**Introduction**

Almost 10% to 20% of all male infertile cases are suffering with azoospermia (1,2). Testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI) are performed for treatment of men diagnosed with obstructive or non-obstructive azoospermia (OA) (3). Testicular spermatozoa are, however, immature physiologically and often are immotile or with finely twitching movement instantly after biopsy (4). Improvement of testicular sperm motility after *in-vitro* culture is the good strategy in azoospermic cases (5). Some procedures for *in vitro* maturation of human testicular spermatozoa have been proposed by researchers (6).

*In vivo* sperm maturation is the process where spermatozoa access to the potential for progressive motility and fertilization. It is the next phase of spermiation stage that spermatozoa are released from the seminiferous tubules and reach the epididymal site (7). Passage of human spermatozoa through epididymis lasts about 10 days which coincides with structural modifications and changes in the lipid and protein composition of the sperm membrane (8,9). It finally leads to their achievement of progressive motility (10,11).

Immediately after testicular biopsy, finding of sufficient numbers of motile sperm cells in azoospermia patients is very difficult. There are some chemical products that are stimulator for *in-vitro* improvements of the testicular sperm motility (12). These are pentoxifylline (13) 2-deoxyadenosine (12) and caffeine (14) that induce...
variations in sperm cyclic AMP contents. However, these drugs may be toxic to the spermatozoa and the resulting embryos in assisted reproduction strategy (15). Also, when spermatozoa are exposed to these products, motility exhibits only for short time, and then become immotile permanently, because depletion of sperm’s metabolic resources occurs rapidly (12). Although, some studies reported that in-vitro culturing of testicular samples can increase the number of motile spermatozoa, but there are no guidelines to suggest the suitable incubation time or temperature used to improve sperm motility in TESE situation. Therefore, determination of appropriate incubation time and temperature for the testicular sperms before ICSI or cryopreservation procedure to yield a proper extracellular environment is critical (16). Therefore, this study was designed to assess the role of different incubation time intervals and temperature on motility feature of spermatozoa extracted from testes biopsies in azoospermia cases.

Methods

Patients

In this prospective study, the subjects were selected between September 2015 and October 2016. Testicular biopsies were obtained from 27 men with OA referred to our infertility center for diagnostic purpose. We collected the specimens from discarded TESE samples for the study. The mean age of the men was 39 years (range, 29–49 years). Informed consent was obtained from each patient and the project was approved by Ethics committee of authors’ institution (IR. SSU.RSI.REC.1394.30).

Testicular biopsy preparation

Briefly, human testicular tissue was obtained surgically (TESE) from patients with OA. The samples were minced mechanically and dissected into small pieces using needle in a sterile Petri dish. The tissue samples were kept moist with Hams F10 medium (Sigma Chemical Co., St Louis, MO, USA) during dissection. Initial examination for the presence of spermatozoa was performed under an inverted phase-contrast microscope (Olympus, Tokyo, Japan). The suspension was then transferred to a test tube, if spermatozoa were noticed in Petri dish. TESE spermatozoa were often accompanied with red blood cells, debris, testicular cells and a large number of dead or immotile spermatozoa that have detrimental effects on existing motile spermatozoa. The samples were centrifuged with RBC lysis buffer for 5 min at 300 g to eliminate these cells. The pellets were then re-suspend in 3 mL of Hams F10 and centrifuged at 500 g for 10 min to wash off the RBC lysis buffer, and to preserve the integrity of the other testicular cells. Finally, the pellet was resuspended in 0.5 mL of Hams F10+20% HSA medium (In Vitro Care Inc., San Diego, CA, USA) (17).

In vitro incubation of testicular tissue

Ham’s F10+20% HSA media is considered as an appropriate medium for sperm culture (18). After preparation, drops of 10 μL were placed in a Petri dish and covered with mineral oil (Sage Biopharma, Bedminster, NJ, USA). Individual spermatozoon was observed carefully with a high magnification under the microscope to evaluate any sign of sperm motility, such as twitching of the head or tail. A total of 100 spermatozoa were observed in all microdrops, and the motility was assessed at day 0 (D0) under an inverted microscope. The first part consisting of sample that was cultured for 3 days at 37 °C in 6% CO₂, 5% O₂ balance N₂. Because, 37 °C is high temperature compared with the physiologic temperature for testes tissue, we cultured the second part for 3 days at room temperature (RT) of 25 °C.

