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Application of Vitality Tests on Asthenozoospermic Semen from Infertile Men

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ABSTRACT

Fresh semen obtained from infertile men occasionally exhibits the total absence of motile sperm (100% asthenozoospermia). Vitality tests may reveal that a proportion of the immotile sperm has functional membranes, which are considered viable. An easy test for the prior determination of sperm viability may assist in sperm selection for the microinjection of oocytes. The main objective of this study was to apply three standard vitality tests on the asthenozoospermia samples from infertile men. A total of 30 semen samples with >50% immotile sperm were obtained for this study. Each semen sample was divided into three equal aliquots following the preliminary evaluation of semen parameters. Three vitality tests of eosin-Y, hypoosmotic swelling (HOS) and pentoxifylline (PX) were applied on all samples. A total of 100 spermatozoa were then evaluated for the sign of viability. The percentage of normal morphology and sperm count were within the normal range of 31.8 ± 15.9% and 55.8 ± 46.6 × 10^6 per ml., respectively. Following the application of eosin-Y, 45.2 ± 15.2% of spermatozoa were unstained (alive). In addition, 51.9 ± 18.6% and 52.7 ± 21.1% of sperm were observed to be viable with HOS and PX tests, respectively. Three samples with total asthenozoospermia demonstrated with 37.61%, 27%, and 33.33% viability after the application of eosin-Y, HOS, and PX tests, respectively. The results indicate that the vitality tests are very useful in differentiating live sperm from dead one, even in the samples with total asthenozoospermia. Also, PX may be a more accurate vitality test with a therapeutic application during the microinjection cycles. It not only has the ability to differentiate live sperm, but also enhances motility. *Iran. Biomed. J. 3 (3 & 4): 77-81, 1999*

Keywords: Vitality test, asthenozoospermia, eosin-Y, hypoosmotic swelling test, pentoxifylline

INTRODUCTION

The treatment of male infertility was revolutionized with the introduction of intracytoplasmic sperm injection (ICSI) in 1992 [1]. This type of technique is primarily aimed at the subjects who have impaired semen parameters and for whom other infertility treatments have already failed. Microinjection with ICSI involves the injection of only one mechanically immobilized, but live, spermatozoon into a mature oocyte (MII) [2, 3, 4]. Therefore, individuals with defects in semen parameters, such as oligo- or asthenozoospermia can benefit from this technique. However, men with severe type of teratozoospermia, such as acrosomeless globozoospermia usually fail the ICSI treatment [4]. In addition, the injection of dead spermatozoon into an oocyte generally results in fertilization failure. Therefore, it is necessary to carefully evaluate the stage of viability of the immotile spermatozoon before starting ICSI [5, 6]. In addition to the ejaculated samples, sperm can be obtained from the testis of obstructive azoospermic patients. Testicular extracted spermatozoa are generally immotile, though their vitality is high [6, 7]. The problem associated with the use of immotile sperm is that the viability of spermatozoon for ICSI is not determined, unless a vitality test is applied accordingly. WHO recommended that vitality tests should be applied for all semen samples with >50% immotility. These types of samples are considered as abnormal [8].

Nowadays, there are several standard tests available for the assessment of the vitality of spermatozoa [5, 6, 7, 8]. One of these tests is based on the principle that dead spermatozoa take up the supravital red stain of eosin-Y, whereas living cells, regardless of their motility stage, will be unstained.

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This is due to the living membrane that actively excludes the dye [9]. Therefore, the eosin-Y staining makes it possible to differentiate the sperm that are non-motile, but alive from those that are already dead. This assay reflects sperm membrane integrity, particularly the head region which takes up the red stain immediately [9, 18].

Jeyendran *et al.* (1984) was the first scientist studying the functional integrity of sperm membranes using a hypo-osmotic solution composed of fructose and sodium citrate [10]. The ability of the sperm tail to swell and/or coil in the presence of the aforementioned solution demonstrates that the influx of water across the membrane occurs normally [5, 10, 11]. This phenomenon indicates the normal plasma membrane integrity of spermatozoa [12]. The hypoosmotic swelling test (HOS test) should not be used as a sperm function test, but can be used effectively as a standard vitality test [8]. Casper and associates (1996) indicated that the HOS test might be used to select sperm with functionally intact membranes in a population of total immotile spermatozoa, obtained from ejaculate or testicular tissue [13].

Recently, the application of Pentoxifylline (PX) has been considered as an effective methodology for diagnosing the viable sperm in the ejaculates or even testis [6, 7]. PX is a methylxanthine derivative in the same pharmacologic class as caffeine, which acts as a phosphodiesterase inhibitor agent [14]. The inhibition of phosphodiesterase results in an increase in intracellular cyclic adenosine monophosphate (cAMP) levels. This, in turn, increases the cellular glycolysis and ATP production in living cells. The elevation of ATP is considered to increase sperm motility [15]. PX can be easily used to differentiate live spermatozoa from dead one by restoring motility, which may also improve the fertilization and embryo formation following ICSI [7].

