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# Comparison of DNA fragmentation rates in sperm samples prepared by microfluidic chip, swim-up and gradient methods

# Mikro akışkan çip, yüzdürme (swim up) ve gradient yöntemleri kullanılarak hazırlanan sperm örneklerinde DNA fragmantasyon oranlarının karşılaştırılması

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## SUMMARY

**Aim:** Sperm preparation methods significantly effect the results of assisted reproduction techniques. Sperm washing removes cells potentially harmful to fertilization and obtains more motile and morphologically better sperm. Conventional sperm washing techniques, include centrifugal, non-centrifugal swim-up and centrifugal gradient methods. Sperm DNA fragmentation is one of the factors suggested for male infertility. Sperm washing methods may effect DNA fragmentation rate. The aim of the current study is to compare sperm DNA fragmentation rates between conventional sperm washing methods and relatively new microfluidic chip method.

**Methods:** Study group consisted of 14 volunteer men. Every sperm sample was allocated and washed separately in 3 categories as; gradient, non-centrifugal swim-up and finally microfluidic chip methods. Sperm DNA fragmentation rates in each category were compared by TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) test.

**Results:** Ratio of TUNEL positive sperm with DNA fragmentation were 12%, 4% and 4,5% by gradient, non-centrifugal swim-up and microfluidic chip methods, respectively. Sperm DNA fragmentation ratio by gradient method statistically significantly increased compared to those with non-centrifugal swim-up and microfluidic chip methods ($p<0.002$). There was no statistical difference between non-centrifugal swim-up and microfluidic chip methods ($p=0.583$).

**Conclusion**: Current study determined increased sperm DNA fragmentation rate by gradient method compared to non-centrifugal swim-up and microfluidic chip methods. Centrifugation may increase oxidative stress, and thus lead to free radical production.

**Keywords:** Sperm,DNA Fragmentation, TUNEL, Sperm Washing

## ÖZET

**Amaç:** Sperm hazırlama yöntemleri, yardımlı üreme tekniklerinin sonuçlarını önemli ölçüde etkiler. Sperm yıkama işlemi, fertilizasyon için zararlı olabilecek hücrelerin ayrıştırır ve daha hareketli ve morfolojik olarak iyi spermlerin elde edilmesini sağlar. Geleneksel sperm yıkama teknikleri, santrifüjlü ve santrifüjsüz swim-up ve santrifüjlü gradient yöntemleridir. Sperm DNA fragmantasyonu, erkek infertilitesi için önerilen faktörlerden biridir. Sperm yıkama yöntemleri DNA fragmantasyonu oranlarını etkileyebilir. Bu çalışmanın amacı, geleneksel sperm yıkama yöntemleri ile yeni bir yöntem olan mikro akışkan çip yöntemi arasında DNA hasarı oranlarını kıyaslamaktır.

**Gereç ve Yöntem:** Çalışma grubu 14 gönüllü erkekten oluşmaktadır. Alınan her semen örneği 3'e ayrılarak gradient, santrifüjsüz swim up ve mikro akışkan çip yöntemleriyle ayrı ayrı yıkandı. Her gruptaki sperm DNA fragmantasyon oranları TUNEL (terminal deoksinükleotidil transferaz-aracılı dUTP işaretleme) testi ile karşılaştırıldı.

**Bulgular:** DNA fragmantasyonlu TUNEL pozitif sperm oranları, gradient, santrifüjsüz swim up ve mikro akışkan çip yöntemleri için sırasıyla %12, %4 ve %4,5 izlendi. Gradient yöntemi ile sperm DNA fragmantasyon oranları, diğer iki yöntemle karşılaştırıldığında istatistiksel olarak anlamlı yüksek bulundu ($p<0.002$). Swimup ile mikro akışkan çip yöntemleri karşılaştırıldığında DNA fragmantasyonu açısında istatistiksel olarak anlamlı bir fark izlenmedi. ($p=0.583$).

**Sonuç:** Mevcut çalışmada santrifüj olmayan swim-up ve microfluidic chip yöntemlerine kıyasla gradient yöntemiyle sperm DNA fragmantasyon oranlarında artış tespit edilmiştir. Santrifüj oksidatif stresi arttırabilir ve böylece serbest radikal üretimine yol açabilir.

