# Adaptation of the Hypoosmotic Swelling Test to Evaluate Membrane Integrity of Boar Spermatozoa

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## ABSTRACT

**The objective of this study was to establish the optimal conditions for hypoosmotic swelling (HOS) test to assess the functional integrity of the membranes of boar fresh or frozen/thawed spermatozoa. When pooled semen sample was incubated for 30 min at 37℃ with different test solution of varied osmolarity, the highest percentage of HOS positive spermatozoa was observed in a 150 mOsmol fructose/Na-citrate solution (33.6%). Incubation time did not affect significantly the score of HOS positive spermatozoa observed in a 150 mOsmol fructose/Na-citrate solution at 37℃, but the osmolarity affected the score of HOS positive spermatozoa under the same condition above. Fresh semen was significantly better than frozen/thawed semen in semen parameters evaluated such as motility, viability, membrane integrity and lipid peroxidation ($p<0.05$). In the relationships of sperm parameters, motility vs viability, motility vs membrane integrity and viability vs membrane integrity were positively correlated (0.82~0.94) but lipid peroxidation vs other estimated factors was negatively correlated (−0.90~−0.98). Among the evaluation methods, motility vs viability, motility vs membrane integrity and lipid peroxidation vs other estimated factors were significantly correlated ($p<0.05$). These results of this study indicate that the optimal condition of HOST in boar spermatozoa is a 150 mOsmol fructose/Na-citrate solution for 30 min incubation at 37℃ and HOST can substitute the examination of motility, viability and lipid peroxidation.**

**(Key words : Hypoosmotic swelling test (HOST), Boar spermatozoa, Spermatozoal plasma membrane, Frozen/thawed semen, Lipid peroxidation)**

## INTRODUCTION

The sperm membrane functional status is particularly importance since an intact and functionally active membrane is required for metabolism, capacitation, acrosome reaction, attachment and penetration of the oocyte (Vazquez *et al.*, 1997; Gadea and Matas, 2000). The assessment of the sperm membrane functional status appear to be a significant marker for the fertilizing capacity of spermatozoa (Jeyendren *et al.*, 1984: Zaneveld *et al.*, 1990).

The hypoosmotic swelling (HOS) test is a one of most commonly used methods for assessing the sperm membrane function in bovine, horse, pig, goat and human (Jeyendren *et al.*, 1984; Correa and Zavos, 1994; Rodriquez-Gil and Rigau, 1996; Caiza *et al.*, 1997). The principle of the test is based on the observation of morphological alterations in spermatozoa exposed to hypoosmotic solutions. During the HOST, spermatozoa with intact plasma membranes will undergo swelling due to the influx of water and subsequently increase in volume to establish an equilibrium between extra and intra components (Nie and Wenzel, 2000). 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 (Francisco *et al.*, 1997). The functional and structural integrity of sperm plasma membrane of the tail needs to obtain a complete picture of sperm quality (Jeyendren *et al.*, 1984). The HOST is a relatively easy and reliable assay method, which has been adapted to test spermatozoa of man and several animals, and being used to evaluate the functional integrity of sperm plasma membranes. The HOST has been used also widely as an additional fertility parameter (Zavos *et al.*, 1994). However, the

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optimal conditions of HOST to evaluate the functional integrity of boar spermatozoal plasma membrane has not yet been completely establish.

The objective of this study was to establish the optimal condition for HOST and to confirm the exactness when compared to other evaluation methods.

## MATERIALS AND METHODS

### Semen Preparation

Sperm-rich fractions were collected from 1~3 crossbred boar of known reproductive history in Youngsuh A.I. center. Just after collection, semen samples were diluted with Modena extender and transported to the laboratory at 17℃ within 2 hr. The collected semen had more than 70% initial sperm motility. All of the treatments were repeated at least 3 times with the semen samples from the different boars. All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

### Hypoosmotic Swelling Test (HOST)

Assay conditions were considered the HOS soulution (sucrose, fructose, Na-citrate, sucrose/Na-citrate and fructose/Na-citrate), incubation time (10~70 min), various osmolarity (50~200 mOsmol) and semen status (fresh or frozen/thawed boar semen). The judgement of total sperm swelling and individual swelling patterns were decided as sperm tail coiled and swollen. A total of 200 spermatozoa per slide were evaluated for coiled tails by counting at least 3 times under a phase contrast microscope at ×400 magnification. Each treatment group was repeated three times with different boar semen.

