Short Communication

Effect of Human Sperm MTT Viability Test on Outcome of Intracytoplasmic Sperm Injection

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Abstract

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Introduction: The aim of this study was to evaluate the effect of human sperm MTT viability assay on outcome of intracytoplasmic sperm injection. MTT is a tetrazolium salt, routinely used for cell proliferation and cytotoxicity assays.

Material and Methods: 50µl of processed semen was treated with MTT solution, while the remaining used as the control. Meanwhile, 109 donated human oocytes (metaphase II) obtained from 12 patients were divided into two groups. Fifty five oocytes were injected using MTT positive sperms, while 54 oocytes were injected with sperms from the control group. Then the injected oocytes were cultured and observed at 18, 42, 66, 90, and 114 hours pos- ICSI. Finally, the fertilization and embryo development rates were compared in both groups.

Results: No significant differences were observed between fertilization and embryo development rates in the MTT and control groups.

Conclusion: After approving that the MTT is not cytotoxic or teratogenic effects, the sperm MTT viability assay may be useful for ICSI in patients with absolute or severe asthenospermia or in patients with highly deformed sperm tails.

Keywords: ICSI, MTT, Human sperm, Viability

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Introduction

Since ICSI was first reported, it has been the first line of treatment for severe male factor infertility (1). This technique has been proven very successful if motile spermatozoa are used. Still, due to limited number of oocytes available during each cycle, the success rate with the use of immotile spermatozoa varies in the literature depending on the source of immotile spermatozoa, the selection procedure of viable sperms, the number of oocytes, and the age of patients (2, 3, 4, 5, 6).

Since sperm viability plays an important role in ICSI outcome, the selection and separation of viable sperms from a population of spermatozoa are essential. There are a few common diagnostic tests for differentiation of viable from nonviable ones, which include eosin & nigrosin (E&N), trypan blue, and hypo-osmotic swelling test (HOST). However, HOST, first reported by Barros et al. (7, 8) is the only test so far available for selection and separation of viable from non-viable ones for ICSI. Consequently, this test or its modified versions called “single sperm curling test” or “water test” have been used for injection of immotile viable sperms into oocytes referred to as HOST-ICSI. The HOST is based on fluid transport across sperm membrane under hypo-osmotic conditions. As a result of fluid influx, the tail expands and bulges in a characteristic pattern considered as hypo-osmotic response, easily identified microscopically (9). Although HOST has been used successfully along with ICSI, there have been some concerns over the use of HOST-ICSI, such as the inability of HOST procedure to distinguish viable from nonviable sperm in semen samples with tail morphological anomalies. Secondly, the plasma membranes of asthenospermic samples are fragile in hypo-osmotic conditions. In other words, although these sperms are HOST-positive, their membrane integrity is lost following the hypo-osmotic shock. However, the extent of

hypo-osmotic damage depends on the type of hypo-osmotic solution used (10). Finally, there are controversial reports regarding beneficial effect of HOST-ICSI on fertilization, implantation, and pregnancy rate (4).

In a recent study (11), we reported a new sperm viability tests called sperm MTT viability assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is among tetrazolium salts, routinely used for cell proliferation and cytotoxicity assays. Sperm MTT viability assay is based on ability of sperm mitochondrial dehydrogenase to convert MTT into purple MTT formazan (12). Since sperm mitochondria are localized to midpiece region, MTT formazan granules can be seen there in viable sperms, and such sperms are considered as MTT positive. Sperm MTT viability assay was standardized and the effects of factors such as time, temperature, MTT concentration, and pH were evaluated (11). In that study, sperm MTT viability assay was compared with HOST and EN viability assays. The results of that study showed significantly high correlations between sperm MTT viability assay and HOST, EN, and sperm motility. In addition, it was shown that sperm MTT viability assay, EN, and HOST have a sensitivity of 97, 98, 99%, and a specificity of 100,100, and 83%, respectively.

The characteristics of sperm MTT viability assay allow selection and separation of viable sperm for ICSI. Therefore, the aim of this study was to evaluate the effect of sperm MTT assay on fertilization and embryo development rates up to blastocyst stage using normal semen samples. We did not aim to perform clinical trails on the efficiency of sperm MTT viability assay in fertilization, development, and implantation rate in moderate or severe asthenospermic samples or samples with severe tail anomalies. Since the main purpose of this study was to evaluate the effect of MTT on fertilization and development rates post-ICSI using MTT positive sperms, motile sperms from normal semen samples were used to exclude the effects of sperm abnormalities. Thus, the processed portion of semen sample with adequate motility was treated with MTT and the remaining used as control. MTT positive sperms and sperms from control group were used for ICSI in donor oocytes. Fertilization and development rates in each group were recorded and compared up to 114 hr post-ICSI.

