**Research Article**

**Efficacy of zeta potential as an effective sperm selection method for ICSI in smokers and obese patients**

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**Abstract**

**Background:** Life style factors and oxidative stress are two key factors which cause male infertility. This work was aimed to study the efficacy of Zeta potential method as a reliable sperm selection technique for ICSI in smokers and obese patients, whose sperms in which oxidative stress and DNA damages are expected to be high.

**Methods:** This is a prospective study performed in 50 smokers and 50 obese patients and compared the zeta potential vs. normal swim down sperm selection method to assess the motility and DNA fragmentation.

**Results:** In smokers, the percentage of motility with swim down was 72.87±4.65 while in zeta it was 76.92±4.69. The DNA fragmentation in swim down vs. zeta was 76.24±6.91 and 67.35±6.05 respectively. In non smokers the percentage of motility with swim down vs. zeta yielded 76.02±6.93 and 78.74±7.4 respectively. The percentage of DNA fragmentation was 74.52±6.4 and 67.99±6.04. In obese patients swim down method yielded percentage of motility with 74.12±4.67 and percentage of DNA fragmentation with 74.52±6.4. The zeta potential yielded 77.10±5.67 percentage of motility and 67.99±6.04 percentage of DNA fragmentation. Student’s t-test showed significant difference between both methods in motility and DNA fragmentation percentage with p-value less than 0.05.

**Conclusion:** Zeta potential method is an effective sperm selection technique in male infertile patients especially in smokers and obese patients whose body contains high reactive oxygen species level. By comparing to other methods of sperm selection, the zeta potential method is easy to perform, less expensive and can be carried out in any basic andrology lab.

**Keywords:** ICSI, Zeta potential, ROS, Motility, DNA fragmentation

1. **Introduction**

Statistical data showed that 7% of men are having the problem of infertility (1). Male infertility has many causes which include physical problems, hormonal imbalance or physiological problems, psychological problems, life style issues etc (2, 3). Among these, the life style plays an important role in male infertility in the current scenario (4, 5). The major life style factors includes, smoking, obesity, alcoholism, use of steroids, inadequate vitamin C and Zinc in the diet, mal nutrition, anaemia, excessive stress, use of tight under wears, exposure to environmental hazards and toxins such as pesticides, led, paint, radiation, radioactive substances, mercury, benzene, boron, and heavy metals etc.

Researches had shown that oxidative stress is also a major cause of male infertility (6-8). It is causing the increase level of reactive oxygen species (ROS) production in the male testis. The reduced level of anti oxidants in the body can enhance the affect of ROS and subsequently leads to infertility. Recently researchers are focusing their eyes to find out the source of ROS production. Among the life style factors smoking and obesity are found to be common sources of ROS production. Research works already proved that tobacco smoking cause damage to DNA (9, 10). The decreased level of anti oxidants in smokers enhances the oxidative damage. The contribution of obesity in ROS production was also established by many research works (11-13). In smokers and obese patients the degree of sperm DNA damage is supposed to be higher even though they may have normal count, motility and morphology compared to non smokers and non obese.

In the current scenario, most of the embryology or andrology laboratories are selecting the sperm on the basis of their shape (morphology) and motility for IVF and ICSI. However visual approaches do not necessarily reflects the quality of

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sperrns like apoptosis, chromatin structure integrity and DNA fragmentation.

Assessment of patient life style and the selection of suitable cheap and reliable sperm selection technique can influence the infertility treatment outcome by selecting a healthy sperm.

In this study we aimed to study the efficacy of Zeta potential method as a cheap and reliable sperm selection technique for ICSI in smokers and obese patients, whose sperms in which oxidative stress and DNA damage are expected to be high.

2. Materials and methods
2.1. Study design and sample collection
This prospective study was performed at an Infertility hospital in Bahrain during the period of July 2014 to September 2014. Questionnaire to the patients were given for the categorization —smokers and obese patients.

Hundred normal semen samples (fifty smokers and fifty obese patients) were collected from men with suspected infertility. The ejaculatory semen samples after 2-6 days of abstinence were only used for this study. Sterile plastic containers were given to patients to directly collect the sample by masturbation. Each collected sample was divided into two for processing by swim down method and Zeta potential method.

