

The Effects of Microfluidic Sperm Sorting, Density Gradient and Swim-up Methods on Semen Oxidation Reduction Potential

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Purpose: To compare the effects of microfluidic sperm sorting, density gradient and swim-up methods on the oxidative reduction potential (ORP) of split semen samples from a single patient population.

Materials and Methods: A prospective controlled study was conducted to compare the effects of three different semen processing methods using split semen samples from the same population of infertile men. The primary outcome was the ORP. Secondary outcomes were the sperm concentration, progressive motility rate and total sperm motility.

Results: A total of 57 split semen samples were included in this study. The ORP was significantly lower in the microfluidic group compared to the density gradient and swim-up groups ($P < 0.05$). The ORP/sperm concentration ratio was significantly lower in the microfluidic and density gradient groups compared to the swim-up group ($P < 0.05$). Total sperm concentration was significantly higher in the density gradient group than the microfluidic and swim-up groups ($P < 0.05$). Motility was significantly higher in the microfluidic and swim-up groups than the density gradient group ($P < 0.05$). The progressive motile sperm rate was significantly higher in the microfluidic and swim-up groups than the density gradient group ($P < 0.05$).

Conclusion: Microfluidic sperm sorting was better for selecting highly motile sperm and yielded a lower ORP than conventional sperm preparation methods.

Keywords: microchip; ORP; ROS; spermogram; male infertility

INTRODUCTION

The main aim of sperm preparation before intrauterine insemination (IUI) is to remove viruses, antibodies, leucocytes and debris from sperm, as well as to remove inhibitors of sperm capacitation factors, such as prostaglandins and reactive oxygen radicals^(1,2). Increased levels of reactive oxygen radicals and lipid peroxidation lead to DNA damage and apoptosis of spermatozoa. This might be related to decreased fertilisation rates, implantation failure and abnormal embryo development⁽³⁾.

The standard sperm preparation techniques are simple washing, density gradient and swim-up procedures. In swim up method, motile sperm swim from a pre-washed pellet up towards a layer of fresh medium for selection^(4,5). In density gradient centrifugation method, sperm are filtered through layers of silane-coated silica particles suspended in nutritive media⁽⁶⁾. Centrifugation is used in both of these methods, and sperm prepared with centrifugation based methods showed a higher generation of ROS and DNA fragmentation in previous reports^(7,8). Therefore, these methods might be harmful to healthy spermatozoa.

Microfluidic sperm sorting is a new sperm preparation method that uses a microfluidic system to select sperm. Microfluidic technology considers the flow of fluid from millimetric microchannels similar to the vaginal

rugae system^(9,10). Most motile and healthy sperm swim through the pores of the membrane and are filtered into the upper part of the system, where they are finally taken from the outlet. Centrifugation and other mechanical methods are not applied to sperm cells; therefore, most functional sperm with high DNA integrity are selected via a physiological sorting system. It has been observed that there was less DNA fragmentation and ROS formation of sperm with microfluidic technology when compared with standard techniques⁽¹¹⁾. Also one study showed that microfluidic sorting of unprocessed semen can be used to select clinically usable, highly motile sperm with nearly undetectable levels of DNA fragmentation⁽¹²⁾.

Oxidative reduction potential (ORP) is a novel marker of oxidative stress and redox imbalance in biological samples^(13,14). It is calculated by measuring the transfer of electrons from a reductant to an oxidant, to determine the balance between total oxidants and reductants in a biological system⁽¹⁴⁾. Therefore, ORP can be used to distinguish abnormal and normal semen, and is also helpful to discriminate sperm from fertile and infertile patients⁽¹⁵⁻¹⁷⁾. Thus, ORP has been suggested as a marker for evaluating semen quality in infertile males⁽¹⁸⁾.

Microfluidic sperm sorting systems are now being used to aid assisted reproduction in many clinics; however, data are currently insufficient to warrant using these systems in routine clinical practice. In addition, there

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Table 1. Basal spermogram parameters of liquefied raw semen of patients.

