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Cooling of canine semen contaminated with urine*

Resfriamento do sêmen canino contaminado por urina

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Resumo

A contaminação do sêmen canino pode ser causada pela micção durante a ejaculação, ou quando o sêmen flui diretamente para a bexiga, que são problemas comuns durante a eletroejaculação para a criopreservação. Os efeitos que a urina causa ao processo de criopreservação, entretanto, não estão completamente entendidos. O presente estudo determinou a resistência do sêmen a diferentes níveis de contaminação por urina após 24h de resfriamento. As amostras de sêmen obtidas de 10 cães foram incubadas em soluções de Ringer, e em diferentes concentrações de urina (0,3,6,13,25,50 e 100%), a 37°C, por 20 min. As amostras foram centrifugadas e os espermatozoides decantados foram ressuspendidos, e resfriados a 5°C por 24h em diluidor a base de leite. A qualidade das amostras de sêmen contaminado por urina diferiu do controle apenas após a exposição de soluções com concentração superior a 13% de urina. Após o resfriamento, apenas os espermatozoides incubados com soluções de urina superiores a 25% exibiram menor atividade que as incubadas na solução simples de Ringer. Em conclusão, o sêmen canino resiste à contaminação com até 13% de urina durante 20 minutos e a motilidade das amostras contaminadas com até 25% recuperam-se de forma semelhante às amostras não contaminadas, se resfriadas a 5°C durante 24 h em um diluidor à base de leite.

Palavras-chave: reprodução, cão, sêmen, coleta, problemas de contaminação.

Abstract

Contamination of canine semen with urine, caused by urination during ejaculation or semen flow into the urinary bladder, is a common problem of sperm collected by electroejaculation for cryopreservation. The effects of urine on sperm cryopreservation, however, are not fully understood. The present study determined the acceptable upper level of contamination with urine for canine semen preservation after 24-h cooling. Semen samples obtained from 10 dogs were incubated with Ringer's solution and different urine concentrations (0, 3, 6, 13, 25 50% and 100%) at 37°C for 20 min. The samples were centrifuged and the decanted sperm resuspended and cooled to 5°C for up to 24 h in a milk-based semen extender. The quality of the contaminated semen samples only differed from that of the control treatment after exposure to urine concentrations above 13%. After cooling, only sperm incubated with urine solutions above 25% exhibited lower activity than those incubated in simple Ringer's solution. In conclusion, canine semen resists contamination with up to 13% urine for 20 min, and the motility of samples contaminated with up to 25% recovers similarly to that of uncontaminated samples if cooled at 5°C for 24 h in a milk-based extender.

Keywords: reproduction, dog, semen, collection, contamination problem.

Introduction

In semen collection protocols, the semen is commonly contaminated with urine, as described for a number of mammals including humans, horses, the forest fox (*Cerdocyon thous*) and brown bears (Makler et al., 1981; Griggers et al., 2001; Souza and Paz, 2011; Gomes-Alves et al., 2014). In dogs, semen can be contaminated with urine in situations such as retrograde ejaculation (Beaufays et al., 2008).

In urine-contaminated environments, the first change that occurs in sperm is decreased motility (Makler et al., 1981; Griggers et al., 2001; Gomes-Alves et al., 2014). With its composition well described in technical reports (Whitbread, 2016), normal urine has detrimental effects on sperm, especially due to differences in pH and osmolarity (Makler et al., 1981; Griggers et al., 2001).

To avoid the deleterious effects of urine on sperm, a number of procedures are applied before or after ejaculation. In humans, ingestion of $NaHCO_3$ solution can buffer urine pH (Brassesco et al., 1988), and in dogs, therapy with alpha-adrenergic drugs, such as phenylpropanolamine, can prevent them (Beaufays et al., 2008). With respect to post-ejaculation procedures, centrifugation of contaminated semen has been applied to equines and brown bears (Griggers et al., 2001; Gomes-Alves et al., 2014). Moreover, the use of milk-based extenders was found to inhibit sperm viability loss and improve semen quality in equines (Griggers et al., 2001), and a similar effect was described using

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commercial extenders to treat brown bear semen (Gomes-Alves et al., 2014). Other studies tested adjustments in urine pH and osmolarity, given their association with the deleterious effects of urine on semen (Makler et al. 1981; Griggers et al., 2001).

