after semen collection (n=9)

Parameter	Volume of sperm-rich fraction (ml)	Concentration (sperm $\times 10^6$ /ml)	Total sperm count (sperm $\times 10^6$)	Sperm morphology (% normal sperm)
Mean $\pm$ SD	3.27 $\pm$ 3.93	253.56 $\pm$ 163.17	493.34 $\pm$ 196.10	77.33 $\pm$ 8.51

Table 2. Mean values $\pm$ SD of spermatozoa motility parameters evaluated by CASA, before and after the freezing-thawing

Semen Treatment	Total Motility (TM) (%)	Progressive Motility (PM) (%)	Path Velocity (VAP) ($\mu\text{m s}^{-1}$)
Fresh semen	83.67 $\pm$ 11.01	53.89 $\pm$ 17.72	140.77 $\pm$ 14.56
Frozen-thawed semen	51.11 $\pm$ 15.39	14.33 $\pm$ 7.16	89.73 $\pm$ 12.36

centrifuged at 1500 $\times$ g (3980 RPM) for 5 min, to eliminate as many as possible of the spermatozoa from this fraction (Igna, 2007). The supernatant was examined microscopically to check if all the spermatozoa had been removed, then it was filtered and transferred to a plastic vial, labeled with the same code as the corresponding sperm-rich fraction of the ejaculate, and frozen at -24 °C until use.

Post-thaw evaluation. The straws were thawed in a waterbath at 38°C for 1 minute. Evaluation of the effect of cryopreservation on sperm motility was done by computer-assisted analysis of the total motility, progressive motility and path velocity.

Post-thaw semen supplementation and spermatozoa motility monitoring. After thawing, 400 μ l of semen were used for each of the three experimental variants that were carried out: 1) semen supplemented 1:1 with insemination media - CaniPlus Enhance (Minitub GmbH, Germany) (S-IM); 2) semen supplemented 1:1 with the autologous prostatic fluid (S-PF) and 3) semen without any addition (S). Samples were incubated at 37° C and sperm motility parameters were assessed after 10, 25, 40 and 50 minutes.

Statistical analysis. Results are expressed as mean $\pm$ SD. Relationships between results were investigated using Mann-Whitney test and Kruskal-Wallis test. We used nonparametric tests because

the number of samples was reduced. Results were considered statistically significant when $P < 0.05$ (Brudiu, 2010).

Results and discussions

The mean values and standard deviation of some parameters (volume, concentration, total spermatozoa number and morphology) for sperm-rich fraction of ejaculate, immediately after semen collection, are shown in Table 1.

In eight of the nine semen collections, the two fractions of the ejaculate - sperm-rich and post sperm-rich (prostate) fractions, were collected separately, and the sperm-rich fractions volume varied between 1.00 and 4.5 ml. Only in one case, separate fractions collection failed, resulting in a 14 ml sperm-rich fraction including a part of prostatic fluid ejaculated.

The concentration values ranged between 30.2 - 568.6 $\times 10^6$ ml⁻¹ and those of the total sperm count on the sperm-rich fraction of ejaculate between 193.1 - 884.1 $\times 10^6$ spermatozoa.

Motility parameters from sperm-rich fraction of ejaculate, assessed by computer-assisted semen analysis, before and after the freezing-thawing procedure are reported in Table 2 (Table 2). Cryopreservation caused an important decrease in all sperm motility parameters analysed. The mean value of total motility (TM) after cryopreservation is very close to that reported by de Rota et al.

Figure 1. Total motility (TM) values recorded in fresh and frozen-thawed semen (n=9)

Figure 2. Progressive motility (PM) values recorded in fresh and frozen-thawed semen (n=9)

(2005) - 50.2 ± 6.0 % as well as that reported by Pop *et al.* (2006) - 30-50%, but below the reported values of Silva *et al.* (2005): 54.6 ± 11.2 and 61.3 ± 9.1 %.

The results reveal a heterogeneity of the impact of cryopreservation on total motility (Figure 1) progressive motility (Figure 2) and velocity (Figure 3), with significant individual differences.

The results recorded during monitoring of spermatozoa motility parameters to frozen-thawed semen samples at different incubation times (10, 25, 40 and 55 minutes), of the three experimental variants, are shown in Table 3.

The highest mean values of total motility (TM) were recorded in semen samples without any addition (S) at 10 and 40 minutes of incubation and in semen samples supplemented with insemination media (S-IM) at 25 and 55 minutes of incubation. The lowest total motility values were recorded in semen samples supplemented with autologous prostatic fluid (S-PF) throughout

the incubation period. Statistical analysis indicated significant differences for total motility values only between groups S and S-PF at 10 minute of incubation ($P < 0.05$).

The addition of insemination media has positively influenced the progressive motility of the spermatozoa, the S-IM group having the highest values compared to the S and S-PF groups. However, no significant differences were found between the three groups (S-IM, S-PF and S) at 10, 25, 40, and 55 minutes of semen incubation.

Post-thaw addition, either of insemination media or prostatic fluid, resulted in a higher velocity than semen without any addition.

