Establishment of Optimal Conditions for the Hypoosmotic Swelling Test to Evaluate the Integrity of Spermatozoal Plasma Membrane in Dog

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ABSTRACT

Hypoosmotic swelling test (HOST) is used for evaluating the plasma membrane function and fertilizing ability in mammal spermatozoa. However, HOS solutions and experimental conditions have not been determined clearly for assessing canine spermatozoa. This study was conducted to examine the HOS solutions and assay conditions, including incubation time (30 to 120 min), storage temperature (4, 17 and 20℃), semen status (fresh and frozen). Maximum spermatozoal plasma membrane swelling was obtained in an 150 mOsm Na-citrate/Fructose solutions with an incubation time for 45 min. The storage temperature and semen status affected the percentage of HOS positive spermatozoa. The HOS test adapted to canine spermatozoa in this study was simple and highly consistent assay with good repeatability. The optimal condition of HOST in canine spermatozoa is an 150 mOsm Na-citrate/Fructose solutions with an incubation time for 45 min regardless of semen storage temperature and semen status.

(Key words : Hypoosmotic swelling test, Spermatozoal plasma membrane, Na-citrate/Fructose solution, Canine spermatozoa)

INTRODUCTION

Although the evaluation of conception rates remains the ultimate test to assess the fertilizing capacity of canine semen, this method is time-consuming and results in the birth of unwanted puppies (Oettle, 1993; Van Soom *et al.*, 2001). Therefore, alternative methods to assess the functional capacity of spermatozoa *in vitro* are required. Until recently, light microscopy was routinely used to evaluate the principal parameters of dog semen, concentration, motility and morphology (Johnson, 1992). Recently, several techniques have been described which may enable more accurate prediction of the fertilizing capacity of a canine semen sample (Hewitt and England, 2001; Van Soom *et al.*, 2001).

Plasma membrane integrity is an important indicator for normal spermatozoal metabolism and function in human and animal (Jeyendran *et al.*, 1984). The role of the plasma membrane in communication between the sperm cell and the external medium is important and involves ion transport across the membrane, the binding of different factors to specific factors, and the maintenance of the membrane potential. The functional and structural integrities of the sperm plasma membranein the tail need to obtain a complete picture of sperm quality (Jeyendran *et al.*, 1984). The hypoosmotic swelling (HOS) test is a relatively new assay being used to evaluate the functional integrity of spermatozoal plasma membranes of men. HOST is a simple, inexpensive and easily applicable technique which has been adapted to test spermatozoa of several species such as bovine, horse, pig and goat. One of the most widely used tests for evaluating spermatozoa membrane status is the hopoosmtic swelling test (HOST), HOST is highly correlated with *in vitro* fertilization and oocyte penetration (Perez-Llano *et al.*, 2001).

The objective of this study was to evaluate the resistance of canine spermatozoa in osmotic stress media and assay conditions, including osmotic pressure in media, incubation time, storage temperature, storage time, and semen source.

MATERIALS AND METHODS

Animal and Semen Preparation

Semen was obtained from two Beagle and one Siberian husky dog that belong to the animal hospital in Kangwon National University with ranging between 2 and 5 years. The dogs were kept in out door kennels, and exercised twice daily.

Semen was collected once or twice weakly by manual stimulation into warmed (37℃) sterile collection bottle.

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Only the sperm-rich fraction of the ejaculates was used. Just after collection, semen samples were diluted with Tris extender and to transport the laboratory at 37℃ within in 20 min. Four experiments were conducted to identify assay conditions that would maximize the canine spermatozoal plasma membrane swelling. Assay conditions considered were evaluated the HOS solution, osmotic pressure, incubation time, storage temperature, storage time and fresh or frozen semen in dog.

Experimental Design

Hypoosmotic solutions were made from 3 sugars (sucrose, fructose and lactose) and 1 salt (Na-citrate) that were evaluated separately and in sugar-salt combinations (sucrose/Na-citrate, lactose/Na-citrate and fructose/Na-citrate) for their ability to induce changes associated with spermatozoal plasma membrane swelling. Seven basic solutions (sucrose, lactose, fructose, Na-citrate, sucrose/ Na-citrate lactose/Na-citrate and fructose/Na-citrate) ranging from osmolality between 50 and 250 mOsmoles (mOsm) in 50 mOsm increments were evaluated. Each sugar or salt initially was mixed to adjust the desired osmolality with double distilled water (dDW). Osmolality was measured for each sugar and salt solution using a freezing point osmometer (Advanced Ins., USA), combined sugar-salt solutions were prepared by mixing equal proportions of the respective sugar and salt with dDW.

