COMPARISON OF DIFFERENT OSMOLARITIES AND SUGAR-SALT SOLUTIONS FOR HYPO-OSMOTIC SWELLING TEST OF FROZEN-THAWED WATER BUFFALO SPERMATOZOA

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ABSTRACT

Hypo-osmotic swelling test (HOST) is a method of assessing the functional integrity of sperm plasma membrane which in turn was claimed as indicator of sperm fertility. Standard protocol of HOST has been established in different species but not in buffaloes. Thus, the current study was conducted to establish the HOST assay for buffalo frozen-thawed sperm cells. Frozen-thawed sperm cells were incubated in different osmolalities (0, 50, 100, 150, 200, 250, and 300 mOsm) of sodium citrate-fructose solution to assess sperm reaction. Then, the effectiveness of sucrose vs. fructose was compared as sugar component of the HOST solution. Results showed higher ($P<0.05$) number of HOS positive (+) spermatozoa in 150 mOsm compared to 0, 250 and 300 mOsm/L though no significant difference was observed with 50, 100, and 200 mOsm/L. Sucrose- and fructose-containing solutions are both effective in enhancing swelling among treated spermatozoa. The results demonstrated that 150 mOsm is the efficient osmolality to effect optimum reaction of frozen-thawed buffalo spermatozoa and that either sucrose or fructose

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could be used for HOST solution to assess the functional membrane integrity of buffalo sperm cells.

Keywords: fructose, HOST, plasma membrane, spermatozoa, sucrose

INTRODUCTION

The reliability of semen quality analysis is important for artificial insemination and in vitro embryo production (IVEP) as these reproductive biotechnologies require good quality semen to produce offspring. In the preparation of semen, cryopreservation is highly important to allow AI and in vitro fertilization (IVF) at the most convenient time. However, assurance of the post-thaw sperm quality is not guaranteed which is one of the major factors affecting the success of AI and IVF. There are a number of techniques available to check sperm viability (Partyka *et al.*, 2012); however, there is a need to explore cheaper methods of reassessing sperm quality of post thawed spermatozoa.

The hypo-osmotic swelling test (HOST) is presently one of the cheapest techniques used for reexamining sperm quality (Santiago-Moreno *et al.*, 2009). In HOST, sperm membrane integrity is examined where the spermatozoa are exposed in hypo-osmotic conditions that cause swelling to functional spermatozoa to achieve osmotic equilibrium. Earlier studies indicate that HOST is more applicable in the prediction of the fertilizing capacity of frozen-thawed semen because sperm membrane integrity in cryopreserved seminal sample is a limiting factor compared to fresh semen (Colenbrander *et al.*, 2003).

In water buffaloes, establishment of HOST in frozen-thawed spermatozoa is still a question. Earlier studies used HOST solutions and procedures adopted from other livestock species. In fresh rabbit spermatozoa, sucrose solution with osmolarity of 60 mOsm/L (Amorim *et al.*, 2009) were used while in equine spermatozoa, fructose, sucrose and lactose at 100, 50, and 25 mOsm, respectively, were utilized (Neild *et al.*,1999). Boar sperm cells incubated in a mixture of fructose and sodium citrate under 100-150 mOsm/L gave an identifiable swelling and coiling of the tails (Vasquez *et al.*, 1997). In rams, 100 mOsm/L sodium citrate

fructose HOS solution (9.0 g fructose and 4.9 g sodium citrate in distilled water) was best (Farshad *et al.*, 2010).

The desired assay conditions specific for water buffaloes is not yet fully established, but HOST solutions used in many species such as the sodium citrate/fructose solution (Lodhi *et al.*, 2008) and sucrose (Amorim *et al.*, 2009) were used in Nili Ravi bulls. Previously, Hufana-Duran *et al.* (2012) reported that 37°C is significantly better that 25°C in enhancing hypo-osmotic swelling test in water buffalo with effect commencing after 15 min exposure. No significant difference in sperm reaction was observed when exposure time was extended until 90 min and slightly decreased when exposure time was extended to 120 min suggesting that extended exposure may cause a reversible reaction.

With these initial results, it is the interest of this study to assess the appropriate osmolality and compare which among the two sugar-salt solutions (fructose-sodium citrate vs. sucrose-sodium citrate) is efficient for the assessment of sperm membrane integrity of frozen-thawed water buffalo spermatozoa to establish the standard HOST in this animal species.