### Comparison of Hypoosmotic Solutions

Solutions made from 2 sugars (sucrose and fructose) and Na-citrate that were evaluated separately and in combination with sugar-salt combinations (sucrose/Na-citrate and fructose/Na-citrate) for their ability to induce changes associated with sperm plasma membrane swelling. Five basic solutions (sucrose, fructose, Na-citrate, sucrose/Na-citrate and fructose/Na-citrate) ranging in osmolality between 50 and 200 mOsmol in 50 mOsmol increments were evaluated. Osmolality was adjusted for each sugar and salt solution using a freezing point osmometer. Each sugar or salt initially was mixed, by weight, in sterile, deionized water to approximate the desired osmolality. Combined sugar-salt solutions were prepared by mixing equal proportions of the respective sugar and salt solution.

### Comparison of Incubation Time

The incubation time of HOST was examined using the semen samples from the different boars. Aliquots of each semen sample were incubated from 10 to 70 min in 10 min increments in hypoosmotic solution at 37℃. Samples were evaluated by the same HOST procedures as described above.

### Comparison of Osmolarity

The osmolarity of HOST was examined using the semen samples from the different boars. Aliquots of each semen sample were examined the various osmolarity from 50 to 200 mOsmol in 50 mOsmol increments. Samples were evaluated by the same HOST procedures under the conditions of fructose/Na-citrate solution for 30 min incubation at 37℃ with various osmolarity.

### Comparison of Semen Status

The fresh or frozen/thawed boar semen were used to test the membrane integrity. Aliquots of each fresh or frozen/thawed semen samples were performed the HOST under the condition of the 150 mOsmol fructose/Na-citrate solution for 30 min incubation at 37℃.

### Quantification of Lipid Peroxidation

Lipid peroxidation was measured of fresh or frozen/thawed boar semen by using the thiobarbiturec acid (TBA) reaction for malondialdehyde. Semen treated with 0.01 M sodium phosphate buffer (pH 7.4) containing 1.15% (w:v) KCl were adjusted in $Ca^{2+}$ and $Mg^{2+}$ free Dulbecco's phosphate buffer saline (DPBS) to give a concentration to $2\times10^6$ spermatozoa/ml. For the increasing of lipid peroxide to malondialdehyde, lipid peroxidation was promoted using a combination of ferrous sulfate and Na-ascobate. Sperm suspension (1 ml) was mixed with 10 μl of 1 mM ferrous sulfate and 10 μl of 5 mM sodium ascorbate, and then incubated for 1 hr at 37℃. The reaction mixture was added 250 μl of 40% trichloactic acid, held for 10 min at 0℃, and centrifuged for 10 min at 2,500 × g. Supernatants (1 ml) mixed with TBA were boiled with in hot water for 10 min. The amount of malondealdehyde produced was quantified against a standard curve at 532 nm wavelength in spectrophotometer.

### Statistical Analysis

All treatments were repeated at least three times with different ejaculators from three boars, respectively. At least 200 spermatozoa per slide were counted with at least three times. The evaluation items of the resistance of boar spermatozoa in osmotic stress media were analyzed statistically. Analysis of variance was performed using the SAS GLM procedure. Duncan's multiple range test was used to compare mean value of each treatment group. Correlations between the differ-

ent methods were calculated using the spearman rank correlation coefficient. A $p$-value below 0.05 was considered significant.

## RESULTS

Table 1 shows the effect of various osmolarity and different hypoosmotic solutions composed of different salts on the plasma membrane swelling in boar spermatozoa. The percentage of spermatozoal plasma membrane swelling in fructose and fructose/Na-citrate groups was higher than other hypoosmotic solution groups. The percentage of HOS positive spermatozoa in 100 mOsmol regardless of hypoosmotic solution was higher than that of other groups except for fructose/Na-citrate solution ($p<0.05$). However, the maximal score of HOS positive spermatozoa was observed in 150 mOsmol fructose/Na-citrate as a hypoosmotic solution ($p<0.05$).

