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Effects of urine and NaCl solutions of different osmolarities on canine sperm

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Abstract

Contamination of canine semen with urine can drastically reduce sperm motility. To assess whether this effect is associated with differences in pH and osmolarity, the present study evaluated canine semen diluted in prostatic fraction, autologous urine, ultrapure water and NaCl solutions containing the following osmolarities: 133, 260, 392, 519 and 860 mOsmol/l. The semen dilutions were incubated for 1 h at 37°C. Total and progressive sperm motility, membrane and acrosomal integrity and sperm morphology were evaluated. Semen and urine pH values were similar (semen = 6.5 ± 0.3; urine = 6.9 ± 0.3). After 1 h of incubation, total and progressive motility in the prostatic fraction were similar to that of sperm in the 260 mOsmol/l solution (nearly 75% for total motility and 26-40% for progressive motility) and higher than observed in the other solutions, which had a total motility lower than 35% and a progressive motility under 3.2%. Compared to sperm in the seminal plasma, membrane integrity was lower in sperm incubated in water, solutions with 133 and 860 mOsmol/l and urine (0-50% integrity), and acrosomal integrity was lower in sperm incubated in water, 860 mOsmol/l solution and urine (0-32% integrity). Morphology changes (bent tail) were detected only in sperm incubated in the most hyposmotic solutions (water and 133 mOsmol/l). In conclusion, pH is most likely not associated with the deleterious effects of urine on semen quality, and although only the most hyposmotic solutions caused morphological changes in sperm, sperm motility is compromised in solutions that are highly hypo- or hyperosmotic, such as urine.

Keywords: dog, osmolarity, semen, urine.

Introduction

Contamination of semen with urine reduces sperm fertility, thereby compromising fertilization. This contamination occurs naturally in canids by retroejaculation and ejaculation accompanied by micturition (Romagnoli, 1999; Watson and Holt, 2001; Silva *et al*., 2004), or during the electroejaculation procedure (Newell-Fugate, 2009).

Damages that urine causes on sperm functionality are mainly associated with the pH and osmolarity changes that it induces in the seminal plasma (Makler *et al*., 1981; Griggers *et al*., 2001). In fact, the osmolarity of canine semen is nearly 300 mOsmol/l (Feldman and Nelson, 1996), whereas urine osmolarity ranges from 50 to over 1000 mOsmol/l (Coles, 1986). The capacity of sperm to resist osmolarity variations is associated with the hydraulic conductivity, i.e., the membrane's capacity to enable water entry and exit (Meyers, 2005), that is controlled by ion channels (Caiza de la Cueva, 1997) and aquaporins, which are specific protein water channels (Preston *et al*., 1992). Semen from some animals, such as stallions, can resist high osmolarity changes (Griggers *et al*., 2001), while canine spermatozoa are sensitive to osmotic stress (Songsasen *et al*., 2002).

Changes in osmolarity have different effects on the functionality of sperm in several animal species, such as fish and mammals. Although hyperosmolarity is less harmful to sperm than hyposmolarity (Makler *et al*., 1981; Griggers *et al*., 2001), a hyperosmotic environment of more than 500 mOsmol/l is enough to reduce sperm motility and plasma membrane integrity of canine sperm (Songsasen *et al*., 2002). Sperm incubated in hyposmotic solutions develop tail swelling, a deformity that is associated with decreased membrane integrity and sperm motility (Kumi-Diaka, 1993). Gao *et al*. (1993) reported that tail swelling also occurred when human sperm was incubated in hyperosmotic solutions by migrating to an environment of lower osmolarity or even to a physiological saline solution.

Despite the evidence presented, information on the effects of urine contamination on the osmolarity of canine sperm is not accurate. Therefore, the present study investigates whether the deleterious effects of urine on sperm are caused by the osmotic differences between them. This was achieved by evaluating the functionality of canine sperm incubated in water, urine, autologous seminal plasma and solutions of different osmolarities.

