Differential Effect of Common Laboratory Treatments on Hypoosmotic Swelling Responses of Human Spermatozoa

AMJAD M. HOSSAIN,^{1,4} RAJESH SELUKAR,² and SALIN BARIK³

Submitted: June 11, 1998

Accepted: July 23, 1998

Purpose: The impact of some of the common laboratory interventions on the hypoosmotic swelling (HOS) responses of human-spermatozoa was investigated.

Methods: The semen samples underwent different laboratory treatments prior to the standard HOS test, which involved incubation of sperm in the hypoosmotic solution. Fresh semen served as a control for all treatment groups and underwent the same HOS procedure. The HOS-reactive spermatozoa and the type of HOS reactions (swelling types) in each group were identified under a phase-contrast microscope for comparison.

Results: All the seven types of HOS responses documented in fresh semen sperm also occurred in the laboratory-processed sperm. The total HOS responses of sperm that underwent cryopreservation, heat shock, and Percoll wash were significantly different from those of the corresponding control. Percoll washing of semen influenced HOS subtypes a and g; cryopreservation affected subtypes a, b, c, and d; and heat shock altered subtypes a, f, and g. In contrast, prolonged postejaculation and cold shock did not affect any of the HOS responses. None of the treatments influenced the d and e responses.

Conclusions: These results indicate that the total HOS response value and specific response subtypes are significantly affected by some of the laboratory treatments but not others.

KEY WORDS: human sperm; laboratory interventions; hypoosmotic swelling response.

INTRODUCTION

The hypoosmotic swelling (HOS) test for investigating the functional integrity and viability of human sperm was introduced by Jeyendran *et al.* in 1984 (1). The rationale of this test is based on the fact that fluid is transported across the sperm tail membrane under hypoosmotic condition until equilibrium is reached. Due to this influx of fluid, the tail expands and bulges in different characteristic patterns, considered the hypoosmotic response (HOS response), which can readily be identified with a phase-contrast microscope (2–4). In damaged or chemically inactive sperm, fluids do pass across the membrane; however, in contrast to healthy sperm, no accumulation of the fluid takes place, and consequently, the cytoplasmic swelling and curling of the tail do not occur (1,3,5,6). Therefore, the HOS response of the tail has been considered an indicator of an intact membrane and, presumably, a normally functioning sperm (1,2,3).

The HOS assay is the simplest test of all the World Health Organization–recommended sperm function tests and, thus, is widely used (2,4,7). Although most of the investigations on the HOS test show good predictive power, some do raise concerns about its validity (8–10). For example, like other sperm function tests, the HOS test in its present form does not provide unequivocal information regarding the fertilizing ability of the sperm (9,11–13). From a recent review of HOS test–related research, it appears that much of the confusion is due to the interpretation of the data rather than to the accuracy of the HOS results (2). The HOS test is usually done by direct incubation of the ejaculate (semen) in hypoosmotic solution within a short period of semen collection (2,14). In reality, however, the ejaculate undergoes one or a combination of laboratory manipulations before recovery of the sperm from the semen for insemination purposes. Some of these manipulations include centrifugation, washing, freeze/thaw, temperature fluctuation, and processing delay.

¹ Department of Obstetrics and Gynecology, Division of Reproductive Endocrinology, University of South Alabama, Mobile, Alabama.

² SPSS, Chicago, Illinois.

³ Department of Biochemistry and Molecular Biology, University of South Alabama, Mobile, Alabama.

⁴ To whom correspondence should be addressed at Department of Obstetrics and Gynecology, Division of Reproductive Endocrinology, University of South Alabama, 307 University Boulevard, No. CCCB/326, Mobile, Alabama 36688.

It is not known how these laboratory interferences influence the natural HOS responses of the spermatozoa. In this study we were, therefore, interested in comparing the HOS responses of sperm of the semen subjected to the aforementioned laboratory treatments. Our study reveals a differential impact of laboratory treatments on the hypoosmotic response pattern of the human spermatozoa. This finding of our study has practical value in quality control of the HOS test.

MATERIALS AND METHODS

Semen specimens from the male partners of infertile couples with no apparent male-factor etiologies were used in the study. The samples were collected in a standard laboratory setup. The liquefied semen was aliquoted separately for cryopreservation, washing, temperature shock and prolonged postejaculation. The aliquot for cryopreservation treatment was frozen using standard semen freezing protocol and the 24-hr postfreeze semen was thawed for the HOS test (7,11). The aliquot for sperm washing treatment was processed by passing through a double-layer (40 and 80%) discontinuous Percoll gradient column (15). In the temperature-shock treatment, the semen was incubated at 25°C below or above the ambient temperature (cold shock or heat shock, respectively) for 10 min. This was achieved by keeping semen on ice (0°C) and in a water bath (50°C). To simulate prolonged postejaculation waiting, an aliquot of the semen was left on the benchtop in a sterile vial at ambient temperature for 24 hr. Fresh semen (30 min postejaculate) was used as a control for all treatment groups in each sample.