2.2. Normal sperm selection by swim down method
In swim down procedure, the natural ability of motile sperm to migrate with the help of gravity is used to select a motile sperm population. The method involves the layering semen over a heavier, more viscous media. Sperm then migrate into and towards the gravitational force, ie, separating motile sperm from the remaining sample, which is left above (14). We performed this method as follows. Initially semen sample was collected into a sterile container. After liquefaction, the sample was analyzed for motility and concentration by Makler counting chamber (Sefi Medical Instruments, Israel). The gradient (100% sperm grade) was kept for incubation at 37°Celsius(C) for 20 minutes. Two milli liters (ml) of gradient was pipette into a centrifuge tube and then gently layered the semen sample over it, without disturbing the media. The sample was then kept for incubation at room temperature 15-20 minutes at 45° angle in a vertical rack. Then the gradient in the lower region was aspirated and mixed it with 6 ml of sperm wash media and centrifuged with 1600 G for 10 minutes. The supernatant was discarded and then 5 ml of sperm wash media was added again to the pellet and kept for centrifugation for 10 minutes at 1600 RCF. Then discard the supernatant and the count, motility and morphology were checked. All these above procedure were carried out in a sterile condition.

2.3. Zeta potential method
The Zeta potential method of sperm selection is based on the negative electro kinetic potential of the sperm. For this study, the Zeta potential method was carried out as per the procedure described by Chan et al. (15).

Immediately after semen liquefaction, the sample was washed in a sterile centrifuged tube with media containing serum. The supernatant was then discarded so that the tube was only with the minimum amount of serum containing medium. After this the pellet was mixed with 1 ml of serum free media. The medium was then exposed to a positive surface charge as follows: the tube was placed inside a latex glove up to the cap. The tube was then grasped by the cap, rotated two or three turns and rapidly removed from the glove. The tube was then grasped at room temperature for 1 min to allow adherence of the charged sperm to the tube wall and then centrifuged at 200g for 5 min. The medium and pellet were then discarded in order to eliminate any non-adhering sperm and other cells. Centrifugation does not alter the net charge on the tube. The tube surface was washed with 200 micro liters of media containing serum in order to neutralize the charge on the tube wall and to detach adhering sperm. The collected medium at the bottom of each tube was used to rinse the wall of the same tube several times in order to increase sperm recovery. Sperm parameters were then examined under microscope.

2.4. Outcome Measures
After processing, each sample was subjected for analysis to assess the motility and DNA fragmentation. The details of tests were as follows.

2.4.1. Motility test
To determine the motility, Makler counting chamber (Sefi Medical Instruments, Israel) was used. After liquefaction 10 micro liter of the semen sample was loaded into the counting chamber. Then the sample was allowed to settle for 1 minute. Objective lens magnification was at 20x. Sperm count was then carried out in 10 squares. The entire grid was counted only if the sperm concentration was less than ten sperm per row. Semen concentration was expressed in millions/milli liter. Then the total count and motility were calculated as per the following formula.

Total count= motile sperm + immotile sperm in 100 squares of Makler counting chamber divided by 10.

The sperm motility was expressed as percent of
motile sperm (sum of rapid progression plus slow progression sperm).

\[% \text{Motility} = \left(\frac{\text{motile sperm}}{\text{Total count}}\right) \times 100\]

2.4.2. DNA fragmentation test

For DNA fragmentation study, the Halo sperm kit (Halotech Dna, SL) was used. The test procedure was based on the sperm chromatin dispersion (SCD) test (17, 18). As an initial step, the lysis solution was kept at room temperature of 22°C. The semen sample was diluted in culture medium to a concentration of 5-10 million per millilitre. The agarose gel was then dissolved by melted in a microwave or in water for 5 minutes at 90-100°C. The agarose eppendorf was transferred to a water bath where temperature was maintained at 37°C and left it for 5 minute until temperature became even. 25 micro liter of semen sample was added to agarose eppendorf and mixed well.14-20 micro litre of cell suspension was taken from agarose eppendorf on to the treated slides and covered with glass cover slip. Care was taken to avoid air bubbles. The slides were kept in horizontal position throughout the process. Then the slides were placed on the cold surface in refrigerator at 4°C and left the sample 5 minute to become gel form. Then acid denaturation (AD) solution was prepared. For this, 80 micro liter of AD was added to 10 milli litre of distilled water and the resultant mixture was placed in the incubation tray. The slides cover was then removed by sliding it of gently with the help of land set. After this, immediately the slides were immersed into AD solution and left to incubate for 7 minutes. Afterwards, placed this in another incubation tray containing 10ml of lysis solution and kept it for incubation for 25 minutes. Then slides were kept horizontally into a tray containing abundant distilled water in order to wash lysis solution. This was kept for incubation of 5 minutes. The slides were then placed horizontally into a tray with 70% ethanol (2min) followed by 90% ethanol (2min) and finally 100% ethanol (2min). Ked the slides for air dry at room temperature followed by diff quick staining. After staining, slides were visualized to analyze the DNA fragmentation.

2.5. Statistical analysis

Basic descriptive statistics (mean ± standard error) were calculated for different parameters such as total motility and DNA fragmentation. Students paired- test was performed to find out the significance in above parameters while comparing swim down vs. Zeta potential method in smokers and obese patients. P-value < 0.05 was considered statistically significant.

