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Integrity of human sperm DNA assessed by the neutral comet assay and its relationship to semen parameters and clinical outcomes for the IVF-ET program

Hee-Jun Chi[1], Da-Yeon Chung[1], Soon-Young Choi[1], Jong-Hyun Kim[2], Gi-Young Kim[2], Jae-Seok Lee[2], Hee-Sun Lee[3], Myung-Hee Kim[3], Sung-Il Roh[3]

[1]ART Research Center, [2]Department of Urology, [3]Department of Obstetrics and Gynecology, Mizmedi Hospital, Seoul, Korea

Objective: To explore potential relationships between sperm DNA integrity and both semen parameters and clinical outcomes.
Methods: Semen analysis of 498 samples was performed according to the 2010 criteria of the World Health Organization. The sperm DNA fragmentation Index (DFI) of the semen samples was assessed using a neutral comet assay.
Results: Sperm DFI showed a significant correlation with semen parameters, including the patient's age, sperm viability, motility, morphology, and number of leukocytes ($p < 0.05$). The sperm DFI values for asthenozoospermic (15.2%), oligoteratozoospermic (18.3%), asthenoteratozoospermic (17.5%), and oligoasthenoteratozoospermic semen samples (21.3%) were significantly higher than that observed in normozoospermic semen samples (10.5%, $p < 0.05$). A sperm DFI value of 14% was used as a threshold of sperm DFI in assessing whether DNA was highly damaged. In 114 IVF-ET cycles, the fertilization rate of the sperm DFI $< 14\%$ group (70 cycles, 61.7%) was significantly higher than that observed for the $\geq 14\%$ group (44 cycles, 55.3%), but there was no difference in the other clinical outcomes between the two groups. In the $\geq 14\%$ group, the pregnancy rates of the ICSI cycles (40.0%) and half-ICSI (44.0%) were higher than conventional IVF cycles (30.7%), but the difference was not statistically significant.
Conclusion: Along with the conventional semen analysis, the sperm DFI assessed using the comet assay was shown to improve the quality of the semen evaluation. To evaluate the precise effect of ICSI on pregnancy rates in the patients who demonstrate high sperm DFI values, further study is necessary.

Keywords: Spermatozoa; Comet Assay; Semen Analysis; Clinical Outcome; Human

Introduction

Traditionally, the diagnosis of male infertility is based on microscopic assessment, including the concentration, motility, and morphology of sperm, but the results of this conventional semen analysis are insufficient as a diagnostic tool in male infertility. Recently, sperm DNA integrity has been regarded as a complementary diagnostic tool and biologic marker of male reproductive health and infertility.

It is well known that DNA damage in spermatozoa occurs during late spermatogenesis as a consequence of endogenous factors present in the testis/epididymis, or due to exogenous factors present after ejaculation. Potential mechanisms for generating DNA damage in sperm have been proposed, and include incomplete chromatin packaging [1], abortive apoptosis [2], oxidative stress by reactive oxygen species (ROS) [3], and an imbalance in endogenous hormone levels [4]. Unlike other cells, spermatozoa are more vulnerable to DNA damage because they do not have the capacity for DNA repair. Therefore,

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Corresponding author: **Hee-Jun Chi**
ART Research Center, 701-4 Naebalsan-dong, Gangseo-gu, Seoul 157-280, Korea
Tel: +82-2-2007-1840 Fax: +82-10-5041-4603 E-mail: ivf129@mizmedi.net

if sperm with damaged DNA are able to successfully fertilize an oocyte, it may result in pathologies such as infertility [3], childhood cancer [5], and imprinting diseases [6,7]. During fertilization, the oolemma acts as a selective barrier and prevents the penetration of anomalous spermatozoa. In ICSI cycles, the sperm selection mechanism of the oocytes is bypassed, which may increase the risk of transmitting damaged DNA. However, there is some debate on the actual risk of ICSI in regard to the selective barrier of the oolemma for the sperm with damaged DNA.

The most widely used methods to detect DNA integrity in individual spermatozoa are the sperm chromatin structure assay (SCSA) [8,9], the terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling (TUNEL) assay [10-12], and the alkaline [13,14] and neutral comet assay [15-17]. Each of these tests determines different aspects of DNA damage. The SCSA and TUNEL assays require the use of expensive instrumentation, such as flow-cytometry equipment for precise assessment. Alternatively, the neutral comet assay is technically simpler to use, is more cost efficient, and has a comparably higher sensitivity for detecting DNA damage. Thus, the latter technique may be more suitable for routine measurements involving the quantification of double-strand (ds) break DNA damage in sperm cells [17].

