EVALUATION OF MEMBRANE INTEGRITY OF BULL FROZEN-THAWED SPERM USING WATER AND HYPO OSMOTIC SWELLING TEST

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ABSTRACT

Assessment of the sperm membrane functional status appears to be a significant marker for the fertilizing capacity of spermatozoa. The hypo osmotic swelling test (HOST) is one of the best methods to evaluate sperm membrane integrity. In the current study, we used DW and hypo osmotic solutions of 50 and 100mOsm/l of dextrose/NaCl, NaCl, sucrose and fructose. Based on the results, Among the dextrose/NaCl, NaCl, sucrose, and fructose solutions and DW, Maximum numbers of swollen of bull frozen-thawed spermatozoa were observed with DW and dextrose/NaCl solution at 50 mOsm with average response by 61.20±8.677 and 47.90±10.181 respectively. The HOST response at 3 and 60 min for all of solutions were positively correlated to each other and there was no significant difference between the responses to the HOST at 3 and 60 min after incubation in all of solutions. The significant correlation was observed between motility and dextrose/NaCl at 50 mOsm, sucrose 50 and 100 mOsm, NaCl 50 mOsm and DW. The high relationship was between motility and DW and dextrose/NaCl at 50 mosm. There was no significant correlation between DW and all of hypoosmotic solutions with staining of the spermatozoa by eosin/nigrosin. In conclusion, the water test can be efficiently used for the evaluation of the functional integrity of the plasma membrane of bull frozen-thawed spermatozoa. The hypoosmular solution of dextrose/NaCl at 50 mOsm is a good medium to evaluate bull frozen spermatozoa. The used of HOST and motility are better tests to evaluate bull frozen thawed sperm than eosin-nigrosin. The short HOST procedure (3 min) is suitable method for evaluating of membrane integrity of bull frozen/thawed spermatozoa.

INTRODUCTION

The hypo osmotic swelling test (HOST) was used for the first time in evaluation of plasma membrane functional integrity in humans (1), rams (2), dogs (3), boars (4), stallions (5) and bulls (6).

The HOST is based on the integrity and stability of plasma membrane of sperm in relation to hypo osmotic solution (7). In the hypoosmolar solution, fluid is transferred into the cell through plasma membrane of spermatozoa. Trying to achieve a balance between intracellular and extracellular spaces, functionally intact membranes begin to swell, starting at the tail of the spermatozoa. Such spermatozoa are denoted as swelled or HOST reactive (HOST+) signifying functionally intact membranes, but spermatozoa with defected membrane do not swell and their tails do not bend so being seen normal (1). The hypo osmotic solution sufficiently provokes plasma membrane without lyses of the sperm membrane (8).

Water only enters in the tail region and creates different types of curls. The appearance of a curl in the tail of a sperm in this test is a sign that water has been transported and plasma membrane has resisted and this indicates that flagellar membrane is intact (9).

Evaluation of sperm membrane status is important since an intact and functionally active membrane is required for metabolism, capacitation, acrosome reaction, attachment and penetration of the oocyte (10). Thus evaluation of the sperm membrane status appears to be a significant marker for the fertilizing capacity of spermatozoa (11). So the use of this inexpensive and simple assay has been recommended as an additional fertility indicator parameter (1, 12).

Different HOST solutions such as dextrose/NaCl, NaCl, sucrose and fructose with different osmolality have been used for this test, but (13) suggested a new test to assess sperm membrane integrity by using distilled water; they called this new test ‘water test’. It was suggested that this test was simpler and faster than other HOST solutions, with a good correlation with routine semen analysis and eosin-Y staining (13).

This study aimed to determine the suitable osmolality, solution and time of HOST required for assessing bull frozen-thawed sperm.

MATERIALS AND METHODS

Sixty frozen semen specimens from 12 Holstein bulls were kindly provided by Jahed Company (Karaj, Iran).

