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# Determination of the Functional Membrane Integrity of Mice Spermatozoa Using the Classic Hypoosmotic Swelling Test (HOST) and Modified Host

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***Abstract:***
*The hypo-osmotic swelling test (HOST) is widely used in determining sperm quality by evaluating membrane integrity in both human and animal spermatozoa. The test measures the swelling ability and membrane integrity of functional sperm in hypo-osmotic solutions. The ability of the sperm membrane to maintain equilibrium between itself and the environment indicates a functional and intact plasma membrane. This functionality is depicted by the coiling and ballooning or "swelling" of the sperm tail when influx of fluid occurs due to hypo-osmotic stress. The modified HOST solution consists of Earle's Buffer Salt Solution (EBSS) while the classic HOST solution consists of sodium citrate and fructose. It has been reported that modified HOST solution show more rapid sperm tail swelling and better viability in human sperm cells. This study aims to compare the swelling ability and viability of mouse sperm upon incubation in classic HOST medium and modified HOST medium. Iso-osmotic solution was used as negative control, while distilled water acts as positive control. Caudal epididymal sperm from twenty mature male mice were collected into Kreb Ringer's Buffer medium and divided into 4 batches, one for each test solution. Sperm were pooled and discarded of cell debris and centrifuged in a discontinuous Ficoll gradient (45% and 90%). Sperm were collected at the pellet and incubated for 60 minutes in the respective test solutions. Tail swelling and viability were observed from smears taken at intervals of 5 minutes. ANOVA analysis showed no significant difference between the classic HOST and modified HOST in sperm 'ballooning' and sperm viability. The results suggests that using modified HOST showed no significant improvement from classic HOST in functional membrane integrity changes in mouse spermatozoa as compared to human spermatozoa.*

***Keywords:*** *Hypo Osmotic Swelling Test (HOST), mice spermatozoa, viability, functional membrane, modified HOST*

## 1. Introduction

Mice models especially the BALB/c strain have been used extensively in studying the mechanisms of human physiology and also drug discovery. However there is little information on the research that was conducted on this particular strain especially for sperm quality assessment using the hypoosmotic swelling test (HOST). In this study, BALB/c mice were used to study the morphology of spermatozoa after incubation in different hypoosmotic test media. Modifications to HOST were made to compare its effectiveness and viability of sperm to the classic HOST. Basically HOST is a test to evaluate sperm quality based on its membrane integrity. HOST was developed using a classic HOST solution by Jeyendran et. al., in1984. It uses the principle of osmotic equilibrium where under hyposmotic stress, cells with a biochemically active membrane will regulate the osmotic movement and experience an increase in size and swelling. In nature, sperm cells experience a hypoosmotic condition when ejaculated sperm from the male reproductive tract enter the female reproductive tract (Joseph, et. al., 2010). In human, migration of sperm to female genital tract experiences a natural osmotic reduction (from 330 mmol/kg to 290 mmol/kg) (Yueng, et. al., 2003: Liu, et. al., 2017). In mice, sperm in the female reproductive tract will experience a sudden decrease in osmolality (415 mmol/kg in cauda epididymis to 310 mOsm in the uterine cavity)(Joseph et. al., 2010; Chen et. al., 2011). To compensate the decrease in osmolality, the regulatory volume decrease (RVD) mechanism will protect the cells from detrimental swelling (Cooper and Yueng, 2003).Ion channel pathway such as CIC-3 chloride channel is found to be highly expressed in the entire flagellum of healthy human sperm (Liu et al., 2017). Water channel proteins or aquaporins also play a role in this RVD and AQP8 was found to regulate the cell volume regulation of sperm (Yeung, 2010)while AQP3 is the key fluid regulator for RVD during the male to female genital tract transition protecting sperm from swelling-induced mechanical stretch (Chen et. al., 2011).Sperm fertilizing capability can be determined by evaluating the membrane integrity under hypoosmotic stress. The classic HOST was further improved in recent years to reduce the incubation time of sperm samples in the hyposmotic solution. A variety of modified HOST have been carried out to determine the best osmolality and incubation time of the hypoosmotic solution in various species (Zubair, et. al., 2015). The sugar-based components of classic HOST which are