A number of studies have shown a relationship between human sperm DNA damage and various semen parameters including sperm concentration, motility, morphology, and leukocyte concentration [18-23]. Sperm DNA damage has also demonstrated a negative effect on the mitochondrial activity of sperm [22,24], the efficiency of fertilization [25-27], embryo development [25,28,29], the chances of a successful pregnancy [26,29-31], and the ability to bring the pregnancy to term [32,33]. Interestingly, a patient's age [17,25] and endogenous hormone level [34] were associated with the integrity of sperm DNA. In contrast, other research groups did not observe a close relationship between sperm DNA integrity and sperm morphology [35], fertilization rate, embryo development [36], or pregnancy outcome for ICSI [10]. The analysis of the sperm DNA integrity is important for improving the accuracy of semen analysis and the diagnosis of male infertility. However, various threshold values of sperm DNA integrity have been reported and differ due to both the variety of methods employed and the inherent variations in results among different research laboratories. Moreover, there is debate regarding the relationship between the sperm DNA integrity and the clinical outcome.

Here we have used a modified neutral comet assay to provide quantitative measurements of DNA damage in human sperm using a large sample size to more accurately approximate the optimal threshold value of sperm DNA integrity. We also explored the relationships among the sperm DNA integrity, semen parameters, and the clinical outcome in IVF programs.

Methods

This study was approved by our Institutional Review Board (the Mizmedi Hospital Research Ethics Committee).

1. Semen samples

A total of 498 semen samples were analyzed using the comet assay after undergoing a freeze-thaw cycle. The comet assay technique was performed on 114 semen samples obtained from male patients who were subjected to the IVF-ET program. The samples were used to ascertain the presence of a possible relationship between the sperm DNA integrity and clinical outcome in the IVF-ET program.

2. Semen analysis

Semen samples were obtained via masturbation after two to five days of ejaculatory abstinence and were analyzed within one hour of collection. After seminal liquefaction, a routine semen analysis was performed using computer-aided semen analysis (CASA; Medical Supply, Seoul, Korea) according to the 2010 World Health Organization (WHO) specifications. The semen samples with a concentration $\geq 15 \times 10^6$ sperm/mL, a motility $\geq 40\%$, a viability $\geq 58\%$, a normal morphology $\geq 4\%$, and leucocytes $< 1 \times 10^6$ cells/mL were considered parameters that characterize normal semen samples. A specific nomenclature is used by andrologists to describe abnormal semen samples encountered during semen analysis. In the present study, abnormal semen samples were characterized by the nomenclature according to the 2010 WHO criteria. An oligozoospermic semen sample means a reduced sperm concentration of $< 15 \times 10^6$ sperm/mL, an asthenozoospermic semen sample means a decreased sperm motility $< 40\%$, and a teratozoospermic semen sample means a decreased number of spermatozoa with normal morphology $< 4\%$. When a semen sample shows abnormal sperm concentration, motility, and morphology, we use combinations of these terms, such as "oligoasthenoteratozoospermic" semen sample.

3. Freeze-thaw cycle of semen samples

The semen samples were collected and cryopreserved in a quantity suitable for three days worth of analysis because the comet assay was performed twice in one week in the interest of efficiency. The semen samples were frozen using ultra-rapid freezing in the absence of a cryopreservative to minimize the cryodamage to the sperm DNA as described previously [15]. The entire semen sample was placed into cryovials and was frozen by static-phase vapor cooling (10 cm above the level of liquid nitrogen [LN_2]; -80°C) for 10 minutes. The samples were then submerged into LN_2 (-196°C) and stored until needed. Prior to experimentation, the samples were removed from LN_2 and the cryovials were left at room temperature for 1 minute before being

submerged into a 37°C water bath. Once the samples were completely thawed, an equal volume of Ca^{2+} and Mg^{2+}- free phosphate buffered saline (PBS) was mixed with the sample and centrifuged at 300 g for 5 minutes to remove seminal plasma and small particles. In the preliminary test, there was no significant difference in the sperm DNA integrity between the fresh semen samples and the freeze-thawed semen samples assessed using the comet assay.

4. Comet assay

The sperm DNA integrity was assessed using the modified neutral comet assay as described previously [37]. Comet assay was performed using a comet assay kit (Trevigen, Gaithersburg, MD, USA). A mixture of semen (5 µL) and low melting agarose (45 µL) was layered onto a comet slide. The slide was transferred to a 4°C refrigerator for 10 minutes. The slide was then submerged into a pre-cooled lysing solution (containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, and 10 mM DL-Dithiothreitol; Sigma, St. Louis, MO, USA) for 2 hours prior to being placed in an electrophoresis buffer (500 mM NaCl, 100 mM Tris, 1 mM EDTA, and 0.2% DMSO; Sigma) for 20 minutes. After electrophoresis for 60 minutes at 10 V and 250 mA, the slide was washed with PBS and a neutralizing solution (50% Ethanol, 20 mM Tris, and 1 mg/mL Spermine; Sigma). After washing with PBS, the slide was stained with SYBR staining solution containing 10 mM Tris, 1 mM EDTA, and 0.01% SYBR Green (Trevigen). Images (400× magnification) were captured using a digital camera imaging system (Nikon digital sight DS-U1) attached to a fluorescence-inverted microscope (Figure 1).