HOST and Water test

The frozen specimens from each bull were thawed at 37°C for 30 seconds (12). Then the specimens were maintained for 1 to 2 min until semen assessment at 37°C. Spermatozoa were assessed for tail abnormality before subjecting to water and short and long HOST.

For the HOST, 20 µ1 of frozen-thawed sperm sample was mixed with 180 µ1 of the hypo osmotic solutions of 50 and 100mOsm/l of dextrose/NaCl, NaCl, sucrose and fructose. The mixtures were incubated for 3 and 60 min. at 37°C and assessment of total sperm swelling and individual swelling patterns were carried out (the percentage of spermatozoa with coiled tails was subtracted from the percentage of spermatozoa reactive to the HOST to obtain the actual proportion of spermatozoa induced to swell after exposure to the HOST diluents).

For water test, 20 µl of frozen-thawed sperm was mixed with 180 µl of distilled water. The mixture was incubated for 5 min. at 37°C and the reaction of sperms to the water was assessed.

Motility

One drop of frozen-thawed sperm was placed on a clean and prewarmed glass slide and two or three prewarmed drops of saline were added and covered with a cover slip. The percentage of progressively motile spermatozoa was estimated subjectively by one observer at 400X magnification using light microscopy (X31 Olympus, Japan).

Vital staining

The vital staining was adopted from that described by (14). Briefly one drop of thawed sperm was mixed with one drop of eosin-nigrosin on a clean and preheated slide and incubated for 30 seconds. A smear was prepared using 10 µl of the above mixture on a glass slide. The proportion of white sperm (unstained or live) and pink or red (colored or dead) were calculated using 400X magnification of light microscopy (X31 Olympus, Japan).

Statistical analysis

The obtained values were expressed as mean ± SD. The mean percentages of swollen spermatozoa obtained with the water and HOST solutions (dextrose/NaCl, NaCl, sucrose and fructose) were compared using one-way ANOVA followed by Duncan's multiple comparison tests. Pearson correlation was performed to evaluate relationship among HOST, water test, and other semen parameters (motility, and live/dead spermatozoa). SPSS 16.0 software was used for all statistical analyses. The results were considered significant at $P < 0.05$.

RESULTS

The results of the present study are summarized in tables 1 and 2. Among the dextrose/NaCl, NaCl, sucrose, and fructose solutions and DW, maximum numbers of swollen bull frozen-thawed spermatozoa were observed with DW and dextrose/NaCl solutions at 50 mOsm with average response of 61.20±8.677% and 47.90±10.181% respectively ($P<0.05$).

The HOST response at 3 and 60 min for all solutions were positively correlated to each other. There was no significant difference between the responses to the HOST at 3 and 60 min after incubation in all solutions ($p< 0.05$).

The Fig. 1 depict the HOST reacted sperms with characteristics coiled tails.

The average value of the motility of the frozen-thawed sperm was 43.89±10.51%. The eosin/nigrosin supravital staining method gave an average of 37.90±7.09% spermatozoa with structurally intact plasma membrane.

Correlation between motility and solutions and DW were evaluated and the significant correlation was observed between motility and dextrose/NaCl at 50 mOsm, sucrose 50 and 100 mOsm, NaCl 50 mOsm and DW. The highest relationship was observed between motility and DW and dextrose/NaCl at 50 mosm.

There was no significant correlation between DW and all hypo osmotic solutions with eosin/nigrosin-stained spermatozoa.

Table 1. Percentage of swollen spermatozoa in frozen-thawed semen using different hypo osmotic solutions and DW (n = 15).