Test animals and sample collection

Two semen samples were collected from each of six healthy male dogs (n = 6) aged between 3 and 7 years (two White Swiss Shepherd dogs, one Basset Hound, one Border Collie, one Blue Heeler, and one mongrel dog). The dogs were housed in the laboratory vicinities, fed commercial food and received water *ad*

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libitum. Semen collection was performed by penis stimulation, without the presence of any bitch in estrus. The sperm rich fraction and prostatic fraction were collected separately. The first fraction of the semen was discarded. The prostatic fraction was used as a control isotonic solution. Autologous urine was collected immediately after semen collection by spontaneous micturition.

Materials and Methods

Determination of seminal and urinary pH of each sample was performed immediately after semen collection using an Oakton (WD-35624-22) pH tester. Osmolarity in the semen and urine samples and in the anisosmotic solutions was measured by a 5004 Microosmett™ (Precision Systems Inc.) osmometer.

Semen dilution

Aliquots of 100 µl of the sperm rich fraction were diluted in 900 µl of one of the 8 solutions tested: prostatic fraction, autologous urine, ultrapure water (Purelab Maxima/Elga) or NaCl solution at 133 (75 mM), 260 (150 mM), 392 (225 mM), 519 (300 mM) or 860 (500 mM) mOsmol/l. Mean concentration of the final solutions was 27.4 x 10^6 spermatozoa/ml. The final solutions were incubated at 37°C for 1 h.

Determination of sperm functionality and morphology

Total and progressive motility were evaluated immediately after semen dilution and after 1 h of incubation at 37°C using the computer system Hamilton Thorne Research CEROS 10.8® with the setup proposed by Iguer-Ouada and Verstegen (2001). The integrity of sperm plasma membrane after 1 h of incubation was evaluated by vital staining with eosin yellow 0.5% and observation under light microscopy (Nikon, model Eclipse 80i) at 1000X magnification (Verheyen *et al*., 1997). The stain was diluted in the NaCl solutions with the same osmolarity as the different semen solutions. Within the 200 spermatozoa evaluated in each sample, those stained red were considered to be dead and those not stained were considered to be alive.

Acrosomal integrity after 1 h of incubation was evaluated using a fluorescent probe PSA-FITC (Pisum sativum Aglutinin, coupled with Fluorescein Isothiocyanate) diluted in a PBS solution (0.139 M NaCl, 0.0027 M KCl, 0.008 M Na_2HPO_4 (H_2O) and 0.147 M KH_2PO_4) at 0.04 mg/ml dilution and propidium iodide diluted in 0.150 M NaCl at the proportion of 0.025 mg/ml (Goodrowe *et al*., 1998). Epifluorescence microscopy (Zeiss Jenalumar microscope) at 1000X magnification was used to evaluate the 200-cell samples and divide the sperm into three categories: intact, semi-damaged, and damaged, as described by Goodrowe *et al*. (1998).

To evaluate sperm morphology, after 1 h of incubation the samples were fixed using 10% formalin solutions, which had the same osmolarity as the samples. A phase contrast microscope (Nikon Eclipse 80i) at 1000X magnification was used to observe the sperm, and morphological defects were classified as proposed by Bloom (1973).

Statistical analyses

After checking for normality by the Shapiro-Wilk test, data on sperm functionality and morphology were compared using analysis of variance, and those that did not fit the normal curve, even after transformations, as arcsine of the square root of a number, were subjected to non-parametric tests (Kruskal-Wallis or Wilcoxon rank-sum test). Significant differences were contrasted using the Tukey HSD multiple comparison test (Lomax, 2007). Statistical tests were performed using the R environment version 2.11.1, 2010. Data are shown as mean ± SEM.

Results

Semen and urine pH values were similar (semen = 6.5 ± 0.3; urine = 6.9 ± 0.3; $P > 0.05$), but semen osmolarity was lower than that of urine (semen = 347.12 ± 26.6 mOsmol/l; urine = 1261.44 ± 88.76 mOsmol/l; $P < 0.05$).