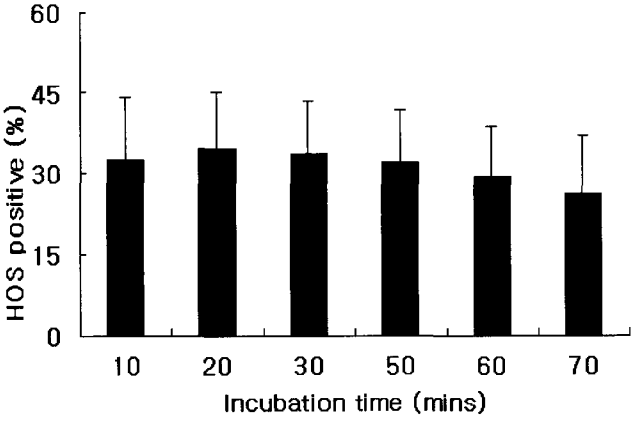


**Fig. 1. Percentage of boar spermatozoa showing swelling in different incubation time assessed by HOST.** Semen sample was incubated in a 150 mOsmol fructose/Na-citrate solution at 37℃ with various incubation time.

**Table 2. Plasma membrane swelling of boar spermatozoa incubated in fructose/Na-citrate solution varied osmolality***

| Osmolarity (mOsmol) | HOS positive (mean%±SD) |
|---|---|
| 50 | 12.0±1.5[b] |
| 100 | 17.8±5.6[ab] |
| 150 | 27.3±4.4[a] |
| 200 | 6.7±0.4[b] |

* HOST performed in a 150 mOsmol fructose/Na citrate solution at 37℃ for 30 min with different osmolarity.

[a,b] Mean values with different superscripts differ significantly ($p<0.05$).

Incubation time also was evaluated using 150 mOsmol fructose/Na-citrate at 37℃ with different incubation time in boar spermatozoa (Fig. 1). The score of sperm plasma membrane swelling in 30 min incubation group (38.8±10.4), was the highest among all experimental groups (32.6±11.4 in 10 min, 34.7±10.4 in 20 min, 33.9 ±9.6 in 40 min, 31.9±9.9 in 50 min, 29.3±9.7 in 60 min and 26.4±10.4 in 70 min, respectively).

The proportion of spermatozoa that showed plasma membrane swelling in fructose/Na-citrate solution for 30 min incubation at 37℃ with various osmolarity was summarized in Table 2. The 150 mOsmol solution (17.8 ±5.8) showed the highest percentage of swollen sperm tails compare to any other groups (12.0±1.5 in 50 mOsm, 17.8±5.8 in 100 mOsmol and 6.7±0.4 in 200 mOsmol, respectively).

The results of sperm characteristics (motility, viability and membrane integrity) and lipid peroxidation were shown the opposite patterns (Fig. 2). The sperm characteristics as motility, viability and membrane integrity were significantly higher than those of frozen/thawed semen (Motility, 94.0±4.0 vs 68.4±2.9; Viability, 78.0±5.0

**Table 1. Plasma membrane swelling of boar spermatozoa after incubation in different hypoosmotic solutions with varied osmolality***

| Osmolarity (mOsmol) | HOS positive(mean%±SD) | | | | |
|---|---|---|---|---|---|
| | Sucrose | Fructose | Na-citrate | Sucrose/ Na-citrate | Fructose/ Na-citrate |
| 50 | 18.1±4.3[b] | 20.3±1.7[c] | 11.5±1.2[b] | 12.3±0.6[bc] | 19.7±1.4[b] |
| 100 | 26.6±2.6[a] | 27.5±1.0[a] | 17.1±1.3[a] | 20.5±0.55[a] | 20.2±0.2[b] |
| 150 | 20.3±1.3[b] | 22.1±1.1[bc] | 14.2±0.4[ab] | 15.4±1.2[b] | 33.6±3.2[a] |
| 200 | 17.8±2.8[b] | 25.6±0.9[ab] | 16.1±0.6[a] | 11.2±1.6[c] | 19.2±0.7[b] |

* Pooled semen samples was incubated for 30 min at 37℃ with different test solution of varied osmolarity. The test replicates three times with different semen samples.

[a~c] Mean values with different superscripts within columns differ significantly ($p<0.05$).

