

Suitability of the hypo-osmotic swelling test for assessing the viability of cryopreserved sperm*†

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Objective: To determine whether the hypo-osmotic swelling test can assess viability of frozen spermatozoa.

Design: Fresh semen specimens were divided into two aliquots: one was not processed and the other was processed by swim-up. Both aliquots were then cryopreserved by the liquid nitrogen vapor method.

Setting: Andrology laboratory at a tertiary care facility.

Patient(s): Eleven volunteers with proven fertility.

Intervention(s): The hypo-osmotic swelling test and the sperm viability assessment by Hoechst 33258 (Sigma Chemical Co., St. Louis, MO) and eosin-nigrosin stains were performed in each aliquot.

Main Outcome Measure(s): The results of hypo-osmotic swelling test as indicated by swelling of sperm tails were compared with the viability results obtained by Hoechst 33258 stain before freezing and by Hoechst-33258 and eosin-nigrosin stains after freezing.

Result(s): The hypo-osmotic swelling test and Hoechst 33258 viability results were correlated highly in unprocessed and processed fresh specimens ($r = 0.95$). After cryopreservation, there was no correlation between hypo-osmotic swelling test and Hoechst 33258 or between hypo-osmotic swelling test and eosin-nigrosin. Prefreeze sperm processing did not influence the post-thaw results. Viability assessed by Hoechst 33258 and eosin-nigrosin was well correlated ($r = 0.72$).

Conclusion(s): The hypo-osmotic swelling test can accurately evaluate viability in fresh human spermatozoa but not in cryopreserved ones. Thus, this test cannot be used to select viable cells in cryopreserved samples for assisted reproductive procedures, such as intracytoplasmic sperm injection. Fertil Steril® 1996;66:798-804

Key Words: Sperm viability, hypo-osmotic swelling test, Hoechst 33258 stain, eosin-nigrosin stain, cryopreservation

Some measure of viability of human spermatozoa is vital in assisted reproduction. Motility alone can be used, especially when assisted reproductive techniques, such as IUI and IVF-ET, are indicated. How-

ever, the evaluation of viability by motility effectively excludes sperm populations that are viable but nonmotile. Cell viability is determined more accurately with DNA (e.g., Hoechst 33258; Sigma Co., St. Louis, MO) (1) or cytoplasmic vital stains (e.g., eosin-nigrosin) (2). However, it is unclear how these stains affect DNA integrity, gamete interaction, and embryogenesis of the exposed sperm in assisted reproduction.

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In selected cases of male infertility, such as patients with spinal cord injury, severe astheno-oligozoospermia, and patients with testicular or Hodgkin's disease, the number of spermatozoa available for IVF-ET is insufficient. In these cases, intracytoplasmic sperm injection (ICSI) appears to be an excellent tool in assisting childless couples (3), and this procedure requires only viable, not necessarily motile, spermatozoa (4). Also, in cases of ICSI failure resulting from egg abnormalities, it may be possible to retrieve fresh spermatozoa with epididymal or testicular aspiration and cryopreserve them for future use. However, despite advances in cryopreservation techniques, post-thaw sperm recoveries remain poor (5, 6). In these extreme cases, assessment of nonmotile but viable cells by a test that permits the same treated spermatozoa to be used for ICSI would be ideal.

Although the hypo-osmotic swelling test originally was described as a test of sperm function that correlated well with the zona-free hamster egg penetration test (7), this correlation has not been confirmed (8–12). However, others have found that the hypo-osmotic swelling test can discriminate between fertile and infertile semen samples (13). Some authors believe that the hypo-osmotic swelling test also can assess sperm vitality because it assesses the osmoregulatory ability of the spermatozoa, an important aspect of membrane integrity (2, 14). Spermatozoa without an intact plasma membrane do not show tail swelling. In addition, abnormal spermatozoa with poor osmoregulatory capacity swell uncontrollably, which rapidly ruptures their overdistended plasma membranes (14). The ability of the hypo-osmotic swelling test to assess the viability of spermatozoa has been demonstrated in fresh specimens (14, 15) but there are few reports where cryopreserved ones were used (16).