5. IVF-ET program

Ovulation induction was achieved after several days of GnRH agonist suppression and was initiated through the administration of recombinant follicle stimulating hormone for a period of 9 to 12 days, followed by 10,000 U of hCG generally administered on the 10th day, when the largest follicular diameter was approximately 18 mm. Unlike the Comet assay, fresh semen was used for the IVF-ET program and motile sperm was prepared using a density gradient. The sperm washing buffer, fertilization medium, and embryo culture medium used for all protocols were GIII series medium (Vitrolife, Göteborg, Sweden). The embryo transfer step was performed on day three and the clinical pregnancy status was confirmed by observation of the uterine sac with a beating heart using ultrasonographic detection. A total of 114 IVF-ET cycles (36 conventional IVF cycles, 23 half-ICSI cycles, and 55 ICSI cycles) were involved in the present study. ICSI was used for the patients who showing male factors, a low number of mature eggs, or a low fertilization rate in the previous conventional IVF cycle. Half-ICSI was used for the patients who underwent the first conventional IVF cycle to prevent the total failure of fertilization. The IVF-ET cycles were divided into two groups according to the threshold of the sperm DNA integrity: 1) the $<14\%$ group and 2) $\geq 14\%$ group.

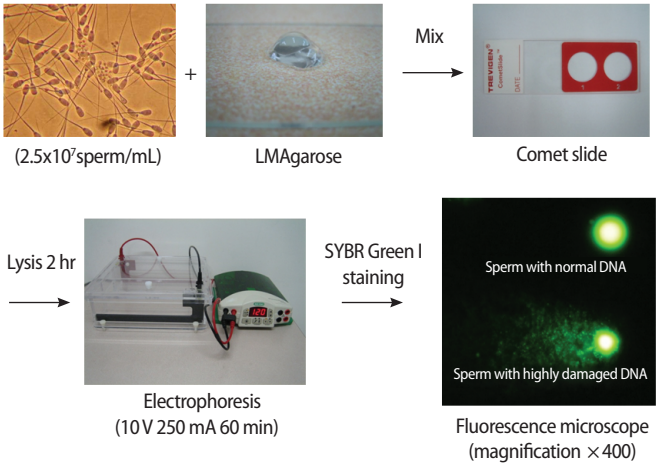

Figure 1. Procedure of neutral comet assay. LMAgarose, low melting agarose.

6. Statistical analysis

The association between the sperm DFI and the semen parameters was explored using Pearson's correlation and a two-tailed Student's *t*-test. These statistical procedures were performed using the SPSS ver. 16.0 (SPSS Inc., Chicago, IL, USA). The clinical outcomes between the experiment and control groups were analyzed using the chi-square test; a *p*-value <0.05 was considered statistically significant.

Results

1. Correlation between sperm DFI and semen parameters

A correlation between sperm DFI and semen parameters was investigated in the 498 semen samples (Figure 2). The degree of correlation was assessed with the correlation coefficient (r) and p-value. Sperm DFI decreased significantly when the sperm viability ($r=-0.605$, $p<0.001$), motility ($r=-0.395$, $p<0.001$), rapid motility ($r=-0.216$, $p<0.001$) or morphology ($r=-0.219$, $p<0.01$) increased, but sperm DFI increased as the patient's age ($r=0.134$, $p<0.01$) or leukocyte concentration in the semen increased ($r=0.138$, $p<0.01$). However there was no significant correlation between sperm DFI and sperm count ($r=0.004$, $p=0.91$) or semen volume ($r=0.028$, $p=0.53$).

2. Sperm DFI of subfertile semen samples

The sperm DFI of subfertile semen samples was compared with that of normal semen samples (Figure 3). The sperm DFI in asthenozoospermic ($15.2\pm9.5\%$), oligoteratozoospermic ($18.3\pm12.2\%$), asthenoteratozoospermic ($17.5\pm13.4\%$), and oligoasthenoteratozoospermic semen samples ($21.3\pm11.2\%$) were significantly higher