Sperm motility immediately after dilution is shown in Table 1. Total motility was lower in sperm samples diluted in urine, in the solutions of 519 and 860 mOsmol/l and in ultrapure water ($P < 0.05$). Total sperm motility in the seminal solution was similar to that obtained in the 260 mOsmol/l solution and higher than that in the 133 or 392 mOsmol/l solutions. Progressive motility was higher in seminal plasma than in the 260 mOsm/l solution, and in both solutions it was higher than in the other solutions ($P < 0.05$).

After 1 h of incubation in the test solutions (Table 2), the highest total and progressive sperm motility was observed in the prostatic fraction and in the 260 mOsmol/l solutions. The highest sperm membrane and acrosomal integrity were in general found in the prostatic fraction, and the lowest in urine and ultrapure water (Table 2; $P < 0.05$). The proportion of sperm with normal morphology was compromised after 1 h of incubation in ultrapure water and in the NaCl solution with the lowest osmolarity, with both treatments showing a high incidence of folded and bent tails.

Table 1. Mean percent value (± SEM) of canine sperm motility immediately after dilution in urine, seminal plasma and solutions of different osmolarities.

Solution/Osmolarity (mOsmol/l)	Motility (%)	
	Total	Progressive
Prostatic fraction / ~347	83.8 ± 2.5[a]	67.5 ± 5.0[a]
Ultrapure water / 0	0[c]	0[c]
NaCl Solution / 133	47.3 ± 5.3[b]	8.6 ± 2.8[c]
NaCl Solution / 260	76.4 ± 4.4[a]	46.7 ± 5.5[b]
NaCl Solution / 392	59.9 ± 4.7[b]	12.1 ± 2.5[c]
NaCl Solution / 519	14.4 ± 4.8[c]	0.3 ± 0.2[c]
NaCl Solution / 860	0[c]	0[c]
Urine / ~1260	14.7 ± 8.6[c]	2.6 ± 2.7[c]

Different letters in the same column indicate a statistical difference (P < 0.05).

Table 2. Mean percent value (± SEM) of canine sperm motility, membrane and acrosomal integrity and spermatozoa with normal morphology after 1 h of incubation in urine, seminal plasma and solutions of different osmolarities.

Solution/Osmolarity	Motility		Integrity		Normal
(mOsmol/l)	Total	Progressive	Membrane	Acrosomal	Morphology
Prostatic fraction / ~347	74.6 ± 6.9[a]	39.9 ± 8.6[a]	91.3 ± 6.5[a]	84.8 ± 7.7[a]	87.5 ± 6.0[a]
Ultrapure water / 0	0[c]	0[b]	5.0 ± 5.5[c]	19.5 ± 9.8[bc]	3.1 ± 1.6[b]
NaCl Solution / 133	32.8 ± 5.9[b]	3.1 ± 2.0[b]	49.4 ± 21.9[b]	38.6 ± 16.6[abc]	1.9 ± 1.2[b]
NaCl Solution / 260	75.1 ± 4.5[a]	25.9 ± 4.5[a]	61.8 ± 23.7[ab]	59.7 ± 16.9[ab]	84.4 ± 12.8[a]
NaCl Solution / 392	34.1 ± 10.7[b]	2.2 ± 1.8[b]	42.7 ± 22.4[bc]	63.4 ± 14.8[ab]	86.1 ± 10.9[a]
NaCl Solution / 519	0[c]	0[b]	55.0 ± 18.9[ab]	62.6 ± 12.2[ab]	83.5 ± 13.4[a]
NaCl Solution / 860	0[c]	0[b]	36.8 ± 27.7[bc]	32.1 ± 16.9[bc]	80.0 ± 11.2[a]
Urine / ~1260	0[c]	0[b]	0[c]	3.3 ± 2.1[c]	81.6 ± 12.7[a]

Different letters in the same column indicate a statistical difference (P < 0.05).