The information available on urine-contaminated sperm is not sufficient to determine the tolerable upper contamination level to maintain the sperm viability rate or estimate how long the cells can resist before decontamination procedures are applied. With a view to contributing to the development of protocols for canine semen preservation, the present study assessed the viability of canine sperm exposed to different urine concentrations and subjected to 24-h cooling.

Materials and methods

Test animals

The semen samples tested were collected from 10 dogs obtained from private breeders as follows: 1 Blue Heller, 3 Labrador Retrievers, 1 Poodle, 1 White Shepherd, 1 Brazilian Terrier and 3 Border Collies. The dogs were aged 1.5-7.0 years (6 ± 1) and weighed 7.0-32 Kg (23 ± 3).

Semen and urine collection

Semen samples from each dog were collected twice a week, but only the second sample was used in the study. We collected the second fraction of 10 ejaculates, by penis stimulation without the presence of any bitch in estrus. Within no more than 30 min post-collection, the samples were taken to the laboratory to undergo the experimental protocols. Autologous urine was collected immediately after semen collection from spontaneous micturition.

Experimental protocol

The experiment was carried out at the Laboratory of Reproduction and Genetic Improvement of the Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, RJ (21°45'47'' S; 41°17'12'' W).

Following a dilution curve, the urine concentrations tested ranged from 0% (simple Ringer's solution) to 100% urine, with intermediary urine in Ringer's solution dilutions at 1:32, 1:16, 1:8, 1:4, 1:2 (3, 6, 13, 25 and 50%, respectively). Aliquots of 400 µL of urine at the different concentrations were incubated with 100 µL of semen in a water bath at 37° C for 20 min. The protocol simulated the *in vivo* conditions of electroejaculation in dogs, allowing the semen to be exposed to urine for a similar time period. After incubation, the samples were centrifuged at 700 x *g* for 7 min (Lopes et al., 2009).

With respect to the cooling protocol, the sperm pellet remaining after supernatant removal was added with 400 µL of milk-based extender (2.4 g milk powder, 4.9 g glucose, 0.75 mg sodium bicarbonate, 20 mg gentamicin, 1000 mL double distillated water, pH 6.8-7.2 and osmolarity of 350-375 mOsm.L^{-1}). The extended samples were cooled in a Minitub® refrigerator at 5°C for 24 h.

Urine and Semen evaluation

Urine samples were evaluated using Uriquest® urinalysis strips (Labtest Diagnóstica S.A.), which allows semi-quantitative determination of bilirubin, ketones, density, glucose, leukocytes, nitrite, pH, protein, blood and urobilinogen. Urine osmolarity was assessed by a 5004-Micro-Osmette™ osmometer (Precision Micro Systems Inc.).

Semen samples from all the treatments were examined fresh and after 24-h cooling for macroscopic assessment of color, appearance and volume. The physicochemical properties of semen were evaluated using universal pH-indicator strips pH 0 - 14 (Merck-Germany) and osmolarity was determined using a 5004-Micro-Osmette ™ osmometer (Precision Micro Systems Inc.).

Semen concentration was measured after the samples were diluted 1 to 200 with formal saline solution. The spermatozoa were counted in a Neubauer chamber under phase contrast microscopy (Nikon Eclipse 80*i*). For morphological analysis, 10 µL of semen sample aliquots were diluted 1 to 10 with formal saline and examined under phase contrast microscopy at 1000x magnification (Nikon Eclipse 80*i*). The cells were categorized according to Oettlé et al. (1993).

Total and progressive sperm motility were evaluated by computer assisted analysis, using a 10.8 Ceros ® Hamilton-Thorn motility analyzer. As in previous studies, this evaluation considered time 0, 20 min and 24 h. The observations were performed using a 7 µL sample aliquot placed on a microscope slide preheated to 37° C and covered with a 22 x 22 mm coverslip (Nöthling and Dos Santos, 2012).

The post-cooling integrity and functionality of sperm membrane was determined from 10 µL aliquots of samples cooled for 24h. The aliquots were then incubated for 45 min in a 60mOsmol/L fructose hypo-osmotic solution. After incubation, the morphology of 200 spermatozoa placed between the slide and the coverslip was observed under phase contrast microscopy (Nikon Eclipse 80*i*) with 1000 x magnification. Spermatozoa that curled their tail after incubation were considered healthy and functional (Kumi-Diaka, 1993). Spermatozoa whose tail had curled before the experimental procedures, as determined by morphological examination, were disregarded in the hypo-osmotic swelling test (HOST).