The samples of the above treatment groups and the corresponding control underwent the HOS test, which was essentially performed according to the original HOS protocol (1). Briefly, a 100- μ l sperm sample was mixed with 1.0 ml hypoosmotic solution (7.35 g sodium citrate and 13.51 g fructose in 1 L distilled water) and incubated at 37°C for 45 min. The HOS-reactive spermatozoa and types of HOS reaction (swelling types) were identified under a phase-contrast microscope at $\times 400$ magnification immediately after incubation. An experienced single observer graded the swelling types except for four samples in which, as the experiment required, two independent observers graded the swellings.

A statistical program called JMP was used to analyze the data. JMP is a modified version of SAS, developed to operate in Microsoft windows. The variations due to semen donors were removed by considering the

Fig. 1. Diagrammatic representation of typical tail swellings exhibited by human spermatozoa exposed to a hypoosmotic solution. (a) No swelling; (b–g) various types of swelling.

semen donors as the blocks. The effect of each treatment on the various swelling types was evaluated by comparing it with that of the control (fresh raw semen) by the paired *t* test. R^2 values were used to check the goodness of fit for the model used. A *P* value of 0.05 or less was considered statistically significant.

RESULTS

To start with, we confirmed and established that all possible types of hypoosmotic responses (swelling types) of human spermatozoa were indeed observed in fresh semen ($n = 26$). The natural sperm population (fresh semen sperm) exhibited all seven (a through g) previously documented types of hypoosmotic swellings at a frequency of $32 \pm 20\%$ (a), $18 \pm 10\%$ (b), $11 \pm 10\%$ (c), $1 \pm 3\%$ (d), $1 \pm 1\%$ (e), $4 \pm 5\%$ (f), and $32 \pm 13\%$ (g), respectively (Fig. 1). In cases where two independent observers graded the swellings, their gradings were very similar (coefficient of variation: $\leq 6\%$) with respect to all HOS types.

The percentage of sperm exhibiting HOS in the different treatment groups and their controls (fresh semen sperm) are shown in Table I. It is clear that the

Table I. Comparison of Hypoosmotic Swelling (HOS) Responses of Spermatozoa Undergoing Different Laboratory Treatments

Treatment	n	HOS-reactive spermatozoa (%)	
		Treatment	Control
Cryopreservation	23	$62 \pm 9^*$	80 ± 13
Percoll wash	47	$84 \pm 6^*$	68 ± 11
Prolonged postejaculation	39	57 ± 14	65 ± 9
Heat shock (50°C)	33	$51 \pm 16^*$	79 ± 9
Cold shock (0°C)	29	69 ± 10	76 ± 12

* Significantly different from the corresponding control ($P < 0.05$).

Percoll wash led to a significant increase (68 vs. 84%), while cryopreservation and heat shock caused significant decreases (83 vs. 62 and 79 vs. 51%, respectively) in the total swelling response compared with that of the corresponding controls ($P \leq 0.05$). Postejaculation delay and cold shock did not produce any significant shift in the HOS response; the total HOS response value of spermatozoa of 24-hr-old semen and those receiving cold shock (0°C for 10 min) were very similar (57 vs. 65 and 69 vs. 76%, respectively) to their respective controls ($P > 0.05$).

All seven types of HOS responses documented in wild-type sperm (raw semen sperm) were also identified in the laboratory-treated sperm (Fig. 1 and Table II), regardless of the treatment. However, a number of response subtypes were significantly altered by some of the laboratory treatments and not by the others. Table II summarizes the differential impacts of the four laboratory interventions on the HOS response pattern. Spermatozoa enriched by a Percoll wash differed from their control (semen sperm) by significant differences in the a and g response subtypes ($P < 0.05$). Significant changes in the a, b, and c types of swelling were caused by cryopreservation ($P < 0.05$). Heat shock (50°C) produced profound ($P < 0.05$) changes in the a, f, and g types of swellings (Table II). None of the treatments influenced the d and e types of swellings ($P > 0.05$).

