An Ideal Sperm Selection Method for the Intrauterine Insemination Treatment of Normozoospermic Infertile Patients*

Normozoospermik İnfertil Hastaların İntrauterin İnseminasyon Tedavisi İçin İdeal Bir Sperm Seçim Yöntemi

Abstract

Aim: In this study, we evaluated the DNA integrity of sperms selected by using the conventional swim-up and density gradient centrifugation techniques and the new nanotechnology-based microfluidic chip method in order to determine the ideal sperm selection method for the intrauterine insemination treatment of normozoospermic infertile patients (NIPs).

Materials and Methods: Semen samples obtained from 20 patients were divided into four equal fractions. Control, density gradient centrifugation, swim-up, and microfluidic chip (MC) groups were created, and the untreated (control) and treated (other) sperm samples from the four groups were examined for DNA integrity. Acidic aniline blue staining and the TUNEL method were used respectively for evaluating sperm chromatin condensation defects and DNA fragmentation. Dichlorofluorescein diacetate and flow cytometry were used to determine the reactive oxygen species levels.

Results: We measured significantly lower values of chromatin condensation defects, DNA fragmentation and reactive oxygen species for the sperms selected with the MC method, compared to the sperms selected with the conventional methods ($p<0.0001$).

Discussion and Conclusion: We found that the new MC method was more effective in selecting sperms with high DNA integrity, compared to the conventional methods. Accordingly, the MC method can be an ideal sperm selection method for use in the intrauterine insemination treatment of NIPs with high DNA fragmentation, apoptosis, and reactive oxygen species levels.

Keywords: DNA integrity; intrauterine insemination; microfluidic chip; sperm selection

Öz

Amaç: Bu çalışmada normozoospermik infertil hastaların (NİH) intrauterin inseminasyon tedavisi için ideal sperm seçim yöntemini belirlemek amacıyla geleneksel yüzdürme ve yoğunluk gradyanlı santrifüjleme teknikleri ve yeni nanoteknoloji bazlı mikroakışkan çip yöntemi ile seçilen spermlerin DNA bütünlüğü değerlendirilmiştir.

Gereç ve Yöntemler: Yirmi hastadan toplanan semen örnekleri dört eşit parçaya bölündü. Kontrol, yoğunluk gradyanlı santrifüjleme, yüzdürme ve mikroakışkan çip (MÇ) grupları oluşturuldu ve bu dört gruba ait işlem görmemiş (kontrol) ve görmüş (diğer) sperm örnekleri DNA bütünlüğü açısından incelendi. Sperm kromatin yoğunlaşma kusurlarını ve DNA kırıklarını değerlendirmek için asidik anilin mavisi ve TUNEL boyaması kullanıldı. Reaktif oksijen türevi seviyelerini tespit etmek için akış sitometrisi ile diklorofloresan diasetat kullanıldı.

Bulgular: MÇ yöntemiyle seçilen spermlerde kromatin kondensasyon defekti, DNA fragmantasyonu ve reaktif oksijen türevi değerleri, geleneksel yöntemlerle seçilen spermlere göre anlamlı şekilde düşük bulundu ($p<0,0001$).

Tartışma ve Sonuç: Yeni MÇ yönteminin geleneksel yöntemlere göre yüksek DNA bütünlüğüne sahip sperm seçiminde daha etkili olduğu görüldü. Buna göre MÇ yöntemi yüksek DNA kırığı, apopitoz ve reaktif oksijen türevi görülen NİH'lerin intrauterin inseminasyon tedavisinde ideal bir sperm seçim yöntemi olabilir.