**Anahtar Kelimeler:** Sperm,DNA Fragmentasyonu, TUNEL, Sperm Yıkama

## INTRODUCTION

Male factor is the main cause in about 20% of infertile couples. The ratio is 30-40% if the combination of male and female factors is included (1, 2). The genetic structure of the sperm and DNA damage effects infertility. Fertilization rate is decreased for in vitro fertilization (IVF) if the genetic damage of spermatozoa is greater than 4% (3). DNA fragmentation rates of spermatozoa were 27,6 % and 13,3 % in infertile and fertile men, respectively, (4). Sperm DNA is suggested to be more significant than the standard results of sperm analysis in determining fertility potential. The cause of sperm DNA damage is not fully explained in literature. Inflammation, apoptosis, smoking and free oxygen radicals (ROS) prevent condensation of sperm chromatin (5). Production and amount of ROS from germ cells and leukocytes are also related to the initiation of apoptosis during spermatogenesis (6). Oxidative stress cause DNA fragmentation in healthy sperms (1,2). Sperm cells with damaged DNA cause repeated IVF and intrauterine insemination (IUI) failures.

DNA fragmentation rates of sperm samples washed using micro-fluid chip, non-centrifugal swim-up and gradient methods will be compared in the current study. There must be sufficient number of sperms with fast progressive motility in order to apply the swim-up method. The swim-up method can be handled with or without centrifugation. Gradient method uses centrifugation. Pipetting is performed to the semen after the liquefication in the swim-up and gradient methods with centrifugation. Sperm is then treated with various mediums and centrifuged. These methods increase DNA fragmentation rate in healthy sperms as they cause oxidative stress (7). Harmful methods such as centrifuging, pipetage, vortexing are not used in micro-fluid chip and non-centrifugal swim-up methods. Aim of the current study, is to compare the methods that are frequently used in assisted reproduction techniques with the micro-fluid chip, a new method, which is thought to be less harmful to sperm DNA. Investigation of the hypothesis is important for increasing success rates in assisted reproductive techniques.

## MATERIAL AND METHODS

The study was conducted with 14 patients' semen. Study group consisted of volunteers without any systemic disease, any history of febrile disease during childhood, or history like testis trauma effectig reproductivce function. The study protocol was approved by the institutional review board of Maltepe University. Consent forms were obtained from the patients.

Each sperm sample was allocated and washed using micro-fluid chip, non-centrifugal swim-up and gradient methods. DNA fragmentation rates of sperm samples washed using 3 different methods were compared by TUNEL assay in the current study.

Sperm analysis, sperm washing techniques, and TUNEL assay were performed by a molecular biologist in order to eliminate subjective effects and standardize the procedures.

### Semen Analysis

Samples were collected following 3-7 days of abstinence. Samples were collected by masturbation and were transferred into clean, wide mouthed glass or plastic containers nontoxic for spermatozoa. The specimen container was placed on the bench or in an incubator (37$^{o}$C) for liquefaction. Both liquefaction and appearance of the semen were assessed. Semen was mixed for homogenization and both volume and pH were measured. Microscopic appearance, sperm motility and dilution required for measuring sperm number were assessed by wet preparation. Semen smears were prepared for morphological examination, air-dried and stained with Diff-Quick. Preparations were fixed in "Diff Quick" fixative for 10 minutes, stained with "Diff Quick" cationic and anionic solutions 1 minute for each, rinsed in water to remove extra strain and air-dried again. The semen morphology was evaluated with 100X magnification at phase contrast microscopy and sperm count were determined.

### Swim-up method without centrifuge

The liquefied semen sample was divided in a conical tube to be approximately 1-2 ml and 1:1 ratio washing media was added slowly added to create a layer. The semen sample was kept with a 45 degree angle in the 37C and 5% CO2 medium incubator for 45 minutes-1 hours. After incubation, approximately 0.5 ml sample was carefully removed from the uppermost layer for processing.

