Sperm MTT Viability Assay: A New Method for Evaluation of Human Sperm Viability

Mohammad Hossein Nasr-Esfahani,^{1,2,4} Roshanak Aboutorabi,³ Ebrahim Esfandiari,³ and Mohammad Mardani³

Submitted March 18, 2002; accepted May 15, 2002

Purpose: MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay is commonly used as a cell proliferation assay. The objective of this study was to evaluate the capability of MTT assay to discriminate between viable and nonviable sperms and compare its efficiency with E&N (eosin and nigrosin) and HOST (hypo-osmotic swelling test).

Methods: MTT assay was modified to obtain optimal result for assessment of sperm viability. After standardization of method, MTT, E&N, and HOST were carried out simultaneously on 57 semen samples from patients referring to Isfahan Fertility and Infertility Center. The correlation coefficient between these tests and sperm motility was calculated using the SPSS statistical program. Specificity and sensitivity of each test was also obtained.

Results: The optimal conditions for sperm MTT viability assay were 2 h after addition of sperm to MTT in HAM'S F10 + 25 mM HEPES + 10% HSA at 37°C and pH 7.4–7.45. Inter- and intra-assay coefficients of variations were 9 and 7%, respectively. The sensitivity and specificity for sperm MTT viability assay, E&N, and HOST were 97, 98, and 99%, and 100, 100, and 83% respectively. High significant correlations were obtained between sperm MTT viability assay, E&N, HOST and motility.

Conclusions: Sperm MTT viability assay can be used as a diagnostic test for discrimination of viable sperms from sperm population.

KEY WORDS: Human sperm; MTT; HOST; E&N; sperm viability.

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is now accepted as an appropriate treatment for patients with male factor infertility, and results in high fertilization and pregnancy rates (1). Because of limited amount of oocytes in ICSI cycles, the selection of viable sperm is essential, especially in cases where no motile sperm can be found in the ejaculate or in the testicular sperm preparation. Different tests or methods have been

developed for differentiation and selection of viable sperms from the sperm population (2). These tests can be divided into two groups: those that have only diagnostic value and those that also have clinical application in ICSI. Dye exclusion tests, including eosin, eosin–nigrosin (E&N), and trypan blue are of the former group and hypo-osmotic swelling test (HOST) is of the latter group. The dye exclusion tests such as eosin–nigrosin are based on the cell permeability and therefore viable spermatozoa remains colorless while nonviable spermatozoa stains accordingly or red. Because of the methodology of these tests, they are not suitable for ICSI in human. Unlike dye exclusion tests, HOST is considered to be a useful method for selecting viable sperms among a population of nonmotile spermatozoa for ICSI (3). During HOST procedure sperms are exposed to a hypo-osmotic

¹ Department of Clinical and Experimental Embryology and Andrology, Royan Institute, Tehran, Iran.

² Isfahan Fertility and Infertility Center, Isfahan, Iran.

³ Department of Anatomy, Isfahan University of Medical Sciences, Isfahan, Iran.

⁴ To whom correspondence should be addressed; e-mail: nasrmhn@yahoo.com.

condition; thus an influx of water results in swelling of the cytoplasmic space and therefore curling of viable sperm tail fibers (4). Because of limited clinical and practical applications of this test, different modifications with different names (single sperm curling test, water test) have been suggested or used for ICSI by different authors (3,5). Lower fertilization rates were obtained from HOST-ICSI as compared with cases where live motile sperms were used. This lower fertilization rate could be due to damage to the sperm or sperm death upon exposure to low osmotic condition. Although ICSI-HOST is considered as an effective method for selecting viable sperm for ICSI, there still appears to be a need for new tests to select viable sperms from immotile sperm population (3).