Materials and Methods

Semen samples

Semen samples were obtained from patients referring to Isfahan Fertility and Infertility Center. The semen samples were analyzed according to WHO (13) standards and then processed using gradient technique. Then, some part of semen samples were treated with MTT according to Nasr-Esfahani (11) and remaining were kept as control.

Sperm MTT viability assay

In summary, 50 µL of processed semen samples were added to 450 of µL 0.5 mg/ml MTT (Labochemi-India) solution in Ham's F10 (Gibco) + 25 mM HEPES + 10% human serum albumin (HSA-France) at 37 °C for 1 to 2 hours. At the end of incubation period, 10 to 20 µL of MTT-treated sperms were removed and added to drops of Ham's F10 + 25 mM HEPES + 10% HSA in microinjection dishes (Falcon 1006) under mineral oil for ICSI.

Oocytes

Oocytes were obtained from patients undergoing superovulation who were at risk of ovarian hyperstimulation and their husbands had no sperm following testicular sperm extraction or they did not want to undergo IVF or ICSI. Patients were consulted regarding hyperstimulation syndrome and consents were obtained regarding the use of their oocytes for experimental purpose.

According to Islamic principles, sperm donation is not allowed in Iran and oocytes from married individuals cannot be donated to other infertile couples. This study was approved by Ethical Committee of the Isfahan Fertility and Infertility center. The oocytes were used in this study could not be used for any clinical purposes as mentioned.

ICSI procedure

In summary, upon removal of cumulus mass, oocytes were treated with hyaluronidase (Sigma). Mature MII oocytes from each patient were randomly divided into two groups and then transferred into micro-drops of Ham's F10 + 25 mM HEPES + 10% HSA in ICSI Falcon dishes containing two drops of PVP (Sigma). MTT-treated or untreated sperms were also added to micro-drops of Ham's F10 + 25 mM HEPES+ 10% HSA in the ICSI dish. ICSI procedure was carried out by means of an inverted microscope equipped with Eppendorf micromanipulator. MTT-positive sperms were washed in Ham's F10 + 25 mM HEPES + 10% HSA twice before injection. Injected oocytes from each group were washed in pre-equilibrated rS1 (Vitro Life) media and then cultured under micro droplets of rS1 media covered with mineral oil (Ferticult). The oocytes were sorted for fertilization 18 hr post-ICSI. The fertilized

oocytes were further cultured for another 48 hr in rS1media and observed daily for embryo development. At the end of 48 hr, the embryos were transferred into drops of prepared dishes containing Vero monolayer in Ham's F10 + 25 mM bicarbonate + 10% FCS (Seromed-Germany) under mineral oil. The development rate of each group was recorded daily for the next two days. The embryos were discarded at this stage.
SPSS statistical software (*t_test*) was used to compare the results in this study.

Results

Figure 1 shows an MTT-positive sperm with normal morphology and Figure 2 shows an MTT-positive sperm with tail anomaly 1.5 hours after co-incubation with MTT solution (0.5 mg/ml) at 37 °C.

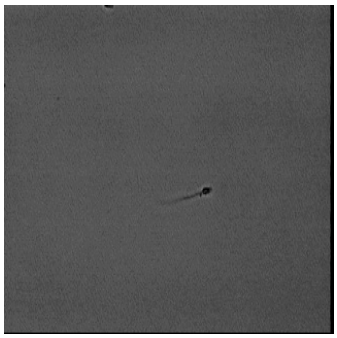

Fig 1: Viable sperm as distinguished by sperm MTT viability assay with normal morphology.

Injected oocytes and embryos were observed at 18, 42, 66, 90, and 114 hours post-ICSI. We did not find any significant differences between the mean number of fertilized oocytes and developmental stage of embryos in MTT-positive sperms and control groups at 18, 42, 66, 90, and 114 hours post-ICSI (Table 1).

Table 1: Fertilization and embryo development rates following ICSI using MTT-positive sperms and sperms from control group.

Hours Post-ICSI	Groups	Mean ± SD	P value
18 hrs	Control	67.09 ± 31.66	.963
Fertilization	MTT	67.42 ± 27.86	NS
42 hrs	Control	100 ± 0	.339
2 ≥ cells	MTT	95.80 ± 14.43	NS
42 hrs	Control	58.05 ± 34.24	.576
4 ≥ cells	MTT	66 95 ± 34 63	NS
66 hrs	Control	81.25 ± 38.60	.833
8-16 cells	MTT	84.71 ± 31.35	NS
90 hrs	Control	73 14 ± 40 55	.673
Morula	MTT	66 65 ± 36 92	NS
114 hrs	Control	54 80 ± 45 36	.272
Blastocyst	MTT	41.15 ± 33.11	NS

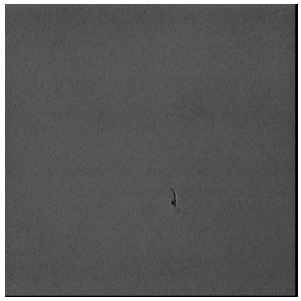

Fig 2: Viable sperm as distinguished by sperm MTT viability assay with tail abnormality.