fructose and sodium citrate dehydrate were replaced with different chemicals to lower the osmolality as reported by Verheyen et. al., (1997). Certain studies also suggested suitable modifications of HOST that suits very low sperm count samples(Ahmadi, 1997). The incubation time of sperm samples in the HOST medium were also reduced as reported by Prinosilova, et. al., (2014). Decrease in incubation time will reduce the irreversible damage to sperm before carrying out further treatment especially for in vitro fertilization (IVF). Thus the osmolality of HOST should be able to induce observable swelling in cells however it should not exceed the threshold of the osmotic stress that could be borne by the sperm to prevent lysis. Even though sperm in every species have the same function which is to fertilize the egg, however, the composition of membrane and structure of sperm varies. Thus, there is no one-fit-all osmolality of HOST even though the procedures of the test could be similar. In this research, comparison between classic HOST with modified HOST which consists of Earle's Balanced Salt Solution and distilled water was carried out on mice spermatozoa. The study is aimed to evaluate the correlation between incubation time and tail swelling from the observation of sperm morphology at each time interval and to determine the optimal time for maximal proportion of tail swelling throughout 30 minutes of incubation.

## 2. Materials and Method

Methods used were according to Verheyen et. al., (1997) and Jeyendran et. al., (2009) with some modification to suit laboratory facilities for the evaluation of the effects of classic hypoosmotic test (HOST) and modified HOST.

Sexually mature male BALB/c mouse were between 15 to 20g with the presence of normal scrotum and testes. A total of 20 mice were used in this experiment and divided into 4 batches equally, one for each test solution. Mice were given mouse pellets, drinking water ad libitum, adequate space for freedom of movement and normal postural adjustments. The beddings in the cages were changed twice weekly to ensure a clean and dry environment.

### *2.1. Preparation of Various Test Media*

There are four hypoosmotic solutions used in this study: (A) Classic HOST solution (Jeyendran et al., 1984) ; (B) Modified HOST solution composed of Earle's Balanced Salt Solution (Verheyen et al., 1997) ; (C) Isoosmotic solution as positive control (Jeyendran et. al., 1984) and (D) Distilled water as negative control.Solution A was prepared by mixing 1g of fructose ($C_6H_{12}O_6$) with 0.7g of tri-sodium citrate 2-hydrate ($C_6H_5Na_3O_7.2H_2O$) in 100mL of distilled water. The osmolality was approximately 150±15 mOSM/kg. Solution B was composed of Earle's Balanced Salt (Sigma Ltd) dissolved in 100mL of distilled water. Osmolality for solution B was approximately 280±10 mOSM/kg. Solution C was made up of 3.7g of tri-sodium citrate 2-hydrate in 100 mL of distilled water, with the osmolality of 300 mOSM/kg (isosmotic medium) which mimic the osmolarity in female mice. All solutions were stored in refrigerator at 4°C and pre-warmed in water bath at 37°C prior to the experiment.

### *2.2. Collection of Cauda Epididymal Sperm*

Mice were sacrificed with chloroform overdose. Both testicles were incised and removed carefully. Cauda epididymis ducts were removed and placed in a petri dish containing Kreb's Ringer Bicarbonate (KRB). KRB serves as a medium that create an isotonic solution relative to the sperm of mice. This could maintain the viability and motility of the spermatozoa. The cauda epididymis ducts were minced using mayo scissors for the spermatozoa to swim out into the buffer. Sperm samples for each test solution were pooled from a batch of mice.

### *2.3. Isolation of Spermatozoa Using Ficoll-Paque PLUS Density Gradient*

Ficoll-Paque PLUS (GE Healthcare Co., USA) is used to separate the motile and immotile spermatozoa according to Highland et al. (2016) who use it for human spermatozoa. This method uses the centrifugation method of semen over density gradients of Ficoll-Paque PLUS which separate cells by their density and the motile spermatozoa swim through the gradient, and settle down as a soft pellet at the bottom of the tube. A two-step discontinuous density gradient was prepared with a 45% (v/v) density top layer and 90% (v/v) Ficoll-Paque PLUS density lower layer. Ficoll was pre-incubated at 37°C to avoid cold shock to sperm. Approximately 2mL of sperm samples was layered on the Ficoll top layer in a Falcon tube. The tube was centrifuged at 300g for 15 minutes at room temperature. The Ficoll fractions (both 45% and 90%) were aspirated, leaving approximately 500µL of the final pellet that contained motile spermatozoa at the bottom of the tube.