Anahtar Sözcükler: DNA bütünlüğü; intrauterin inseminasyon; mikroakışkan çip; sperm seçimi

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INTRODUCTION

The male factor is associated with almost half of all infertility cases. Although routine semen analyses are still valuable evaluation methods, about 15% of infertile males show normal semen parameters complicating the final diagnosis (1,2). It has been reported that 8% of males with normal semen parameters have sperm DNA damage (3). Currently there are no established rules for functional sperm selection, for which clinics often need to determine their own criteria (4). The conventional semen parameters (volume, sperm count, motility, and morphology) cannot identify the *in vitro* blastocyst and fertilization rates (5,6). Furthermore, sperm DNA integrity and chromosomal abnormalities are also important factors in the treatment and diagnosis of male infertility (6). The method used for sperm selection might affect sperm DNA integrity and thus reduce the chances of successful fertilization (7). Various methods have been developed for sperm preparation prior to the use of assisted reproduction techniques (ARTs) in clinical practice (8,9). The swim-up (SU) and density gradient centrifugation (DGC) methods, based on motility and morphology, are commonly used for sperm selection. However, both methods involve repetitive centrifugation and pipetting procedures, which reduce sperm quality with higher reactive oxygen species (ROS) levels and DNA fragmentation (10–12). Use of the best sperm selection method and removal of damaged sperm are critically important for success with ARTs.

The microfluidic chip (MC) (Fertile Plus® Koek Biotechnology, Izmir, Turkey) is a new, nanotechnology-based method developed in order to eliminate the centrifugation and pipetting steps. It has a membrane with micropores of different diameters, which select the functional sperms by motility (13). Low DNA integrity and a high ROS ratio are regarded as the underlying causes of infertility in normozoospermic infertile patients (NIPs) with normal semen parameters when using ART. The mechanisms that can lead to these defects in semen are apoptosis, chromatin condensation and oxidative stress in which ROS production occurs. During processes like centrifugation and pipetting oxidative stress in sperm increases and DNA integrity deteriorates (10,13). Routine semen parameters are not helpful in determining the appropriate method to select the most functional sperm content in NIPs, for whom the determination of the ideal method is a priority for successful treatment.

In this study, we aimed to assess the DNA integrity of spermatozoa selected with the conventional SU, DGC, and new nanotechnology-based MC methods, and determine the ideal sperm selection method for the intrauterine insemination (IUI) treatment of NIPs by taking into account the underlying causes, such as chromatin condensation defects, DNA fragmentation, and ROS. After the initial semen evaluation, the samples were divided into four groups, and the chromatin condensation defects, DNA fragmentation, and ROS levels were assessed.

MATERIALS AND METHODS

Experimental design

This study was approved by the Ethics Committee for Clinical Studies of the Bolu Abant Izzet Baysal University, Faculty of Medicine (no. 2015/70). Informed consent was obtained from all patients. Semen samples obtained from infertile patients (age: 22–49 years) who visited the Gynecology and Obstetrics Clinic at the Medical Faculty Hospital of the Bolu Abant Izzet Baysal University. The samples were obtained after sexual abstinence for 2–7 days and left on a heating surface (37°C) for 30 minutes. Routine semen analyses and sperm concentration, motility and morphology assessments were performed according to the 2010 World Health Organization (WHO) criteria (13). A Makler counting chamber was used to analyze the samples for concentration and motility. Evaluation of sperm morphology was performed according to Kruger's strict criteria by counting 200 sperms with an immersion objective at x100 magnification in smears stained with Spermac (14,15). After the evaluation, 20 NIPs were

Table 1. Descriptive statistical values

Parameters	Values
Concentration (xM/ml)	86.45±35.732 (32–174)
Total motility (%)	62.40±10.10 (38–78)
Rapid progressive motility (%)[a]	12.30±6.23 (5–22)
Slow progressive motility (%)[b]	42.00±7.30 (29–54)
Normal morphology (%)[c]	11.55±4.89 (4–25)

[a] Class A motility; [b] Class B motility; [c] Kruger's strict criteria; M: million