### Gradient Method

Medium containing silica particles was used for the gradient method. Two layers were created by placing 90% and 50% gradient solutions into a 15 ml conical tube as 1 ml from bottom to top. About 2 ml of liquefied ejaculate was left on the 90% layer and centrifuged at 200-500 g for 5 min. The supernatant formed at the top after centrifugation was discarded. Pellet sections of 90% and 50% layers were transferred in two separate tubes, mixed with the washing medium at 1: 1 ratio and centrifuged at 200-500 g for 5 min again. After the centrifugation, uppermost layer

was discarded and semen samples accumulated at the bottom part was diluted with 0,5 ml washing solution and were ready for processing.

**Micro-Fluid Chip Method**

The semen sample was liquefied in 37 °C incubators. After pipetting 13 $\mu$l solution from the inlet hole of 5 channels in the micro-fluid chip, 2 $\mu$l sample from the liquefied semen was pipetted slowly from the inlet hole. 2 $\mu$l mineral oil was added to each of the outlet holes and 1 $\mu$l of mineral oil was added to the inlet holes. The prepared solution was incubated at 37 °C for 30 min. The motility of sperms were examined under a microscope and incubation time was extended if sperm motility was slow. 20 $\mu$l was collected with micropipette from the liquid containing the motile sperm that accumulated in the last exit reservoirs.

Detection of sperm DNA fragmentation by Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

The presence of DNA strand breaks was evaluated by TUNEL assay using In Situ Cell Death Detection kit with fluorescein (Roche Diagnostics®, Mannheim, Germany), according to the manufacturer's protocol described below.

Sperm pellet containing 1-2 million sperm was washed with Dulbecco's phosphate-buffered saline without calcium chloride and magnesium chloride (PBS) and centrifuged at 800x g for 5 minutes. Seminal plasma was seperated and pellet was washed twice with PBS containing 1% bovine serum albumin (BSA). Pellet was diluted with 100 $\mu$L PBS containing 1% BSA. Pellets were fixed with 3,7% paraformaldehyde in PBS for 1 hour at room temperature. After washing with PBS,10 $\mu$L of the sperm pellet was pipetted onto slide and dried at room temperature. Dried slides were washed with PBS. The cell permeability was done with 0.1% Triton X-100 on ice for 2 minutes, and then incubated with the TUNEL reaction mixture (50 $\mu$l) in a humidified chamber and dark room at 37°C for 1 hour. After washing in PBS, they were counterstained with 1 mg/ml 4',6-diamidino2-phenylindole (DAPI) II (Abbott Laboratories, Salt Lake City, UT, USA, 06J50-001). For each slide, approximately 100 spermatozoa were analyzed with a fluorescence microscope. Each spermatozoon was classified as either a normal (blue nuclear fluorescence due to DAPI II) or fragmented DNA (green nuclear fluorescence). The final percentage of spermatozoa with fragmented DNA was reported for each sample.

**Determination of sperm DNA fragmentation by TUNEL assay**

The percentage of spermatozoa with fragmented DNA from 100 spermatozoa in each slide were calculated. Sperm DNA fragmentation by TUNEL assay were detected for micro-fluid chip, non-centrifugal swim-up and gradient methods and statistically analyzed.

**Statistical Analysis**

All data were analyzed by using the SigmaStat 3.0 (Systat Software Inc., Erkrath, Germany) package software. The data were analyzed by the repeated variance analysis (oneway repeated measures ANOVA). The ShapiroWilk test was used to determine if the quantitative variables had a normal distribution. Sperm DNA fragmentation data had non-normal distribution. "Paired test" was used to determine the difference between the methods. The study data were expressed as the median, min-max and number of cases. Percentages of median values were included. Results were presented as mean ± standard error (SEM). P values equal to or less than 0.05 were considered statistically significant. ($P < 0.05$).

## RESULTS

Our study was conducted with 14 volunteers between the ages of 25 and 45. The median age of the individuals was calculated as 34. The demographic data of 14 semen samples including sperm characteristics for the volume, concentration, progresssive motility, morphology, vitality are demonstrated in Table 1.

**Table 1.** The demographic data of 14 semen samples.

| | |
|---|---|
| Volume (ml) | 3 (2,2-4,5) |
| Concentration (mil/ml) | 44 (7-76) |
| Progresssive motility (% a+b) | 39 (20-57) |
| Morphology (% normal) | 2 (0-4) |
| Vitality (%) | 50 (40-65) |

Data are presented as median and (range).

Sperm samples were prepared by different washing methods and DNA fragmentation rates have been determined.100 sperm were counted with fluorescent microscopy for each method and rate of DNA fragmentations was determined.