About 0.5 mL of the upper layer of Hams F10+20% HSA was changed with the same volume of the fresh medium daily. The culturing samples were assessed daily for percentage of motile spermatozoa using the same method as the initial collection by an inverted phase-contrast microscope (Nikon Diaphot 300; UK Ltd., Kingston). The PH and temperature of the culture medium and incubator were controlled daily and maintained throughout the study.

Statistical analysis

SPSS software package was used for data analysis. The Paired Student’s t-test was performed to compare the differences in the parametric, and Mann Whitney U test was used in non-parametric groups. Statistical significance was set at P≤0.05. The data showed as mean ± SD.

Results

The findings indicated that very few spermatozoa demonstrated twitching tail in samples from collecting biopsy tissue on D0. After culture to D1, a maximum range
of sperm cells began demonstration of twitching or wavy flagellar movement in both groups. Even, spermatozoa in some of the cases were seen to be swimming at the periphery of the culture droplets. However, the numbers of motile spermatozoa in incubator culturing condition at D1 were significantly lower than RT condition (67.85±13.2 vs. 76.3±10.5; P ≤ 0.05). With further culturing, the spermatozoa lost their progressive motility, but maintained the twitching motility after 48 h of culturing (D2). However, when in-vitro culturing was continued for three days (D3), the sperm motility rate was decreased significantly in both groups (Table 1).

Discussion

Successful pregnancies and healthy babies were achieved after TESE followed by ICSI (19,20). In some countries, such as the United Kingdom, practice guidelines stipulate that only documented motile sperms can be injected into oocytes. According to these guidelines, only motile sperms have to be injected into MII oocytes in IVF facilities. Other reports declared that the injection of immotile sperm to the oocyte may lead to the failure of the fertilization (21,22). Motility is a safe, practical and necessary method to estimate the sperm viability in ICSI program (4). However, it was reported that only >3% of the testicular spermatozoa are motile after a biopsy (15). Some studies suggested the testicular sperm culturing technique to stimulate the sperm motility (23-25). The exact mechanism of improving the motion of testicular sperms after in vitro culture is still not clear. In the current study, our samples were prepared with simple wash method before culturing and all of the testicular cells, such as sertoli, leydig and germ cells were present during the culturing along with the sperm cells. The interaction between testicular somatic and germ cells and even the interaction among germ cells may enhance the sperm maturation process, presumably by providing a coculturing system (12). It was suggested that having access to a progressive motility is the sign of sperm maturation (5,26). Another factor in the beginning of sperm motility may be due to the elimination of inhibitory factors with simple wash before culturing. The activation or synthesis of essential factors assists in motility improvement (12). Gradual degeneration of the testicular somatic cells after a long-term culturing may decrease the sperm motility.

Adding chemical components, including 2-deoxyadenosine and pentoxifylline (27) or hypo-osmotic swelling test (HOST) (28,29) can stimulate the motility or viability of the testicular sperms in vitro condition. In conditions that only twitching spermatozoa are available, in vitro culturing is more effective. Using motile spermatozoa after this method can be a preferable optimal alternative, rather than chemical components and HOST. We found that a small number of sperms has a typical motile immediately after testicular biopsy analysis, but after culturing them in RT condition, sperm motility was significantly increased in comparison with culturing at 37 °C after 24 h. It should be noted that motile spermatozoa were still present in both conditions at D2 of culture, but their motility begins to decline significantly.