DISCUSSION

The HOS test was introduced in the human system in 1984 by documenting the seven distinct HOS

responses mentioned previously. Subsequent studies confirmed the existence of the response subtypes (4,6), thus establishing the authenticity of the technique. In a previous study, we showed that HOS response evaluation using shorter incubation times may help in detecting the cytoskeletal variability in the sperm population (16). In the present study we have produced evidence indicating that laboratory treatments alter the hypoosmotic response pattern of human spermatozoa. It seems to us that such changes in the HOS of spermatozoa probably occurred due to the direct influence of the laboratory interventions on the cytoskeletal assemblies of sperm.

As mentioned, in reality, the ejaculate undergoes a variety of interventions associated with its transport and/or processing. However, the potential impact of these interventions on the HOS responses of spermatozoa has never been critically addressed. For example, it is not unlikely that the semen will experience considerable temperature fluctuation during transport to the laboratory or during laboratory processing. By applying controlled temperature shocks (50 and 0°C) for a short duration (10 min), we were able to demonstrate that temperature elevation (heat shock), but not reduction (cold shock), influences the HOS responses of spermatozoa. The profound impact of heat shock demonstrates the importance of maintaining an optimum sample temperature in obtaining true HOS response. The original investigation by Jeyendran *et al.* in 1984 (1) shows a reduction of tail swellings due to heat treatment (56°C for 30 min). In our study, we showed a similar impact on total swellings by an even shorter exposure

Table II. Impact of Different In Vitro Treatments on the Hypoosmotic Tail Swelling Responses of Human Spermatozoa^a

Treatment	Hypoosmotic swelling type						
	a	b	c	d	e	f	g
Cold shock (n = 29)	-3.21 ± 1.3 0.87	-0.5 ± 0.9 0.91	0.2 ± 0.7 0.92	0.3 ± 0.4 0.47	-0.1 ± 0.2 0.63	0.2 ± 0.4 0.91	0.6 ± 1.3 0.85
Heat shock (n = 33)	-17 ± 2.9* 0.79	3.7 ± 2.1 0.62	0.4 ± 1.8 0.76	0.1 ± 0.3 0.70	0.1 ± 0.1 0.38	1.5 ± 0.5* 0.70	6.7 ± 2.3* 0.67
Freeze/thaw (n = 23)	-12.2 ± 1.5* 0.88	6.4 ± 1.5* 0.74	3.9 ± 1.3* 0.62	0.3 ± 0.1 0.60	0.1 ± 0.3 0.31	0.8 ± 0.5 0.75	-1.5 ± 2.4 0.49
Percoll wash (n = 47)	8.6 ± 1.6* 0.87	1.5 ± 1.3 0.80	1.3 ± 1.2 0.69	0.2 ± 0.3 0.72	0.0 ± 0.2 0.70	0.5 ± 0.6 0.61	-12.8 ± 2.0* 0.78

^a Top and bottom values indicate $\frac{1}{2}$ [control - treatment] ± standard deviation and R^2 , respectively.

* P values are less than 0.05 when the treatment is compared with the control.

at a lower temperature (50°C for 10 min). In addition, we found that response subtypes a, f, and g were specifically affected by high temperature (Table II). Studies of HOS responses of sperm obtained from varicocele patients show a similar effect of heat shock *in vivo* (17). Varicocele development is known to alleviate scrotal temperature. Sperm of males with this condition exhibit a significantly lower HOS response. These results lend further support to our findings and document the relevance of the *in vitro* heat-shock experiments. A large decrease in the HOS response was also observed following cryopreservation of the semen. This is probably due to a significant damage to the sperm tail caused by freezing and thawing, which diminished the membrane's ability to swell. Indeed, a few previous studies have reported a considerable number of tail-swollen sperm in semen subjected to freeze/thaw without any hypoosmotic treatment (7,11,18). The comparable HOS response values of raw semen sperm and frozen-thawed sperm after Percoll wash probably suggest that the majority of the cryodamaged sperm are eliminated by washing, which further supports the beneficial value of the Percoll wash.

Practically all *in vitro* fertilization protocols require the preparation of sperm free of seminal plasma so that they are sterile and exhibit good morphology and motility. Several sperm wash techniques are currently available for such preparations (15). It has been generally assumed, albeit without direct experimental evidence, that such washings result in the enrichment of sperm with better membrane characteristics. Our results show that this may be true, since a substantially higher total HOS response (84%) was exhibited by sperm following Percoll wash. In future investigations, it will be worth comparing the HOS pattern of the sperm population selected by the different sperm wash techniques practiced in various laboratories. Finally, when most of the laboratory interventions show either a positive or a negative influence on HOS responses of spermatozoa, it is interesting to find that the HOS pattern of semen 24 hr postcollection is no different from that of the fresh semen. By this time, however, a large proportion of the sperm (>80%) loses its motility. The seminal fluid must, therefore, contain ingredients that stabilize the membrane of the sperm for at least some time, even when the motility of the sperm is lost.