The non-toxic vitality tests are particularly important in instances where immotile sperm are the only available source for ICSI. In this situation, ICSI either has been cancelled or a vitality test has been used to select a viable spermatozoon for injection [13]. The direct injection of dead spermatozoon into an oocyte would generally fail the fertilization process (total fertilization failure). Therefore, the objective of this study was to examine the relationship between the eosin-Y, HOS test and PX (tests that examine plasma membrane integrity through different approaches) in a selective group of infertile with >50% immotile sperm.

MATERIALS AND METHODS

Semen Samples. A total of 30 semen samples from men aged 21-56 years, who were enrolled for infertility treatment, were prospectively collected for this study. The selected samples were asthenozoospermia with >50% immotility. The semen were collected in sterile wide-mouth cups after 3 to 5 days of sexual abstinence, and allowed to liquefy for 30-40 minutes at 37°C. The volume of the semen along with the parameters of count, type of motility, and morphology were studied by one individual (F. M-R). Sperm count and motility were analyzed using Mackler counting chamber (Sefi-Medical Instrument Inc.), while the percentage of normal morphology was examined by WHO criteria [8].

Vitality Tests. Three different vitality tests were done on each semen sample at the same time. For this, each sample was divided into equal portions in three sterile tubes (Falcon Co., USA).

1. Eosin-Y. The eosin-Y staining (0.5% wt/vol) was performed by mixing 10 m l of semen with 10 m l of the stain on a microscope slide and covered with a 22 × 22 mm coverslip. A total of 100 spermatozoa were then counted within 2 min after the addition of the stain [8]. The results were expressed as the percentage of unstained (live) sperm (Figure 1).

2. HOS test. The HOS test was done by mixing 10 ml of semen and 100 ml of warm hypoosmotic solution. This solution was prepared by dissolving 7.35 g of sodium citrate and 13.5 g of fructose in 1 liter of deionized distilled water filter sterilized and frozen in 1 ml aliquots for use as required [8]. The mixture was incubated for 45-50 minutes at 37°C before it was examined with a microscope at 400× magnifications. One hundred spermatozoa were examined, and the percentage of sperm that showed coiled tails (sign of live) was calculated accordingly (Figure 2). The coiling representing an intact flagellar membrane was evaluated according to the WHO guidelines [8].

3. PX. In all samples, PX (Sigma chemical Co., St Louis) was used at a standard concentration of 1 mg/ml (3.6 mM) in Ham's F10 medium (Biochrom Co., Germany) [16]. This was prepared weekly and kept refrigerated at 4°C until used. The PX test was performed by mixing 250 ml of semen and 10 ml of

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the aforementioned solution. The mixture was then incubated for 30 minutes at 37°C before it was examined at 400× magnifications. The percentage of spermatozoa showing motility was calculated as living cells.

Paired t-test was applied to compare the results using SPSS for windows version 6.0 and the significance differences were expressed at the level of $p<0.05$.

Fig. 1. Light microscopy of spermatozoa stained with eosin-Y. Arrows pointing non-stained live sperm. 400.

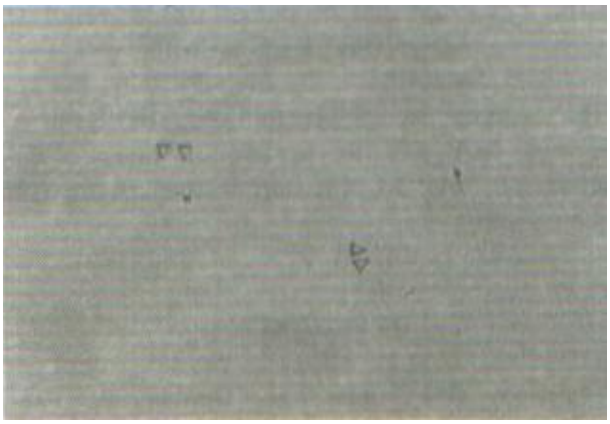

Fig. 2. Light microscopy of sperm following HOS test. Arrows showing coiled-tail (live) sperm. X400.

RESULTS

The semen analysis data and the results from three sperm vitality tests are shown in Table 1 and Fig. 3, respectively. The difference between the percentage of live sperm detected by eosin-Y and HOS tests, as well as eosin-Y and PX were statistically significant ($p<0.01$). However, the results from HOS test and PX were insignificant ($p>0.05$). Only three samples were shown with complete asthenozoospermia (0% motility), which demonstrated with 37.61%, 27%, and 33.33% viable sperm, after the application of eosin-Y, HOS, and PX tests, respectively. The other samples (total of 27) were asthenozoospermia with 52% to 90% immotile spermatozoa. All vitality tests were successful in detecting live sperm from all the semen samples.

Table 1. Summary of semen parameters according to the WHO criteria (1999) (Values are mean ± SD).