The aim of this study was to investigate the diagnostic value of MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) for selection of viable spermatozoa. Initially in 1983, Mosmann (6) used MTT tetrazolium salt for assessment of cellular viability and also for lymphocyte proliferation and cytotoxicity assay. The yellowish MTT tetrazolium salt is a substrate of mitochondrial dehydrogenase enzyme, which upon removal of the bromide converts the MTT to purple MTT formazan. Purple MTT formazan products called dye grains or spikes can be seen in the cytoplasm or traversing the cell membrane (7). This study hopes to present a new diagnostic test using MTT for sperm viability test.

MATERIALS AND METHODS

Human ejaculated spermatozoa were obtained by masturbation from either fertile normozoospermic donors or infertile patients attending the Isfahan Fertility and Infertility Center andrology laboratory. Semen samples were collected in sterile plastic containers after 2–5 days of sexual abstinence. Analyses of semen samples were performed within 30 min of the delivery of semen samples to laboratory according to WHO standards (2). Semen samples were processed twice with HAM'S F10 (Biochrom, Germany) + 10% human serum albumin (HSA: LFB-France) for 10 min at 2500 rpm. E&N, HOST, and MTT were carried out on the processed semen samples as explained below.

Eosin and Nigrosin (E&N)

E&N staining was carried out according to WHO standards (2). Briefly, 1% eosin (Merck) and 10% Nigrosin (Merck) was prepared in distilled water. A

1:2 volume of semen was mixed with 1% eosin. After 30 s, an equal volume of nigrosin was added to this mixture and then thin smears were prepared and observed by light microscopy at 1000X magnification. Viable sperm remained colorless while nonviable sperm stained red.

HOST

HOST was carried out according to Jiaen Liu (8). In order to obtain a hypo-osmotic solution with approximately 150 mOsmol, 0.9% NaCl was mixed with equal volume of distilled water. A 1:2 ratio of washed semen sample was mixed with hypo-osmotic solution and then the HOST reaction was observed under light microscope at 400X magnification after 30 min. Different HOST sperm-tail patterns were taken as HOST-positive and then percentage of live sperms were recorded.

Sperm MTT Viability Assay

In routine MTT assay for somatic cells, MTT is dissolved in PBS (phosphate-buffered saline) at 5 mg/mL and then filtered in order to sterilize and remove small amounts of insoluble residues present in some batches of MTT. Then MTT is added to the mixture of somatic cells and culture media to obtain a final concentration of 0.5 mg MTT per mL of media (6,7). In order to obtain the suitable media for sperm MTT viability assay, HAM'S F10 + 10% HSA with different buffering bases were used (HAM'S F10 + 25 mM Sodium Bicarbonate, HAM'S F10 + 10 mM Sodium Bicarbonate + 15 mM HEPES, HAM'S F10 + 25 mM HEPES). Initial studies showed that to obtain optimal results, pH and temperature should be kept constant throughout the assay; therefore HAM'S F10 + 25 mM HEPES (Gibco) was shown to be the most suitable media for the sperm MTT viability assay (data not shown). Thus routinely MTT (Loba Chemie, India) were dissolved in HAM'S F10 + 25 mM HEPES at 0.5 mg/mL and then pH was adjusted to 7.4–7.45. Then the MTT solution was filtered (0.22 μ m, Millipore) and sterile HSA was added to filtered MTT solution at 10% concentration. MTT solution was kept at 4°C in the dark for a maximum of 1 week. For routine MTT assay 50 μ L of washed sperm (5–10 millions) were added to 450 μ L of MTT solution, which was warmed to 37°C, 30 min before addition of sperm.

In order to obtain the optimal time for sperm MTT viability assay, in each of the 17 samples the sperms were observed every 30 min between 1 and

Fig. 1. MTT formazan dye grains formation in the midpiece region (X1000).

4 h after addition of sperm to MTT. Before observations the MTT–sperm mixture was slowly mixed. Sperm with grains or, rarely, with spikes in the midpiece region were taken as viable and sperm without any signs of residue were considered to be nonviable (Fig. 1). Sperm MTT viability assay was performed under sterile conditions. After analysis of the results in the 17 samples, 2 h was taken as the optimal time for sperm MTT viability assay (Fig. 2). In the remaining 40 samples observation was carried out only 2 h after addition of sperm.