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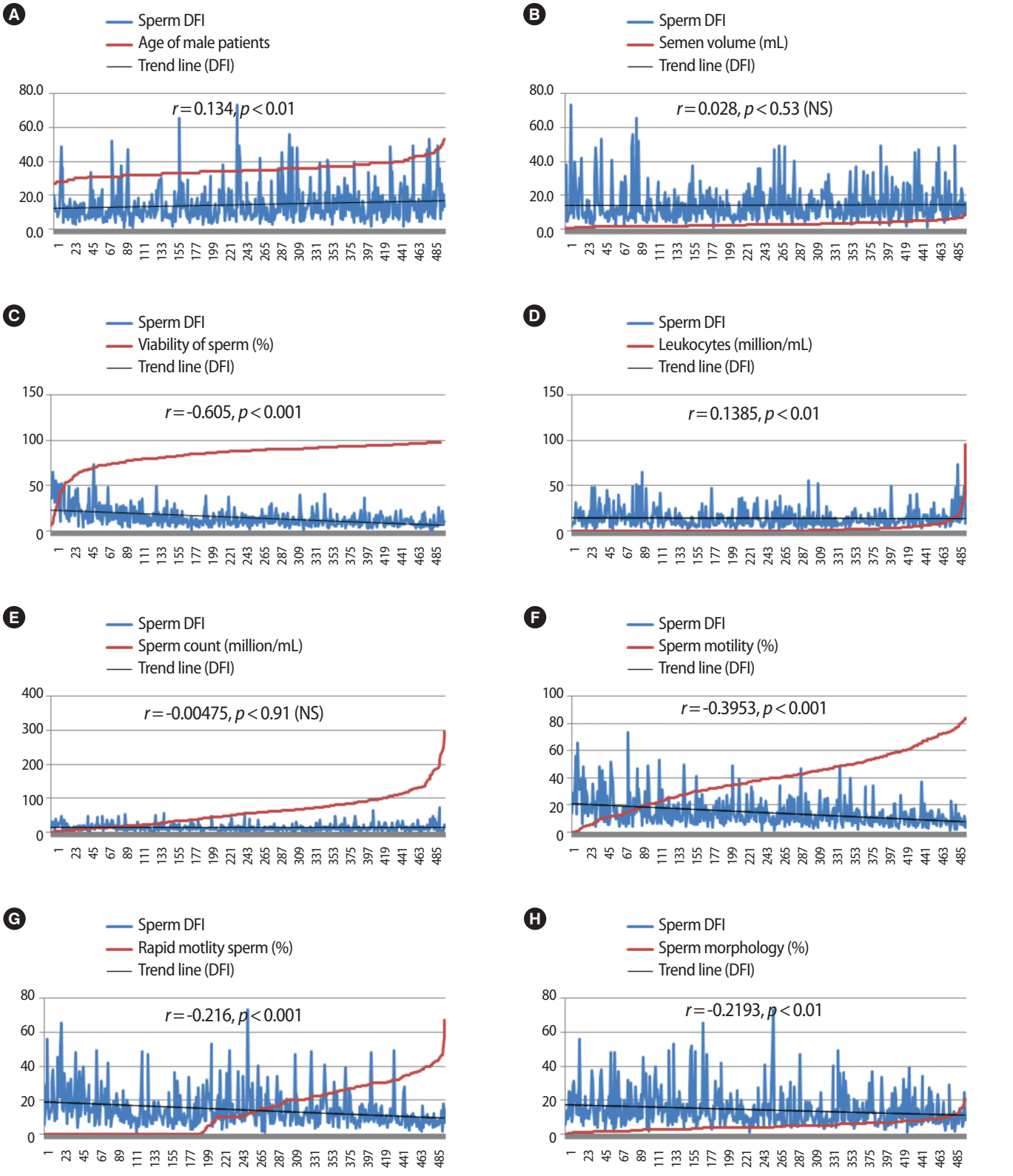

Figure 2. Correlation between semen parameters and sperm DNA fragmentation index (DFI) assessed using neutral comet assay in 498 semen samples. $p < 0.05$ was considered statistically significant, r = coefficient. NS, not significant.

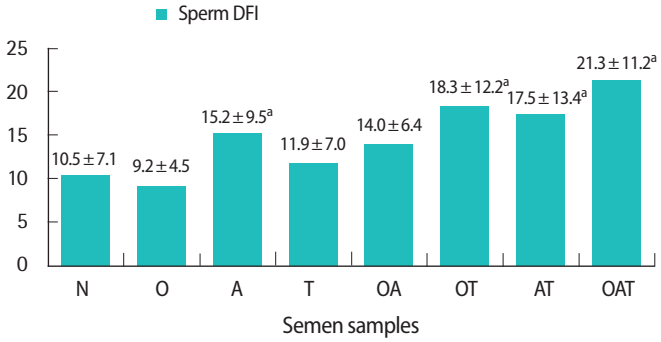

Figure 3. Comparison of sperm DNA fragmentation index (DFI) between subfertile semen samples and normal semen samples. [a]The values (DFI) of subfertile semen samples were significantly higher than the value of normozoospermic samples. Values are presented as mean ± SD. $p < 0.05$ was considered statistically significant. N, normozoospermic; O, oligozoospermic; A, asthenozoospermic; T, teratozoospermic; OA, oligoasthenozoospermic; OT, oligoteratozoospermic; AT, asthenoteratozoospermic; OAT, oligoasthenoteratozoospermic.