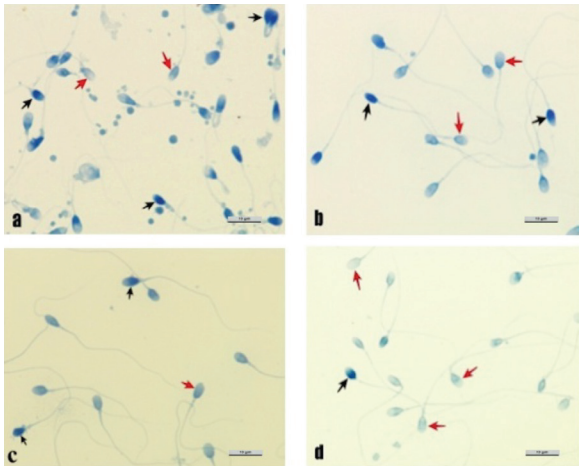

Figure 1. Acidic aniline blue staining. Sperm heads with abnormal chromatin condensation (AB+) (black arrow) and with normal chromatin condensation (AB-) (red arrow). The light micrographs for the semen (a), DGC (b), SU (c), and MC (d) samples (x100).

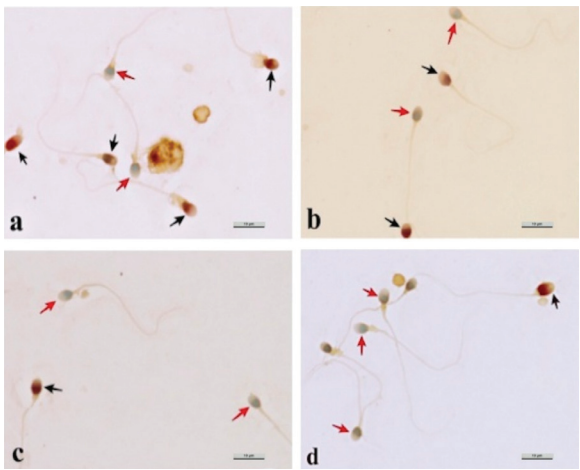

Figure 2. The TUNEL method. Sperm heads with DNA fragmentation (TUNEL [+]; black arrow) and without DNA fragmentation (TUNEL [-]; red arrow). The light micrographs for the semen (a), DGC (b), SU (c), and MC (d) samples (x100).

included in the study. Then each semen sample was divided into four equal fractions and used for the DGC, SU, MC and control (neat semen) groups.

Sperm selection methods

Density gradient centrifugation (DGC): Using a sterile Pasteur pipette, 1 ml of 90% PureSperm (Nidacon, Gothenburg, Sweden) was transferred into a 15 ml conical tube and 1 ml of 40% PureSperm was slowly transferred to form an upper layer. Then, 1 ml liquefied semen sample was slowly layered on the top of gradient solutions. After centrifugation at 1600 rpm for 15 min, the supernatant containing poor quality sperm and semen residues was removed. The pellet was resuspended with 5 ml PureSperm Wash medium (Nidacon) and centrifuged at 1200 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 0.8 ml medium for further analysis (16).

Swim-up (SU): The liquefied semen sample was first taken into a conical tube and mixed by pipetting with 9 ml of PureSperm Wash (Nidacon) and centrifuged at 1200 rpm for 5 minutes. After the supernatant was removed, 1 ml of medium was slowly layered on top and incubated at 37°C for 30 min with 45° angle in an incubator (6% CO2). During this period, the motile sperms moved on top, to the surface of the medium. Then, the upper layer of the medium, in which high-quality sperm was present, was taken for further analysis (17).

Microfluidic chip (MC): By the microfluidic chip (Fertile® Plus Chip) method, an environment was provided for sperm to flow through a microfluidic system toward the membrane which separates sperms by consequent polycarbonate filters with the 3-, 5- and 8-µm diameter micropores. Briefly, 850 µL of the liquefied semen sample was given slowly through the inlet of the Fertile® Plus Chip until the area under the membrane was completely filled. The Fertile® Chip outlet pool was filled by 850 µl Fertile® Plus sorting solution (human tubal fluid [HTF] + 1% bovine serum albumin [BSA]). Then, the chip was incubated at 37°C for 30 minutes. The motile sperms swam through the micropores, and 700 µl solution containing high quality sperms was collected from the outlet pool (18). The selected sperm sample was used for further analysis.

