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Effect of post-thaw dilution with caffeine, pentoxifylline, 2'-deoxyadenosine and prostatic fluid on motility of frozen-thawed dog semen

C. Milani [a,*], A. Fontbonne [b], E. Sellem [c], C. Stelletta [a], O. Gérard [c], S. Romagnoli [a]

[a] Department of Veterinary Clinical Sciences, Faculty of Veterinary Medicine, Agripolis, 35020 Legnaro- PD (Italy)
[b] C.E.R.C.A- Alfort National Veterinary College, 7, avenue du général de Gaulle, 94700 Maisons-Alfort cedex- France
[c] U.N.C.E.I.A- Département R&D,13 rue Jouët, 94704 Maisons-Alfort cedex- France

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Abstract

The aim of the experiment was to evaluate the motility pattern of frozen-thawed canine semen to which pentoxifyilline (PTX), caffeine (CAF), 2'-deoxyadenosine (DX), and prostatic fluid (PROST) were added after thawing. Semen evaluations were performed using computer-assisted sperm analysis (CASA) at thawing and during 120 min of incubation at 37 °C. Three experiments were conducted: 1) to establish which concentrations of stimulants work best; 2) to investigate the interaction between thawing rate and addition of CAF 5 mM, PTX 2.5 mM and PROST; 3) to evaluate the effect of PTX 7.5 mM and DX 5 mM on semen motility after thawing. In experiment 1, ALH and VCL were enhanced at thawing by CAF 7.5 mM (CAF 7.5: 9.1 ± 0.5 μm; control: 6.7 ± 0.4 μm) and DX 5 and 7.5 mM (DX 5: 199.1 ± 12.8 μm/s; DX 7.5: 197.3 ± 13.9 μm/s; control: 162.5 ± 8.4 μm/s), while PTX 2.5-5-7.5 mM improved TOT after 120 min of incubation. In experiment 2, PROST lowered ALH values throughout incubation ($P < 0.05$) with respect to the other treatments, in particular when compared to CAF at Time = 30 and at Time = 60. In experiment 3, PTX 7.5 mM improved VAP (PTX: 101.6 ± 6.8 μm/s; control: 81.9 ± 10.5 μm/s), VSL (PTX: 82.9 ± 6.4 μm/s; control: 65.9 ± 9.8 μm/s), VCL (PTX: 214.3 ± 13.3 μm/s; control:167 ± 15.7 μm/s), ALH (PTX: 10.5 ± 0.3; control: 7.3 ± 1.4 μm), PM (PTX: 11.3 ± 4.2%; control: 7.7 ± 3.9%) and TOT (PTX: 20.1 ± 5.3%; control:15.6 ± 5.6%) at Time = 120, while DX 5 mM influenced VCL at Time = 60 (DX: 218.3 ± 14.3 μm/s; control: 188.5 ± 7.5 μm/s, $P < 0.05$). Motility stimulants may be useful for enhancing motility of canine frozen-thawed spermatozoa without affecting sperm longevity.

Keywords: Canine; Frozen semen; Motility stimulants; Prostatic fluid; Computer-assisted semen analysis

1. Introduction

Sperm cryopreservation continues to gain importance in assisted canine reproduction, as it allows semen exchange among canine semen banks placed in different countries or continents, without moving stud dogs or females for breeding. Freezing and thawing procedures reduce the life-span of spermatozoa after thawing, and this is due to the fact that cryopreservation induces capacitation-like changes to spermatozoa [1]. Pre-freezing dilution and extender [2–4], cryoprotectant [5–8], freezing rate [2,9], freezing technique [10–13], thawing rate [10,14,15], as well as individual factors among ejaculates of different dogs [12,16], are all factors that can affect sperm motility and viability after thawing.

* Corresponding author. Tel.: +39 049 8272947; fax: +39 049 8272954.
E-mail address: chiara.milani@unipd.it (C. Milani).