The results of various studies, especially those related to the addition of prostatic fluid to frozen-thawed semen, are quite difficult to compare due to different experimental conditions. Here are some of the variation elements in the studies on this area: - *the origin of the canine spermatozoa used*: epididymal spermatozoa (Korochkina *et*

Figure 3. Path velocity (VAP) values recorded in fresh and frozen-thawed semen (n=9)

Table 3. The mean values $\pm$ SD of total motility (TM), progressive motility (PM) and path velocity (VAP) of frozen-thawed semen at different incubation times (10, 25, 40 and 55 minutes), for the three experimental variants: 1) S+IM (semen + insemination media), 2) S-PF (semen sample + prostatic fluid) and 3) S (semen samples - without the addition of any fluid)

	Total Motility (TM) (%)	Progressive Motility (PM) (%)	Path Velocity (VAP) ($\mu\text{m s}^{-1}$)
10 min			
S-IM	51.75 $\pm$ 13.40	20.00 $\pm$ 14.82	102.84 $\pm$ 13.27
S-PF	38.88 $\pm$ 12.65	11.63 $\pm$ 6.44	93.20 $\pm$ 10.07
S	52.88 $\pm$ 13.84	15.25 $\pm$ 9.11	90.61 $\pm$ 7.99
25 min			
S-IM	47.00 $\pm$ 12.31	14.00 $\pm$ 11.24	97.79 $\pm$ 13.85
S-PF	33.13 $\pm$ 15.42	8.63 $\pm$ 5.26	91.47 $\pm$ 12.47
S	40.38 $\pm$ 12.89	8.38 $\pm$ 6.23	84.22 $\pm$ 10.56
40 min			
S-IM	26.88 $\pm$ 15.51	5.88 $\pm$ 7.00	91.86 $\pm$ 10.72
S-PF	20.25 $\pm$ 12.80	2.75 $\pm$ 1.909	82.63 $\pm$ 11.10
S	28.63 $\pm$ 12.58	3.75 $\pm$ 3.92	81.15 $\pm$ 7.60
50 min			
S-IM	18.25 $\pm$ 15.14	3.50 $\pm$ 5.53	84.45 $\pm$ 13.56
S-PF	12.25 $\pm$ 10.11	1.500 $\pm$ 1.414	72.2 $\pm$ 31.1
S	16.13 $\pm$ 12.86	2.375 $\pm$ 2.669	78.85 $\pm$ 8.54

al., 2014), spermatozoa from sperm-rich fraction collected separately (Nöthling *et al.*, 1993, Nöthling *et al.*, 2005) and spermatozoa from the complete ejaculate collected (Rota *et al.*, 2007); - *the origin of prostatic fluid* : prostatic fluid collected from the same donor dog used in the study, usually collected separately during

the same ejaculation (autologous PF) (Nöthling *et al.*, 1993, Stănescu *et al.*, 2012) or the whole ejaculate collected and then separated the prostatic fluid by centrifugation (Rota *et al.*, 2007); prostatic fluid collected from a dog other than the sperm donor used in the study (homologous PF) (Nöthling *et al.*, 2005, Korochkina *et al.*, 2014) or

prostatic fluid collected from different dogs (others than sperm donors) and mixed together (mixture of homologous PF) (Milani *et al.*, 2010); - *prostatic fluid processing regime, especially centrifugation parameters' values* (g force or revolutions per minute and centrifugation time) which differ almost in each study: one step of 3000 rev min⁻¹ for 5 min (Nöthling et Volkmann, 1993), 300 g for 5 min (Milani *et al.*, 2010); 1000 x g for 10 min (Nöthling *et al.*, 2005), 1118 x g 10 min (Stănescu *et al.*, 2012), or two step centrifugation when the prostatic fluid was separated from the complete ejaculate: 700 g for 6 min then a second time 1200 g for 6 min. (Rota *et al.*, 2007), 700x g for 6 minutes, the supernatant removed and centrifuged again at 3468 x g for 10 minutes (Korochkina *et al.*, 2014); - the dilution rate of frozen-thawed semen with prostatic fluid: 1:1 (Korochkina *et al.*, 2014), 1:2 (Rota *et al.*, 2007, Stănescu *et al.*, 2012), 1:3 (Nöthling *et al.*, 2005); - *incubation condition*: in vivo (artificial insemination of the bitch) (Nöthling et Volkmann, 1993, Nöthling *et al.*, 2005) or in vitro at 37°C (Milani *et al.*, 2010, Korochkina *et al.*, 2014) or 38°C (Rota *et al.*, 2007).

Some older (Bartlett, 1962, Mann, 1954) and more recent studies (Mogielnicka-Brzozowska *et al.*, 2014, Mogielnicka-Brzozowska *et al.*, 2015, Souza *et al.*, 2007) aim to identify the composition of seminal plasma, the origin of its components, and its relation to spermatozoa and process of fertilization. Continuing research on this area is necessary to identify the nature and composition of an addition fluid in artificial insemination with frozen-thawed semen, for improving fertility.

Conclusion

This study reveals the beneficial effect of the added insemination media, post-thawing, on motility parameters of dog spermatozoa. Addition of prostatic fluid had a negative effect on the total motility and progressive motility values during incubation and caused a slight increase in spermatozoa velocity.

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