Sperm parameters*	Basal
Volume (ml)	3.24 ± 1.57
Concentration (106/ml)	55.63 ± 37.12
Motility (%)	59.05 ± 14.96
Progressive motility (%)	15.15 ± 9.02
TPMSC	97.35 ± 94.39
ORP	39.24 ± 19.95
ORP/conc	1.40 ± 1.68

*data are presented as mean ± SD or number(percent)

Abbreviations: ORP: Oxidation Reduction Potential; TPMSC:- Total motile sperm count; conc:concentration

are insufficient data on the effects of standard semen preparation methods and microfluidic sperm sorting systems on sperm quality and oxidative stress. Therefore, in the present study, we compared the effects of the microfluidic sperm sorting, density gradient and swim-up methods on ORP levels in split semen samples obtained from a single patient population.

MATERIALS AND METHODS

This prospective study was a laboratory evaluation of split semen samples obtained from a single patient population; the samples were discarded after a routine semen analysis. This study was conducted at the In Vitro Fertilisation Unit of Izmir Medical Park Hospital (Izmir, Turkey). Bahçesehir University institutional review board approval was obtained for this study.

Semen preparation procedure

Semen samples were obtained by masturbation after 2–5 days of abstinence into a sterile, labelled container. All semen samples were incubated at 37°C for 30 min.

Density gradient technique

The density gradient technique was performed according to the following steps. First, a gradient column was prepared by placing 1 mL of 80% gradient media (Origio/Medicult Media) in a centrifuge tube with an additional 1 mL of 55% gradient media layered on top. Next, 3 mL of semen was layered on top of the 55% layer and centrifuged at 1,400 rpm for 10 min. The supernatant and gradient medium just above the sperm pellet were removed and discarded. The sperm pellet was washed with 3 mL of sperm wash media and centrifuged at 1,600 rpm for 10 min. The supernatant was collected and resuspended to the final volume in 0.5 mL of sperm wash medium.

Swim-up technique

A liquefied semen sample was placed in a tube and diluted 1:1 with sperm washing medium. The mixture was centrifuged for 10 min at 1,200 rpm. The supernatant was extracted and 1 mL fresh culture medium

was layered above the pellet. The tube was placed on a stand, tilted at a 45° angle and incubated for 1 hour at 37°C. After incubation, 0.6 mL of the supernatant was placed into an empty tube for evaluation.

Microfluidic technique

Microfluidic sperm sorting was performed using the Fertile Plus chip (Koek Biotechnology, Izmir, Turkey), which is a flow-free, dual-chambered microfluidic single-use chip. The first collection chamber is the sample inlet, and fluid channels are separated from the second collection chamber by a microporous membrane. An untreated 850 µL semen sample was injected into the inlet chamber, and 700 µL sperm wash medium heated to 37°C was added to the microporous membrane (outlet chamber); the chip was incubated for 30 min at 37°C. The processed 650 µL sperm sample was collected from the outlet.

Oxidation reduction potential

The ORP was evaluated by a galvanostat-based system that measures redox potential using the Male Infertility Oxidative System (MIOXSYS; Aytu Bioscience Inc., Englewood, CO, USA). The system consists of a MIOXSYS analyser and a sensor strip. In total, 30 µL of a completely liquefied semen sample was loaded on the sample port and measured in millivolts (mV) for 4 min. The ORP values were normalised by the sperm concentration and expressed as mV/106 sperm/mL. ORP values > 1.37 mV/106 sperm/mL are indicative of oxidative stress⁽¹³⁻¹⁵⁾.

Outcome measures and statistical analysis

The primary outcome measure was the ORP of the semen samples. Secondary outcome measures were the total sperm concentration and motility. The statistical analysis was performed using SPSS software (version 20.0; SPSS Inc., Chicago, IL, USA). For the statistical methods, for a comparison between k-related samples Friedman test was used. For paired comparison between groups Wilcoxon signed rank test was used. A two-sided *p*-value < 0.05 was considered significant.