The purpose of this study was to determine whether the hypo-osmotic swelling test can assess accurately the viability of frozen, as effectively as fresh spermatozoa and whether sperm preparation before freezing affects viability.

MATERIALS AND METHODS

TES and Tris (TEST)-yolk buffer freezing medium and sperm washing medium (Biggers-Whitten-Whittingam [BWW] with 0.3% human serum albumin) were purchased from Irvine Scientific (Santa Ana, CA). Hoechst 33258 (bis-benzimide), eosin, and nigrosin were obtained from Sigma Chemical Company.

The Hypo-osmotic swelling solution was prepared by mixing 7.35 g of sodium citrate and 13.51 g of fructose in 1 L distilled water (7). A stock solution

of Hoechst 33258 was prepared by dissolving 1 mg of Hoechst 33258 in 1 mL Dulbecco's phosphate-buffered saline. Ten-microliter aliquots of the stock solution were frozen at -20°C in microfuge tubes and covered with aluminum foil to protect them from light.

Sperm Preparation and Cryopreservation Procedure

Semen samples from healthy donors ($n = 11$) were selected based on the World Health Organization criteria (17) for normal semen specimens. Approval of this study was obtained from the Institutional Review Board. All subjects were asked to observe 2 to 3 days of sexual abstinence before donating. Semen samples were produced by masturbation into sterile specimen cups and allowed to liquefy at 37°C before the evaluation of sperm count and motility. After initial analysis, each specimen was divided into two aliquots. The first aliquot (the unprocessed sample) was frozen without any further treatment. The second aliquot (the processed sample) was prepared by the swim-up method to select a highly motile and morphologically normal population of spermatozoa (18) and then frozen. TEST-yolk buffer with glycerol was used for cryopreservation. An aliquot of the freezing medium equal to 25% of the original specimen volume then was added to the specimen and gently mixed for 5 minutes using a Hema-Tek aliquot mixer (Miles, Elkhart, IN). This procedure was repeated to give a final 1:1 (vol:vol) ratio of freezing medium to sperm sample. Cryovials containing the specimens were frozen at -20°C for 8 minutes and thereafter in liquid nitrogen vapor at -79°C for 2 hours. The vials finally were transferred to liquid nitrogen at -196°C for long-term storage. For analysis, cryovials were thawed at room temperature for 5 minutes and then incubated at 37°C for 20 minutes. The cryomedium was removed by washing with BWW and centrifuging the samples at $250 \times g$ for 10 minutes. The sperm pellet was reconstituted in BWW.

Assessment of Viability

Prefreeze viability was evaluated by the hypo-osmotic swelling test and by Hoechst. Post-thaw viability was evaluated by the hypo-osmotic swelling test and Hoechst and eosin-nigrosin stains. Simultaneous assessment of viability by hypo-osmotic swelling test and Hoechst stain was done by adding $0.1 \mu\text{L}$ of sperm suspension to 1 mL of the hypo-osmotic swelling test solution and incubating for 1 hour at 37°C . After this period, spermatozoa were pelleted by centrifugation at $250 \times g$ for 5 minutes; resuspended in $100 \mu\text{L}$ of Hoechst solution ($1 \mu\text{g}/\text{mL}$), and

Table 1 Percentage of Viable Spermatozoa Before and After Cryopreservation, With and Without Processing With the Swim-up Technique, as Measured by the Hypo-Osmotic Swelling Test, the Hoechst Staining Test, and the Eosin-Nigrosin Staining Test in Samples From 11 Normal Men*

Sperm preparation	Prefreeze		Post-thaw		
	Hypo-osmotic swelling test	Hoechst stain	Hypo-osmotic swelling test	Hoechst stain	Eosin-nigrosin staining test
	%	%	%	%	%
Unprocessed	80.2 ± 14.2	74.8 ± 22.1	49.0 ± 8.2	31.3 ± 15.6	27.6 ± 16.1
Processed	88.1 ± 10.8	84.5 ± 14.4	45.0 ± 12.6	29.1 ± 13.9	24.4 ± 11.1