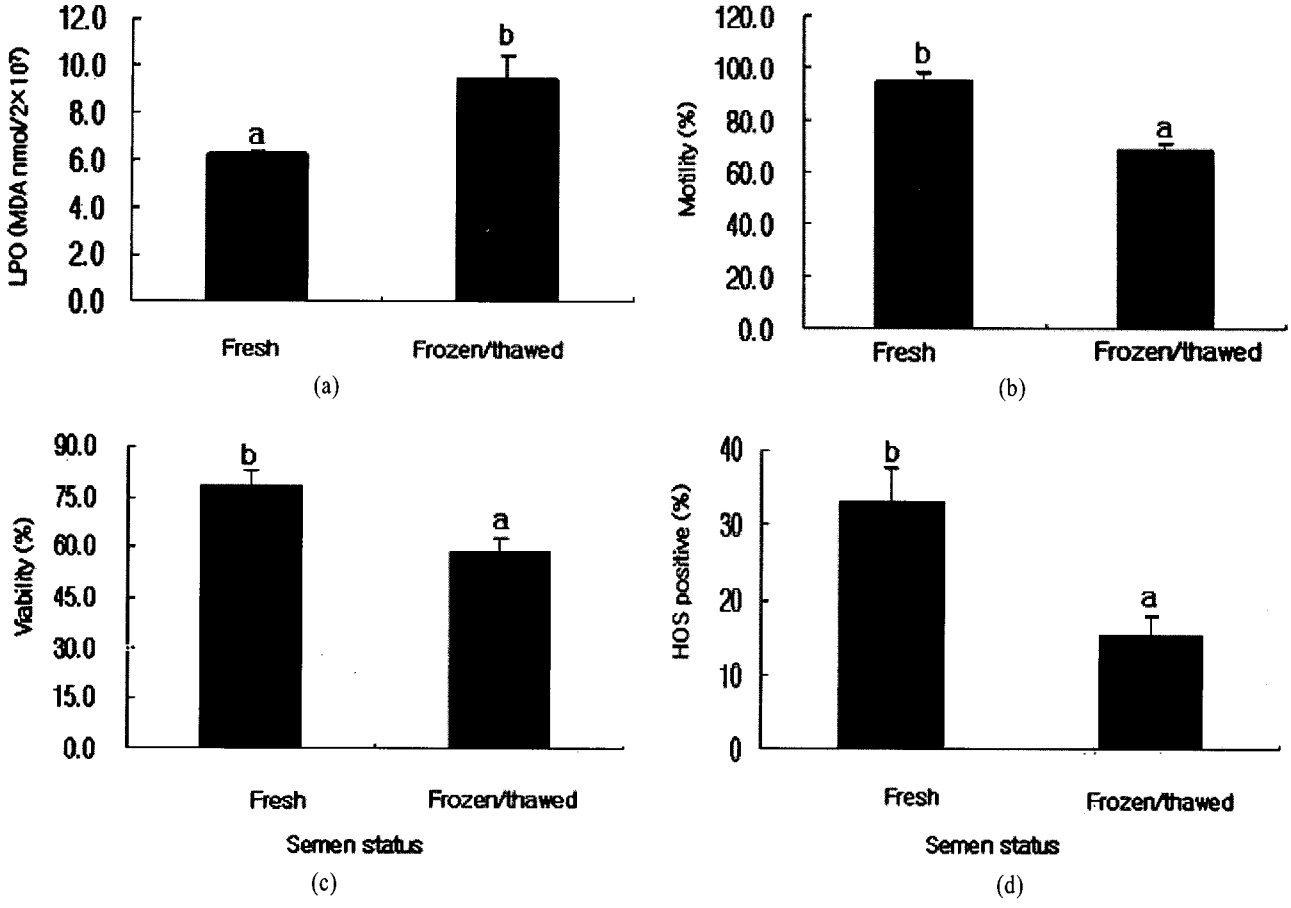


**Fig. 2. Comparison of semen evaluation methods (lipid peroxidation, motility, viability and HOST) on fresh or frozen/thawed boar semen.**
[a,b] Mean values with different superscripts differ significantly ($p<0.05$).

vs 59.0±3.6; Membrane integrity, 33.0±4.6 vs 15.3±3.6, respectively). MDA concentration (LPO score) was 6.27 ±0.1 nmol/l × $10^6$ in fresh semen and 9.5±0.9 nmol/l × $10^6$ in frozen/thawed semen.

Relationships of evaluation methods for sperm viability were investigated by the examination of motility, viability, membrane integrity and LPO (Table 3). Among evaluation methods, motility vs viability, motility vs membrane integrity and viability vs membrane integrity were positively correlated (0.82~0.94), but LPO vs motility, viability and membrane integrity were negatively correlated (−0.90~−0.98).