Volume (ml.)	4.56 ± 2.23 (1-8)
% Normal morphology	31.80 ± 15.89 (15-55)
Concentration ($\times 10^6$/ml)	55.79 ± 46.65 (1-180)
% Progressive motility	21.23 ± 14.44 (0-40)
% Immotility	67.13 ± 20.4 (52-90)

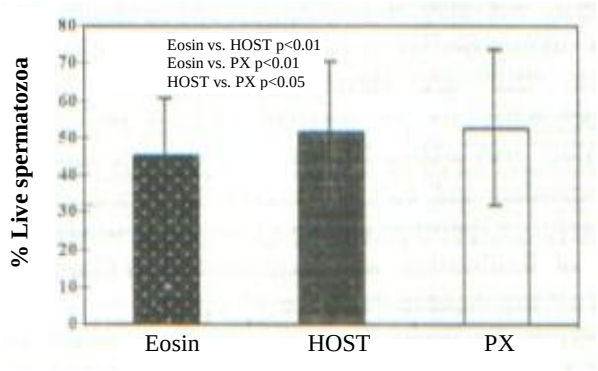

Fig. 3. The percentage of live sperm as assayed by three vitality tests.

DISCUSSION

The vitality tests for investigating the functional integrity of human sperm have been introduced in assisted reproductive technology (ART) laboratories for the last several years. The mechanism of these tests is different from each other, but the rationale is to differentiate live from dead sperm within a short period of time [5, 7, 8, 17, 18]. The eosin-Y staining technique for the assessment of sperm viability results in the death of the sperm [9]. Therefore, it cannot be used in the microinjection-ICSI cycles. Recently, Angelopoulous *et al.* reported that 56.3% of sperm recovered from testis of five infertiles were non-stained (alive) with Eosin-Y [6]. Our results revealed that only 45.23% of seminal spermatozoa were alive after the addition of eosin-Y. This test is

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simple with easy application, but may fail to detect all the viable sperm in ejaculates. The HOS test is also simple, but superior to the eosin-Y test, which has been recommended by WHO [8]. It is considered as one of the standard tests, with easy application; thus, it is widely used [17]. Although, most of the investigations on the HOS test show good predictive power, some do raise concerns about its validity [19, 20]. For example, like eosin-Y, the HOS does not provide unequivocal information regarding the fertilizing ability of the sperm [5]. It, however, should be pointed out that Casper *et al.* (1996) demonstrated fertilization and cleavage rates of 43% and 39%, with HOS selection of viable sperm from complete asthenozoospermia samples [13].

Recently, PX has been added to the list of vitality tests [6, 7]. This drug can be added to the semen samples not only to evaluate the sperm vitality, but also to improve sperm motility. It is interesting that sperm that are devoid of midpiece (where mitochondria are located) will not show any type of motility after adding PX [6]. In a previous study by Dimitriadou and colleagues (1995), PX was shown to improve sperm progressive motility; however, the rate of fertilization and resulting embryo formation was not increased in 97 IVF cycles [15].

Our results demonstrate that vitality tests can be used to select viable sperm even in samples with 100% immotile sperm. Only three samples showed 100% immotility, with viable sperm of 37.61%, 27%, and 33.33%, when tested with eosin-Y, HOS, and PX, accordingly. In addition, the results confirm that a good correlation between the HOS and PX tests exists (51.90% vs. 52.75%). In this study, over 50% of spermatozoa were shown to be alive following the application of the HOS test. Therefore, these two tests, comparing to eosin-Y, not only are more successful in evaluating sperm vitality, but also will not damage the sperm cell membrane. As a result, the same reacted sperm with HOS or PX can be used for ICSI cycles [6, 7, 13, 14, 15, 16, 17, 21]. Casper *et al.* (1996) noticed that only 31.1% of samples with total sperm immotility were reacted with HOS test [13]. These findings also suggest that PX is an adequate replacement to the HOS test; it not only functions as a vitality test, but also can improve the sperm motility within a short period of time [7]. Furthermore, Tasdemir and associates (1998) studied the effect of PX on immotile testicular sperm of infertile men [7]. They observed the initiation of motility in all samples after 30 min of incubation with the PX. Therefore, they considered PX as a superior test to HOS, which not only differentiates between dead and live sperm, but also initiates or improves sperm motility. It also has been proposed that PX acts as an antioxidant by removing peroxides formed from free radicals and thus preventing acrosomal membrane and maintaining acrosomal reaction [16].

In conclusion, the vitality tests are beneficial in detecting live sperm in ejaculates or from other sources such as epididymis, testicular tissue, or frozen-thawed specimens. It should be noted that Eosin-Y is not suitable for ICSI cycles, as it will immediately kill the sperm [8, 9]. However, the HOS and the PX tests are presently the best methods in choosing the live sperm for microinjection of the oocytes [6, 7, 13, 15, 16, 21]. As an advantage over HOS, motility enhancers such as PX may have dual role of distinguishing viable sperm, in addition to enhancing the motility. None-the-less, PX should be used with caution during ICSI cycles, since it may be toxic to the oocytes or resulting embryos [21].

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