* Values are means ± SD.

incubated for 10 minutes in the dark. Spermatozoa then were washed in phosphate-buffered saline solution to remove the excess stain, centrifuged at $250 \times g$ for 5 minutes, and the pellet resuspended in $50 \mu\text{L}$ of ice-cold ethanol for permeabilization. Ten microliter aliquots subsequently were smeared on a microscope slide in triplicate, air dried, and stored in the dark at -4°C . These slides were scored within 48 hours of staining. The slides were examined using a Leitz Orthoplan fluorescence microscope (Leitz, Wetzlar, Germany) at $\times 1,000$ magnification in the presence of an anti-quenching agent (Cargille immersion oil, type DF; Fisher Scientific, Pittsburgh, PA) to minimize the loss of fluorescence. For the hypo-osmotic swelling test, the slides were placed under phase-contrast illumination to assess the sperm tail

swelling. Swelling in the tail indicated that the spermatozoa were viable with intact plasma membrane. In contrast, absence of swelling indicated nonviable spermatozoa with damaged membrane. Hoechst stain was used to assess the fluorescence pattern of the same spermatozoa using Ploemopak epi-illumination module with an A2 filter cube (Leitz). Hoechst stains the nuclei of damaged cells and is excluded from viable cells. Spermatozoa fluorescing pale blue were classified as alive, whereas those appearing as bright blue were classified as dead. A total of 200 spermatozoa were scored in each sample. A small aliquot of each cryopreserved specimen was thawed and scored with the eosin-nigrosin stain to validate the Hoechst results in cryopreserved specimens.

Cryoinjuries induced during the freeze-thaw process, such as swelling of the sperm membrane, were assessed in each specimen by scoring the percentage of swollen spermatozoa in the absence of hypo-osmotic swelling test solution. This value was subtracted from the total hypo-osmotic swelling test scores to give a corrected result. The corrected hypo-osmotic swelling test scores thus equal the percentage of swollen sperm exposed to the hypo-osmotic swelling test solution minus the percentage of swollen sperm after cryopreservation without exposure to the hypo-osmotic swelling test solution.

Statistical Analysis

The Pearson correlation coefficient was used to assess the relationship between the viability results of the hypo-osmotic swelling test and Hoechst stain before freezing and between the hypo-osmotic swelling test and Hoechst and eosin-nigrosin stains after thawing. The paired Student's *t*-test was used to analyze the viability results in the unprocessed and the processed specimens before and after cryopreservation. A *P* value < 0.05 was considered significant.

RESULTS

The mean percentage scores of the hypo-osmotic swelling test, Hoechst staining, and eosin-nigrosin

