The Indian Journal of Animal Reproduction; 23(1): 8-11; June 2002,

Research Article

The hypo-osmotic swelling test : an assay of cell membrane integrity and quality of frozen thawed buffalo semen

H.C. PANT[1], A.K. MITTAL[2], S.H. PATEL[3], H.R. SHUKLA[4], R. KASIRAJ[5] AND J.H. PRABHAKAR[6]

Sabarmati Ashram Gaushala, Lali-387120, Kheda, Gujarat

Received : February 7, 2001
Accepted : January 18, 2002

ABSTRACT

Frozen-thawed semen from 10 buffalo bulls was subjected to hypo-osmotic swelling test (HOST) by incubating for 60 minutes at 37°C in a fructose/sodium citrate solution with an osmolarity of 100 mOsm kg^{-1}. On examination with a phase-contrast microscope (x400), a proportion of cells with intact membranes were found swollen characterized by bending, coiling or shortening of the tail. Some of these cells showed non-progressive flickering of the middle piece. Of the 10 bulls, one which had the lowest mean value for swollen spermatozoa following HOST also had the lowest fertility. It is concluded that the HOST could be useful in evaluating fertility of buffalo bulls in conjuction with other semen analysis parameters. Further studies should involve separate assessment of both inactive and active swollen cells and their relationship to fertility.

Key words : Hypoosmotic test, buffalo, semen quality, fertility

Routine semen evaluation methods (i.e. total number of spermatozoa, progressive motility and morphology) have limited accurcy in predicting the fertility of a semen samples (Bishop *et al.*, 1954; Linford *et al.*, 1976; Soderquist *et al.*, 1991). Accordingly, attempts are being made to develop *in vitro* tests capable of predicting fertility with greater accuracy. One such test is termed hypo-osmotic swelling test (HOST) which was initially developed to evaluate biochemical activity of the physically intact human sperm membrane (Jeyendran *et al.*, 1984). This test is dependent upon the positive correlation between coiling of the flagellum in response to lowered osmotic pressure and high fertilizing ability of the semen (Van der Ven *et al.*, 1986). This test has been developed for diverse mammalian species such as cattle (Correa and Zavos, 1994; Revell and Mrode, 1994), horses (Caiza de laCueva, 1997) and pigs (Gadea *et al.*, 1998). There was no report when this work was started on HOS test for buffalo spermatozoa. The objectives of this study were to develop a simple procedure for the HOS test for buffalo spermatozoa and to assess its effectiveness in predicting the fertility of frozen buffalo semen.

Corresponding author - [1]344 Jiwaji Nagar, Thatipur, Gwalior - 474 011, [2]Manager, [3]Deputy Manager, [4]Officer Grade I, Sperm Station, [5]Deputy General Manager Technical and [6]General Manager

MATERIALS AND METHODS

Semen from 10 Murrah buffalo bulls which was being collected once weekly for routine freezing was used for the HOS test. One to two 0.5 ml straws were thawed at 37°C for 1 minute. Samples of semen (100 µl) were incubated at 37°C for 1 hour in 1 ml of a hypo-osmotic solution having an osmotic strength of 100 mOsm kg^{-1}. An aliquot of semen under test was similarly incubated in an isoosmotic solution (control) with an osmotic strength of 300 mOsm kg^{-1} (Table 1). Following incubation, sperm swelling was estimated by examining a drop of sample on a glass slide covered with a cover

Table 1. Composition of solutions for HOS test

	HOS test solution	Control solution
Fructose (g)	0.99	5.4
Trisodium citrate (g)	0.49	2.94
Millipore water to	100 ml	100 ml
Osmolarity	100 m Osm kg^{-1}	300 ml Osm kg^{-1}

slip. A total of at least 200 spermatozoa were counted using a warm stage, under a phase contrast microscope at x 400 magnification. During the initial stage of standardization another drop of same semen was examined similarly. The total proportion of swollen spermatozoa was calculated by dividing the number of reacted cells by the total spermatoza counted in the same area and multiplying the figure by 100. The proportion of swollen spermatoza from a control sample was subtracted from this value.

Statistical analysis : The precision of the assay was examined by the standard deviations of replicate determinations from their means. This was calculated from the result of sixteen semen samples counted in duplicate and analysed by the method of Snedecor (1952) according to the formula :

$$\text{Estimate of S.D. ('S')} = \sqrt{\frac{\varepsilon d^2}{2n}}$$

Where 'd' is the difference beween the two values in a duplicate determination, and 'n' is the number of duplicate determination performed.