In our study, two observers independently evaluated the HOS response of the same sperm sample, and in such cases, the results obtained by them were

nearly identical. We are not aware of any other sperm function tests in which the interobserver variation is so low (coefficient of variance < 6%). We believe that this reproducibility combined with the ease with which the test is done underlies the reason for such widespread use of the HOS test in clinical andrology all over the world.

In summary, by showing very negligible interobserver variation in scoring HOS, we have reconfirmed the previous claim (1–4) that the HOS test is highly reproducible. Most important, our studies documented that common laboratory factors may affect the HOS responses of spermatozoa both positively and negatively. These findings will be extremely useful in controlling the quality of the HOS test and, hence, ensuring that the test results are free from artifactual variations. Furthermore, this will serve to augment the reliability of the highly popular HOS test even further.

REFERENCES

1. Jeyendran RS, Perez-Pelaez M, Crabo BG, Zaneveld LJD: Development of an assay to assess the functional integrity of the human sperm membranes and its relationship to other semen characteristics. *J Reprod Fert* 1984;70:219–228
2. Jeyendran RS, Van der Ven HH, Zaneveld LJD: The hypoosmotic swelling test: An update. *Arch Androl* 1992;29:105–116
3. Mladenovic I, Micic S, Genav O, Papic N: Hypoosmotic swelling test for quality control of sperm prepared for assisted reproduction. *Arch Androl* 1995;34:163–169
4. Takahashi K, Uchida A, Kitao M: Hypoosmotic swelling test of sperm. *Arch Androl* 1990;25:225–242
5. Casper RF, Cowan L, Meriano JS, Lucato ML, Jarvi KA: The hypo-osmotic swelling test for selection of viable sperm for intracytoplasmic sperm injection in men with complete asthenozoospermia. *Fertil Steril* 1996;65:972–976
6. Mordel N, Shemesh A, Dano I, Schenker JG, Epstein-Eldan M, Laufer N: Novel parameters of human sperm hypoosmotic swelling test and their correlation to standard spermatogram, total motile sperm fraction, and sperm penetration assay. *Fertil Steril* 1993;59:1276–1279
7. Check ML, Check JH: Poor hypo-osmotic swelling test results from cryopreserved sperm despite preservation of sperm motility. *Arch Androl* 1991;26:37–41
8. Chan SYW, Wang C, Fox EJ, Tang LCH, Chan MMC, Tang GWC: The relationship between the human sperm hypoosmotic swelling test, routine semen analysis, and the human sperm zona-free hamster ovum penetration assay. *Fertil Steril* 1985;44:668–672
9. Fuse H, Kazama T, Katayama T: Relationship between hypoosmotic swelling test, semen analysis, and zona-free hamster ovum test. *Arch Androl* 1991;27:73–78
10. Jager S, Kremer J, Mijchman J: Hypoosmotic sperm swelling test does not assess fertilizing capacity of human spermatozoa. *Arch Androl* 1991;26:195–197
11. Check DJ, Kiefer D, Katsoff D, Check JH: Effect of pentoxifyl-

- line added to freezing media on subsequent post-thaw hypoosmotic swelling test and other semen parameters. *Arch Androl* 1995;35:161–163
12. Kiefer D, Check JH, Katsoff D: The value of motile density, strict morphology, and the hypoosmotic swelling test in in vitro fertilization embryo transfer. *Arch Androl* 1996;37:57–60
 13. Vantman D, Zinaman M, Sherins R: Dissociation of hypoosmotic tail swelling patterns in sperm from fertile and infertile men. *J Androl* 1987;8 (Suppl):24
 14. Fuse H, Okumura M, Kazama T, Katayama T: Correlation of seminal plasma transferrin concentration on hypoosmotic swelling of sperm. *Arch Androl* 1994;32:95–100
 15. Hossain A, Huff C, Rizk B: Fractions of Percoll eliminated sperm: increased Sperm yield in Percoll wash. *Arch Androl* 1996;37:189–195
 16. Hossain A, Barik S, Rizk B, Thorneycroft IH: Evidence of time course morphological changes in human sperm tails when exposed to hypo-osmotic treatment. *Hum Reprod* 1998;13 (in press)
 17. Fuse H, Kazama T, Katayama T: Hypo-osmotic swelling test in patients with varicocele. *Arch Androl* 1991;27:149–154
 18. Check JH, Lurie D, Baker A, Katsoff D, Benfer K: Transfer of cryopreserved embryos improved pregnancy rates in patients with damage to the functional integrity of the sperm membrane as measured by the hypo-osmotic swelling test. *Fertil Steril* 1996;65:1241–1244