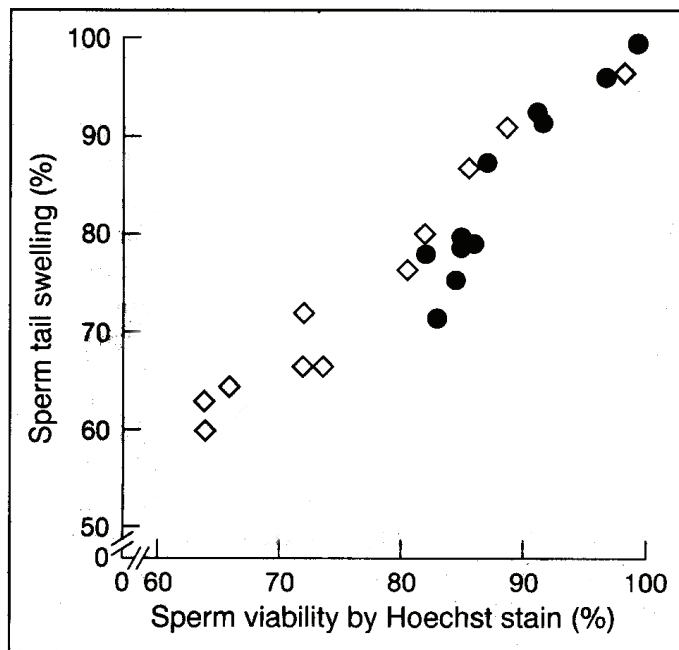
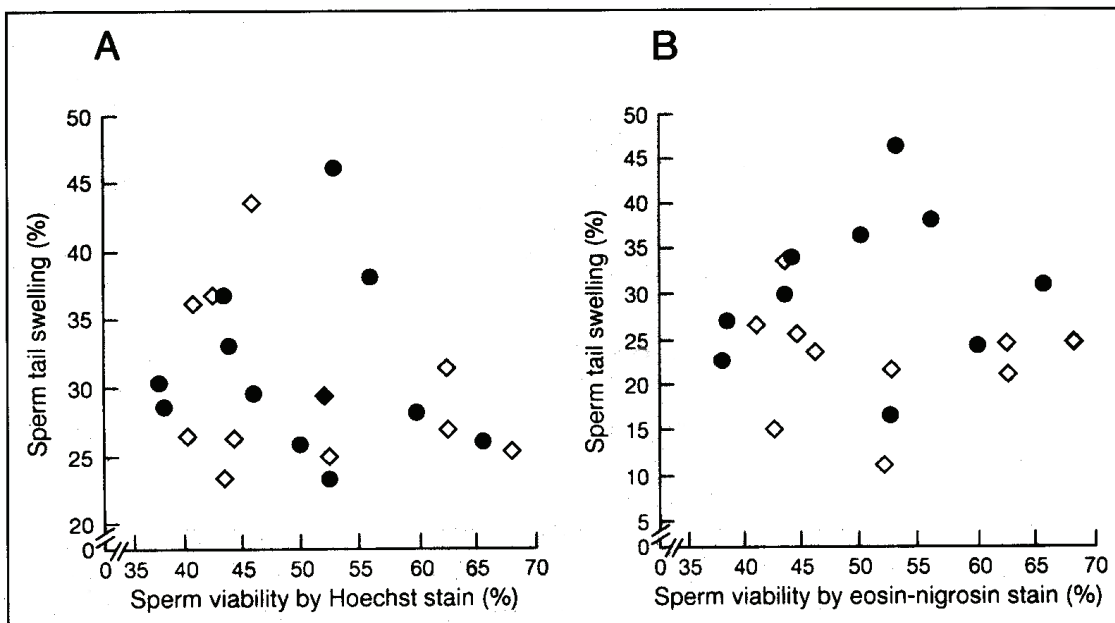


Figure 1 Correlation of viability results before cryopreservation between sperm tail swelling and Hoechst 33258 (●, unprocessed specimens, $n = 11$; ◇, specimens processed by swim-up, $n = 11$). Strong correlation seen between hypo-osmotic swelling test indicated by sperm tail swelling and Hoechst 33258 staining in both unprocessed ($r = 0.95$; $P < 0.0001$) and processed specimens ($r = 0.94$; $P < 0.0001$).

Figure 2 Correlation of post-thaw viability results (A) between sperm tail swelling and Hoechst 33258 stain and (B) between sperm tail swelling and eosin-nigrosin results (●, unprocessed specimens, n = 11; ◇, specimens processed by swim-up, n = 11). No correlation was present either between hypo-osmotic swelling test as indicated by sperm tail swelling and Hoechst 33258 in unprocessed ($r = 0.11$; $P = 0.70$) and processed ($r = 0.13$; $P = 0.64$) or between hypo-osmotic swelling test and eosin-nigrosin in both unprocessed ($r = 0.19$; $P = 0.52$) and processed specimens ($r = 0.33$; $P = 0.29$).



stain in both processed and unprocessed specimens are shown in Table 1. In fresh sperm specimens in both unprocessed and processed groups, the tail swelling after the hypo-osmotic swelling test was $80.2\% \pm 14.2\%$ and $88.1\% \pm 10.8\%$ (mean \pm SD; $P = 0.03$). The majority of live spermatozoa fluoresced pale blue after Hoechst staining ($74.8\% \pm 22.1\%$ and 84.5 ± 14.4 , respectively; $P = 0.038$). The results of hypo-osmotic swelling test strongly correlated with those obtained by Hoechst staining in both unprocessed ($r = 0.95$; $P < 0.0001$) and processed specimens ($r = 0.94$; $P < 0.0001$) (Fig. 1).