Discussion

The findings of the present study corroborate the hypothesis that differences between urine and prostatic fraction osmolarity have deleterious effects on canine sperm quality. Sperm quality was maintained only in the prostatic fraction and in the physiological solution that had the closest osmolarity to the seminal liquid (260 mOsmol/l), but sperm functionality and morphology were compromised when it was diluted in anisosmotic solutions and urine. This damage is most likely not associated with pH since it was similar between semen and urine, as reported in earlier studies (Coles, 1986; Feldman and Nelson, 1996).

Earlier studies have shown that because of osmolarity differences, urine contamination compromises human semen motility (Makler *et al*., 1981) and in dogs it impairs semen cryopreservation (Songsasen *et al*., 2002). In human semen, Crich and Jequier (1978) found 10% sperm motility after 5 min of incubation in urine (548 mOsmol/l) and 0% after 20 min. In addition to the previous data, the present study showed that urine mixed with canine semen decreased total and progressive sperm motility to nearly 16% immediately after incubation.

Data on the motility, integrity and morphology of sperm incubated in urine had a high variance. This is a reflex of osmolarity variation among the samples, i.e, although mean urine osmolarity was nearly 1260 mOsmol/l, sperm motility was mostly unaffected in some samples that reached ~500 mOsmol/l. Nikolettos *et al*. (1999) also found a wide variation of 0 to 34% in the motility of sperm diluted in different urine samples. Although urine osmolarity oscillates throughout the day (Coles, 1986) as a function of physiological processes and conditions of the dog, it is usually hyperosmotic in relation to semen, and therefore it can be deleterious to sperm. Other studies found that human (Gao *et al*., 2003) and equine (Pommer *et al*., 2002) sperm are resistant to highly hyperosmotic solutions, but as shown in the present study, this is not the case with canine sperm, which is more sensitive to solutions over 860 mOsmol/l.

Although the extremely high and low osmolarity conditions of urine and water compromised sperm integrity, cell morphology of canine sperm changed drastically only when it was submitted to hyposmotic solutions (0 and 133 mOsmol/l). The main alteration observed was tail swelling and folding due to water entering the sperm, which reflects a physiological effort to maintain osmotic balance of the cell. This phenomenon was also observed and described by Kumi-Diaka (1993) for canine sperm and Gao *et al*. (1993) for human sperm. Gao *et al*. (1993) observed by scanning electron microscopy that a hyperosmotic NaCl solution (2650 mOsmol/l) causes shrinkage of human sperm, but this was not observed in the present study.

In conclusion, the main effects of urine on

canine semen are associated with osmolarity changes. Although only the more hyposmotic solutions caused morphological changes in sperm, the highly anisosmotic solutions, including urine, compromised sperm motility. These findings are novel for canine semen and corroborate studies on other animal species that show a reduction of sperm motility in anisosmotic solutions. Different urine concentrations may have different effects on sperm viability, and urine components may account for spermatic damage along with osmolarity. These issues are matter of future studies.

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References

Bloom E. 1973. The ultrastructure of some characteristic sperm defects and a proposal for a new classification of the bull spermiogram. *Nord Vet Med,* 25:383-391.

Caiza de la Cueva FI, Pujol MR, Rigau T, Bonet S, Miró J, Briz M, Rodriguez-Gill JE. 1997. Resistance to osmotic stress of horse spermatozoa: the role of ionic pumps and their relationship to cryopreservation success. *Theriogenology,* 48:947-968.

Coles EH. 1986. Renal function tests. *In:* Coles EH (Ed.). *Veterinary Clinical Pathology*. 4th ed. Philadelphia, PA: WB Saunders. pp. 178-179.

Crich JP, Jequier AM. 1978. Infertility in men with retrograde ejaculation: the action of urine on sperm motility, a simple method for achieving antegrade ejaculation. *Fertil Steril,* 5:572-576.

Feldman EC, Nelson, RW. 1996. *Canine and Feline Endocrinology and Reproduction.* 2nd ed. Philadelphia, PA: WB Saunders. pp. 673-690.