Each of canine semen was used to test incubation time. Aliquots of each spermatozoal samples were prepared and incubated at 37°C for 30 to 120 min in 15 min increments. Samples were evaluated by the same HOS test procedures as previously described with the exception of incubation time. Also, each of canine semen was used to test storage temperatures (4, 17 and 20℃)and storage times (0~48 hrs) and semen source (fresh and frozen semen).

Hypoosmotic Swelling Test (HOST)

As a starting point to adapt the HOS test for canine spermatozoa the following procedures, as described by Jeyendran *et al.* (1984), were used to evaluate various assay conditions. After semen collection, 100 μl of canine semen were added to 1.0 ml of prewarmed HOS solution (37℃) in a 1.5 ml microcentrifuge tube. Each samples were incubated for different incubation times at 37°C. After incubation, a small drop of sample was placed on a glass slide and was covered with cover slip. Slides were examined at ×400 using phase contrast microscopy. A total of 100 spermatozoa were observed for changes associated with swelling in tail. The percentage of positive (+) spermatozoa (number of spermatozoa with coiled tails per total number of spermatozoa examined ×100) was recorded for each sample.

Statistical Analysis

The SAS mixed linear model program was used to analyze the data. Treatment means were compared for differences through use of Duncan's modified multiple range tests. The differences were considered statically significant at $P<0.05$.

RESULTS

Effects of Solusion and Osmolality

Spermatozoal plasma membrane swelling was similar in sucrose, lactose and Na-citrate, and plasma membrane swelling in 50~150 mOsm was higher than other osmolarity groups ($P<0.05$). However, maximal membrane swelling occurred in 150 mOsm Na-citrate/fructose as HOS (Table 1).

Effect of Incuvation Time

Table 1. Effects of different solution and varied osmolality on the swelling of canine spermatozoa*

Osmotic pressure (mOsm)	% of HOS positive spermatozoa (Mean±SE)						
	Sucrose	Fructose	Lactose	Na-citrate	Na-citrate / Sucrose	Na-citrate / Fructose	Na-citrate / Lactose
50	70.2±1.48[a]	64.5±5.83	74.3±4.48[a]	76.3±1.33[a]	62.2±5.36[ab]	66.0±4.50[ab]	69.3±4.86[a]
100	73.7±4.04[a]	64.8±2.16	70.3±5.57[a]	73.8±8.00[a]	75.8±6.45[a]	71.2±4.91[ab]	69.3±1.20[a]
150	67.7±1.45[a]	67.5±4.64	71.3±7.49[a]	68.3±2.61[ab]	63.0±4.33[ab]	78.3±1.16[a]	55.0±5.34[bc]
200	54.7±1.74[b]	59.8±3.16	46.2±1.83[b]	59.7±3.52[b]	59.7±1.96[b]	58.5±4.80[b]	61.5±3.75[ab]
250	40.8±3.44[c]	74.3±5.81	50.3±1.66[b]	55.2±4.00[b]	49.5±2.08[b]	68.5±3.25[ab]	46.2±0.92[c]

* Pooled semen samples were divided into 35 different aliquots and each sample was incubated with the indicated solutions at 37℃ for 30 min. The test was repeated 3 times with different pooled semen samples.

[a~c] Values with different superscripts within same column differ significantly ($P<0.05$).

Table 2. Effect of incubation time on hypoosmotic swelling score of canine spermatozoa*

Incubation times (min)	% of HOS positive spermatozoa (Mean±SE)
30	77.3±1.74[a]
45	78.0±3.00[a]
60	68.9±0.88[bc]
75	74.2±2.02[ab]
90	64.2±1.83[c]
105	63.5±2.30[c]
120	65.3±1.36[c]

* Pooled semen samples were incubated for different incubation times with 150 mOsm Na-citrate/fructose solution at 37℃. The test was repeated 3 times with different pooled semen samples.
[a~c] Values with different superscripts differ significantly ($P<0.05$).

Incubation time also was evaluated using 150 mOsm Na-citrate/fructose as HOST solution. Proportions of HOS positive spermatozoa in 30 and 45 min incubation groups (77.3±1.7 and 78.0±3.0) were higher than those of other groups ($P<0.05$), except a 70 min incubation time group (Table 2).