After cryopreservation, a poor correlation was seen between hypo-osmotic swelling test and Hoechst 33258 stain and between the hypo-osmotic swelling test and eosin-nigrosin stain in both unprocessed and processed specimens (Fig. 2A and B). However, a small percentage of unprocessed and processed spermatozoa were swollen before exposure to the hypo-osmotic swelling test solution ($20.4\% \pm 8.2\%$ and $16\% \pm 6.4\%$, respectively). Even after correcting the hypo-osmotic swelling test results for the swelling expected from cryopreservation, the viability scores measured by the hypo-osmotic swelling test and Hoechst stain showed a poor correlation in both unprocessed and processed specimens (Fig. 3A and B). Results of eosin-nigrosin and Hoechst staining after thawing significantly correlated in both unprocessed ($r = 0.72$; $P = 0.004$) and processed groups ($r = 0.60$; $P = 0.02$; Fig. 4). There was no significant difference in the post-thaw viability results measured by these two staining methods in unprocessed and processed groups ($P = 0.07$).

Both Hoechst 33258 and eosin-nigrosin stains can be used as gold standards of viability. The Hoechst

33258 stain in the present study was used to evaluate the viability status of the sperm population before and after cryopreservation. The eosin-nigrosin stain was used only after cryopreservation. Therefore, we used Hoechst 33258 stain as the "gold standard" to calculate the positive and negative predictive value of the hypo-osmotic swelling test. The positive and negative predictive values of the hypo-osmotic swelling test to assess viability in both unprocessed and processed fresh sperm populations was very high (Table 2). After cryopreservation, although similar positive predictive value was seen, the negative predictive value was low. This indicates the poor specificity of the hypo-osmotic swelling test in the evaluation of viability in frozen sperm specimens.

DISCUSSION

One of the properties of the cell membrane is its ability to permit the selective transport of molecules. Human spermatozoa swell under hypo-osmotic conditions as a result of influx of water followed by expansion of the cell membrane. The sperm tail appears to be particularly susceptible to hypo-osmotic conditions (19). These osmotic changes induce typical morphological alterations characterized by the presence of a swollen area, which is clearly observed under phase-contrast microscopy. These morphological changes are thought to indicate membrane integrity and normal functional activity of human spermatozoa (7).

Some investigators believe the hypo-osmotic swelling test to be clinically important because it assesses the osmoregulatory ability of the spermato-