Statistical analysis

Data were organized in a Microsoft Excel spreadsheet for mean and standard error calculations. Percentage data underwent arcsine square root transformation (y=arcsin (x^{-2})).

Data were assessed using Kruskal-Wallis one-way analysis of variance and the treatments contrasted applying the post hoc Newman-Keuls Student procedure, using BioEstat 5.0 free software, Brazil. Statistical differences were considered at $P < 0.05$. Data are expressed as mean ± standard error.

Results

Fresh semen showed a mean volume of 2 ± 0 mL, 285 ± 83 million spermatozoa.mL^{-1} and 79 ± 7% total motility.

Urine composition differed among the dogs in terms of osmolarity. The other urine parameters were similar, particularly the categorical outcomes (table 1).

Table 1: Urinalysis outcomes (n = 10)

Variables	Outcomes
Bilirubin	normal
Urobilinogen	normal
Ketones	negative
Glucose	normal
Protein	77 ± 12
Blood	negative
Nitrite	negative
pH	6± 1
Density	1.026 ± 0.003
Leukocytes	negative
Osmolarity (mOsmol.L^{-1})	515 to1455

After the sperm was incubated in a water bath at 37°C for 20 min, motility was similar between the treatments with Ringer's solution and urine contamination up to 13%, but decreased in treatments with 25% or more urine (table 2).

After incubation with the different urine concentrations, the semen was cooled at 5°C for 24 h in a milk-based extender (table 3). Sperm viability was maintained in treatments with up to 25% contamination with urine, indicating the efficiency of the extender in improving total and progressive motility (BCF, VAP and VSL). These variables were lower in sperm samples contaminated with than 50% or more urine ($p < 0.05$). Sperm integrity, indicated by the HOST value, did not differ among treatments ($p>0.05$).

Discussion

The simple Ringer's saline solution was chosen because, as reported in earlier studies, it is an appropriate medium to maintain sperm functions. Its simple composition (NaCl, KCl and $CaCl_2$), is similar to the Cheng medium and intravesical application poses no potential risks (Tsai et al., 1990). In fact, after 20 min of semen incubation with pure Ringer's solution, total sperm motility (table 2) was similar to that described by Griggers et al. (2001) for horse semen contaminated with 0, 5, 10, 33 and 50% urine in 0.9% NaCl.

The urinalysis results were within the normal range for dogs (Whitbread, 2016), but the urine osmolarity varied significantly, from 515 to 1455 mOsmol.L^{-1} (Table 2). This result indicates that urine is a difficult-to-control variable in studies investigating its effects on sperm viability.

Table 2: Fresh sperm motility (mean ± standard error). Canine semen was incubated at 37°C for 20 min with different urine concentrations (n = 10)

Sperm parameters	Ringer´s solution	Urine concentration					
		3%	6%	13%	25%	50%	100%
Total motility	63 ± 10^{a}	61 ± 7^{a}	47 ± 7^{ab}	46 ± 9^{abc}	26 ± 6^{bcd}	13 ± 4^{d}	8 ± 3^{d}
Progressive motility	29 ± 5^{a}	34 ± 6^{ab}	30 ± 5^{abc}	30 ± 7^{abc}	14 ± 4^{acd}	5 ± 2de	0 ± 0^{e}
BCF (Hz)	22.3 ± 1.8	21.3 ± 1.2	21.4 ± 1.3	21.8 ± 1.7	18.2 ± 2.7	22.6 ± 4.1	10.2 ± 4.3
STR (%)	85 ± 3	83 ± 3	83 ± 2	84 ± 4	76 ± 9	71 ± 12	42 ± 14
VAP (μm.s^{-1})	74.8 ± 9.5^{a}	75.6 ± 7.5^{ab}	67.7 ± 4.9^{abc}	69.8 ± 7.0^{abc}	45.5 ± 8.3^{cd}	19.9 ± 4.3^{de}	10.0 ± 3.6^{e}
VSL (μm.s^{-1})	63.1 ± 9.2^{a}	64.7 ± 8.1^{a}	57.5 ± 5.4^{a}	59.9 ± 6.8^{a}	38.5 ± 7.3^{ab}	17.3 ± 3.5^{bc}	8.6 ± 3.1^{c}
Osmolarity (mOsmol.L^{-1})	282 ± 2^{a}	311 ± 4^{a}	336 ± 7^{b}	388 ± 15^{b}	488 ± 29^{c}	650 ± 55^{d}	1047 ± 110^{d}

Different letters in a same row indicate a statistical difference between the treatment means ($p < 0.05$)
BCF = beat cross frequency, STR = straightness, VAP = velocity average path, VSL = velocity straight line.