Discussion

ICSI has been proved to be a very successful method for fertilization. The outcome of ICSI depends on the quality of oocytes and the viability of sperms with normal morphology (15). It is not always possible to obtain viable motile sperms in a small group of patients and random selection of non-motile ejaculated sperms have resulted in lower fertilization rates. Nagy (16) reported lower fertilization with injection of non-motile testicular sperms compared with motile testicular sperms. Literature studies reveal that HOST-ICSI is the only procedure available for selection and injection of non-motile but viable sperms for intracytoplasmic sperm injection into oocytes. During HOST-ICSI procedure, sperms are introduced into hypo-osmotic solution (2). The viable sperm tail swells and forms different subtypes; these sperms are considered as HOST-positive (17). The HOST-positive sperms are immediately washed in an iso-osmotic medium and then used for injection into oocytes (3). Since the initial reports of first pregnancy with HOST-ICSI, this procedure has been successfully used for treatment of patients with severe or absolute asthenospermia. Because of disadvantages of this procedure in certain patients (sperms with tail anomalies), the purpose of this study was to evaluate the effect of MTT- positive sperms on the fertilization and embryo development rates post-ICSI. The results in Table 1 showed no significant difference between fertilization and embryo development rates up to blastocyst stage. As sperms with good motility were used for injection of oocytes in both MTT-positive and control groups, the only difference between the two group was treatment of sperms with MTT. Therefore, our results suggest that incubation of sperms with MTT solution and the presence of MTT granules in the midpiece region of injected sperms have no inhibitory effect on fertilization and development rates of embryos up to blastocyst stage *in vitro*.

Even though the MTT-ICSI procedure might have certain advantages over HOST-ICSI especially in patients with severe sperm tail anomalies or sperm sensitive to hypo-osmotic conditions, there are certain drawbacks of MTT-ICSI procedure as follows: (1) a gradual loss of MTT granules upon introduction of sperms into drops of media under mineral oil and (2) a reduction in sperm motility upon gradual exposure of sperms with MTT solution. These phenomena are possibly due to solubility of MTT granules in mineral oil and cytotoxic effect of MTT or inhibition of sperm mitochondrial dehydrogenase.

In conclusion, MTT viability test is not only a good diagnostic test for distinguishing viable sperm from non-viable ones (11), but also can be potentially used along with ICSI procedure, as demonstrated by our results.

However, it is necessary to perform further animal studies concerning the toxicity and teratogenic effects of MTT on embryo development post-blastocyst stage before clinical application of MTT-ICSI in patients with severe asthenospermia or in semen samples with tail anomalies.

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مقاله کوتاه

اثر روش ارزیابی حیاتی اسپرم انسانی با روش MTT، بر نتایج حاصل از تزریق درون سیتوپلاسمی تخمک

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چکیده

دریافت مقاله: ۸۴/۱۰/۲۵، پذیرش مقاله: ۸۴/۱۲/۱

* **هدف:** بررسی اثر به کارگیری روش MTT در ارزیابی حیاتی اسپرم انسانی، بر نتایج حاصل از تزریق درون سیتوپلاسمی تخمک

* **مواد و روش‌ها:** ۱۰۹ تخمک به دو گروه تقسیم شدند. ۵۵ تخمک توسط اسپرم های ارزیابی شده؛ با روش MTT به طور هم‌زمان و ۵۴ عدد با اسپرم هایی از همان نمونه ها بدون مجاورت با محلول MTT مورد تزریق قرار گرفتند (گروه کنترل). تخمک‌ها در زمان‌های ۱۸، ۴۲، ۶۶، ۹۰ و ۱۱۴ ساعت پس از تزریق مورد مشاهده قرار گرفتند. درصد لقاح و رشد جنین‌ها در دو گروه مقایسه شد.

* **یافته‌ها:** در بین دو گروه مورد آزمایش و گروه کنترل از نظر درصد لقاح و میزان رشد جنین ها اختلاف معنی‌داری مشاهده نشد.

* **نتیجه‌گیری:** در صورت اثبات عدم وجود اثرات تراتوژنیک MTT، می توان از روش ارزیابی حیاتی اسپرم توسط MTT، در روش درمانی ICSI جهت انتخاب اسپرم زنده، در بیمارانی که دچار آستنواسپرمی شدید شده و یا دارای دم اسپرم آمورف می باشند، استفاده کرد.

کلیدواژگان: اسپرم انسان، ارزیابی حیاتی، ICSI، MTT