Although, Wood et al. proved that there is not a difference in motility between immediate and cultured testicular sperms (30), some studies have confirmed our results (24,31-33). Since, after 24–48 h of culturing, adequate motile spermatozoa may exist in the culture media, a period of 24–48 h as an optimal time for the development of motility is suggested (4,24). They suggested that the sperm testicular biopsy is achieved 1 or 2 days before the oocyte retrieval. Also, Bin et al. showed that this finding was eligible in the frozen-thawed TESE specimens. They reported that culturing frozen-thawed TESE samples for 24 h, presented a similar motility with fresh TESE.

| Incubation time | Motility at RT (%) | Motility at IT (%) | P value |
|-----------------|-------------------|-------------------|---------|
| Before incubation | 13.22±3.6         | 13.22±3.6         | NS      |
| Day 1           | 76.3±10.5         | 67.85±13.2        | 0.012*  |
| Day 2           | 43.70±11.9        | 38.40±11.7        | NS      |
| Day 3           | 15.33±6.9         | 12.03±4.3         | NS      |

Values are mean ± SD. *, on day 1, sperm motion were significantly different between RT and IT (P<0.05), as indicated by the t-test. RT, room temperature (25 °C); IT, incubator temperature (37 °C); NS, not significant.
samples (4). The sperm in vitro culturing approach is associated with aging in long-term culturing. Due to the presence of high unsaturated fatty acid in its membrane and deficiency of the antioxidant in its cytoplasm (34), sperms may be vulnerable to harmful effects of reactive oxygen species (ROS) which may lead to sperm DNA damages (12). Recently, Schiewe concluded that the elevated ROS and DNA fragmentation index (DFI) after in vitro culturing are not harmful, and they may be due to the presence of dead sperms in solution and using them does not have any impaired clinical outcomes (32). Indeed, ROS production in small amounts, the by-product of electron transfer chain in sperm cells, is necessary for sperm vital activities (35). However, if these products aggregate during the culturing, they will gradually become toxic to the spermatozoa (36).

The applied media and the culturing duration are two main factors in sperm preparation prior to ART techniques. Hams F10 is a common media in ART laboratories that was used for sperm culturing. Also, adding serum supplementation may help enrich the culturing media. If sperm culturing is performed in a simple and un-supplemented media (23,24,37) or the media is not changed or gassed with CO₂ (31), it may result in the deficiency of energy and nutritious substrates. Using suitable and changeable mediums can be supportive for sperm maturation. In this study, we cultured the samples in Hams F10+HSA that are an isotonic medium to ensure sperm survival by avoiding osmotic shock and pH alterations, and it provides the optimal buffering capacity. Adding serum to the culture media acts as a powerful antioxidant with an important role in preserving sperms from oxidative stress-induced damages, helping its motility and preventing lipid peroxidation (38). Also, proteins of HSA are a nutrient in the culture media; it seems that their existence has some effects on the sperm motility (39).

In the current study, we changed the culturing medium daily, which caused the preservation of sperm motility. Our data showed that the majority of testicular sperms acquired motility and became mature after culturing, if they were normal. A decline in motion after prolong culturing may be due to the sperm cells death that may be induced by necrosis and apoptosis. Also, the evaluation of testicular sperm with electron microscope reported a structural anomaly in midpiece, which disturbs the motility, even after in vitro culture (12).

There is a main concern that incubator temperature is beyond the physiologic temperature of testis function and sperm culturing at low temperature may offer a good result in ART programs. Although the spermatozoa culturing at 37 °C is a routine approach in ART laboratories, we found that testicular sperms enhanced their motility better at RT in comparison with culturing at 37 °C. Some sperm functions, like capacitation are temperature dependent processes and the cellular mechanisms that involved in these functions, can be controlled by culturing temperature. Some studies demonstrated that sperm culturing at RT caused a temporary “quiescent” state in the capacitation process (40). Regarding to this feature, sperm culturing at RT may be beneficial for delayed treatment of infertility, especially in cases with azoospermia. It is concluded that testicular sperm culturing at RT for 24 h was appropriate to improve sperm motion in azoospermic patients. Long-term incubation or suboptimal conditions may damage the sperm motion and increase the structural/chromosomal abnormalities (41). Further studies are needed to evaluate the sperm DNA integrity after in-vitro culture of TESE cases.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: Informed consent was obtained from each patient and the project was approved by Ethics committee of authors’ institution (IR.SSU.RSI.REC.1394.30) and written informed consent was obtained from all patients.

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