Assessment of the sperm chromatin condensation

The suspension of selected sperm was placed on a microscope slide and smears were prepared. The smears were fixed with 3% gluteraldehyde for 30 minutes, and stained with 5% aqueous acidic aniline blue in 4% acetic acid (pH 3.5) for 5 min (19). In the samples, which were examined by the immersion objective (x100), the sperm heads with abnormal chromatin condensation were stained blue (AB+) while the sperm heads with normal chromatin condensation were not stained (AB-). For each sample, at least 200 sperms were examined and the percentage of AB+ sperm was calculated.

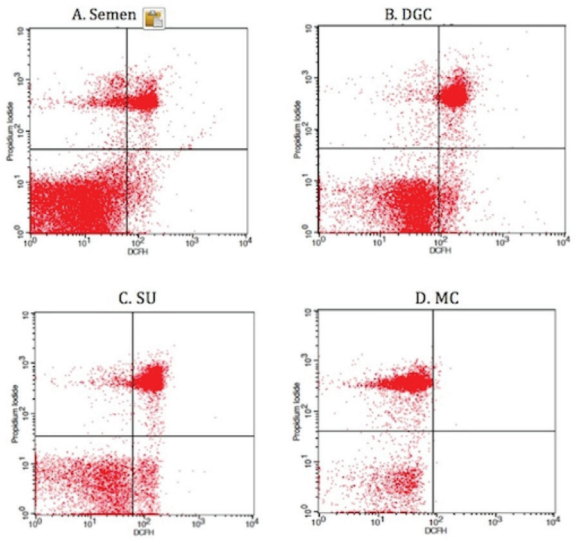

Figure 3. The flow cytometric histograms of the intracellular ROS measurements of sperms stained with DCFH and PI.

Assessment of DNA fragmentation

The smears were examined for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining with the In Situ Cell Death Detection Kit (Merck Millipore, Darmstadt, Germany) by the immersion objective (x100 magnification). The smears were fixed in methanol for 30 minutes in accordance with the TUNEL (Millipore) staining protocol. The brown-stained sperm heads were assessed as TUNEL (+) and the TUNEL (+) apoptotic sperm percentages were calculated by counting 200 sperms for each sample (20).

Flow cytometric assessment and analysis of ROS

Sperm samples were diluted at the concentration of $5x10^6$ sperm/ml. Intracellular ROS concentrations were measured using 25 mg/ml DCFH-DA (Sigma-Aldrich, St. Louis, MO, USA) in dimethyl sulfoxide (DMSO). Propidium iodide (PI) (1 mg/mL; BD Biosciences, San Jose, CA, USA) was used as a counter stain. Five µl DCFH-DA (10 mM) and 3 µl PI were added to 492 µl diluted sperm samples. The sperm suspensions were incubated at 37°C for 30 min and mixed just before the analysis. The amount of DCFH-positive and PI-negative sperms and the DCFH fluorescence intensity were measured by FACSCalibur (BD Biosciences) by using argon laser beam. A minimum of 10,000 spermatozoa were assessed at a flow rate of approximately 100 cell/second for each sperm sample. Following the diffusion into the sperm cell, the nonfluorescent DCFH-DA was converted into fluorescent DCFH by interacting with intracellular H_2O_2. Then, the green fluorescence intensity was assessed at 500 and 530 nm. PI counterstain, which is a nucleic acid dye, was used together with DCFH staining, and apoptotic sperms were excluded from the assessment. PI red fluorescence was analyzed in the FL-2 channel. The percentage of PI-positive cells and mean fluorescence were calculated and analyzed by the CellQuest Pro Software (BD Biosciences) (21).