Standardization of Results

During preliminary experiments, semen samples from donors or infertile patients were used for evaluation of coefficient of variation (CV) for inter- and intra-assay variations. Intra-assay variations were determined by evaluating 100 sperm on 10 different microscopic fields (a total of 1000 cells) from the same semen specimen. Interassay variations were accomplished by evaluating the percentage of viable sperm in five different specimens from the same sperm donor. CV was calculated as suggested by Mortimer (9) for both intra- and interassay values using the following formula: $CV (\%) = (\text{standard deviation}/\text{Mean}) \times 100$. The CV during standardization of results was 7 and 9% for intra- and inter-assay variation respectively.

Sensitivity and Specificity

Sensitivity and specificity of the three tests were carried out. Ten good semen specimens with good

motility were selected for evaluation of sensitivity and specificity. Density gradient percoll processing was carried out on these samples and the time for centrifugation was adjusted to obtain sperm preparations with >99% motility. These samples were taken as gold standards for viable sperm. Part of the same samples were frozen and thawed in liquid nitrogen in absence of any cryoprotectant. This procedure was carried out 3 times in order to make sure that no sperm remain alive. As suggested by Ahmadi (10), during this procedure all cellular components, including mitochondria and cell membrane, are destroyed. These samples were taken as gold standards for nonviable sperm. Both samples in equal volumes and concentrations were mixed in order to obtain a sample with 50% live and 50% nonviable sperm. Then E&N, HOST, and MTT assays were carried out on these two groups. The sensitivity and specificity of each test was calculated using the following formulae:

- Sensitivity: percentage of viable sperm in each test /total live sperm in each sample
- Specificity: percentage of nonviable sperm in each test/total nonviable sperm in each sample

RESULTS

Initial results from co-incubation of sperm with MTT showed formation of MTT formazan granules and, rarely, spikes around the midpiece (Fig. 1). Thus any sperm showing these features was taken as MTT-positive or live, otherwise they were taken as nonviable sperm. Since MTT formazan formation is enzyme-dependent, the results in Fig. 2 showed that MTT formazan formation is time-dependent, and the optimal time for MTT formazan reading was between 1.5 and 2.5 h after addition of sperm to MTT. Since the maximum percentage of live sperm was observed at 2 h after addition of sperm to MTT, this was taken as the suitable time for sperm MTT viability assay.

In order to assess the credibility of this assay, calculations of the coefficient of variation for inter- and intra-assay were carried out: the results were 9% for the former and 7% for the latter. Sensitivity and specificity of these three sperm viability assays (MTT, E&N, and HOST) were calculated as afore-mentioned (Table I). MTT, E&N, and HOST were carried out simultaneously on 57 semen samples. The means of percentages of live sperm along with standard deviation for MTT, E&N, and HOST

Table I. Sensitivity and Specificity of E&N, HOST, and MTT assay

	Sensitivity, %	Specificity, %
E&N	98	100
HOST	99	83
MTT	97	100

Note. E&N: eosin and nigrosin; HOST: hypo-osmotic swelling test; MTT: 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

were 59.59 ± 28.88 , 66.45 ± 22.63 , and 71.91 ± 24.26 , respectively.

In order to obtain the relation between MTT assay, E&N, HOST and percentage motility, correlation coefficient between these assays and motility were calculated using the SPSS statistical program. The results showed a good significant correlation between these assays, as shown in Table II.