### *2.4. Incubation of Spermatozoa in Various Test Media*

Washed sperm pellet were mixed with each of the hypo-osmotic solutions and incubated at a temperature of 37°C for 30 minutes. Morphology and viability of spermatozoa was assessed at 5 minute intervals.

### *2.5. Eosin- Nigrosin Staining for Sperm Viability*

At each time interval, approximately 1µl of sperm suspension was placed on a glass slide and stained with 1µl of Eosin-Nigrosin. This staining method was chosen as it is suitable to assess the vitality of sperm sample with motility less than 25%. Nigrosin's role is to increase the contrast between the sperm and background which ease the visualization process; Eosin stains the dead sperm dark pink while live sperm remain white (Agarwal et. al., 2016).. The slide was smeared at the angle of 45°, air-dried and viewed under light microscope. Smear slides for each time interval were triplicated for assessment of the percentage of viability and tail morphology.

*2.6. Evaluation of Tail Swelling Morphology*

Approximately 100 spermatozoa for each time interval were observed carefully at 400x magnification under compound microscope (B-350 Series, Optika Co., Italy). Interpretation of tail swelling morphology was based on Jeyendran, et. al., (1984).

*2.7. Statistical Analysis*

All data for each group were analyzed statistically by using IBM® SPSS® Statistics software (version 22 for Windows). Data were expressed as means ± standard error (%). Skewness and Kurtosis of variables were tested for normality distribution, Analysis of Variance (ANOVA) with multiple variances were used for statistical analysis for the significant difference between groups. Statistical significance was set at $P<0.05$. Pearson's correlation was used to determine correlation between viability and time.

**3. Results and Discussions**

Approximately 100 sperms were counted for each solution every 5 minutes up to 30 minutes. According to Jeyendran et al. (2009), normal ejaculates should contain more than or equal to 60% of HOS-positive spermatozoa and abnormal ejaculates should contain less than 50% of HOS-positive spermatozoa.

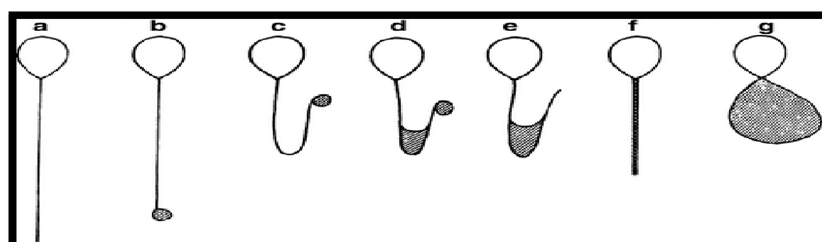
*3.1. Morphology of Tail Swelling*

A few categories of abnormalities has been observed from the samples, A) Head abnormalities which included decapitated heads, blunt hooks and amorphous head; B) Distal cytoplasmic droplets and proximal cytoplasmic droplets; C) Tail abnormalities including shortened tail piece, folded tails and other abnormalities exclusive of the "ballooning" and coiled tail sperm. All these abnormalities were not counted as HOS positive sperm reaction. HOS positive sperm were counted by referring to the sperm patterns according to Jeyendran et. al., (1984) and Ramu and Jeyendran, 2013). Mice sperm reactions were slightly different as compared to human spermatozoa, but quite similar to that in rat spermatozoa. This might be due to the difference in sperm and membrane structure.

*3.2. Reaction in HOS Medium*

HOS positive sperm tails include the "balloon" at the tip of the tail, and coiled tail sperms. Figure 1shows type of tail swelling of human spermatozoa in HOST medium according to Ramu and Jeyendran, 2013 while Figure 2 shows type of tail swelling in paddy rat spermatozoa in HOST medium (Ong and Sabrina unpublished data) where type I is the normal sperm tail, type II is shortened sperm tail, type III and IV are the optimal tail swelling referred to as "ballooning" and type VI to VIII are coiled sperm tails and are irreversible responses. Type III until type VI are the most common HOS positive reaction types observed in mice sperm samples under 100x magnification.