Effect of Storage Temperature and Period

The proportion of spermatozoa that showed plasma membrane swelling was varied according to the storage temperature and period in 150 mOsm Na-citrate/fructose as HOS solution (Table 3). The proportion of HOS positive spermatozoa at 4, 17 and 20℃ were shown 70.8±2.40, 79.9±1.89 and 82.3±1.24, respectively. The 17℃ storage group was shown the highest percentage of swollen sperm tails. The score of HOS positive spermatozoa at 4 ℃ group was sharply dropped from 2 hrs post incubation, but 17℃ and 20℃ storage groups did not show the similar results.

Table 3. Effects of storage temperature and periods on hypoosmotic swelling score of canine spermatozoa*

Storage periods (hr)	% of HOS positive spermatozoa (Mean±SE%)		
	4℃	17℃	20℃
0	88.0±2.5[a]	88.0±2.5[a]	88.0±2.5[a]
2	73.5±4.2[b]	84.6±2.5	84.1±2.7[ab]
6	71.1±2.1[b]	83.1±1.2[a]	83.6±2.9[bc]
12	68.9±3.7[b]	82.0±1.3[a]	84.1±1.5[ab]
24	65.3±2.4[bc]	73.1±0.7[b]	78.3±1.3[c]
48	58.4±1.0[c]	68.7±5.4[b]	76.2±3.0
Overall	70.8±2.4[B]	79.9±1.9[A]	82.3±1.2[A]

* Pooled semen samples were divided into 18 different aliquots and each sample was incubated with 150 mOsm Na-citrate/fructose solutions at 37℃ for 45 min. The test was repeated 3 times with different pooled semen samples
[a~c] Values with different superscripts within same column differ significantly ($P<0.05$).
[A, B] Values with different superscripts differ significantly ($P<0.05$).

Table 4. Comparison of fresh and frozen/thawed semen on hypoosmotic swelling score in dog

Semen source	% of HOS positive spermatozoa (Mean±SE)
Fresh	78.3±1.2[a]
Frozen/thawed	44.0±1.2[b]

[a,b] Values with different superscripts differ significantly ($P<0.05$).

Effect of Semen Source

Proportions of HOS positive spermatozoa were 78.3±1.2 in fresh and 44.0±1.2 in frozen/thawed semen, respectively ($P<0.05$, Table 4).

DISCUSSION

The plasma membrane integrity is essential for the fertilizing capacity of spermatozoa. Until recently, the membrane intactness of canine spermatozoa was routinely assessed by means of light microscopic stains, such as eosin/nigrosin (Bangham and Hancock., 1955; Dott and Foster., 1972) or trypan blue (Risoparton *et al.*, 2002). The major problem with those techniques is that spermatozoa may show partial staining, making interpretation difficult (Hancock, 1957). The plasma membrane swelling test is a simple, inexpensive and easily applicable technique which has been adapted to test spermatozoa of several species such as bovine, horse, pig, goat and human. The structural and functional integrity of the sperm membrane is very important to analyze the sperm function because these characteristics are crucial for the viability and fertilizing ability of spermatozoa. However, HOS solutions and methodology used for evaluation of human spermatozoa may not provide the optimum conditions for evaluation of canine semen.

In this study we investigated a range of osmolarity, various HOS solutions and incubation time that induce the optimal spermatozoal plasma membrane swelling. Our results indicated that maximal membrane swelling occurred in 150 mOsm Na-citrate/fructose as HOS solution.

This was similar trends that the sodium citrate/fructose solution at 150 mOsm reported for human spermatozoa (Jeyendran *et al.*, 1984). Moreover,in our results, incubation time did alter the percentage of spermatozoal plasma membrane swelling.

A sodium citrate/fructose solution was tested for their ability to induce swelling of the spermatozoa. A sodium citrate/fructose and incubation at 150 mOsm for 45 min conditions gave the optimal and respectable results. Jeyendran *et al.* (1984) suggested that at the same osmolality (150 mOsm), solution (fructose and sodium citrate) affect the sperm membrane differently, but presumable sugars and electrolytes have a different influence on the influx of water through the sperm membrane. Therefore, compounds appear to have a different effect on the ability of spermatozoa to swell in a hypoosmotic solution.

The result indicates that fructose and sodium citrate HOS solution and incubation at 150 mOsm for 45 min were optimal condition for the HOS test in canine semen, and HOS test may be a useful indicator of the fertilizing ability of spermatozoa for *in vivo* and *in vitro* fertilization in dog.

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