Seminal Tumor Necrosis Factor-alpha Level and Sperm Nuclear DNA Integrity in Healthy Donors

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정액 내 Tumor Necrosis Factor-alpha 농도와 정자 DNA 손상과의 관련성

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목 적: 정액 내 tumor necrosis factor-alpha (TNF-α) 농도와 정자 DNA 손상 및 정액 검사 소견과의 관련성을 평가하고자 하였다.

연구방법: 정액 표본은 45명의 건강한 남성에서 자위에 의하여 획득하였다. 정자의 상태는 컴퓨터 정액 분석기를 이용하여 판정하였으며, 두부의 DNA 손상은 TUNEL 분석방법에 의해 측정하였다. TNF-α 농도는 동결-융해된 정장액에서 ELISA법으로 측정하였다.

결 과: 정자 DNA 손상율은 1.9%에서 53% (mean ± SD, 12.4±9.6%)로 매우 광범위하게 나타났다. 단변량분석에 의하면 DNA 손상 정도와 정자의 농도, 운동성과는 관련이 없었으나, 직진운동성 (linearity)과는 음의 상관 관계를 나타내었으며 (r=-0.325, p=0.03) 연구 대상 남성의 연령과는 양의 상관 관계를 나타내었다 (r=0.484, p=0.001). 정액 내에 존재하는 TNF-α (>1 pg/mL)는 연구 대상 남성의 73.3% (33/45)에서 검출되었으며 평균 농도는 4.9 pg/mL, 범위는 1.1에서 22.6 pg/mL이었다. 정액 검사 상의 정자 상태와 정자 DNA 손상과는 유의한 관련성이 나타나지 않았다.

결 론: 본 연구에서는 정자 DNA의 손상이 남성의 연령과 관련성이 있음을 확인하였으나, TNF-α와의 관련성은 확인할 수 없었다.
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중심단어: 정자, 핵 통합성 (nuclear integrity), DNA 분절 (fragmentation), TUNEL, 종양 괴사 인자-α (tumor necrosis factor-alpha)

Previous study demonstrated that sperm DNA fragmentation >30~40% was incompatible with fertility, irrespective of sperm concentration, morphology and motility.[1] It was also reported that no pregnancy was achieved after standard IVF and ICSI, when sperm DNA fragmentation was >27% in raw semen.[2] Sperm DNA damage is more common in infertile men; whilst ~13% of sperms have DNA fragmentation in fertile men, ~28% of sperms exhibit DNA fragmentation in infertile men.[3,4]

DNA damage in the male germ line is known to be associated with poor semen quality, low fertilization rates

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or impaired preimplantation development.[5] Sperm DNA fragmentation has been reported to affect the outcome of assisted reproduction in multiple ways, including effects on sperm motility and morphology, fertilization rates, embryo cleavage and pregnancy rates.[6~8] However, the causes of this DNA damage are still uncertain but the major candidates are oxidative stress and aberrant apoptosis.[5]

Cytokines, released by various cells in the male urogenital tract, have a deleterious effect on sperm function and fertility.[9,10] Their production occurs in response to foreign antigens, pathogens, and chronic inflammation.[11] Tumor necrosis factor-alpha (TNF-α), a proinflammatory cytokine, is able to induce apoptosis and act as a pro-apoptotic factor. An in vitro study demonstrated that co-incubation of TNF-α with sperm decreases total motility and produces DNA fragmentation in a concentration- and time-dependent manner.[12,13] Buch et al.[14] also reported that TNF-α could induce lipid peroxidation by reactive oxygen species (ROS) generated within spermatozoa. This might be a potentially important pathologic mechanism of sperm damage by cytokines.

TNF-α is present in the seminal plasma of normal men at the concentration of 62.2±16.4 fmol/mL[15] and its levels in seminal plasma correlate negatively with sperm motility and morphology, but not with total sperm counts in fertile and infertile men.[16,17] Nonetheless, in asymptomatic subfertile males attending infertility investigation, seminal TNF-α was not associated with semen quality but correlated with leukocyte counts.[18] To date, seminal concentration of TNF-α relevant to sperm nuclear DNA integrity has not been studied. The present study aimed to evaluate seminal concentration of TNF-α in correlation with sperm parameters and nuclear DNA integrity in asymptomatic healthy donors.