DISCUSSION

Since 1992, ICSI is considered as a suitable method for treatment of male infertility (1). In order to carry out ICSI, suitable live sperms are required; however, in some cases of absolute or severe asthenospermia or sperm extracted from testicular tissue, selection of live suitable sperm is of importance. In these cases embryologists are usually confronted with difficulties in the selection of a suitable live sperm. Usually percentage of fertilization is lower in such cases as compared with when suitable motile sperms are present in ejaculates (8). Considering the prevalence of these cases among an infertile population (1 in 5000) (11), it has been suggested that a suitable procedure for selection of live sperm from a population of sperm is required and it is of paramount importance for the treatment of such infertility cases. Two usual procedures suggested for selection of such cases are the induction

of motility using pentoxifylline (12) and the selection of viable sperm for ICSI through HOST (13). Although both procedures are used for the treatment of such infertility cases, some researchers believe that during the HOST procedure, cell membranes can be damaged (14,15), and this might account for the lower than expected fertilization rates (16). Therefore introduction of a new sperm viability test that is based on different sperm functional characteristics might be useful as a diagnostic test for the discrimination of viable sperm from nonviable sperm, and such procedures might be helpful in the treatment of male infertility due to severe asthenospermia.

So, this study was to initially introduce sperm MTT viability assay as a new diagnostic test for the discrimination of live sperm from dead sperm. Formation of MTT formazan granules or spikes around the sperm midpiece show that sperm mitochondria contains dehydrogenase, which is capable of converting MTT to MTT formazan, and the presence of MTT formazan granules in the midpiece region identifies that sperm as a live sperm.

HAM'S F10 with a different buffering base was taken as the medium for carrying out MTT assay. The results showed that keeping pH constant is important throughout this assay. Thus HAM'S F10 + 25mM HEPES + 10% HSA with pH adjusted at 7.40–7.45 were considered suitable for this assay. Figure 2 shows that the MTT formazan formation is time-dependent, and the optimal time for sperm MTT viability assay was 2 h after the addition of sperm to MTT solution, which is similar to that for other somatic cells (7). The decrease in sperm motility and percentage of positive MTT spermatozoa observed after 2 h could be due to MTT formazan toxicity or the inhibitory effect of MTT or MTT formazan on mitochondrial dehydrogenase, and if this is the case the sperm MTT viability assay might have only diagnostic value. Therefore clinical usage of this assay needs further evaluation. In order to assess the credibility of this test, inter- and intra-assay coefficients of variation were calculated, and the values were 9 and 7% respectively. Since these values are low, they give credit to this test to be used for assessment of sperm viability. Another important factor for a test to have diagnostic value is to have high sensitivity and specificity. The results in Table I show that the sensitivity and specificity of MTT assay is high and is close to that of E&N, which is usually taken as the routine gold standard method for assessment of viable sperm. However, the specificity of this test was higher than that of HOST, but compared with the HOST procedure, which requires about

Table II. Correlation Coefficient^a of Variations Between E&N, HOST, MTT assays and Sperm Motility

	E&N	HOST	MTT	Motility
E&N	1.00	0.89	0.75	0.60
HOST	0.89	1.00	0.73	0.59
MTT	0.75	0.73	1.00	0.40
MOTILITY	0.60	0.59	0.40	1.00

Note. E&N: eosin and nigrosin; HOST: hypo-osmotic swelling test; MTT: 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

^a $p = 0.00$ in each case.

Fig. 2. The relation between percentage of viable sperms and the time after addition of sperm to MTT.

30 min, the MTT assay needs a longer time. This result confirms previous results which suggested that during HOST procedure hypo-osmotic shock on its own induces membrane damage and therefore increases the percentage of false positive sperm, which means that the percentage of dead sperm is higher than the expected value (14).

E&N is based on sperm functional aspects of cell membrane, HOST is based on integrity of sperm membrane, and sperm MTT viability assay is based on sperm mitochondrial activity; and since the outcome of each test is to measure the sperm viability, a high significant correlation coefficient should be observed between these tests and sperm motility. However sperm motility showed lower correlation with sperm MTT viability assay as compared with the correlation between sperm motility and E&N or HOST: this could be due to inhibitory effects of MTT, on mitochondrial dehydrogenase, or due to cytotoxicity of these products, which needs further evaluation, as pointed above.

