Original article:

Evaluation of Semen Samples Before and After 'Swim Up' Technique with Mitotracker

Cetinkaya Karabekir Seda[1], Aydan Özgörgülü[2]

Abstract:

Objectives: Infertility problems are seen in nearly 15% of married couples, and almost 50% of these involve male infertility. The sperm mitochondrion plays an important role in sperm motility. The morphology of sperm, sperm preparation techniques, and the number and motility of sperm during insemination are important parameters that affect the rate of pregnancy with intrauterine insemination. The aim of the study was to observe the changes in sperm mitochondrial activities and numbers with the Mito Tracker Red 580 kit at 30th, 45th and 60th minutes after swim up. ***Materials and Methods*:** In this study, we evaluated 20 oligospermic and 20 normospermic samples. The "swim-up" technique was applied to these oligospermic and normospermic samples, and changes in sperm mitochondria and number at 30, 45, and 60 min were observed using MitoTracker Red 580 staining. ***Results:*** The numbers of swimming sperm in oligospermic and normospermic samples were higher at 30 min than at 45 and 60 min. No marked change was detected in the mitochondrial activities of swimming sperm in oligospermic and normospermic samples at 30, 45, and 60 min after the swim-up. Our findings demonstrate that the number of swimming sperm decreased with time, but no change was seen in the mitochondrial activity of the sperm by the swim-up technique. ***Conclusion*:** According to findings, mitochondrial movement is not exactly related to the 30th, 45th and 60th minutes. Therefore, it is thought that it needs to be investigated in mechanisms other than mitochondrial activity.

Keywords: sperm,swim-up method; MitoTracker 580; oligospermia

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Introduction

Intrauterine insemination (IUI) is a part of *in vitro*fertilization (IVF)technology. This technological improvement has come to the fore with the development of sperm preparation techniques. In recent years, it has become a successful treatment method in terms of both results and costs when applied to appropriate fertile patients[1].

One of the most commonly used methods in preparing sperm by washing for the IUI procedure is the "swim-up" method. Although many progressive motile sperm can be obtained through swim-up, the number of sperm decreases[2]. Sperm with greater movement capacity are separated from the pellet produced by centrifugation by moving toward the culture medium, separating the sperm from the seminal plasma. One of the effects of centrifugation is the production of oxygen free radicals (ROT) by subpopulations of cells in semen[4,5]. It has been reported that ROT formed in the centrifugation process can cause permanent DNA damage[3]. However, it has also been demonstrated that low levels of ROT increase sperm capacitation, acrosome reactions, and oocyte fusion. ROT production by spermatozoa is a normal physiological phenomenon, serving as a significant mediator in the regulation of sperm capacitation and

1. Seda Cetinkaya Karabekir, Assistant Professor Seda Cetinkaya Karabekir, University of KTO Karatay, Faculty of Medicine, Department of Histology and Embryology, Fetih neighborhood, Alaaddin Kap Street. Number: 130 Konya, TR 42020, Turkey. Email:sedacetinkaya03@gmail.com, seda.cetinkaya@karatay.edu.tr
2. Prof. Aydan Özgörgülü, University of Necmettin Erbakan, Faculty of Meram Medicine, Department of Histology and Embryology, Konya, Turkey. Email:acanbilen@konya.edu.tr

Correspondence to: Seda Cetinkaya Karabekir , Assistant Professor Seda Cetinkaya Karabekir, University of KTO Karatay, Faculty of Medicine, Department of Histology and Embryology, Fetih neighborhood, Alaaddin Kap Street. Number: 130 Konya, TR 42020, Turkey, **Email**:sedacetinkaya03@gmail.com, seda.cetinkaya@karatay.edu.tr

facilitating the acrosome reaction, sperm-oocyte interaction, and signal transduction mechanisms[3-5]. Under normal conditions, ROT are removed continuously by antioxidant mechanisms to maintain them at appropriate levels, allowing sperm to perform their normal functions[3,5]. However, excessive ROT production leads to oxidative stress, resulting in the disruption of spermatozoa and exceeding the antioxidant capacity of seminal plasma. Furthermore, high levels of ROT disrupt the inner and outer mitochondrial membranes[5-7].