The data was subjected to analysis of variance after initial transformation to Arcsin (Steel & Torrie, 1960).

RESULTS AND DISCUSSION

The precision of the method examined by standard deviations of replicate determinations from their means is listed in Table 2 which shows the values 'S' and the coefficients of variations for 16 semen samples at two ranges. The precision expressed as coefficient of variation, for two ranges of HOS values ranged from 3.2 to 6.3 percent with an overall of 4.3 per cent.

The mean percentage of spermatozoa with swollen tails is listed in Table 3. The mean values for ten bulls ranged from 22.6±3.9 to 58.8±6.3 with an overall mean of 45.0±1.4. Analysis of variance of the data revealed significant difference between ejaculates ($P<0.05$) and between bulls ($P<0.01$). These results cearly indiate that frozen-thawed buffalo spermatozoa react to hypo-osmotic swelling test in a manner similar to that reported for human (Jeyendran *et al.*, 1984), bovine (Correa and Zavos, 1994; Revell and Mrode, 1994), equine (Neild *et al.*, 1999) and canine (Rodriguez-Gill *et al.*, 1994) spermatozoa. Our results are in agreement with a recent report of buffalo spermatozoa

Table 2. Precision of duplicate estimations of HOS responsive spermatozoa

Range (%)	No. of samples	Mean	S.D.	Coefficient of variation (%)
20-55	8	40.6	2.55	6.3
56-90	8	68.8	2.18	3.2
Overall	16	54.7	2.38	4.3

Table 3. Mean percentage of frozen-thawed buffalo spermatozoa swollen to hypo-osmotic swelling test (HOST)

Bull No.	Mean±S.E.M.	n	Range
1	47.5±3.9	10	21.1-64.0
2	47.5±6.1	10	16.5-69.0
3	30.5±3.8	10	14.0-52.6
4	47.7±3.1	10	22.2-57.5
5	22.6±3.9	10	07.8-51.1
6	57.5±4.4	5	45.3-71.0
7	55.2±5.5	5	42.0-72.2
8	58.8±6.3	10	16.1±71.5
9	52.3±3.9	5	39.4-62.1
10	45.4±3.5	5	36.1-52.0

(Pratap *et al.*, 2000). These investigators examined the reactivity of both fresh and frozen-thawed Murrah buffalo spermatozoa to HOST. Their mean percentage of swollen spermatozoa following HOST post-thaw were 45.0±0.44, 57.5±0.48 and 45.5±0.41 following conventional freezing, moderate rate and slow rate of programmable, freezing, respectively. However, unlike present study, these workers did not report variation between bulls or between ejaculates within a bull.

In our study we employed a solution of an osmolarity of 100 mOsm as against 150 mOsm mg^{-1} used by Pratap and associates (2000). Others have shown that a 100 mOsm solution of fructose and sodium citrate is appropriate for hypo-osmotic swelling test of frozen thawed bovine spermatozoa (Correa and Zavos, 1964; Revell and Mrode, 1994), while a 150 mOsm solution is suitable for neat semen (Revell and Mrode, 1994).

Our overall mean percentage of swollen spermatozoa was similar to that reported by Pratap *et al.* (2000) despite the fact that these workers did not employ a control sample in which an aliquot of semen sample has to be incubated with an iso-osmolar solution and the proportion of swollen spermatozoa in the control are substrated from the calculations (Correa and Zavos, 1994). It is therefore reasonable to speculate that a 100 mOsm kg^{-1} solution may be more appropriate for conducting a HOST on frozen-thawed buffalo spermatozoa. Further studies are needed to determine the effectiveness of HOST together with supravital test as a means of evaluating the functional integrity of frozen-thawed buffalo spermatozoa using solutions of osmolarities varying from 50 to 300 mOsm. This would help to determine the correct osmolarity of the solution that would result in maximal number of clearly identifiable swollen spermatozoa.

In the present study, in all semen samples examined, some of the swollen spermatozoa were found to show non-progressive motility characterized by varying intensity of a flickering of the midpiece. This is in agreement with an earlier report for bovine spermatozoa (Revell and Mrode, 1994). It is suggested that in future studies separate count may be done for active and inactive swollen sperm as a high proportion of active swollen spermatozoa may show higher correlation with fertility.