## DISCUSSION

Evaluation of sperm characteristics *in vitro* is impor-

**Table 3. Correlation coefficients among different testing methods to evaluate the sperm quality in pig**

| Evaluatuion methods | Motility | Viability | Membrane integrity | Lipid peroxidation |
|---|---|---|---|---|
| Motility | - | 0.94** | 0.93** | −0.98** |
| Viability | 0.94** | - | 0.82* | −0.90** |
| Membrane intergrity | 0.93** | 0.82* | - | −0.91** |
| Lipid peroxidantion | −0.98** | −0.90** | −0.91** | - |

* $p<0.05$, ** $p<0.01$.

tant for research on sperm preservation, artificial insemination and *in vitro* fertilization in pig industry. Many scientists have endeavored to develop laboratory assays to accurately predict the fertility potential of a male semen (Cerolini *et al.*, 2000; Iguer-Ouada and Verstegen, 2001). The ultimate goal of semen quality examination is to find the proper laboratory methods to accurately measure the fertilizing ability of a semen. Visual sperm characteristic tests such as concentration, motility, viability and abnormality by microscopic examination is still limited, mainly due to the difference of permeability to stain and different biochemical function of spermatozoa and also influenced by the conditions and the evaluator's skills, leading the high variability among laboratories and observers (Iguer-Ouada and Verstegen, 2001). The evaluation of sperm motility and spermatozoa membrane status is the most frequently assessed characteristics of sperm viability. Various tests are used to investigate the viability of boar sperm, including visual motility assessment, staining method using chemical compound, membrane integrity by HOST, lipid peroxidation of plasma membrane and so on (Leonardo *et al.*, 2003). The visual motility assessment of boar spermatozoa, which widely used in practice, is very subjective method. Also measuring the lipid peroxidation is one of efficient method to evaluate the sperm viability, but it is a time-consuming and high technique remanding method (Cerolini *et al.*, 2000). Currently one of the most widely used tests for evaluating spermtozoal membrane integrity is HOST, which reported highly correlated with *in vitro* fertilization results in human spermatozoa (Perez-Llano *et al.*, 2001). HOST is one of reliable methods and is a simple, inexpensive and easily appliable technique which has been adapted to test spermatozoa of several species such as bovine, horse, pig, goat and human (Jeyendren *et al.*, 1984; Correa and Zavos, 1994; Rodriquez-Gil and Rigau, 1996; Caiza *et al.*, 1997). 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 (Nie and Wenzel, 2000). However, HOS solutions and methodology used for evaluation of human spermatozoa may not provide the optimum conditions for evaluation of boar semen.

In this study we investigated to establish the optimal condition of HOST that evaluate the changes of spermatozoal plasma membrane swelling, including a range of osmolarity, varied HOS solutions and incubation times in boar spermatozoa. Our results indicated that maximal membrane swelling in HOST of boar spermatozoa occurred in 150 mOsmol fructose/Na-citrate as HOS solution. This is similar to the results of Jeyendran *et al.* (1984) that they reported a highest results in 150 mOsmol fructose/Na-citrate solution in HOST of human spermatozoa. But, in our results (see Table 2), incubation time did not significantly alter the percentage of spermatozoal plasma membrane swelling observed under the conditions of 150 mOsmol fructose /Na-citrate solution at 37℃. And, the 30 min incubation time was superior to any other incubation time groups.

Hypoosmotic solutions made from 2 sugar (sucrose and fructose) and Na-citrate that were evaluated separately and in sugar-salt combinations were tested for their ability to induce swelling of the boar spermatozoa in this study. HOST conditions of a 150 mOsmol fructose and Na- citrate for 30 min at 37℃ were optimal, showing repeatable results. Jeyendran *et al.* (1984) suggested that at the same osmolality (150 mOsmol), solution (fructose and Na-citrate) affect the sperm membranes differently, but presumable sugars and electrolytes have a different influence on the in flux 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 evaluation methods (motility, viability, membrane integrity and LPO) used in this study for sperm viability were highly correlated in boar semen irrespective of status (fresh and frozen/thawed).

The results of this study indicate that the optimal conditions of HOST in boar spermatozoa is a 150 mOsmol fructose/Na-citrate solution with 30 min incubation at 37℃ and HOST might be utilized to evaluate sperm quality instead of motility, viability and lipid peroxidation.

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