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Effects of sperm preparation techniques on sperm survivability and DNA fragmentation

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Abstract

Objective: This study was performed to determine the effect of swim-up (SU) and density gradient centrifugation (DGC) on sperm survival and DNA fragmentation.

Methods: Individual semen samples were analyzed before each was divided into two aliquots (half for SU and half for DGC) for calculation of sperm survival and the DNA fragmentation index (DFI). Sperm DNA fragmentation was determined using the sperm chromatin dispersion test.

Results: The DFI of the 63 semen samples processed using both procedures was lower than that of the fresh semen samples. The DFI was significantly lower for samples processed using the SU than DGC method. In the sperm survival test, the SU technique was associated with increased sperm motility and vitality following preparation. After 24 hours, however, the concentration and percentage of surviving sperm were significantly lower in the SU than DGC group.

Conclusions: Both semen preparation techniques help to minimize sperm DNA fragmentation; however, when the DFI is $<$30%, the SU technique is more appropriate than DGC. While DGC may be superior for intrauterine insemination, the SU method may be preferable for in vitro fertilization or maturation.

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Keywords
Swim-up, density gradient centrifugation, sperm survival, sperm DNA fragmentation, DNA fragmentation index, sperm preparation

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Introduction

Infertility is defined as the failure to conceive a child after 1 year of regular sexual activity, and it affects 8% to 15% of reproductive-aged couples worldwide; male factors account for approximately half of infertility cases.[1,2] Although semen analysis is widely acknowledged as a standard procedure for assessing male fertility, the results do not provide evidence of sperm function. Because the sperm parameters evaluated in semen analysis are insufficient for evaluating male fertility, it is impossible to anticipate the outcome of assisted reproductive technology.[3]

Infertility can occur even when semen analysis results are normal, and it can be caused by sperm DNA fragmentation.[4–6] Sperm DNA integrity is crucial for effective transmission of genetic information. Higher DNA fragmentation in sperm can have a detrimental impact on embryo morphokinetic parameters and may help to predict pregnancy outcomes following intracytoplasmic sperm injection and in vitro fertilization (IVF).[7,8] In reproductive cycles, the DNA fragmentation index (DFI) is inversely correlated with embryo development and the implantation rate but positively correlated with the miscarriage rate.[9] The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay,[10] single-cell gel electrophoresis (commonly known as the comet assay), and the sperm chromatin structure assay[11] are only a few of the techniques used to assess DNA fragmentation. After removing DNA-linked proteins, sperm with unfragmented DNA products display a typical halo in the sperm chromatin dispersion (SCD) assay; the absence of halos or the presence of tiny or degraded halos indicates DNA damage.[12]

Male fertility and the success of assisted reproduction procedures, particularly intrauterine insemination (IUI), are associated with sperm survival. Sperm viability in the uterine cavity and sperm motility in the female reproductive tract are the most important factors for successful fertilization.[13] The sperm survival test (SST) is used to measure the motility and survivability of sperm at 24 hours post-ejaculation following swim-up (SU) and density gradient centrifugation (DGC) processing.

DGC and SU are currently the most frequently utilized techniques for semen preparation. In recent years, comparative studies of sperm preparation procedures have been conducted to evaluate outcomes such as conventional semen parameters or recovery rates.[14,15] Increasingly, studies have shown how different preparation methods affect molecular markers such as the DFI, sperm telomeres, and cell apoptosis. Additionally, research has shown that the SU method, but not the DGC technique, produces a significantly lower ratio of DNA fragmentation than when using fresh samples.[16] According to other studies, SU is the best method because it can significantly reduce sperm DNA damage and may have a predictive value in IUI.[17–19] In contrast, Wang et al.[20] observed that the DFI is decreased following DGC except in cases of severe oligozoospermia. Moreover,

both SU and DGC have been demonstrated to reduce sperm deformity, with DGC being more effective in reducing DNA fragmentation.[21] Furthermore, compared with fresh semen, the average sperm telomere length significantly increases after sperm preparation; nonetheless, the difference between the two techniques is not significant.[22] The impact of various sperm preparation procedures on sperm function remains unknown. We therefore performed the present study to determine how the DGC and SU techniques affect sperm survival and the DFI.