In conclusion, Sperm MTT viability assay can be taken as a good diagnostic test for discrimination of viable sperm from nonviable sperm, and if sperm MTT viability assay proves to be nontoxic then it might have the potential to be used for ICSI; however the effect of MTT on fertilization and embryo development needs to be studied.

ACKNOWLEDGMENTS

We would like to thank the infertility specialists working in Isfahan Fertility and Infertility Center, especially Dr. S. M. Ahmadi, Dr. S. A. Kalantari, Dr. B. Foruham, and Dr. Z. Behdadipour for their full support and F. Molavi, F. Molavi, A. Hesami, and A. Salehi for their technical supports. We also like to thank the Royan Institute, Tehran, Iran, for their financial support, Project No. P/79/404.

REFERENCES

1. Palermo G, Joris H, Devroey P, Van Steirteghem AC: Pregnancies after intracytoplasmic injection of a single spermatozoon into an oocyte. *Lancet* 1992;340:8-17
2. WHO Laboratory Manual for the Examination of Human Semen and Sperm Cervical Mucus Interaction, 3rd edn, Cambridge University Press, 1992
3. Tsai YL, Liu J, Garcia JE, Katz E, Compton G, Baramki TA: Establishment of an optimal hypo-osmotic swelling test by examining single spermatozoa in four different hypo-osmotic solutions. *Hum Reprod* 1997;12(5):1111-1113
4. Hossain AM, Rizk B, Barik S, Huff C, Thorneycroft IH: Time course of hypo-osmotic swellings of human spermatozoa: Evidence of ordered transition between swelling subtypes. *Hum Reprod* 1998;13(6):1578-1583
5. Ahmadi A, Ng SC: The single sperm curling test, a modified hypo-osmotic swelling test, as a potential technique for

- selection of viable sperm for intracytoplasmic sperm injection. *Fertil Steril* 1997;68(2):346–350
6. Mosmann T: Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55–63
 7. Hansen MB, Nielsen SE, Berg K: Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods* 1989;119:203–210
 8. Liu J, Tsai YL, Katz E, Compton G, Garcia JE, Baramki TA: High fertilization rate obtained after intracytoplasmic sperm injection with 100% nonmotile spermatozoa selected by using a simple modified hypo-osmotic swelling test. *Fertil Steril* 1997;68(2):373–376
 9. Mortimer D: Technician Training and Quality Control Aspect in Practical Laboratory Andrology, Oxford University Press, New York, 1994
 10. Ahmadi A, Ng SC: Developmental capacity of damaged spermatozoa. *Hum Reprod* 1999;14(9):2279–2285
 11. Eliasson R, Mossberg B, Camner P, Afzelius BA: The immotile-cilia syndrome: A congenital ciliary abnormality as an etiologic factor in chronic airway infections and male sterility. *N Engl J Med* 1977;297:1–6
 12. Tasdemir I, Tasdemir M, Tavukcuoglu S: Effect of Pentoxifyline on immotile testicular spermatozoa. *J Assist Reprod Genet* 1998;15:90–92
 13. Sallam HN, Farrag A, Amaya AF, Ezzeldin F, Eid A, Sallam A: The use of a modified hypo-osmotic swelling test for the selection of viable ejaculated and testicular immotile spermatozoa in ICSI. *Reproduction* 2001;16(2):272–276
 14. Ramirez JP, Carreras A, Mendoza C: Sperm plasma membrane integrity in fertile and infertile men. *Andrologia* 1992;24:141–144
 15. Jeyendran RS, Van der Ven HH, Perez-Pelaez M: Development of an assay to assess the functional integrity of the human sperm membranes and its relationship to other semen characteristics. *J Reprod Fertil* 1984;70:219–228
 16. Casper RF, Meriano JS, Jarvi KA, Cowan L, Lucato MN: The hypo-osmotic swelling test for selection of viable sperm for intracytoplasmic sperm injection in men with complete asthenospermia. *Fertil Steril* 1996;65:972–976