MATERIALS AND METHODS

Seminal sample

Frozen semen of water buffalo bulls processed and distributed by the Semen Processing Laboratory of the Philippine Carabao Center at Carranglan, Nueva Ecija and is stored at Reproductive Biotechnology Laboratory for in vitro fertilization purposes were used in this experiment.

General procedure

The semen samples were thawed at 39°C for 15 sec, and the cryoprotectant was washed by layering the thawed semen in 39°C pre-warmed Brackett and Oliphant medium (BO medium, Brackett and Oliphant, 1975) and subjecting it to centrifugation at 1,500 rpm for 5 min. The supernatant was discarded leaving the sperm pellet which was then disturbed to mix well and then subjected to various treatments. To determine the quality of the semen sample used in the experiments, percent motility, percent live and dead and percent abnormality were examined after thawing and after centrifugation. The latter two abnormalities were determined by eosin-nigrosin staining technique.

Assessment of sperm motility. To assess sperm motility post-thawing, pre-warmed (37°C) microscope slides, glass coverslips, disposable glass or plastic pipettes and a vial of phosphate buffered saline was used and these were prepared and equilibrated at 37°C prior to thawing of the frozen semen. After thawing at 39°C, a 10 µl sample of the semen was taken by a pipette dispenser and analyzed for motility.

Assessment of sperm viability. Sperm viability was assessed to provide information on the percent live and dead spermatozoa in the seminal sample. This was done by the eosin-nigrosin staining technique as earlier described by Hufana-Duran *et al.* (2010).

Assessment of sperm abnormality. Percent abnormal sperm was taken from the untreated seminal sample to provide baseline information on the incidence of sperm abnormality. This data was deducted from the data gathered in the analysis of hypo-osmotic swelling test. Using the eosin-nigrosin stained seminal sample, sperm abnormality was recorded. Usually 200 sperm cells were counted and the total percentage morphologically normal sperm was recorded.

Effect of osmolality of the solution on swelling of the sperm cells. To determine the effect of osmolality of the solution on HOS reaction of the sperm cells, sperm cells were exposed to 0, 50, 100, 150, 200, 250, and 300 mOsmol fructose and sodium citrate solution at 37°C and 15 to 90 min exposure. The osmolality of solution that gave the highest HOS reaction was used in the succeeding steps.

Effect of sucrose or fructose in combination with sodium citrate as HOST solution. To determine the effect of sugar in the HOST solution, sucrose and fructose were compared. Using the best osmolality of the solution determined previously at 37°C after 15 to 45 min of exposure, the sperm cells were exposed to two treatments, *i.e.* sucrose vs. fructose in combination with sodium citrate and the HOS reaction were evaluated.

Hypo-osmotic swelling test (HOST)

Preparation of the combination of sugar-salt solution was conducted by mixing the same proportions of sugar and salt solution. The 300 mOsmol/L sodium citrate (MW=294.1) and fructose (MW= 180.16)-based HOS solution was made by dissolving 14.7 g of sodium citrate and 27.02 g fructose in 1000 ml of distilled water. Meanwhile, 300 mOsmol/L sodium citrate-sucrose was made by combining 14.7 g of sodium citrate and 51.345 g of sucrose (MW=342.3) for every 1000 ml of distilled water. Using serial dilutions with double distilled water, preparation of different osmolality on HOS solutions were made. Of the dense sperm sample after centrifugation and removal of supernatant, 10µl was taken and was added to 40µl of pre-warmed (37°C) HOS solution in a 1.5 mL microcentrifuge tube. For the HOST analysis, a small drop of sample was placed on a clean glass slide pre-soaked in ethanol overnight at refrigeration temperature and was covered with cover slip. Slides were examined at 400x using phase contrast microscopy (Nikon E200 phase contrast microscope, Nikon Instruments, Tokyo, Japan). A total of 200 spermatozoa were observed for changes associated with swelling based on the appearance of swelled sperm cells described by Foncesca *et al.* (2005). The percentage of HOS (+) spermatozoa (number of spermatozoa with coiled tails out of swelling and not due to abnormality, per total number of spermatozoa examined x100) was recorded for each sample. Figure 1 presents the appearance of HOST reactive sperm cells.