Methods

Selection of patients

From April to July 2020, patients seeking infertility treatment at Hue University Hospital in Vietnam were enrolled in this cross-sectional study. All patients underwent semen analysis as a routine test, and the remaining semen samples were used in the study with the patients' permission. A sperm concentration of at least 5 million/mL was required for inclusion, as was patient agreement to participate in the study. Patients who lacked sperm DNA fragmentation and SST results, had an inability to ejaculate, had surgically extracted sperm, had retrograde ejaculation, or had azoospermia were excluded from the study. Written informed consent was obtained from all participants, and the study protocol was approved by the institutional review board of the University of Medicine and Pharmacy, Hue University, Vietnam (approval number H2020/448 issued on 30 September 2020). In total, 63 semen samples were collected. Each sample was divided into two aliquots, one of which was treated with SU and the other with DGC.

Sample collection and preparation

Semen samples were collected via masturbation after 2 to 7 days of sexual abstinence and then liquefied in an incubator at 37°C. The semen parameters were analyzed by two experienced embryologists and classified according to the 2010 World Health Organization criteria. Each sample was then divided into two halves for DGC and SU treatment.

SU procedure

Each 1-mL semen sample was placed in a round-bottom tube. Next, 1 mL of a washing medium (FertiCult Flushing Medium; FertiPro, Beernem, Belgium) was added to the semen sample. The tube was placed at an angle of 45° at a temperature of 37°C for 30 minutes. The supernatant was then aspirated into a 5-mL tube and centrifuged for 5 minutes at $300 \times g$. The final pellet was resuspended in 0.3 mL of FertiCult Flushing Medium.

DGC procedure

Each 1-mL semen sample was added above the density gradient of a sperm preparation medium (Sil-Select Plus; FertiPro) (1.5 mL of upper layer 45% and 1.5 mL of lower layer 90%) and centrifuged for 15 minutes at $350 \times g$. The sperm pellet was then washed in 3 mL of FertiCult Flushing Medium and centrifuged twice for 10 minutes at $350 \times g$. The final pellet was resuspended in 0.3 mL of FertiCult Flushing Medium.

Measurement of sperm DFI

A diagnostic kit for DNA fragmentation (Halosperm kit; Halotech DNA SL, Madrid, Spain) was used to test the sperm DNA integrity. This kit works on the principle that sperm with intact DNA generate

a halo of dispersed DNA loops that can be observed under a microscope, whereas sperm with fragmented DNA do not generate such a halo. The procedure was carried out according to the manufacturer's instructions. To prevent gelation, agarose was melted in an Eppendorf tube and held at 37°C; 20 μL of washed sperm was then added to 40 μL of melted agarose and evenly mixed. Next, a 10-μL aliquot of the agar–cell suspension was placed on a super-coated slide and placed in a refrigerator at 4°C for 10 minutes to harden the agarose. The slide was then immersed in an acid denaturant for 7 minutes before being incubated in lysis solution for 25 minutes at 37°C. The slides were stained with Giemsa stain after washing the sample in distilled water for 5 minutes and dehydrating it in ethanol. The DFI was calculated using at least 500 sperm cells per slide for each semen sample. Sperm were observed and classified into five groups: those with a large halo (halo width equal to or larger than the diameter of the core), those with a medium halo (halo width equal to one-third the diameter of the core), those with a small halo (halo width less than one-third the diameter of the core), those without a halo, and those that were degraded. The DFI was calculated as follows:

$$\mathrm{DFI}\,(\%) = \frac{\text{Number of spermatozoa with fragmented DNA (small halo + without halo + degraded)}}{\text{Number of spermatozoa counted}} \times 100$$

The results were calculated as the average of the results obtained by two embryologists. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to capture photographs and measure the sizes of the halos in the sperm DNA fragmentation assay.

SST

After 24 hours of incubation, the SST was performed, which involved analyzing changes in sperm parameters. At 0 and 24 hours after incubation at 37°C, the vitality and progressive motility of sperm in the post-preparation semen samples were assessed. The following formulas were used to determine the sperm vitality index (SVI) and sperm motility index (SMI):

$$\mathrm{SVI}\,(\%) = \frac{\text{Vitality at 24 hours}}{\text{Vitality at 0 hours}} \times 100$$

$$\mathrm{SMI}\,(\%) = \frac{\text{Progressive motility at 24 hours}}{\text{Progressive motility at 0 hours}} \times 100$$

Statistical analysis

The statistical analysis was carried out using IBM SPSS Statistics for Windows Version 22.0 (IBM Corp., Armonk, NY, USA). Data are expressed as mean ± standard deviation. The paired Student's *t*-test was used to compare data from the two sperm preparation methods. A p value of <0.05 was considered statistically significant. Graphs were created using R programming software.

Results

The participants' general characteristics are shown in Table 1. In total, 63 semen samples were collected in this study. The mean age of the participants was 34.63 ± 6.36 years (range, 25–53 years). A total of 20 (31.7%) participants exhibited normal semen characteristics, while 43 (68.3%) had at least one abnormal characteristic. The mean percentage of progressive motility was 31.32% ± 6.90%, the percentage of morphologically normal spermatozoa was 58.7%, and the mean DFI was 27.25% ± 18.98%.