Gao DY, Ashworth E, Watson PF, Kleinhans FW, Mazur P, Critser JK. 1993. Hyperosmotic tolerance of human spermatozoa: separate effects of glycerol, sodium chloride, and sucrose on spermolysis. *Biol Reprod,* 49:112-123.

Goodrowe KL, Hay MA, Platz CC, Behrns, SK, Jones MH, Waddell WT. 1998. Characteristics of fresh and frozen-thawed red wolf (*Canis rufus*) spermatozoa. *Anim Reprod Sci,* 53:299-308.

Griggers S, Paccamonti DL, Thompson RA, Eilts BE. 2001. The effects of pH, osmolarity and urine contamination on equine spermatozoal motility. *Theriogenology,* 56:613-622.

Iguer-Ouada M, Verstegen JP. 2001. Evaluation of the "Hamilton Thorn computer-based automated system" for dog semen analysis. *Theriogenology*, 55:733-749.

Kumi-Diaka J. 1993. Subjecting canine to the hypoosmotic test. *Theriogenology,* 39:1279-1289.

Lomax RG. 2007. Selected multiple comparison procedures. *In*: Lomax, RG. *An introduction to statistical concepts*. 2nd ed. Mahwah, USA: Lawrence Erlbaum Associates. pp. 240. Available on: http://books.google.com.br/books?id=EZjaQBpLmxsC&pg=PA240&dq=kruskal-wallis+Tukey&hl=ptBR&ei=CgjxTJvPHMT58AbDpenRDA&sa=X&oi=book_result&ct=result&resnum=5&ved=0CD0Q6AEwBA#v=onepage&q=kruskal-wallis%20Tukey&f=false. Accessed on: Nov. 25th 2010.

Makler A, David R, Blumenfeld Z, Better OS. 1981. Factors affecting sperm motility. VII. Sperm viability as affected by change of pH and osmolarity of urine specimens. *Fertil Steril,* 4:507-511.

Meyers SA. 2005. Spermatozoa response to osmotic stress. *Anim Reprod Sci,* 89:57-64.

Newell-Fugate AE. 2009. *The effects of two formulations of deslorelin on the reproduction of male African wild dogs* (Lycaon pictus). Pretoria, South Africa: University of Pretoria. Master's Dissertation. Available from: http://upetd.up.ac.za/thesis/available/ etd-02192009-154836/. Accessed on: November 25th, 2010.

Nikolettos N, Al-Hasani S, Bauklok V, Schöpper B, Demirel LC, Baban N, Sturm R, Rudolf K, Tomalak K, Tinneberg HR, Diedrich K. 1999. The outcome of intracytoplasmic sperm injection in patients with retrograde ejaculation. *Hum Reprod,* 14:2293-2296.

Pommer AC, Rutllant J, Meyers SA. 2002. The role of osmotic resistance on equine spermatozoal function. *Theriogenology,* 58:1373-1384.

Preston GM, Carroll TP, GugginoWB, Agre P. 1992. Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. *Science,* 256:385-387.

Romagnoli S. 1999. Retrograde ejaculation in the dog. *In*: WSAVA Congress, 1999, Lyon, France. Lyon: WSAVA. 4 pp.

Silva AR, Morato RG, Silva LDM. 2004. The potential for gamete recovery from non-domestic canids and felids. *Anim Reprod Sci,* 81:159-175.

Songsasen N, Yu I, Murton S, Paccamonti DL, Eilts BE, Godke RA, Leibo SP. 2002. Osmotic sensivity of canine spermatozoa. *Cryobiology,* 44:79-90.

Verheyen G, Joris H, Crits K, Nagy Z, Tournaye H, Van Steirteghem A. 1997. Comparison of different hypo-osmotic swelling solutions to select viable immotile spermatozoa for potencial use in intracytoplasmic sperm. *Hum Reprod Update,* 3:195-203.

Watson PF, Holt WV. 2001. Cryopreservation of gametes and embryos of canidae and felidae. *In:* Watson PF, Holt WV (Ed.). *Cryobanking the Genetic Resource*: Wildlife conservation for the future? London: Taylor & Francis. pp. 363-390.