### 3. Results

The results in table1 indicate that, in smokers, the percentage of motility and percentage of DNA fragmentation in swim down method was 72.87 and 76.24 with standard deviation of 4.65 and 6.91 respectively. At the same time the Zeta potential method yielded motility percentage of 76.92 and DNA fragmentation percentage of 67.35 with standard deviation of 4.69 and 6.05 respectively.

Table2 shows the analysis results of non smokers. The percentage of motility and DNA fragmentation in swim down method was 76.02 and 66.73 with standard deviation of 6.93 and 4.64 respectively. In the zeta potential method, the values were 78.74 and 62.55 with standard deviation of 7.4 and 5.37 respectively. In case of obese patients (table3), the swim down method yielded the values of 74.12 % motility and 74.52% DNA fragmentation with standard deviation of 4.67 and 6.4 respectively. The Zeta potential yielded the values of 77.1 and 66.99 with standard deviation of 5.67 and 6.64 respectively. In non obese patients (table4) motility in swim down method was 80.20 % with standard deviation of 3.69 while in zeta potential; this was 84.60 % with standard deviation of 3.74. The percentage of DNA fragmentation in swim down method was 70.79 with standard deviation of 4.06, but in zeta method it was 67.26 with standard deviation of 4.46. The students paired t-test shows significant difference between swim down method and zeta potential method for percentage of motility and DNA fragmentation with p-value less than 0.05 in all the patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Swim down method (Mean±SD)</th>
<th>Zeta potential method (Mean±SD)</th>
<th>P-Value (student paired t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility (%)</td>
<td>72.84±4.65</td>
<td>76.92±4.69</td>
<td>0.00</td>
</tr>
<tr>
<td>DNA Fragmentation</td>
<td>76.24±6.91</td>
<td>67.35±6.05</td>
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<td>0.00</td>
</tr>
<tr>
<td>DNA Fragmentation</td>
<td>66.73±4.64</td>
<td>62.55±5.37</td>
<td>0.00</td>
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</table>

Table 1. Comparison of swim down vs. Zeta potential method in terms of total motility and DNA fragmentation in smokers

Table 2. Comparison of swim down vs. Zeta potential method in terms of total motility and DNA fragmentation in non smokers.
Table 3. Comparison of swim down vs. Zeta potential method in terms of total motility and DNA fragmentation in obese patients.

<table>
<thead>
<tr>
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<th>Zeta potential method (Mean±SD)</th>
<th>P-Value (student paired t-test)</th>
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</thead>
<tbody>
<tr>
<td>Motility%</td>
<td>74.12±4.67</td>
<td>77.10±5.67</td>
<td>0.00</td>
</tr>
<tr>
<td>DNA Fragmentation%</td>
<td>74.52±6.4</td>
<td>67.99±6.05</td>
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</table>

Table 4. Comparison of swim down vs. Zeta potential method in terms of total motility and DNA fragmentation in non-obese patients.

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>Zeta potential method (Mean±SD)</th>
<th>P-Value (student paired t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility%</td>
<td>80.20±3.69</td>
<td>84.06±3.74</td>
<td>0.00</td>
</tr>
<tr>
<td>DNA Fragmentation%</td>
<td>70.79±4.06</td>
<td>67.24±4.41</td>
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</table>

4. Discussion

Many studies were published related to the use of DNA damage test as an additional useful test to the standard semen analysis (18-20). Although the WHO standard is considered to be the gold standard for the diagnosis of male infertility, it has been argued that the parameters measured according to the manual are insufficient or in some cases irrelevant, especially following the introduction of ICSI. This is because they only assess gross abnormalities such as head and tail defects. Indeed none of these assessments determine the relative health of sperm with less DNA fragmentation.

Study results showed that, recurrent abortion after ICSI, miscarriages, lower quality of embryos, malformations or deformities to the fetus are correlated to the DNA integrity of the sperms which are selected for the ICSI (21-24).

In these situations choosing a suitable sperm selection method plays an important role in the selection of sperms with less DNA fragmentation. Each IVF lab has to assess the patient lifestyle and has to select an appropriate sperm selection method especially for the kind of patients whose body contains excess amount of ROS level.

Our results indicate that by comparing the swim down method, the Zeta potential method is found to be very effective and efficient method to select sperms with motility and less DNA fragmentation. Better results of zeta method can be due to its peculiar way of identification of sperms by imparting the electrical potential to the mature, less DNA fragmented sperms during the time of processing.

Conclusion

Zeta potential method is an effective sperm selection technique in the male infertile patients especially in smokers and obese patients whose body contains high reactive oxygen species level. By comparing to other methods of sperm selection, the Zeta potential method is easy to perform, less expensive and can be carried out in any basic andrology lab.

References

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