Table 2 shows the effects of the sperm preparation procedures on sperm survival

Table 1. General characteristics of fresh semen samples (n = 63).

Characteristics	Mean ± SD (range) or n (%)
Age, years	34.63 ± 6.36 (25–53)
pH	7.29 ± 0.45 (6.5–8.5)
Sexual abstinence, days	4.25 ± 1.39 (2–7)
Semen volume, mL	2.63 ± 0.93 (2–6)
Sperm concentration, $\times 10^6$/mL	37.68 ± 15.33 (15–73)
Total sperm number, $\times 10^6$	93.47 ± 62.22 (28–358)
Sperm vitality, %	83.97 ± 6.82 (61–93)
Progressive motility	31.32 ± 6.90 (11–48)
<32%	31 (49.2%)
≥32%	32 (50.8%)
Number of sperm progressive motility, $\times 10^6$	29.41 ± 20.91 (7.98–110.83)
Normal sperm morphology	3.68 ± 1.46 (1–7)
<4%	26 (41.3)
≥4%	37 (58.7)
DFI, %	27.25 ± 18.98 (6–81)

SD, standard deviation; DFI, DNA fragmentation index.

Table 2. Outcomes of SU and DGC techniques (n = 63).

Outcomes	Post-preparation by SU	Post-preparation by DGC	P-value
Concentration, $\times 10^6$/mL	14.29 ± 7.64	24.33 ± 10.36	<0.001
Sperm vitality, %			
0 hour	95.70 ± 3.09	90.90 ± 4.61	<0.001
24 hours	57.25 ± 19.82	66.16 ± 15.65	0.001
SVI	59.80 ± 20.50	72.92 ± 17.47	<0.001
Progressive motility, %			
0 hour	90.63 ± 4.36	85.11 ± 5.22	<0.001
24 hours	29.37 ± 16.58	38.25 ± 12.59	<0.001
SMI	32.40 ± 18.19	45.07 ± 15.02	<0.001

Data are presented as mean ± standard deviation.
P-value: SU vs. DGC.
SU, swim-up; DGC, density gradient centrifugation; SVI, sperm vitality index; SMI, sperm motility index.

and semen parameters. At 0 hours, the mean ratios of sperm vitality and progressive motility were significantly higher in the SU group than in the DGC group (95.70% ± 3.09% vs. 90.90% ± 4.61% and 90.63% ± 4.36% vs. 85.11% ± 5.22%, respectively; $p < 0.05$). At 24 hours, however, the DGC group had significantly higher sperm vitality (66.16% ± 15.65%) and progressive motility (57.25% ± 19.82%) than the SU group ($p < 0.05$). The SVI and SMI were significantly higher in the DGC group than in the SU group (72.92% ± 17.47% vs. 59.80% ± 20.50% and 45.07% ± 15.02% vs. 32.40% ± 18.19%, respectively; $p < 0.05$).

To determine the effects of DGC and SU on the DFI and sperm characteristics, the DFI, progressive motility, and morphology of all 63 samples were analyzed as shown in Table 3. Analysis of the DFI group, which consisted of 45 participants with a normal

Table 3. Comparison of DFI, sperm progressive motility, and sperm morphology after using the SU and DGC techniques (n = 63).

Outcomes	n	DFI (%) Post-preparation by SU	DFI (%) Post-preparation by DGC	P-value
DFI, %				
DFI < 30	45	5.33 ± 4.17	8.18 ± 3.78	<0.001
DFI ≥ 30	18	8.28 ± 4.76	8.83 ± 3.47	0.532
Sperm progressive motility				
Normal progressive motility	32	4.63 ± 3.18	7.66 ± 3.33	<0.001
Abnormal progressive motility	31	7.77 ± 5.14	9.10 ± 3.93	0.057
Sperm morphology				
Normal morphology	37	5.65 ± 4.11	7.38 ± 3.55	0.014
Abnormal morphology	26	6.92 ± 5.01	9.50 ± 3.67	0.001

Data are presented as mean ± standard deviation. P-value: SU vs. DGC.
DFI, DNA fragmentation index; SU, swim-up; DGC, density gradient centrifugation.