The middle section of spermatozoa, which is rich in mitochondrial membranes, is the primary target of ROT. Rapid loss of intracellular adenosine triphosphate (ATP), causing axonemal damage and decreased sperm motility, is regarded as the major mode of action of ROT[8].

The duration of sperm motility and mitochondrial activity are significant indicators of fertilization potential[9]. Therefore, in this study, the mitochondrial activity in patients with oligospermia and normospermia after the swim-up technique and how their sperm numbers were affected at 30, 45, and 60 min were evaluated with the MitoTracker kit.

Materials and methods

This study was performed with samples from 40 patients (20 with oligospermia and 20 with normospermia) selected from patients at the Necmettin Erbakan University, Meram Faculty of Medicine, Assisted Reproductive Techniques Unit from June 2012 to August 2012. Semen samples were taken after 3 days of sexual abstinence; motility assessment and viability examination of the samples were then carried out.

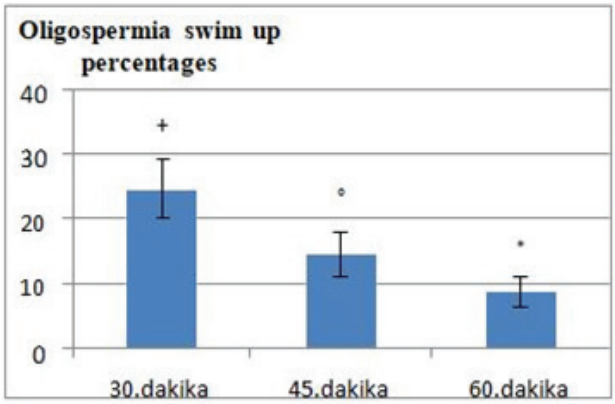
Each semen sample was homogenized in medium at a 1:1 ratio to a total volume of 3 mL.Next, 1 mL was taken from the homogenized mixture and placed in three microcentrifuge tubes, and 100 µL of sperm flotation medium was added. The tubes were incubated in a 45° inclined position in a 5% carbon dioxide environment at 37°C for 30, 45, and 60 min. Following the incubation period, the 100-µL top layer was removed with a micropipette and centrifuged. Sperm deposited after centrifugation were spread on poly-lysine-coated slides, stained with 50 µl of mitotracker red 580 (Invitrogen,Cat. no. M22425), and incubated for 1 h. After 1 h, sperm mitochondria and changes in sperm numbers were examined under dark ambient conditions with fluorescence microscopy.

The data were analyzed using SPSS software (PASW Statistics for Windows, Ver. 18.0; SPSS, Inc., Chicago, IL, USA). A dependent *t*-test was used to assess related samples, and p values of <0.05 were considered to indicate statistical significance. A t-test between the groups was performed in comparing the oligospermicand normospermic samples.

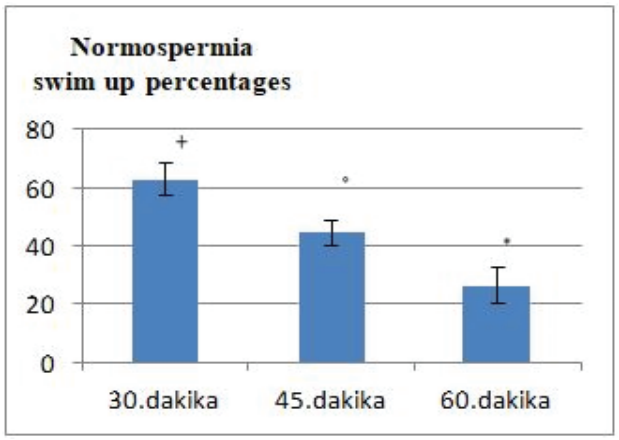
Ethical clearance: The study was conducted in accordance with the WHO 2010 criteria (ethics committee decision no. 2012/08).