Figure 1: Morphological Changes of Human Spermatozoa Subjected to Hypoosmotic Stress. Adapted from Ramu & Jeyendran (2013).




*. Figure 2: Morphological Changes of Paddy Rat Spermatozoa Subjected to Hypoosmotic Stress Adapted from ONG and Sabrina (Unpublished Data)*

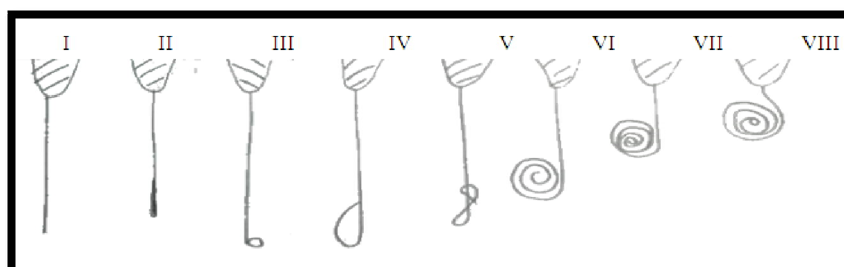



*Figure 3*

3.2.1. Comparison of HOS Positive Spermatozoa between Test Solutions

From Figure 4, both classic HOST medium and modified HOST medium had a bell-shaped pattern for HOS positive reactivity throughout the incubation time. Sperm incubated in classic HOST medium reached a peak at 10 minutes and decreased with prolonged incubation; while for sperm in modified HOS medium reached the peak at 15 minutes and decreased gradually. The same pattern could be seen in swollen tail sperm (ballooning tail) for both classic HOS medium

and modified HOS medium. However, as time increases, percentage of HOS positive sperm value did not show any significant correlation.

3.2.2. Comparison of Swelling Spermatozoa between Test Solutions

Type III and type IV (Figure 2) showed the best tail swelling. Both test solutions did not show remarkable difference in number of welling spermatozoa throughout the 30 minutes incubation. Based on the Figure 4, sperm incubated in classic HOS medium showed highest tail swelling number at 10 minutes, and later on fluctuated with prolonged incubation. While for modified HOS medium, sperm showed the highest number of tail swelling at 0 minute and 15 minutes. No correlation was found between these two HOSTS solutions in terms of sperm swelling as the difference of optimal incubation time was only 5 minutes.

3.2.3. Optimal HOS Test Incubation Time

Optimal incubation time for each medium was taken based on the highest tail swelling number throughout the 30 minutes incubation time. Classic HOS medium had a shorter incubation time (10 minutes) compared to modified HOS medium (15 minutes). In other words, classic HOS medium is better than modified HOS medium.