Table 3: Post-cooling sperm motility (mean ± standard error). Canine semen was incubated with different urine concentrations at 37°C for 20 min and cooled at 5ºC for 24 h in a milk-based extender (n = 10)

Sperm parameters	Ringer´s solution	Urine concentration					
		3%	6%	13%	25%	50%	100%
Total motility	53 ± 7^{a}	66 ± 5^{ab}	63 ± 6^{abc}	59 ± 6^{ab}	40 ± 8^{acd}	27 ± 7de	6 ± 2^{e}
Progressive Motility	27 ± 4^{a}	32 ± 4^{ab}	33 ± 5^{ab}	29 ± 5^{abc}	15 ± 4^{acd}	9 ± 3de	1± 0^{e}
BCF (Hz)	22.4 ± 1.4	20.2 ± 1.7	20.2 ± 1.6	20.6 ± 1.7	21.3 ± 3.6	18.6 ± 3.5	15.8 ± 4.0
STR	77.2 ± 2	77 ± 1	78 ± 2	79 ± 2	69 ± 8	65 ± 11	58 ± 13
VAP (μm.s^{-1})	82.0 ± 6.5^{a}	87.1 ± 7.2^{a}	86.9 ± 6.6^{a}	82.8 ± 6.9^{ab}	59.5 ± 11.3^{abc}	46.8 ± 10.2^{cd}	21.6 ± 5.8^{d}
VSL (μm.s^{-1})	62.2 ± 4.5^{a}	67.1 ± 5.6^{a}	68.8 ± 5.2^{ab}	65.0 ± 5.5^{ab}	45.4 ± 8.4^{ac}	37.3 ± 7.7^{cd}	17.0 ± 4.2^{d}
HOST (%)	62.5 ± 6.4	66.9 ± 6.7	65.8 ± 6.6	63.4 ± 6.3	64.4 ± 5.7	58.4 ± 6.2	55.2 ± 6.5

Different letters in the same row indicate astatistical difference between the treatment means ($p < 0.05$)
BCF = beat cross frequency, STR = straightness, VAP = velocity average path, VSL = velocity straight line.

In the treatments subjected to urine contamination, fresh sperm motility was compromised when urine concentration was 13% or more. The osmolarity in these treatments was close to or greater than 500 mOsmol.L^{-1}, which, according to Songsasen et al. (2002), is the upper limit before sperm motility declines. In a study on horses, Griggers et al. (2001) found that an osmolarity of 322 mOsmol/L affected motility after semen incubation with urine for 1h. It is important to underscore that the effects of urine on sperm motility also depend on the length of time the sperm remains in an environment that is incompatible with its physiological needs (Makler et al., 1981).

The results obtained suggest that centrifugation did not have a negative effect on the functionality of sperm contaminated with urine, corroborating Griggers et al. (2001) and a study with brown bear semen (Gomes-Alves et al., 2014) However, centrifugation alone does not mitigate the effects of urine, and the use of a milk-based extender is essential to maintain cell functionality. In the present study, it protected semen from up to 13% urine contamination and allowed sperm functionality to be restored in the much more hostile environment containing 25% urine.

In a study on canine sperm, Martins et al. (2009) retrieved them from epididymis and diluted them in lactate-free Ringer's solution before freezing them in Tris/citric acid/amicacin/Orvus® WA Paste. Total motility, progressive motility, VAP, VCL, STR and LIN were similar to those reported here. Although the authors did not record pre-freezing data, it can be assumed that canine semen in Ringer's solution and contaminated with 25% urine may reach sperm-oocyte binding rate of 50%.

Conclusions

In canine semen diluted in simple Ringer's solution and incubated for 20 min at 37°C, sperm response is not compromised if the sample is contaminated with up to 13% urine. After cooling with a milk-base extender at 5°C for 24 h, even the samples treated with up to 25% urine showed sperm quality similar to that of the uncontaminated control treatment. Based on these results, it can be affirmed that semen contaminated with up to 25% urine remains viable for at least 24 h when diluted in simple Ringer's solutions and cooled at 5°C in a milk-based extender.

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