Solutions mOsm/L	Time(min)	Mean	Std. Deviation
Dex/NaCl 50	3	46.10 ^{de}	9.960
	60	47.90 ^{e}	10.181
Dex/NaCl 100	3	44.50 ^{de}	5.543
	60	45.10 ^{de}	5.587
Sucrose 50	3	44.90 ^{de}	8.837
	60	46.30 ^{de}	11.295
Sucrose 100	3	37.70 ^{bc}	7.334
	60	40.50 ^{cd}	7.906
NaCl 50	3	43.90 ^{cd}	7.752
	60	46.50 ^{de}	8.059
NaCl 100	3	42.90 ^{cd}	9.291
	60	45.20 ^{de}	9.438
Fructose 50	3	36.60 ^{ab}	5.254
	60	38.10 ^{ab}	6.740
Fructose 100	3	35.70 ^{a}	7.454
	60	37.80 ^{ab}	8.297
DDW	5	61.20 ^{f}	8.677

^{ad}Values with the same superscript within columns are not significantly different (P>0.05)

Table 2. Correlation between percentages of swollen spermatozoa under different solutions and motility and Eosin/nigrosin results (n = 15).

Parameters mOsm/L	Time (min)	Eosin/nigrosin	Motility
Dex/NaCl 50	3	r =0.445^{ns}	r = 0.726 *
	60	r = 0.421	r = 0.745 *
Dex/NaCl 100	3	r = 0.278	r = 0.162
	60	r = 0.308	r = 0.187
Sucrose 50	3	r = 0.218	r = 0.725 *
	60	r = 0.170	r = 0.731 *
Sucrose 100	3	r = 0.142	r = 0.715 *
	60	r = 0.123	r = 0.661 *
NaCl 50	3	r = 0.155	r = 0.720 *
	60	r = 0.619	r = 0.718 *
NaCl 100	3	r = 0.632	r = 0.635
	60	r = 0.628	r = 0.651
Fructose 50	3	r = 0.374	r = 0.511
	60	r = 0.280	r = 0.521
Fructose 100	3	r = 0.264	r = 0.546
	60	r = 0.161	r = 0.468
DW	5	r = 0.485	r = 0.852 *

* Significant at P < 0.05. ^{ns} Not significant for all HOS tests

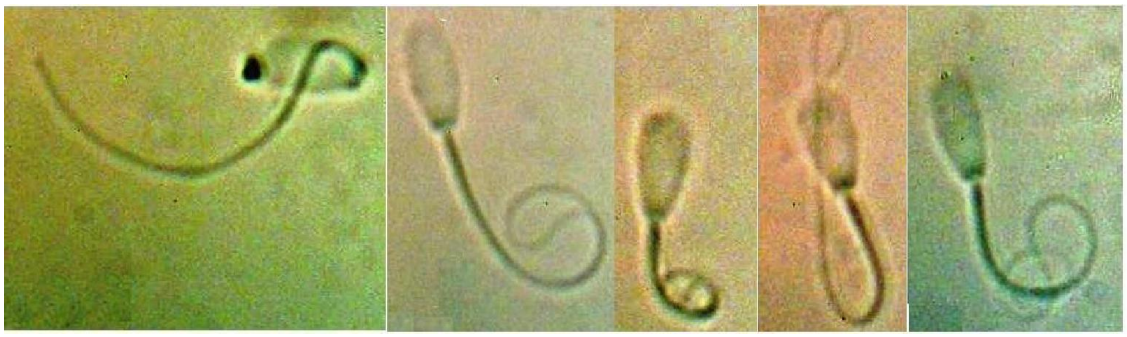

Figure 1. Morphological changes typical of the swelling observed in frozen-thawed bull sperm (samples were incubated in dextrose/NaCl at 50 mosm).

DISCUSSION

The functional and structural integrity of sperm membrane are crucial for the motility and viability of spermatozoa (15). Many authors consider that the best evaluation of fertility can be achieved by evaluation of sperm membrane integrity (16). The membrane integrity of spermatozoa is damaged during freezing and thawing. The HOST is an ideal method by being quick, simple functional tests which assesses membrane integrity (6, 17). The HOST is reported to have much better efficacy than supravital (eosin/nigrosin) staining. The evaluation of functional status of the sperm membrane by the HOST is a better indicator of their fertilization capacity (18). The HOST can indicate whether the sperm membrane is biochemically active or damaged during the process of freeze-thawing (19).