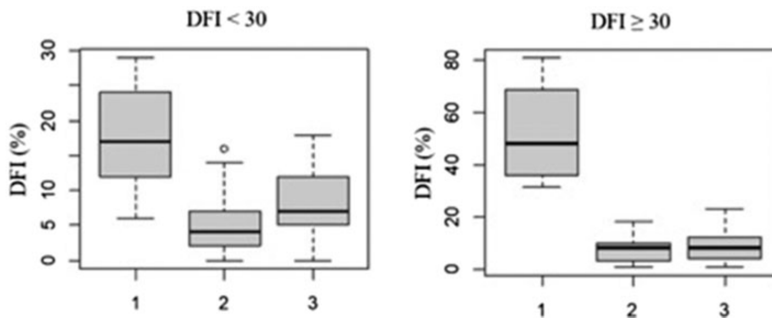

Figure 1. Box and whisker plots for the DFI in different groups of patients showing the median and interquartile ranges. (1) DFI of fresh semen samples. (2) DFI after performing the SU technique. (3) DFI after performing the DGC technique. The dots indicate values outside the range. DFI, DNA fragmentation index; SU, swim-up; DGC, density gradient centrifugation.

DFI (DFI < 30%) and 18 participants with an abnormal DFI (DFI ≥ 30%), showed that among participants with a normal DFI, the SU approach reduced the DFI to a significantly lower value than did the DGC method (5.33% ± 4.17% vs. 8.28% ± 4.76%, respectively; $p < 0.001$). The DFI was decreased following both SU and DGC. Among participants with an abnormal DFI, the difference in the DFI between the two techniques was not statistically significant (Figure 1). The SU approach resulted in a significantly lower reduction in the DFI than did the DGC method in terms of sperm progressive motility and morphology.

Discussion

The quality of spermatozoa is critical for successful reproduction. The techniques employed to prepare human spermatozoa are important in the field of reproductive medicine; however, their impacts on sperm quality remain unknown. The present study was performed to investigate whether sperm

preparation procedures have an effect on sperm quality as determined by the sperm survival rate and DFI. To our knowledge, this is the first study to show a relationship between sperm survival and sperm preparation approaches such as SU and DGC. Additionally, each individual semen sample underwent both SU and DGC. This study design enabled us to compare the two techniques objectively and effectively.

The sperm DFI is an excellent functional indicator of male fertility.[23] When compared with the DFI of fresh semen samples, the DFI was significantly lower following both SU and DGC. In particular, the SU technique was associated with a significantly lower DFI than the DGC technique. This result corroborates the findings of an earlier study by Zini et al.,[16] who found that when samples are processed using the SU technique, the percentage of DNA fragmentation was dramatically lower than that in fresh semen samples; however, the DGC technique did not result in markedly lower fragmentation. Kim et al.[24] concluded that the SU technique is superior for enriching genetically competent sperm, and Jayaraman et al.[25] found no change in the DFI after utilizing various techniques. In contrast, few studies have shown that the DFI of DGC-prepared samples is lower than that of SU-prepared samples.[21,26,27]

These disagreements regarding the DFI might be explained by the use of different sperm DNA fragmentation tests, such as TUNEL,[17,19,25] the SCD assay,[18] or the Halosperm assay.[24] The SCD assay is a DNA integrity test for sperm nuclear condensation. The halo size disparity allows us to determine the degree of sperm DNA fragmentation. The application of imaging analysis software, which takes pictures from slides and measures the halo objectively, can overcome the restriction of subjective assessment by laboratory staff. In addition, the SCD test has a number of advantages over the standard TUNEL approach, such as simple equipment, highly repeatable results, and high homogeneity. Jayaraman et al.[25] found that the DFI does not change when different techniques are utilized. Kim et al.[24] assessed DNA damage using the Halosperm assay and discovered that the SU technique is superior for enriching genetically competent sperm. After performing SCD assays to evaluate 65 subfertile individuals, Oguz et al.[18] demonstrated that the SU method significantly lowered sperm DNA damage and may even have some predictive relevance in IUI.

Several reports have described contradictory effects of various media on the DFI following DGC. Whereas no difference in the DFI was observed in fractions obtained using SpermGrad medium (Vitrolife, Englewood, CO, USA) and PureCeption medium (Sage Biopharma, Bedminster, NJ, USA),[28] the DFI was significantly lower in the post-wash step when using PureSperm (Nidacon Laboratories, Gothenburg, Sweden) and Sil-Select and significantly higher when using SpermGrad; however, the Sil-Select Plus preparation yielded sperm samples with the lowest DFI .[29] Lee et al.[30] compared the percentage of morphologically normal spermatozoa, the DFI, and the hyaluronic acid-binding ability in four different media with various silica concentrations. The four media tested were PureSperm (40%/80%), PureCeption (40%/80%), SpermGrad (45%/90%), and Sydney medium (40%/80%) (William A. Cook, Queensland, Australia). The DFI was found to increase following management with Sydney medium and to decrease following treatment with PureCeption, SpermGrad, and PureSperm media. In this investigation, we used two layers of media with variable silica concentrations (45% and 90%) to retain seminal plasma, exotic cells, and dead sperm. Viable sperm cells with good

motility were able to get past the separators and reach the bottom of the centrifugation tube.