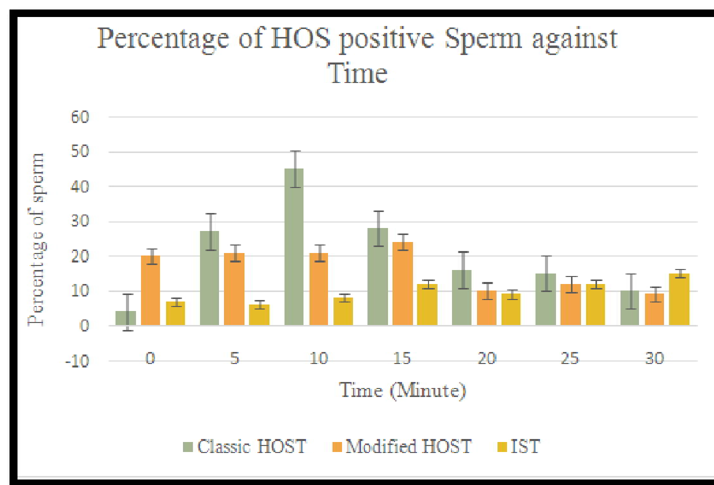



*Figure 4: Sperm HOS Positive Percentage in Various Test Media*

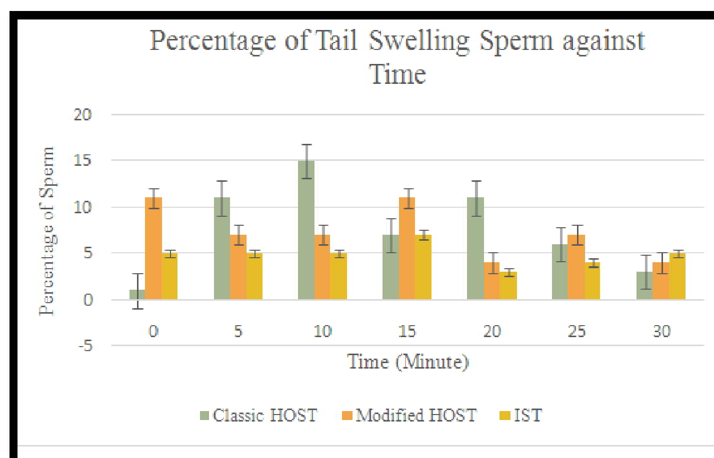



*Figure 5: Tail Swelling Percentage against Time in Various Test Media*

*3.3. Evaluation and Interpretation of Non-Viable and Viable Spermatozoa*

Eosin-Nigrosin staining was used to observe the sperm viability and morphology. Well-functioned sperm membrane would not take up the staining while non-viable sperm would be stained a dark pink colour. Some of the sperm heads were stained partially indicating the sperm were dying. Only fully-stained sperm head were counted as dead spermatozoa, and fully clear or unstained sperm head were counted as live spermatozoa.

3.3. 1. Correlation between Time Factor and Sperm Viability

The result suggests that the 30 minutes exposure significantly is associated with the reaction of sperm in all the solutions ($p<0.05$) as seen in Figure 5. Sperm viability of classic HOST was 48.29±7.5; modified HOST was 32.71±6.4 and for positive control was 17.86± 1.7. By using Pearson's correlation test, viability against time showed the value of r= -0.494

indicating that 49.4% of the decrease of sperm viability may be due to the increase of time in general. Nevertheless, when the correlation was compared between solutions, only modified HOST showed correlation between time and sperm viability.