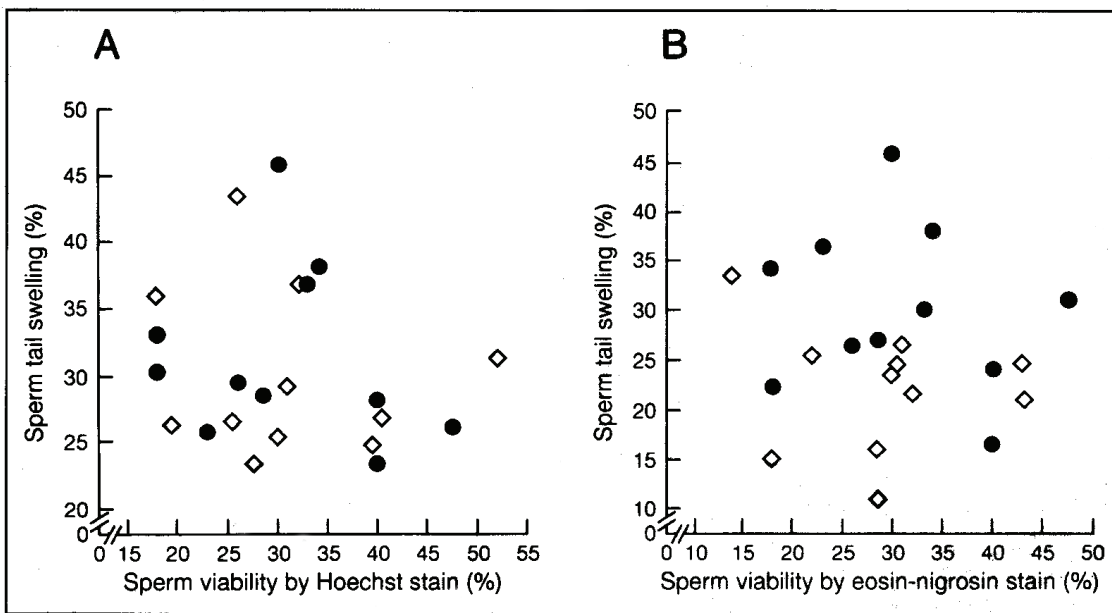


Figure 3 Correlation of post-thaw viability results (A) between corrected sperm tail swelling (corrected hypo-osmotic swelling test, see text) and Hoechst 33258 stain and (B) between corrected hypo-osmotic swelling test and eosin-nigrosin (●, unprocessed specimens, $n = 11$; ◇, specimens processed by swim-up, $n = 11$). No correlation was seen between corrected hypo-osmotic swelling test and Hoechst 33258 in unprocessed ($r = 0.22$; $P = 0.43$) and processed ($r = 0.04$; $P = 0.89$) or between hypo-osmotic swelling test and eosin-nigrosin in both unprocessed ($r = 0.44$; $P = 0.12$) and processed specimens ($r = 0.37$; $P = 0.19$).

zoa, an aspect of membrane integrity and viability. However, the relevance of the hypo-osmotic swelling test as a functional sperm test has not been validated (8–12). Van den Saffele et al. (14) showed that the capacity of fresh spermatozoa to react in a hypo-osmotic environment provided the same information as the viability test using eosin-nigrosin stain, which evaluates the capacity of the head membrane to exclude dye (14). The measurement of sperm viability and membrane integrity by a combined eosin-nigro-

sin stain and the hypo-osmotic swelling test showed a negative correlation between the abnormal hypo-osmotic swelling test results and sperm penetration assay scores in 70% of the samples analyzed by other processing methods (15). These investigators also concluded that sperm washing by centrifugation altered the hypo-osmotic swelling test result, whereas the swim-up method selected a population of spermatozoa with intact head and tail membranes. Our results are in agreement with those of McLaughlin et al. (16), who also measured sperm membrane integrity in fresh spermatozoa with the hypo-osmotic swelling test and Hoechst stain and found a significant correlation ($r = 0.80$) between the two methods.

The hypo-osmotic swelling test is a physiological and nondeleterious test. This test can assess accurately the viability of fresh human spermatozoa and is not only useful for diagnostic purposes, but also clinically crucial as a tool in identifying nonmotile but viable spermatozoa for micromanipulation procedures, such as ICSI, in which sperm motility is not important. In the present study, sperm viability was measured accurately by Hoechst staining before and after cryopreservation. Also, eosin-nigrosin stain correctly identified viable spermatozoa after cryopreservation. However, the implications of using stained spermatozoa on DNA integrity, gamete interaction, embryogenesis, and the implications of using stained spermatozoa for assisted reproduction are unknown.