Based on the manufacturer's recommendation, the DFI reference value was set to 30% in this study. In the normal group (DFI < 30%), the DFI following SU preparation was significantly lower than that after DGC. In the abnormal group (DFI ≥ 30%), the difference in DNA fragmentation between the two approaches was not statistically significant. This implies that current preparatory procedures are unable to reduce the DFI when the DFI is abnormal. According to the preceding description, the SU technique may be more efficient than the DGC technique at reducing sperm DNA fragmentation, especially in samples with a DFI of <30%.

The SST findings in the present study revealed that the SU technique was associated with better outcomes the DGC technique, notably in terms of sperm motility and vitality. After 24 hours, however, the concentration and percentage of surviving sperm cells were significantly higher in the DGC group than in the SU group. These differences may have been due to prolonged SU incubation, which has a detrimental impact on sperm function. In addition, the SU process requires pelleting of spermatozoa, which results in close cell-to-cell contact between spermatozoa, leukocytes, and cell debris, resulting in the production of a large amount of reactive oxygen species.[31] When the concentration of reactive oxygen species or free radicals exceeds the antioxidant capacity of the cell, oxidative stress occurs.[32] As a result of the oxidative stress, DNA fragmentation occurs via lipid peroxidation of the sperm membrane.[33] In addition, an extended incubation period can reduce sperm vitality and motility. Therefore, the DGC method should be utilized in treatments such as IUI, which requires a high concentration of sperm with good vitality. In IUI, the sperm selection process naturally occurs in the fallopian tube so that sperm DNA fragmentation is not a priority in sperm preparation. Although the SU approach resulted a lower recovery rate and sperm vitality rate in the present study, the sperm quality at 0 hours post-preparation and the number of sperm with a low DFI were superior to those obtained with DGC. As a result, the SU technique may be appropriate for assisted reproductive techniques such as IVF, which requires only a small number of spermatozoa.

Although several studies have compared the two sperm preparation techniques, our study based on the DFI and survivability assessment revealed further details regarding the differences in the effects of the SU and DGC techniques, which can provide more clinical options for infertility treatment. Although the small sample size of this study should be considered as a limitation, both SU and DGC were used on the same sample (each semen sample was separated into two aliquots). As a result, the statistical analysis was appropriate. Furthermore, the Halosperm assays were assessed using ImageJ software to capture photographs and measure the halo, which resulted in a decrease in subjectivity and an increase in precision. Another strength of this study is that we evaluated survivability at both 0 and 24 hours. Although the sperm progressive motility and morphology at 0 hours were better in the SU group than in the DGC group, these traits were reversed after 24 hours, favoring the DGC group. To the best of our knowledge, this is the first report to show a relationship between sperm survival and sperm preparation techniques in the literature. Further research of alterations in sperm structure (e.g., acrosomes, mitochondria) and function caused by reactive oxygen species is needed.

In conclusion, both sperm preparation procedures decreased the DFI, but SU preparation had a more favorable

processing impact. The SU technique should be used to prepare sperm with a low DFI (<30%). In terms of motility and viability, both techniques produced spermatozoa that were comparable to those obtained from fresh samples (at 0 hours). Notably, at 24 hours, the DGC group had a significantly higher concentration and percentage of surviving spermatozoa than did the SU group. In practice, however, SU should be the preparation of choice for both IUI and IVF because the success of fertilization is less significant than the embryo's development and future well-being.

Consent for publication

Written consent for publication was obtained from all participants.

Availability of data and material

All data are available in the submitted manuscript.

Competing interests

The authors have no competing financial or other interests to declare in relation to this manuscript.

Ethics approval

This study was approved by the Ethics Committee of the University of Medicine and Pharmacy, Hue University (approval number H2020/448). All information and data were encrypted and confidential. All participants provided informed consent. All samples were strictly handled and processed as stipulated by an approved local review board protocol.

Author contributions

Conceptualization, methodology, and supervision: M.T.L., H.N.T.D., T.T.T.N, Q.H.V.N., and N.T.C. Data collection, formal analysis, original draft, review, and editing: M.T.L., H.N.T.D., T.T.T.N., and T.V.N. All authors contributed to the interpretation of the data and approved the final manuscript.

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