RESULTS

A total of 57 split semen samples were evaluated in this study, and three sperm processing groups (microfluidic, density gradient and swim-up groups) were compared. Raw liquefied semen samples were evaluated for each patient. The basal spermogram parameters and basal ORP levels are shown in **Table 1**. The spermogram parameters and ORP levels of the three sperm processing groups are shown in **Table 2**.

When the ORP and ORP/sperm ratio were compared between in all groups (raw sample, microfluidic, density gradient and swim-up groups) there was a significant difference between all groups. To investigate the

Table 2. Comparison of spermogram parameters and ORP levels in microfluidic sperm sorting, density-gradient and swim-up groups.

Sperm parameters	Microfluidic	Density-gradient	Swim-up	<i>p</i>
Concentration (106/ml)	20.29 ± 19.01	35.70 ± 20.97	15.00 ± 13.33	0.007
Motility (%)	98.57 ± 1.42	75.30 ± 14.32	95.33 ± 9.59	0.000
Progressive motility (%)	60.00 ± 20.81	24.90 ± 6.26	59.55 ± 16.21	0.000
TPMSC	12.29 ± 11.25	15.40 ± 10.90	7.60 ± 6.74	0.386
ORP	84.38 ± 26.19	259.83 ± 13.64	248.63 ± 23.27	0.000
ORP/conc	8.52 ± 7.33	10.17 ± 7.57	57.53 ± 84.42	0.000

*data are presented as mean ± SD or number(percent)

Abbreviations: ORP: Oxidation Reduction Potential; TPMSC:Total motile sperm count; conc:concentration

difference between each group paired comparison were established in each group separately. Basal level of ORP and ORP/sperm concentration ratio were found to be significantly lower in raw semen sample than three other groups ($P < 0.05$). Also ORP levels were significantly lower in the microfluidic group than the density gradient and swim-up groups ($P < 0.05$). The ORP/sperm concentration ratio was significantly lower in the microfluidic and density gradient groups than the swim-up group ($P < 0.05$).

Total sperm concentration, motility, progressive motile sperm rate and total motile sperm count were significantly different between raw semen sample and three sperm processing groups ($P < 0.05$). When each group was evaluated by paired comparison, total sperm concentration was significantly higher in the density gradient group than the microfluidic and swim-up groups ($P < 0.05$). Motility was significantly higher in the microfluidic and swim-up groups than the density gradient group ($P < 0.05$). The progressive motile sperm rate was significantly higher in the microfluidic and swim-up groups than the density gradient group ($P < 0.05$). Total motile sperm count was not significantly different among the groups ($P = 0.386$).

DISCUSSION

Assisted reproductive technologies have improved very rapidly over the last decade. However, sperm processing and selection methods have shown few changes during this time. It is clear that selecting healthy spermatozoa is imperative to ensure a successful pregnancy and healthy offspring. Moreover, using the optimal semen processing method should provide the healthiest spermatozoa for assisted reproductive treatments.

Reactive oxygen species (ROS) are vital for sperm maturation and capacitation, and for the acrosome reaction and oocyte fusion^(19,20). However, excess ROS can harm spermatozoa DNA and cause apoptosis, which leads to reduced fertilisation, implantation failure, embryonic developmental problems and poor pregnancy outcomes^(21,22). Therefore, the ORP is extremely important during sperm maturation and processing. Conventional spermogram parameters (concentration, motility and morphology), which are related to pregnancy rates, can vary within the same individual at different times, and among different populations^(23,24). Interobserver variability is also an important issue during spermogram analysis⁽²⁵⁾. The ORP can function as an advanced and independent marker of semen quality in infertile males⁽¹⁸⁾. Thus, we compared the effects of the two most common conventional sperm processing methods (density gradient and swim-up) to those of the microfluidic sperm sorting technique, in terms of basic spermogram parameters and the ORP.