Result

In terms of sperm number, the WHO criteria define oligospermia as 10 to 20 million/mL and normospermia as >20 million/mL. In this study, we examined samples from men with both oligospermia and normospermia. We sought to observe sperm mitochondria and changes in the numbers of sperm in semen samples after swim-up using the MitoTracker Red 580 Kit at 30, 45, and 60min.(Graph 3.1, 3.2)



Graph 3.1. Swim-up percentages at 30, 45, and 60 min in oligospermic samples as assessed using a dependent t-test (+: statistical significance between 30 and 45 min, °: statistical significance between 45 and 60 min, *: statistical significance between 30 and 60 min). A dependent t-test was used to compare oligospermic samples at 30 vs. 45, 45 vs. 60, and 30 vs. 60 min. Statistically significant decreases were observed between the time periods (p <0.001).



Graph 2. Swim-up percentages at 30, 45, and 60 min in normospermic samples as assessed using a dependent t-test (+: statistical significance between 30 and 45 min, °: statistical significance between 45 and 60 min, *: statistical significance between 30 and 60 min). A dependent t-test was used to compare the normospermic samples at 30 vs. 45, 45 vs. 60, and 30 vs. 60 min. Statistically significant decreases were observed between the time periods ($p < 0.001$).

An independent t-test was used to compare the oligospermic and normospermic samples at 30, 45, and 60 min. A statistically significant difference was found at 30, 45, and 60 min between the oligospermic and normospermic samples (all $p < 0.001$). Significant differences were also found in comparisons of the 30-, 45-, and 60-min sperm numbers between the oligospermic and normospermic samples after swim-up.

A radiation difference was observed in the treatment of sperm mitochondria from oligospermic patients at 30, 45, and 60 min with the MitoTracker Red 580 dye. No radiation difference was observed in the treatment of sperm mitochondria from normospermic patients at 30, 45, and 60 min with MitoTracker Red 580. Radiation in the sperm mitochondria of normospermic samples after swim-up was higher than that in the oligospermic samples, indicating higher mitochondrial activity in the normospermic samples.

An immotile semen sample and a +2 leukocyte semen sample from the MitoTracker Red 580 kit were used for control purposes. No signal was present, indicating that there was no mitochondriala ctivity. (Figure 1, 2)

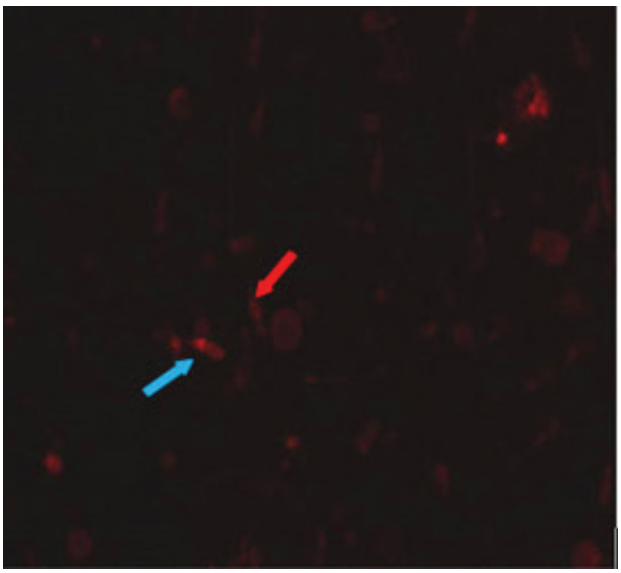


Fig 1.Sperm in the +2 leukocyte semen sample stained with MitoTracker Red 580. A photomicrograph indicated no staining (the blue arrow indicates a stained sperm cell, and the red arrow indicates an unstained sperm cell).

Discussion

Sperm motility assessment is a part of semen analysis when evaluating the fertility potential of a patient. However, there is no agreement on which test is optimal for showing fertility potential, and some publications have claimed that various newly proposed methods are inadequate in showing fertility potential[10].

It has been emphasized that adequate sperm motility is crucial for passing the cervical mucus and consequently reaching the ovum for fertilization[11,12]. Additionally, it has been reported that the duration of sperm motility is a significant indicator of fertilization potential and that tests should be performed over a 24 -h period in an appropriate culture medium[9].