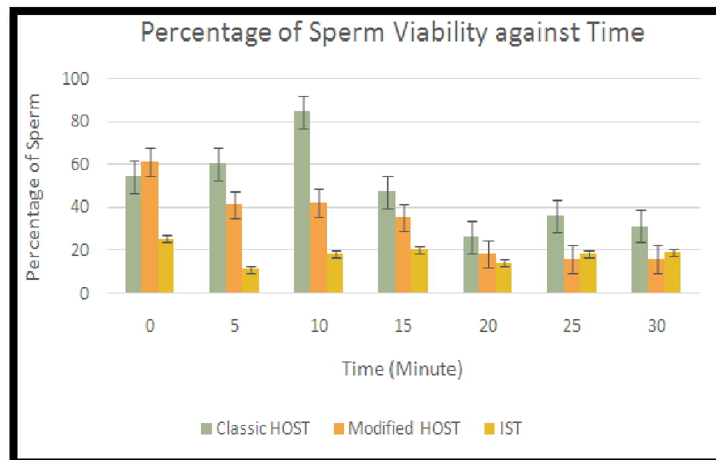



*Figure 6: Sperm Viability Percentage in Various Test Media*

The results indicated that all sperm undergo maximum tail swelling by 30 minutes incubation ($p<0.05$)in both classic HOST and Modified HOST solutions. The optimal incubation time of solutions were determined by the highest number of best swelling (type III and type IV) spermatozoa. The result suggests that classic HOST (10 minutes for maximum tail swelling)is better than modified HOST (!5 minutes for maximum tail swelling) with a difference of 5 minutes. Functional sperm membranes have the ability to coil and swell under hypoosmotic stress. The tail swelling and coiling were caused by the spherical expansion of tail piece, inducing the flagellum of inner membrane to coil (Jeyenderan et. al., 1984). Sperm with a non-intact membrane do not have the regulatory volume mechanism that served to maintain the osmotic equilibrium of the inner membrane. As the incubation time prolonged, it is believed that the sperm membrane will be gradually damaged and result in leakage where the coiled tail occurred as a reaction to empty the membrane. A long period of hypoosmotic stress will cause irreversible effects on the sperm cell and eventually lead to cell death (Buckett, 2003). Sperm viability is also significantly ($p<0.05$) related to time. There was a moderate correlation between time and sperm viability indicating the sperm viability decrease along the incubation time. However, there is no significant relationship between Classic HOST and Modified HOST in terms of sperm viability. Sperm viability was assessed by the Eosin-Nigrosin staining method. Eosin-Nigrosin was known for its effective staining of slides where Nigrosin stained the background to ease the observation process and Eosin stained dead cells (Agarwal et. al., 2016). Nevertheless, numerous swelling tail pieces were observed in dead spermatozoa. The staining method may be accounted for this condition where stain might dissolved into the spermatozoa with prolong staining period, indirectly affecting the association between sperm viability and types of solution subsequently giving a false negative result (Ong& Sukardi, unpublished data). Interpretation of result for viability due to the nature of the acrosome structure of mice sperm (hooked shape) makes interpretation of dead sperm quite tricky. The plane of the sperm acrosome upon staining may be folded or flattened and this may cause possible errors in interpretation.

Ficoll gradient (45% and 90%) was used to separate the viable spermatozoa from cell debris and formed a pellet at the bottom of the tube (Highland et al., 2016). This method was used for human sperm. Usually a two-step discontinuous Percoll gradient is used for rat or mouse sperm in most papers reviewed. The centrifugation process might somehow induce certain level of damage to the sperm samples. From the slides, certain sperm pieces were observed in which the sperm were torn apart leaving only the head or tail piece. Swelling tail pieces were found but without the sperm head therefore they were excluded in the count. Smearing of the slides too may be the cause of tail piece detached from the sperm and lead to false positive or false negative result.

Earle's Balanced Salt Solution used in this study should be replace with Earle's Medium solution as used in Verheyen's study (1997). The Earle's medium consists of 1% human serum albumin and 0.48% (v/v) of HEPES, which results in a final osmolality of 139 mosmol/kg (Verheyen et al., 1997). For mice species, human serum albumin could be replaced with bovine serum albumin with some modifications in the osmolality of solutions. HOST is a simple test which can be performed almost in every laboratory and provides crucial information of the fertilizing capacity of spermatozoa. HOST evaluates the membrane integrity of sperm membrane which is a better information than classic parameters of semen analysis (Jeyendran et al., 1984). Sperm membrane integrity is not only crucial as a divider between intracellular and extracellular spaces, but also an intimation of DNA stability (Bassiri et. al., 2013). Therefore, this test is able to create value not only in human andrology but also in animal anthology. For further improvisation of modified HOST on mice species, a combination of several tests targeting different aspects of sperm structural and biochemical functions would produce more reliable results. For mice species, most cauda epididymal sperm samples have a low sperm count and HOS medium might dilute the sperm causing insufficient sperm volume to perform ICSI. With the Single Sperm Curling (SCC) test, it could help in the viable sperm selection in low sperm count samples (Ahmadi, 1997).

A high percentage of standard error in the variables were due to a small sample size used in each batch which may be the factor of increased variation especially in quantitative study and produce false positive or false negative results. This suggests that the statistical result may not reflect the accuracy of the test solutions. A bigger sample size should be used in further study in mice species to reduce the possible statistical error.

**4. Conclusions**

The sperm viability of mice spermatozoa in classic HOST and modified HOST is reduced with prolonged incubation time. Modified HOST results showed a correlation between time and sperm viability whereas other solutions did not show any significant correlation. Compared to classic HOST, sperm viability in modified HOST decreased more rapidly throughout the 30 minutes of incubation. Sperm tail swelling increased until an optimal time and then decreased gradually. Sperm tail swelling is significant at 10 minutes for classic HOST and 15 minutes for modified HOST i.e., curling is much more resistant in modified HOST medium rather than in classic HOST medium. The results suggests that using modified HOST showed no significant improvement from classic HOST in functional membrane integrity changes in mouse spermatozoa as compared to human spermatozoa.

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