Although in this study semen samples with considerable variation in their HOST reactivity were not used for a fertility trial nevertheless we compared the overall mean HOST values with the fertility of these bulls assessed on a field-based progeny testing programe. Interestingly, bull # 5 which had the lowest mean value of 22.6±3.9 per cent for swollen spermatozoa following HOST had the lowest fertility (pregancy rate 44.0 per cent as against the pregnancy rate ranging between 51.0 to 62.0 per cent for the remaining bulls). In the homosapiens, Jeyendran *et al.* (1984) found a close correlation between the percentage of swollen sperm cells and the percentage of denuded hamster oocytes penetrated by capacitated spermatozoa of the same sample. In the bovine, Revell and Mrode (1994) found a high correlation (0.79) between HOS test results and fertility (expressed as non-return rate) when the bull effect was removed. However, Rota *et al.* (2000) failed to find any correlation between *in vitro* fertility and HOS test results of bovine spermatozoa. The HOS test evaluates membrane function, while several variables may be involved in fertility potential. This may account for the discrepancy in the results of fertility trial referred above. Further studies are needed to ascertain if the HOS test is effective in predicting the fertility potential of bulls of extremely high or low fertility.

In conclusion, the HOS test is a simple procedure which could be useful in evaluating quality of frozen buffalo bull semen and in predicting its fertility in conjunction with other semen analysis parameters as suggested earlier for cattle (Correa and Zavos, 1994; Revell and Mrode, 1994). Further studies may involve assessment of both active and inactive swollen cells and their relationship to fertility.

REFERENCES

Bishop, M.W.H., Campbell, R.C., Hancock, J.L. and Walton, A. (1954). Semen characteristics and fertility in the bull. J. Agric. Sci., 44: 227-248.

Caiza de la Cueva, F.I., Rigau, T., Bonet, S., Miro, J., Briz, M. and Rodriguez-Gill, J.E. (1997). Subjecting horse spermatozoa to hypoosmotic incubation : effects of incubation. Theriogenology, 47: 765-784.

Correa, J.R. and Zavos, P.M. (1994). The hypoosmotic swelling test : its employment as an assay to evaluate the functional integrity of the frozen-thawed bovine sperm membrane. Theriogenology, 42: 351-360.

Gadea. J., Matas, C. and Lucas, X. (1998). Prediction of porcine fertility by homologous *in vitro* penetration (hIVP) assay. Anim. Reprod. Sci., 54: 95-108.

Jeyendran, R.S., Van Der Ven, H.H., Perez-Pelaez, M., Carbo, B.G. and Zaneveld, L.H.D. (1984). Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characterisitics. J. Reprod. Fert., **70**: 219-228.

Linford, E., Glover, F.A., Bishop, C. and Stewart, D.L. (1976). The relationship between semen evaluation methods and fertility in the bull. J. Reprod. Fert., **47**: 283-291.

Neild, D., Flores, M., Mora, N., Beconi, M. and Aguero, A. (1999). Hypoosmotic test in equine spermatoza. Theriogenology, **51**: 721-727.

Pratap, N., Reddy, V.N.V., Sarma, P.A. and Honnappa, T.G. (2000). Employment of the hypoosmotic swelling test (HOST) to evaluate sperm membrane integrity of fresh and frozen buffalo (Bubalus bubalis) bull semen. Buffalo J., **16**: 207-213.

Revell, S.G. and Mrode, R.A. (1994). An osmotic resistance test for bovine semen. Anim. Reprod. Sci., **36**: 77-86.

Rodriguez-Gill, J.E., Monserrat, A. and Rigau, T. (1994). Effects of hypoosmotic incubation on acrosome and tail structure of canine spermatozoa. Theriogenology, **42**: 815-829.

Rota, A., Penzo, N., Vincentil, L., and Mantovani, R. (2000). Hypoosmotic swelling (HOS) as a screening assay for testing *in vitro* fertility of bovine spermatozoa. Theriogenology, **53**: 1415-1420.

Snedecor, G.W. (1952). Determination of precision of a chemical assay method. Biometrics, **8**: 85-86.

Soderquist, L., Janson, L., Larsson, H. and Einarsson, S. (1991). Sperm morphology and fertility in AI bulls. J. Vet. Med. Asso., **38**: 534-543.

Van der Ven, H.H., Jeyendran, R.S., Al-Hasani, S., Perez-Pelaez, M., Didericj, K. and Zeneveld, L.J.D. (1986). Correlation between human sperm swelling in hypoosmotic medium (hypoosmotic swelling test) and *in vitro* fertilization J. Androl., **7**: 190-196.