Statistical analysis

This observational cohort study included NIPs, in accordance with the 2010 WHO criteria. Variance analysis was performed for the repeated measurements and the (control, DGC, SU, and MC) groups were examined by the Bonferroni test. $p<0.01$ was considered statistically significant. The IBM SPSS (v. 21) software was used for the calculations.

RESULTS

The mean patient age was 32.75±6.95 (22–49) years. The descriptive statistical values of the study parameters were expressed as mean±standard deviation (minimum and maximum) in Table 1.

Sperm concentration

Before washing, the mean sperm concentration for the 20 NIPs was calculated to be 86.45±35.73 M/ml. In terms of concentration, a significant difference was observed when the values of semen parameters were compared with the post-washing values of DGC, SU, and MC ($p<0.0001$).

There was a significant concentration difference between the groups ($p<0.0001$), although the SU and MC methods demonstrated no significant difference. More sperm was obtained by the DGC (55.55 M/ml) method, compared to the SU (45.65 M/ml) and MC (46.90 M/ml) methods.

Sperm motility

The post-wash motile sperm percentages were significantly higher in all three selection methods than in the semen group ($p<0.0001$). The mean motility percentages were calculated to be 83.80%, 86.15%, and 90.20% for the DGC, SU, and MC methods, respectively. When the total motility percentages for all three methods were compared, there was no significant difference between the DGC and SU methods although a

statistically significant difference was found between the other groups (p<0.0001). All groups showed significant difference in terms of rapid progressive (Class A) motile sperm percentages (p<0.0001). While the mean percentages of Class A motility were 29.70%, 33.30%, and 35.60% for the DGC, SU, and MC methods, respectively, it was calculated to be 12.30% in the pre-wash semen group. While the rate of increase in Class A motility was 41% after the DGC and 75% after the SU methods, it was determined to be the highest with 91% after the MC method.

Sperm morphology

The mean percentages of sperms with normal morphology were calculated to be 11.55±4.89 (S), 16.30±5.79 (DGC), 17.50±6.21 (SU), and 19.45±7.27 (MC). When the percentages were compared, all groups showed a significant difference (p<0.0001), but no significant difference was found between the DGC and SU methods.

Sperm chromatin condensation

The chromatin condensation defect percentages were calculated to be 42.60% (DGC), 33.90% (SU), and 26.80% (MC), according to acidic aniline blue staining. When the selection methods were compared, a significant difference was found between all groups (p<0.0001). The chromatin condensation defect percentage was lowest with the MC method (Figure 1).

Sperm DNA fragmentation (SDF)

The mean percentages of SDF assessed by TUNEL staining were calculated to be 30.1±13.86 (S), 9.25±3.97 (DGC), 5.95±2.93 (SU), and 3.30±2.27 (MC). There was a significant difference between all groups (p<0.0001) when compared to each other, and the SDF percentage was lowest with the MC method (Figure 2).

Flow cytometric ROS analysis

According to the DCFH (H_2O_2) staining results, there was a significant difference between the groups (p<0.0001). The percentage of sperms stained with DCFH was calculated to be highest (23.74%) with the SU and lowest (7.45%) with the MC methods. With the DGC and SU methods, the ratio of intracellular ROS increased by 20.43% and 23.74%, respectively, compared to the semen group (12.47%).

The dot graphics of the intracellular ROS measurements are presented for each group in Figure 3. The lower left and right quadrants show the unstained live sperms and the live sperms with high intracellular ROS, respectively. The upper left quadrants show the apoptotic cells in which ROS was not detected, and the upper right quadrants show the apoptotic sperms in which high ROS was detected. The mean DCFH fluorescence intensity of the DCFH-positive and PI-negative sperms in the lower right quadrants was calculated.