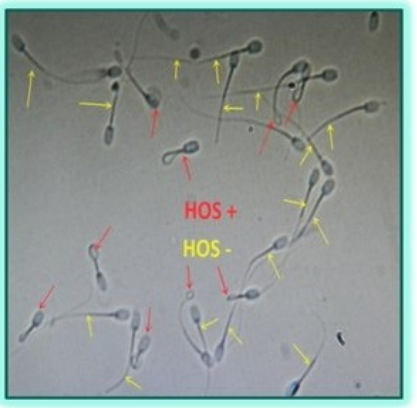

Figure 1. Appearance of HOST reactive sperm cells. Red arrow defects HOST(+) sperm cells (with coiled tails). Blue arrow are non-reactive sperm cells or HOST (-) sperm cells.

Statistical analysis

Data were analyzed using ANOVA for a completely randomized design to determine the effect of different osmolality of the HOS solutions on the seminal sample. Tukey's Multiple Comparison Test was used to compare the means among the two sub-studies and determine the statistical difference among treatment groups. Averages, frequencies, percentages and standard deviations were used to express the results.

RESULTS AND DISCUSSION

Initial semen quality analysis showed an average of 43.5±2%, 70.19±8.7%, and 26.05±8.2% post-thawing motility, livability and abnormality, respectively (Table 1). After 5 min of centrifugation at 1,500 rpm, a noticeable increase in the percent abnormality (29.74±6.7%) and decrease in the sperm motility (27.6±7%) and livability (62.52±13.5%) were observed. The results showed the quality of the seminal sample used in the present study.

Table 1. Percent motility, livability, and abnormality post-thawing and after centrifugation of frozen-thawed water buffalo spermatozoa used in the experiment.

Study	Post-thawing, %			After centrifugation, %		
	Motile	Live	Abnormal	Motile	Live	Abnormal
1	45.0	76.3	20.1	32.5	72.1	25.0
2	41.9	64.1	31.9	22.7	53.0	34.5
Ave.	43.5±2.0	70.2±8.7	26.1±8.2	27.6±7	62.5±13.5	29.7±6.7

Exposure of the sperm cells to increasing level of osmolalities showed optimum swelling of the treated sperm cells at 150 mOsm with 52.4% reactive sperm cells, which is higher (P<0.05) than at 0, 250 and 300 mOsm/L but not different to 50, 100 and 200 mOsm/L (Figure 2).

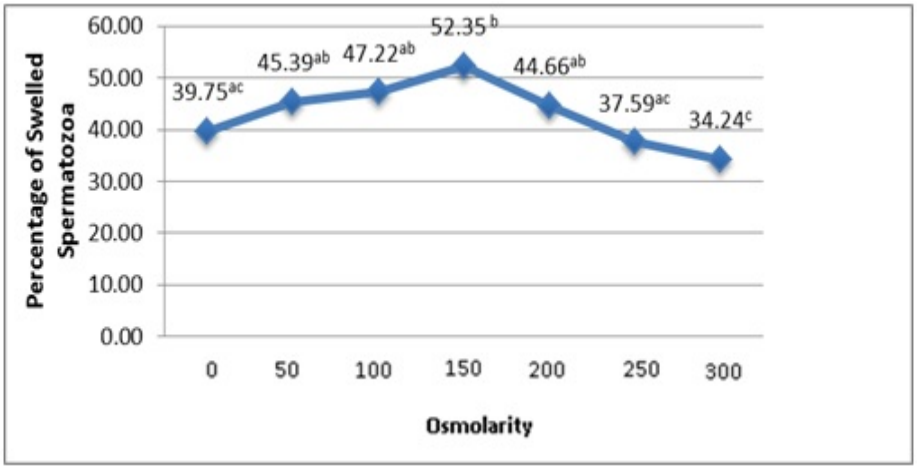

Figure 2. Effect of osmolality of solution on the swelling reaction of buffalo spermatozoa.

The least percent swelling was observed at 300 mOsm with 34.2% of HOS reactive sperm cells. This suggests that as the osmolality increases beyond the optimum concentration of non-permeable solutes in the ooplasm of the sperm cell, the number of spermatozoa with coiled tail decreases. These results support the earlier finding that the osmolality of water buffalo sperm cell is 261 mOsm/L (Khan and Ijaz, 2008) because osmolality higher than the amount of solutes in the cell would cause shrinking of the cell and not swelling. This is because the osmotic pressure outside the cell is higher causing the water inside the sperm cell diffuse to the external environment. This is why different osmolalities suited for various species were being identified. The highest percentage of sperm tail coiling was observed at 100 to 150 mOsm in boars (Vasquez *et al*., 1997) whereas 125 mOsm is optimum for fresh goat spermatozoa (Foncesca *et al*., 2005).