Based on the Jeyendran *et al*. (1984), the osmolarity of the solution should be sufficient to induce the best effect without causing any harm to the sperm (1).

The results of the present study showed that no significant differences were observed between the HOST responses recorded 60 min. after incubation compared to that of 3 min. after being placed into the hypo osmotic solution. This should facilitate the use of this technique by veterinarians so that the HOST assay adopted for routine use in clinical examinations.

Based on the results of (20 ,6 ,21 ,8 and 12), the largest number of swelled spermatozoa (HOST reactive) was observed in the hypoosmolar solution of fructose and Na-citrate. However, according to the reports of (22 and 17), the high response of spermatozoa was observed in the hypoosmolar solution of sucrose but (23), have been found high responses of

spermatozoa in fructose, sucrose and lactose hypoosmolar solutions respectively. However, our results showed that the best HOST solution used to evaluate bull frozen sperm was dextrose/NaCl solution.

The difference observed in the percentage of spermatozoa swellings in the sodium citrate and sugar solutions observed in this study, could have resulted from different influences on the availability of water for transport through the plasma membrane (23).

Studies on water permeability of some mammalian sperm membranes of ram and human (24) and bull (25) show that the osmotic water permeability coefficient is very high and that the associated activation energy is very low, which suggests a porous membrane and the presence of water channel proteins. In ram and human sperm membranes, glucose transporters may have a secondary water channel function (24).

(20,8 and 12) have been found the majority of HOST reactive bull spermatozoa in a hypoosmolar solution at 100 mOsm/l and similarly, (22, 17and 260, have been found maximum response of equine spermatozoa in a 100 mOsm/l. But(23) did not find any differences between hypo osmotic solutions at 100, 50 and 25 mOsm/l in bull spermatozoa (23). The best response in hypo osmotic solution of 60 mOsm/l in boar (6), and canine sperm (27) have been reported. (28) have been found high response of goat spermatozoa in 125 mOsm/l solution (28). But our result showed that the suitable osmolality was 50 mOsm/l solutions.

According to our findings, there was no significant difference in incubation time of 3 and 60 min. at 37ºC. So our results agreed with the results of (22, 29,8,and 30), who did not find any difference between short and long incubation time using hypo osmotic solutions.

In our research high positive correlations were observed between the HOST and sperm motility. This is in agreement with the previous study. (6 ,31 and 12), , who obtained a high correlation between HOST reactive, normal morphology and progressive motility of sperm. The sperm motility partially depends on the functional integrity of the membrane and partially on other biochemical activities such as sperm metabolism (1).

In the present study there was no significant correlation found between all HOST test and eosin/nigrosin staining. According to the (20) the HOST has much better efficacy than supravital (eosin/nigrosin) staining to evaluate membrane integrity. Our results agrees with (21) who did not found any significant correlation between eosin/nigrosin and water and HOST tests.

The evaluation of functional status of the sperm membrane by the HOST is a better indicator of their fertilization capacity (18) and the HOST can indicate whether the sperm membrane is biochemically active or not (19). Although motility is one of the best indicators of viability of sperm, the combination of the motility and HOST could be the best laboratory test to evaluate frozen-thawed sperm.

The high response of spermatozoa in DW and dextrose/NaCl in 50 mOsm solutions in our study is in agreement with the previous study. (27) who used DW and HOST to evaluate canine spermatozoa. Our results, however, were in contrast to the finding of (31) who found the percentage response of buck spermatozoa to water test was significantly lower than that obtained by the HOST.

In conclusion, it has been demonstrated that the water test can be efficiently used for the evaluation of the functional integrity of the plasma membrane of bull frozen-thawed spermatozoa. The hypoosmolar solution of dextrose/NaCl at 50 mOsm is a good medium to evaluate bull frozen spermatozoa. Application of HOST and motility are better tests to evaluate bull frozen-thawed sperm than only eosin-nigrosin staining. The fast and relatively inexpensive short time procedure (3 min) is a noticeable advantage of HOST for evaluation of bull frozen/thawed spermatozoa.

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