Table 1. Characteristics of 498 semen samples divided by the threshold value (14%) of sperm DFI

Semen parameters	Characteristics of semen samples		*p*-value
	Sperm DFI < 14% group	Sperm DFI ≥ 14% group	
Age (yr)	34.7 ± 3.9	36.0 ± 4.6	< 0.001
Count (x10^6/mL)	62.5 ± 44.8	58.0 ± 49.1	0.147
Motility (%)	44.2 ± 18.5	31.1 ± 19.2	< 0.001
Viability (%)	88.6 ± 7.6	76.7 ± 18.7	< 0.001
Morphology (%)	5.26 ± 3.1	4.36 ± 2.6	< 0.001
Leukocyte (x10^6/mL)	1.93 ± 6.2	2.64 ± 6.2	0.010

Values are presented as mean ± SD.
$p < 0.05$ was considered statistically significant.
DFI, DNA fragmentation index.

than the percentage in normozoospermic semen samples (10.5 ± 7.1%, $p < 0.05$).

3. Threshold of sperm DFI

On the basis of the potential correlation of sperm DNA integrity with semen analysis, we used a DFI of 14% as a threshold of the sperm DFI in assessing whether DNA was highly damaged. When the 498 semen samples were divided into two groups (< 14% and ≥ 14% groups) according to the threshold value of DFI (14%), the mean values of all the semen parameters in the < 14% group were significantly higher than those of the ≥ 14% group ($p < 0.05$), with the exception of the sperm count (Table 1).

4. Association between sperm DFI and clinical outcome in IVF-ET cycles

Unlike with semen parameters, the sperm DNA integrity did not demonstrate a close correlation with the clinical outcome in 114 IVF-ET cycles (Table 2). The fertilization rate (61.7%) of the sperm DFI < 14% group was significantly higher than the rate of the ≥ 14% group (55.3%, $p < 0.05$). However, there were no differences in the percentage of good embryos and the pregnancy rate between the < 14% group (32.2% and 35.7%) and the ≥ 14% group (33.6% and 38.6%). The abortion rate in the < 14% group (5.7%) was lower compared to the ≥ 14% group (11.3%), but a statistically significant difference was not observed due to the small number of IVF-ET cycles.

Table 2. Association between the sperm DFI and clinical outcomes in 114 IVF-ET cycles

Characteristics of IVF-ET cycles	Sperm DFI < 14% group	Sperm DFI ≥ 14% group	*p*-value
No. of IVF-ET cycles	70	44	
Age of male	35.4 ± 3.9	38.0 ± 5.8	0.005
Age of female	33.3 ± 3.8	35.5 ± 3.9	0.004
No. of egg retrieved	8.9 ± 5.5	9.2 ± 5.7	0.770
Endometrial thickness (mm)	10.4 ± 1.4	10.1 ± 1.6	0.330
No. of embryos transferred	2.48 ± 0.7	2.50 ± 0.9	0.920
No. of fertilized/oocytes	388/628 (61.7)	226/408 (55.3)	0.040
No. of good embryos	125/388 (32.2)	76/226 (33.6)	0.780
No. of pregnancy cycles	25/70 (35.7)	17/44 (38.6)	0.900
No. of implanted/transferred	28/174 (16.1)	22/110 (20.0)	0.490
No. of abortion cycles	4/70 (5.7)	5/44 (11.3)	0.460

Values are presented as mean ± SD, or number (%).
$p < 0.05$ was considered statistically significant.
IVF-ET, *in vitro* fertilization-embryo transfer; DFI, DNA fragmentation index.

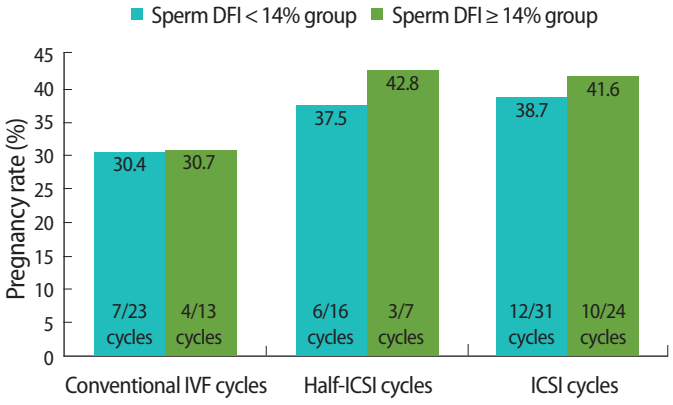

Figure 4. Association between the sperm DFI and conventional IVF and ICSI cycles. There was no significant difference in the pregnancy rates between the two groups. DFI, DNA fragmentation index; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection.