No randomized controlled study has yet evaluated the various sperm preparation methods: the gradient, swim-up, and wash-centrifugation methods. It has been suggested that from the results of studies comparing semen parameters, the gradient method can be recommended; however, additional studies are required before making any definitive conclusions regarding this recommendation[13]. The swim-up method is routinely used in the clinical setting and has become an established technique.

The methods of preparing sperm for insemination are known to affect pregnancy success. When IUI was performed with processed sperm versus unprocessed sperm in a study by Goldenberg et al.,[14] higher pregnancy rates were obtained with processed sperm. However, variable and conflicting data on the success of sperm preparation techniques have also been reported. For example, Monqautetal.[15] recommended the selection of a sperm preparation method according to intended use (IUI, IVF, or ICSI). They determined that less vacuolization was present in the sperm selected using the swim-up method.

These and similar studies indicate that all methods have advantages and disadvantages relative to one another; there is no optimal method. Selecting the method according to patient and process to be performed and making modifications to these methods based on the patient seem to be appropriate for good sperm selection.This is the first reported study to assess mitochondrial activity and floating sperm numbers at 30, 45, and 60 min after swim-up with the MitoTracker Red 580 kit innormospermic and oligospermic samples. Normospermic samples had higher fluorescence signals than oligospermic samples upon evaluation of the fluorescence signals that depend on mitochondrial activity.