DISCUSSION AND CONCLUSION

Intrauterine insemination is a common, easy, and cost-effective ART. Successful fertilization requires selection of fast-moving, morphologically normal sperms and separation of as much of the ejaculate content as possible, which inhibits sperm fertilization ability (22–26). A study comparing conventional and advanced selection methods found that a higher pregnancy rate was obtained with advanced selection methods (27). When the semen contains motile sperm with normal concentration (normozoospermia), SU is the most commonly preferred method in IVF laboratories. The DGC method, which provides a higher amount of sperm after washing, is preferred in patients who suffer from conditions like oligozoospermia, teratozoospermia, or asthenozoospermia (3,12). In this study, the semen samples obtained from the infertile patients included showed normal concentration, morphology, and motility. The most commonly used sperm selection methods were compared with the new MC method.

Better spermiogram results were obtained with the three selection methods, compared to the pre-wash semen samples. The mean concentration of the semen group exhibited a decrease after the use of the DGC, SU, and MC methods. Although the decrease was less with the DGC method, the sperm concentrations obtained with the other two methods were not found low enough to affect the success of the fertilization treatment. Since less semen was washed in the MC method, a lower number of sperms was obtained after washing, compared to the other methods. However, when assessed by number, high DNA integrity, and sperm ratio with a low ROS rate, it was determined that a sufficient number of high-quality sperms was obtained with the MC method. Although the motile sperm per-

centage was calculated to be higher with the SU than with the DGC method, the difference was not statistically significant. The percentage increase in motility was significantly higher with the MC method, as in the study of Asghar et al. (18), and the rate of increase in class was seen to be highest after using the MC method. It was also reported that a higher rate of motile sperms was obtained with the MC than with the SU method (28). Unlike the results of other studies, we found no significant difference between the morphological values of the DGC and SU groups (29,30), although the motility increase with the MC method was, similarly, greater than that obtained with the SU and DGC methods (18). Also, mechanical damage is less with the MC method, and the selection of high-quality sperms with no exposure to chemicals is based on sperm movement through a special membrane with micropores of different diameters (3, 5, and 8 μm) in optimum time. Similar results have been reported in the literature (18,31,32). As a result of the acidic aniline blue staining for sperm chromatin condensation assessment, a significant decrease was observed with the use of the selection methods. The minimum and maximum chromatin condensation defect values were observed with the MC and DGC methods, respectively, which is consistent with the literature (18,33).

In normozoospermic patients, a SDF percentage >30% is considered to indicate advanced damage and thus a significant decrease in the fertilization rates (1). Despite the apparently normal sperm parameters, high SDF ratios are interpreted that the underlying cause of infertility may be related to sperm DNA integrity. In our study, although similar pre-wash SDF values were measured for the samples, the values decreased significantly after the use of the three washing methods, the most significant decrease occurring with the MC method.

Sperm selection using the conventional methods requires one- or two-stage centrifugation, which may lead to damage and pressure on sperm and increased ROS levels that cause SDF (10,34,35). In addition, the DGC and SU methods are time-consuming with a low motile-sperm recovery rate, and thus only a small number of sperms can be retrieved with the actual quality due to the lack of specificity (36). As a result, using the conventional DGC and SU methods can decrease the fertilization and pregnancy rates (37). With the MC method, the selection of high-quality sperm was performed without centrifugation and the damage due to it and we obtained sperms with lower SDF. Furthermore, the sperms were exposed to less stress. We found that the ROS levels significantly differed between the groups. The number of sperms stained with DCFH was highest with the SU method and lowest with the MC method. Also, the ROS ratio was found to have increased in the DGC sample. These results suggest that the MC method is more suitable for sperm selection with low ROS levels in NIPs.

Furthermore, our results show that, compared to the conventional SU and DGC methods, the MC method is more effective in selecting spermatozoa with DNA integrity, providing a sufficient number of high-quality sperms. This is important because the selection of functional sperm with better DNA integrity has a critical role in the IUI treatment of NIPs by taking into account the underlying causes. Further studies to focus on pregnancy rates are needed to confirm these promising results obtained with the MC method.

Conflict of Interest and Financial Disclosure

The authors declare that they have no conflict of interest to disclose. The study was supported with a grant from the Bolu Abant Izzet Baysal University Scientific Research and Projects Unit (2015.08.03.970).

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