The median rate of TUNEL positive sperm was 12%, 4% and 4.5% with gradient, non-centrifugal swim-up and micro-fluid chip methods, respectively. Statistically significant difference was detected between the gradient method and the other two methods in terms of DNA fragmentation ($p<0.002$) (Table 2). Comparison of DNA fragmentation between non-centrifugal swim up and microfluidic chip method revealed no statistically significant difference ($p=0.583$).

**Table2.** DNA fragmentation rates after sperm washing methods

| Groups | n | Median | Median %25 - 75 |
|---|---|---|---|
| Age | 14 | 34 (26-41) | 28,0 - 39,25 |
| Gradient Method | 14 | 12,5(2-27) | 4,5 - 17,75 [a] |
| Swim-Up Method | 14 | 4 (0-15) | 1,0 - 7,0 [b] |
| Micro-Fluid Chip Method | 14 | 4,5(0-15)* | 1,0 - 7,5 [c] |

## DISCUSSION

Sperm preparation methods significantly effect the results of assisted reproduction techniques. Repetitive centrifugation stages are applied in the traditional techniques for the sperm preparation including gradient and centrifugation swim-up methods. The purpose of these techniques is to separate sperm from seminal plasma.

The sperms with high movement capacity are separated by their flow from the pellet formed by centrifugation towards the culture medium (8). One of the consequences of centrifugation is the production of reactive oxygen species (ROS) by the subpopulation cells in the semen which may lead to permanent DNA damage (8, 9). 25% of infertile men have high levels of ROS. High levels of ROS are toxic for membrane structure of spermatozoa, proteins and DNA. On the other hand, low amounts of ROS molecules have a role in signal transmission for physiological reactions and support fertilization (2). They are involved in capacitation, acrosome reaction, hyperactive motility and physiological modulation of phagocytosis (10). Source of ROS in semen are neutrophils and spermatozoa (11,12). Addition of H2O2 at low dose stimulates capacitation whereas catalase as an antioxidant prevents this process (6). Formation of the superoxide anion is the first reaction during capacitation, peaks in 15-20 minutes and then falls (13, 14). Production of ROS meanwhile, has a direct role in the activation of adenylate cyclase and tyrosine kinase (13,15).

Superoxide anion stimulates the condensation and hyperactive motility, whereas the antioxidant superoxide dismutase enzyme prevents these processes (16). Hyperactive motility is also stimulated with increasing O2 concentration while the antioxidant capacity of semen for O2 is below 37% (17).

High ROS rates are produced by abnormal sperm and semen leukocytes, resulting in sperm dysfunction. DNA-damaged sperm fails to fertilize the oocyte. Animal experiments have shown that sperm with damaged DNA leads to poor embryo development (18).

Current study compared sperm DNA fragmentation rates between gradient, non-centrifugal swim-up and microfluidic chip methods. Sperm DNA fragmentation ratio by gradient method statistically significantly increased compared to those with non-centrifugal swim-up and microfluidic chip methods. We did not observe any statistical difference between non-centrifugal swim-up and microfluidic chip methods.

Asghar et al observed significantly lower DNA fragmentation by microfluidic chip method compared to centrifugal swim-up method (19). The centrifugal step appears to increase the DNA fragmentation rate. Centrifugation based sperm swim-up, and density gradient separation methods reduce sperm quality during the repetitive centrifugation steps (7). Concentrated sperm in solid pellets, often with incidental inflammatory cells demonstrates higher ROS generation and DNA fragmentation. Significant improvement in DNA fragmentation is observed in sperm sorted using macrofluidic sperm sorter chip compared to unsorted semen sample and swim-up method (20).

In conclusion, statistically significant increase in DNA fragmentation rate is observed with gradient method compared to microfluidic chip and non-centrifuged swimup washing methods. It is also important analyze the cost of the procedures for the laboratory. The cost of gradient and swim-up methods are known to be more appropriate than the micro-fluid chip method. Although the cost of the gradient method is low, higher rates of DNA fragmentation is observed. Such studies are important for clarifying the advantages and disadvantages of the sperm washing methods. More efficient sperm washing process in assisted treatment techniques can minimize DNA fragmentation.

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