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From the early days of assisted canine reproduction until the mid 90's, the various protocols used for freezing produced thawed semen with a very low motility and thermoresistance, and, as a consequence, very low (<50%) results in term of *in vitro* and *in vivo* fertility [17]. The addition of some detergents to the cryopreservation extenders, like Orvus ES paste [18] and Equex STM paste [10,19] significantly improves post-thaw survival of canine spermatozoa. As semen storage in liquid nitrogen allows semen preservation for an extremely long period, the oldest canine frozen semen banks may have semen samples which were frozen according to old and less successful protocols. Such semen samples often show very low motility rates at thawing and longevity which *in vitro* is frequently < 2 h even at 22 °C. Therefore, it may be of interest to find a thawing protocol that could enhance the possibilities of a low-motility frozen semen to reach the oviduct and therefore to fertilize the oocyte.

Dilution of thawed semen has two important functions, namely to allow penetrating cryoprotectants to flow out of the cell, as well as to provide additional substrate for sperm energy metabolism. A positive effect of post-thaw dilution on sperm longevity has been reported [20].

Motility stimulants such as the two methylxanthines pentoxifylline and caffeine, are already known to enhance the motility of ejaculated sperm by an inhibiting phosphodiesterase activity, thus enhancing cAMP at the level of the sperm tail. 2'-Deoxiadenosine, an analogue of adenosine, has a similar effect. Methilxanthines and adenosine analogues have been used in fresh and frozen-thawed semen of different species such as human [21,22], hamster [23], goat [24], boar [25], cat [26], bull [27] and horse [28]. In the dog species, pentoxifylline has been proven to have a significant beneficial effect on progressive motility of fresh semen and, in frozen-thawed semen, its addition at the moment of thawing is beneficial [29]. Also the addition of prostatic fraction at the moment of thawing has been considered, but although *in vivo* studies report improved conception and pregnancy rates, [30], *in vitro* studies demonstrated no difference on motility, longevity, and acrosome integrity during incubation of frozen-thawed semen with autologous prostatic fluid [31].

Motility is one of the most important features of spermatozoa in order to reach the oocyte. An objective assessment of sperm motility made by computer assisted sperm analysis (CASA) allows a rapid evaluation of a large number of tracks left by motile spermatozoa; this leads to assessment of total and progressive motility as well as different characteristics of sperm velocities, which are indicative of the quality of the movement of spermatozoa, such as, for example, sperm hyperactivation [1]. Different analysers have been validated for dog semen, such as Hamilton Thorne Analyzer [32], Sperm Quality Analyzer [33], and Sperm Vision [34], and are now largely used for the assessment of fresh, chilled, and frozen-thawed semen.

The aim of this work was to evaluate the effect of pentoxifylline, caffeine, 2'-deoxyadenosine, and heterologous prostatic fluid added at the time of thawing on motility parameters of spermatozoa frozen using the Norwegian protocol; in particular we wanted to evaluate: a) the concentration of each motility stimulant which works better; b) the interaction between thawing rate and post-thawing addition of pentoxifylline, caffeine, and heterologous prostatic fluid; c) the effect of pentoxifylline and 2'-deoxyadenosine at post thawing and during 30, 60, and 120 min of semen incubation time.

2. Materials and methods

2.1. Frozen semen

Semen straws frozen at the Small Animal Reproduction Study Centre (C.E.R.C.A.) of the Alfort National Veterinary College—Maisons Alfort (France) were used for this study. Semen was frozen during the period 1983–1997 in a Tris-fructose-citric acid extender according to the Norwegian freezing protocol [17] and kept stored for commercial and owner's purposes. Former owners of such samples did not have any further intent in using their dog's frozen semen, and had signed a waiver form.

2.2. Chemicals

Caffeine, Pentoxifylline, and 2'-Deoxyadenosyne, as well as chemicals used for the thawing medium, were purchased from Sigma-Aldrich Chemical Company, Saint Quentin Follovier (France).

2.3. Experiment 1

Straws of 0.5 ml of 7 stud dogs (1 Boxer, 1 Dogue de Bordeaux, 1 Saluki, 1 Labrador, 1 Shar-pei, 1 Spaniel Pechinese, 1 Elkhound) were thawed in a waterbath at 37 °C for 1 min; the content was then mixed, divided into 10 aliquots and diluted 1:1 with a Tris-Glucose-Citric acid (TGC) based medium as control [19] or with TGC supplemented with either Caffeine, Pentoxifylline, or