5. Association between sperm DFI and conventional IVF and ICSI cycles

Of the 114 IVF-ET cycles, 36 conventional IVF cycles, 23 half-ICSI cycles and 55 ICSI cycles were performed according to the semen quali-

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ty, oocyte maturity, and previous IVF outcome of the patients (Figure 4). In the sperm DFI < 14% group, the pregnancy rates of the ICSI, the half-ICSI, and the conventional IVF cycles were 40.0%, 35.7%, and 30.4%, respectively, whereas the pregnancy rates of the ≥ 14% group were 40.0%, 44.4%, and 30.7%, respectively. There was no significant difference in the pregnancy rates between the two groups due to the small number of the IVF-ET cycles.

Discussion

Although many methodologies are available to assess DNA fragmentation in spermatozoa, we conclude that the neutral comet assay is a technically simple and cost-efficient method to measure the ds DNA breaks in sperm cells [17]. The neutral comet assay seems to be more sensitive to and reliable for the detection of ds DNA breaks than the alkaline comet assay because the alkaline conditions inherently induce the formation of DNA damage, a phenomenon that is not observed with the neutral comet assay [38].

In the present study, we had to freeze the semen samples for the comet assay because the comet assay was performed only twice a week. The semen samples were frozen using an ultra-rapid freezing method in the absence of a cryopreservative to minimize the cryodamage to the sperm DNA integrity as described previously by Duty et al [15]. They reported that flash-freezing in LN_2 in the absence of a cryoprotectant most closely reproduced the results obtained using fresh semen samples. In our preliminary test, there was also no difference in the sperm DNA integrity between the fresh and the freeze-thawed semen samples assessed using the comet assay. This result is supported by the observation that DNA of the spermatozoa obtained from fertile men was found to be unaffected by the cryopreservation process [21].

In the present study, the sperm DFI was significantly increased in the subfertile semen samples compared to normal semen samples (Figure 3). Shamsi et al. [23] reported a similar statistically significant increase in DNA damage in oligozoospermic (20%), asthenozoospermic (24%), teratozoospermic (28%), and oligoasthenoteratozoospermic (OAT) semen samples (43%) when compared to control samples (8%). In addition, other authors reported that oligozoospermic [28], teratozoospermic [39], leukocytospermic [22], and OAT semen samples [14] showed a significant increase in the amount of DNA damaged sperm.

Various threshold values of sperm DFI have been proposed according to the various protocols and criteria used to determine them. The SCSA method has defined a 27% to 30% DFI as the point at which an individual is placed into the sub-fertile group [40], and the neutral comet assay method has suggested that ds DNA breaks are present in 15% to 25% of native sperm [16]. It is certainly reasonable that the threshold value of the sperm DNA integrity will be adjusted by ongoing studies. In the present study, the mean value of sperm DNA integrity in 498 semen samples was 14.1%, and 14% as a cutoff value of sperm DFI showed a significantly closer relationship between the sperm DFI and various semen parameters compared to any other cutoff values. Thus we decided to use 14% as a threshold of sperm DFI to assess whether DNA was highly damaged. Moreover, a previous study of the sperm chromatin dispersion test used 15% as the threshold of fertile sperm DFI [41], which supports our decision to use a threshold of 14%. The 498 semen samples were divided by the threshold into two groups (a < 14% group and a ≥ 14% group). We observed that age correlates with an increase in the percentage of sperm with highly damaged DNA, as indicated in previous reports [17,19,25]. The mean values of sperm motility, morphology, and viability in the < 14% group were significantly higher than the values in the ≥ 14% group. These observations were similar to the reports that lower levels of semen quality are characterized by higher levels of DNA damage [10,39].

Extensive sperm nuclear DNA fragmentation has been positively correlated with lower fertilization rates [41], poorer embryo development [25], lower pregnancy rates and an increased incidence of spontaneous abortion [31,34]. In contrast, some authors reported that there was no correlation between sperm DNA integrity and the clinical outcomes in ICSI cycles [1,10,36]. In the present study, we divided 114 IVF-ET cycles into the < 14% (70 cycles) and the ≥ 14% group (44 cycles) based on the sperm DFI threshold value. Although the fertilization rate of the < 14% group was significantly higher than the rate of the ≥ 14% group, there were no differences in the other clinical outcomes between the two groups (Table 2). The comparison of the clinical outcomes between the two groups may be an incomplete analysis because the IVF-ET cycles were divided into two groups by the threshold value that was only based on the semen parameters. Moreover, we could not perform a proper statistical comparison due to the small number of IVF-ET cycles. Therefore, many additional IVF-ET cycles are needed to more thoroughly explore the relationship between sperm DNA integrity and the clinical outcome, with a threshold based on the clinical criteria.