In the present study, the positive and negative predictive values of the hypo-osmotic swelling test were very high, thus confirming that this test accurately can assess sperm viability in fresh specimens, as shown by others (14–16). Further, sperm preparation by the swim-up method did not influence the

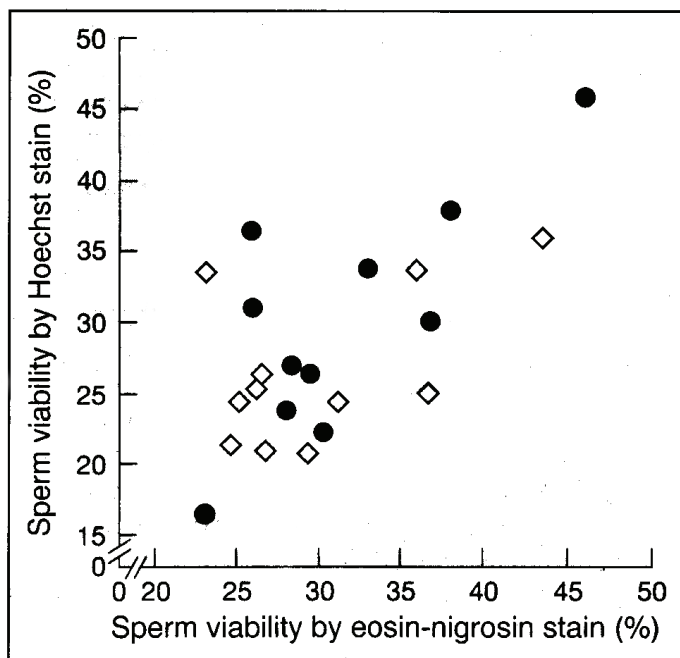


Figure 4 Correlation of post-thaw viability results between Hoechst 33258 and eosin-nigrosin stains (●, unprocessed specimens, $n = 11$; ◇, specimens processed by swim-up, $n = 11$). Strong correlation seen between Hoechst and eosin-nigrosin in both unprocessed ($r = 0.72$; $P = 0.004$) and processed specimens ($r = .60$; $P = 0.02$).

Table 2 Positive and Negative Predictive Values of Hypo-Osmotic Swelling Test in Assessing the Viability of Unprocessed and Processed Sperm Specimens Before and After Cryopreservation*

Cryo-preservation	Positive predictive value		Negative predictive value	
	Unprocessed	Processed	Unprocessed	Processed
Before	0.96	0.98	0.80	0.78
After	0.86	0.93	0.33	0.32

* Hoechst 33258 stain was considered as a "gold standard."

viability scores measured by the hypo-osmotic swelling test or by Hoechst staining in fresh specimens, and these results were correlated highly in both unprocessed and processed specimens. Even though the hypo-osmotic swelling test showed high sensitivity (positive predictive value) in assessing viability of fresh and frozen sperm, it had very low specificity in frozen specimens, limiting its use as a good predictor of viability in cryopreserved human spermatozoa. The hypo-osmotic swelling test, Hoechst stain, and eosin-nigrosin stain scores were not correlated, and sperm preparation by the swim-up technique did not improve the results. We observed sperm tail swelling in 16% to 20% of sperm population after thawing as a result of freezing and freeze-thaw process. Correction of the hypo-osmotic swelling test scores did not improve the correlation between the viability scores assessed by hypo-osmotic swelling test, Hoechst stain, or eosin-nigrosin stain.

An intact sperm tail membrane does not indicate that the membrane in the head is also intact. The membranes in the head and tail regions are differently labile. Cryopreservation and the freeze-thaw process disrupts the outer and inner interaction membrane, resulting in loss of interaction contents (20–22); however, this does not necessarily affect the tail region. These differences in the results between fresh and frozen specimens suggest that the determining factor in sperm survival during cryopreservation is the sperm head membrane, whereas the hypo-osmotic swelling test measures the tail membrane swelling.

In conclusion, the hypo-osmotic swelling test is a simple and nondeleterious assay that can evaluate accurately viability and membrane integrity in fresh human spermatozoa. Sperm preparation before cryopreservation by the swim-up method does not improve significantly post-thaw viability. In cryopreserved samples, the hypo-osmotic swelling test is inaccurate in predicting damage to the sperm membrane. Both Hoechst and eosin-nigrosin stains are better predictors of viability in cryopreserved samples. We therefore strongly believe that the hypo-

osmotic swelling test is not clinically useful for selecting viable cryopreserved spermatozoa for assisted reproductive procedures.

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