The absence of substantial difference between 50, 100, 150, and 200 mOsm could be due to the use of cryopreserved seminal sample compared to the fresh semen used in the previous studies (Amorim *et al*., 2009; Vasquez *et al*., 1997; Foncesca *et al*., 2005; Mandal *et al*., 2003; Iqbal *et al*., 2009; and Lodhi *et al*., 2008). The data imply that frozen-thawed water buffalo spermatozoa reacts easily to HOS solution and that HOST can be carried out using osmolality ranging from 50 to 200 mOsm/L with best reaction at 150 mOsmol/L.

In determining the best sugar for HOST, no significant difference in the percentage of HOS (+) spermatozoa was observed between fructose and sucrose (Table 2). Though fructose gave numerically higher reaction among treated spermatozoa, the lack of differences with sucrose could be due to the fact that they have the same properties. Fructose is commonly present in the semen and several studies conducted on HOST used sodium citrate-fructose as HOST solution that induced optimum swelling on fresh goat sperm cells (Foncesca *et al*., 2005), rams (Farshad *et al*., 2010), and human spermatozoa (Jeyendran *et al*., 1984). However, sucrose is also used as HOST solution in species such as stallion (Mansour, 2009; Nie and Wenzel, 2001) and fresh rabbit spermatozoa (Amorim *et al*., 2009). With nearly the same percentage of dead and mild swelling for both solutions, the results suggest that either of these sugars could be used in examining the functional integrity of the sperm plasma membrane.

Table 2. Comparison of the effect of sucrose and fructose in combination with sodium citrate as HOST solution.

HOST solution	Motile, %	Live, %	Swelled, %		HOST (+), %
			Mild, %	Severe, %	
Sucrose	25.55	37.80	14.92	21.73	36.65
Fructose	14.00	37.67	14.17	34.16	48.33

The mean percentage of HOS (+) sperm cells of the present study (48.33 %) is lower than 85.25% of HOS reactive sperm cells of Nili Ravi bulls observed by Lodhi *et al.* (2008) using the same osmolality and solution. Difference in the results may be due to the variation in the seminal sample used; Lodhi *et al.* (2008) used fresh semen while frozen semen was used in this study. Furthermore, the initial quality of the sperm cells used in the current study had high abnormality (29.74±6.7) and only 27.6±7% motile and 62.52±13.5% live sperm cells which contributed to such differences. Results also suggest that frozen-thawed water buffalo spermatozoa have lower functional membrane integrity compared to fresh bull semen (Lodhi *et al*., 2008), which confirms earlier claims (Bailey *et al.*, 2000) that cryopreservation can decrease the fertilizing capacity/fertility of the spermatozoa.

Interestingly, equating the 48.3% HOST(+) sperm in fructose-sodium citrate solution and the 62.52±13.5% live and 29.74±6.7% motile sperm observed from the initial semen quality

analysis shows that the percentage of HOST (+) sperm is higher than the motile sperm but lower than the live sperm cells. Since HOST is a test for membrane function that indicates the fertilizing capacity of spermatozoa (Jeyendran *et al.,* 1984; Smith *et al.*, 1992), the overall mean of HOS (+) spermatozoa revealed that some sperm cells with intact membrane are not motile. Rota *et al.* (2000) indicated that HOST does not appear to be sufficiently sensitive to discriminate between semen samples of intermediate fertility. Since the semen sample used in this experiment had high abnormality, the results confirmed that not all sperm cells that have intact membrane are motile. This suggests that the claim of sperm membrane integrity as an indicator for sperm fertility is still subject for further verification.

Based on the results, it was concluded that 150 mOsm of either fructose or sucrose containing solutions is optimum for HOST assay of post-thawed water buffalo sperm cells. Osmolality of 300 mOsm is high for HOST in water buffalo sperm cells while 50 to 200 mOsm solutions are acceptable. Since HOST was used to evaluate sperm fertility, it could be an effective and practical tool in screening bulls used for AI and IVF in water buffalo species. This technique could be used as routine analysis for semen samples of the bull and breeding farms conveniently. Results of the present study, however, need to be confirmed using data gathered on actual fertility record of bulls to evaluate its efficiency in predicting the fertility of bulls.

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