In ICSI cycles, the sperm with damaged DNA can be used for fertilization by bypassing the sperm selection mechanism of the oocytes. Nevertheless there was no correlation between the sperm DFI and the ICSI outcomes [1,10,36]. Furthermore, it was suggested that ICSI might help overcome the diminished pregnancy prognosis with high sperm DFI [40]. In the present study, although there was no statistical significance, the pregnancy rates of ICSI cycles were higher than conventional IVF cycles (Figure 4). This result suggests the possibility that ICSI may be better than conventional IVF for maintaining a stable pregnancy rate even in the high sperm DFI group. ICSI may be a bet-

ter method to exclude sperm with high DFI values because most embryologists strive to select only those sperm showing sufficient motility and normal morphology for injection. As mentioned above, these semen parameters demonstrated a close relationship with the sperm DNA integrity. In conclusion, we confirmed that the modified neutral comet assay is a simple, valuable, and reliable method for assessing sperm DNA integrity. Sperm DNA integrity was correlated with semen parameters and 14% was the optimal threshold of the sperm DFI on the basis of these aforementioned characteristics, at least in this study. Therefore, sperm DNA integrity may be a good biomarker to evaluate semen quality and to diagnose male infertility in conjunction with semen analysis. Although we observed a relationship between sperm DNA integrity and the fertilization rate in the IVF-ET cycles, we did not observe a relationship with other clinical outcomes. Further studies on the relationship between sperm DNA integrity and clinical outcome are necessary.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

References

1. Nasr-Esfahani MH, Salehi M, Razavi S, Anjomshoa M, Rozbahani S, Moulavi F, et al. Effect of sperm DNA damage and sperm protamine deficiency on fertilization and embryo development post-ICSI. Reprod Biomed Online 2005;11:198-205.
2. Sakkas D, Mariethoz E, St John JC. Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. Exp Cell Res 1999;251:350-5.
3. Aitken RJ, Krausz C. Oxidative stress, DNA damage and the Y chromosome. Reproduction 2001;122:497-506.
4. Lo KC, Lamb DJ. The testis and male accessory organs. In: Strauss JF, Barbieri RL, editors. Yen and Jaffe's reproductive endocrinology: physiology, endocrinology, and clinical management. 5th ed. Philadelphia, PA: Elsevier Saunders; 2004. p. 367-87.
5. Lewis SE, Agbaje IM. Using the alkaline comet assay in prognostic tests for male infertility and assisted reproductive technology outcomes. Mutagenesis 2008;23:163-70.
6. Cox GF, Bürger J, Lip V, Mau UA, Sperling K, Wu BL, et al. Intracytoplasmic sperm injection may increase the risk of imprinting defects. Am J Hum Genet 2002;71:162-4.
7. Ørstavik KH, Eiklid K, van der Hagen CB, Spetalen S, Kierulf K, Skjeldal O, et al. Another case of imprinting defect in a girl with Angelman syndrome who was conceived by intracytoplasmic semen injection. Am J Hum Genet 2003;72:218-9.
8. Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. Fertil Steril 2004;81:1289-95.
9. Bungum M, Humaidan P, Axmon A, Spano M, Bungum L, Erenpreiss J, et al. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. Hum Reprod 2007;22:174-9.
10. Caglar GS, Köster F, Schöpper B, Asimakopoulos B, Nehls B, Nikolettos N, et al. Semen DNA fragmentation index, evaluated with both TUNEL and Comet assay, and the ICSI outcome. In Vivo 2007;21:1075-80.
11. Benchaib M, Braun V, Lornage J, Hadj S, Salle B, Lejeune H, et al. Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. Hum Reprod 2003;18:1023-8.
12. Young KE, Robbins WA, Xun L, Elashoff D, Rothmann SA, Perreault SD. Evaluation of chromosome breakage and DNA integrity in sperm: an investigation of remote semen collection conditions. J Androl 2003;24:853-61.
13. Agbaje IM, McVicar CM, Schock BC, McClure N, Atkinson AB, Rogers D, et al. Increased concentrations of the oxidative DNA adduct 7,8-dihydro-8-oxo-2-deoxyguanosine in the germ-line of men with type 1 diabetes. Reprod Biomed Online 2008;16:401-9.
14. Nili HA, Mozdarani H, Aleyasin A. Correlation of sperm DNA damage with protamine deficiency in Iranian subfertile men. Reprod Biomed Online 2009;18:479-85.
15. Duty SM, Singh NP, Ryan L, Chen Z, Lewis C, Huang T, et al. Reliability of the comet assay in cryopreserved human sperm. Hum Reprod 2002;17:1274-80.
16. van Kooij RJ, de Boer P, de Vreeden-Elbertse JM, Ganga NA, Singh N, Te Velde ER. The neutral comet assay detects double strand DNA damage in selected and unselected human spermatozoa of normospermic donors. Int J Androl 2004;27:140-6.
17. Singh NP, Muller CH, Berger RE. Effects of age on DNA double-strand breaks and apoptosis in human sperm. Fertil Steril 2003;80:1420-30.
18. Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, et al. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. Fertil Steril 2003;79 Suppl 3:1597-605.
19. Trisini AT, Singh NP, Duty SM, Hauser R. Relationship between human semen parameters and deoxyribonucleic acid damage assessed by the neutral comet assay. Fertil Steril 2004;82:1623-32.
20. Schmid TE, Kamischke A, Bollwein H, Nieschlag E, Brinkworth MH. Genetic damage in oligozoospermic patients detected by