Immature and abnormal spermatozoids, leukocyte

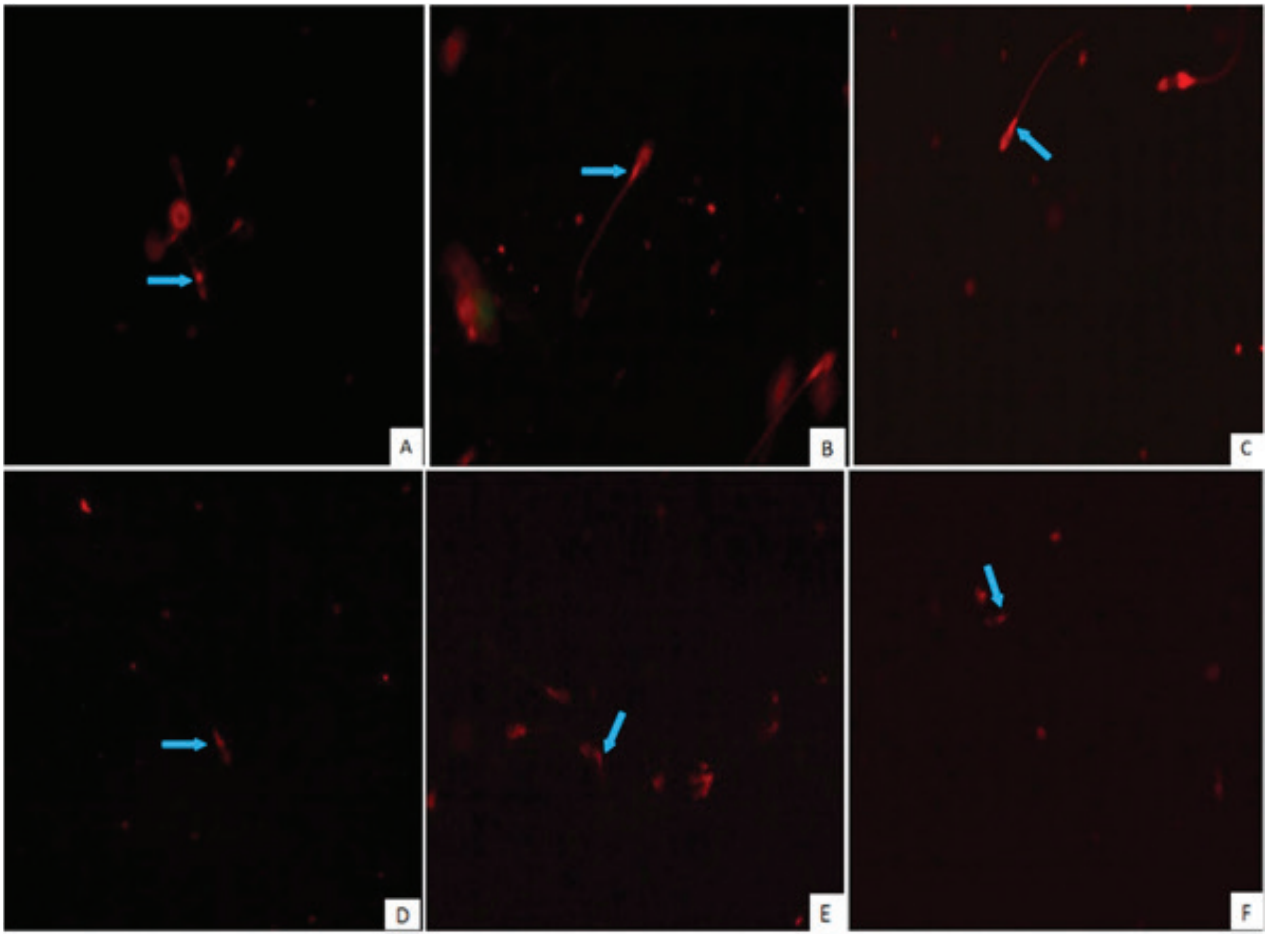


Fig 2. Semen samples after swim-up (the blue arrows indicate stained sperm cells). Normospermic sample stained with MitoTracker Red 580 (A: 30 min, B: 45 min, C: 60 min) and oligospermic sample (D: 30 min, E: 45 min, F: 60min).

contamination, and *in vitro* spermatozoid preparation processes (centrifugation and cryopreservation) cause high ROT production in addition to low amounts of antioxidants in serum, seminal plasma, and sperm preparation solutions, resulting in oxidative stress[16]. In a study by Wid lansky et al.,[17] the MitoTracker probe showed a decrease in mitochondrial mass under diabetic conditions in skeletal muscle, and mitochondrial fission increased secondary to a decrease in ATP production and cellular growth and an excessive increase in reactive oxygen species in mitochondria.

Based on the lower signals in the oligospermic samples than the normospermic samples in this study, we consider that mitochondrial fission may have increased due to the increase in reactive oxygen species in the oligospermic samples and that the signal decreased depending on the reduction in the accumulated mass of mitochondria. When the MitoTracker Red stain was applied to the +2 leukocyte control semen samples, which were known to have lower sperm motility, the neck portion of the sperm was not stained; that is, there was no mitochondrial activity.

These results are consistent with those in a study by Ferramosca et al.,[18]who established a connection with sperm mitochondrial respiration by evaluating variations in sperm motility and oxygen consumption through a polarographic method. When they compared an as the nospermic (reduced sperm mobility) sample with a normospermic sample, they identified a significant decrease in mitochondrial respiration.

In one retrospective study of the effect of the interval between sperm preparation and IUI on pregnancy rates in 102 patients, there was no difference in pregnancy rates between 30 and 31–60 min[19]. The findings of this study suggest no change in mitochondrial activity at 30, 45, or 60 min following swim-up, but lower numbers of sperm could affect pregnancyrates.

Conclusion

In conclusion, we found no significant change in the mitochondrial activity of floating sperm at 30, 45,

and 60 min with the swim-up method for the IUI procedure. However, the larger number of floating sperm at 30 than at 45 and 60 min is expected to be important in pregnancyrates.

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Author's contribution:

Data gathering and idea owner of this study: Cetinkaya Karabekir S, Aydan Özgörgülü

Study design: Cetinkaya Karabekir S, Aydan Özgörgülü

Data gathering: Cetinkaya Karabekir S, Aydan Özgörgülü

Writing and submitting manuscript: Cetinkaya Karabekir S, Aydan Özgörgülü

Editing and approval of final draft: Cetinkaya Karabekir S, Aydan Özgörgülü

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