Sperm concentration was higher in the density gradient group than the swim-up and microfluidic groups. At first glance, this would seem to be advantageous; however, the pellet includes both immotile and motile sperm after density gradient centrifugation. Thus, swim-up and microfluidic sperm sorting were superior with respect to sperm motility than the density gradient technique. The proportion of motile sperm was significantly higher in specimens that underwent the microfluidic and swim-up techniques versus the density gradient technique. In addition, the progressive motile sperm rate was significantly higher in the microfluidic and swim-up groups

than the density gradient group. The number of progressive motile spermatozoa inseminated is one of the most important prognostic factors for pregnancy after IUI⁽²⁶⁾. Thus, we conclude that the microfluidic system is a good alternative to conventional methods, yielding a high motile sperm rate during IUI cycles.

It is clear that a high ORP exposes the sperm to DNA damage⁽²⁷⁾. DNA integrity might be the most important factor in sperm processing, as it directly affects the DNA of the embryo, and the subsequent offspring. Normal spermogram parameters do not always indicate healthy spermatozoa, and high DNA fragmentation rates have been detected even in normozoospermic male partners in unexplained infertile couples undergoing IUI^(28,29). Sperm DNA damage is correlated with a lower pregnancy rate and longer time to pregnancy during both natural and IUI cycles⁽³⁰⁻³⁴⁾. In addition, significantly lower clinical pregnancy and delivery rates were reported in the context of high DNA fragmentation rates, in both IVF and IUI cycles^(32,34). Sperm chromatin assay parameters have been reported to be related to spontaneous abortion rates, where sperm DNA damage may adversely affect the quality of post-implantation embryos⁽³⁵⁾.

Based on these findings, sperm preparation techniques might be an important factor in the DNA fragmentation rate. Conventional sperm preparation techniques depend on sedimentation and migration to separate spermatozoa, which exposes the sperm to DNA-damaging ROS⁽³⁶⁾. The results of previous studies are conflicting and there are limited data on this subject. Amiri et al. reported higher levels of DNA fragmentation in swim-up versus density gradient samples⁽³⁷⁾. Another report found no significant difference in the amount of apoptotic sperm recovered between the density gradient and swim-up methods⁽³⁸⁾. In contrast, improved DNA fragmentation was reported after processing sperm using both the swim-up and density gradient methods in teratozoospermic men⁽³⁹⁾.

Few data are available on microfluidic sperm sorting (11,40-41). Recently some studies noted that microfluidic-sorted sperm showed significantly less ROS and DNA fragmentation compared to those treated by the conventional swim-up method^(11,41). Also, Quinn et al. reported that microfluidic sorting of unprocessed sperm was associated with nearly undetectable levels of DNA fragmentation compared to the density gradient centrifugation and swim-up methods⁽¹²⁾. Our results support the aforementioned studies by showing that the ORP was lower after microfluidic sperm sorting compared to the density gradient and swim-up methods.

The advantages of microfluidic technology lie in the selection of higher concentrations of highly motile sperm, but with a shorter processing time, while also preserving overall sperm DNA quality and integrity without a centrifugation step. No special technical skills or equipment are needed for the procedure. Reduced variability due to human error and less potential for environmental contamination are other possible advantages⁽¹¹⁾.

A limitation of this study was its laboratory-based design; we did not evaluate the effects of these sperm processing methods in the clinical setting. Therefore, it was not possible to draw definitive conclusions regarding the clinical effects of microfluidic sperm sorting based on our results. However, this is the first study to compare the effects of the microfluidic sperm sorting,

density-gradient centrifugation and swim-up methods on the ORP of semen. The adverse effects of centrifugation were demonstrated in the present study, and the ORP was lower in unprocessed semen than in all of the processed semen samples.

CONCLUSIONS

As a conclusion; microfluidic sperm sorting allows for the selection of highly motile sperm with a lower ORP than conventional sperm preparation methods. However, randomised controlled studies are needed to evaluate the effects of this procedure in the clinical setting.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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