doi: 10.5653/cerm.2011.38.1.10

fluorescence in-situ hybridization, inverse restriction site mutation assay, sperm chromatin structure assay and the Comet assay. Hum Reprod 2003;18:1474-80.

21. Donnelly ET, Steele EK, McClure N, Lewis SE. Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. Hum Reprod 2001;16:1191-9.
22. Fariello RM, Del Giudice PT, Spaine DM, Fraietta R, Bertolla RP, Cedenho AP. Effect of leukocytospermia and processing by discontinuous density gradient on sperm nuclear DNA fragmentation and mitochondrial activity. J Assist Reprod Genet 2009;26:151-7.
23. Shamsi MB, Venkatesh S, Tanwar M, Talwar P, Sharma RK, Dhawan A, et al. DNA integrity and semen quality in men with low seminal antioxidant levels. Mutat Res 2009;665:29-36.
24. Donnelly ET, O'Connell M, McClure N, Lewis SE. Differences in nuclear DNA fragmentation and mitochondrial integrity of semen and prepared human spermatozoa. Hum Reprod 2000;15:1552-61.
25. Morris ID, Ilott S, Dixon L, Brison DR. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. Hum Reprod 2002;17:990-8.
26. Lewis SE, Aitken RJ. DNA damage to spermatozoa has impacts on fertilization and pregnancy. Cell Tissue Res 2005;322:33-41.
27. Aitken RJ, Koopman P, Lewis SE. Seeds of concern. Nature 2004;432:48-52.
28. Tomsu M, Sharma V, Miller D. Embryo quality and IVF treatment outcomes may correlate with different sperm comet assay parameters. Hum Reprod 2002;17:1856-62.
29. Borini A, Tarozzi N, Bizzaro D, Bonu MA, Fava L, Flamigni C, et al. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. Hum Reprod 2006;21:2876-81.
30. Agarwal A, Allamaneni SS. The effect of sperm DNA damage on assisted reproduction outcomes. A review. Minerva Ginecol 2004;56:235-45.
31. Aitken RJ, De Iuliis GN. Value of DNA integrity assays for fertility evaluation. Soc Reprod Fertil Suppl 2007;65:81-92.
32. Carrell DT, Liu L, Peterson CM, Jones KP, Hatasaka HH, Erickson L, et al. Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. Arch Androl 2003;49:49-55.
33. Lewis SE, Agbaje I, Alvarez J. Sperm DNA tests as useful adjuncts to semen analysis. Syst Biol Reprod Med 2008;54:111-25.
34. Meeker JD, Singh NP, Hauser R. Serum concentrations of estradiol and free T4 are inversely correlated with sperm DNA damage in men from an infertility clinic. J Androl 2008;29:379-88.
35. Chan PJ, Corselli JU, Patton WC, Jacobson JD, Chan SR, King A. A simple comet assay for archived sperm correlates DNA fragmentation to reduced hyperactivation and penetration of zona-free hamster oocytes. Fertil Steril 2001;75:186-92.
36. Abu-Hassan D, Koester F, Shoepper B, Schultze-Mosgau A, Asimakopoulos B, Diedrich K, et al. Comet assay of cumulus cells and spermatozoa DNA status, and the relationship to oocyte fertilization and embryo quality following ICSI. Reprod Biomed Online 2006;12:447-52.
37. Singh NP, Stephens RE. X-ray induced DNA double-strand breaks in human sperm. Mutagenesis 1998;13:75-9.
38. Chi HJ, Kim JH, Ryu CS, Lee JY, Park JS, Chung DY, et al. Protective effect of antioxidant supplementation in sperm-preparation medium against oxidative stress in human spermatozoa. Hum Reprod 2008;23:1023-8.
39. Kalthur G, Adiga SK, Upadhya D, Rao S, Kumar P. Effect of cryopreservation on sperm DNA integrity in patients with teratospermia. Fertil Steril 2008;89:1723-7.
40. Evenson DP, Wixon R. Clinical aspects of sperm DNA fragmentation detection and male infertility. Theriogenology 2006;65:979-91.
41. Ahmad L, Jalali S, Shami SA, Akram Z. Sperm preparation: DNA damage by comet assay in normo- and teratozoospermics. Arch Androl 2007;53:325-38.