MATERIAL AND METHOD

We recruited forty-five healthy donors with age ranged from 25 to 50 years. All of the subjects had no presenting signs or a history of genital inflammation. Twenty-four donors were married state and fertility was proven in nine donors. The Institutional Review Board of Seoul National University Bundang Hospital approved this study.

After avoiding coitus for at least three days, semen samples were obtained by masturbation. After liquefaction for 30 min at room temperature, routine sperm quality was immediately assessed by computer-assisted semen analysis (CASA). Using SAIS (Semen Analysis Image System, version 10.1, Medical Supply, Korea), the concentration and motility of sperms, velocity of curved line (VCL), velocity of straight line (VSL), average path velocity (VAP), mean linearity (LIN), amplitude of lateral head displacement (ALH) and straightness (STR, [VSL/VAP]) were assessed. Hyperactivated sperms (HYP) were defined as VCL ≥100 μm/s, LIN <60% and ALH ≥5 μm. The semen analyzer was set as followings; Image acquisition rate 30 frame/sec, tract sampling time 1 sec, minimum sampling for motility 5 frames, minimum sampling for velocity 10 frames, minimum sampling for ALH 12 frames, maximum velocity 250 μm/s, minimum velocity 10 μm/s, cell size range 10~250 pixels. All of the subjects had normal sperm concentration (mean ± SD, $64.8 \pm 27.4 \times 10^6$/mL). Sperm motilities were ranged between 22.7% and 86.3%.

Nuclear DNA integrity was measured by the TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labeling (TUNEL) assay in raw semen. Sperms were separated from seminal plasma by centrifugation (3,000 rpm for 5 min), then smeared on silane-coated slide (DAKO, Glostrup, Denmark) and air-dried. The sperms were fixed with 4% neutral buffered formalin

for 1 hour at 15~25℃, and then washed with PBS. The sperms were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate (Sigma, St. Louis, MO). A commercial apoptosis detection kit was used (In Situ Cell Death Detection Kit, cat. no. 11 684 795 910, Roche Diagnostics GmbH, Germany). The remaining procedures were performed as guidance directed within the kits. Counterstaining was performed using mounting medium with 4',6-diamidino-2-phenylindole (DAPI, cat. no. H-1200, Vector Laboratories, Inc., Burlingame, CA). The sperms with fragmented DNA had their nuclei stained in green, whereas the other cells nuclei were blue. At least 500 sperms were counted per person and the percentage of sperms with fragmented DNA was thus determined.

The separated seminal plasmas were immediately frozen at -20℃. TNF-α concentrations were measured by enzyme-linked immunosorbent assay (ELISA) in thawed seminal plasmas. A commercial test kit (TNF-α-IRMA, Biosource, Nivelles, Belgium) was used according to the manufacturer's instructions. The test kit is a solid-phase ELISA on a microplate coated with a monoclonal antibody specific for TNF-α. Briefly, the samples were diluted 1:2 and a part of samples (500 μL) were used for the analysis. After addition of a second polyclonal horseradish peroxidase labeled antibody, 3,3',5,5'-tetramethyl-benzidine (TMB) was reacted as chromogenic substrate. The optical density of the colored end

Table 1. Basic semen characteristics in forty-five healthy donors according to sperm motility

	Sperm motility <50% (n=17)	Sperm motility ≥50% (n=28)	P
Age of men (years)	33.8±6.0	34.6±6.2	NS
Volume of semen (mL)	2.8±1.1	2.8±1.0	NS
Sperm concentration (×10^6/mL)	40.7±13.6	79.5±22.8	< 0.001
Total motile sperm count (×10^6)	45.0±30.6	153.9±103.3	< 0.001
DNA fragmentation by TUNEL (%)	9.1±3.6	14.5±11.4	0.065
TNF-α level in seminal plasma (pg/mL)	5.0±3.3 (not detectable in 3)	4.8±5.0 (not detectable in 9)	NS
VCL (μm/s)	59.9±5.5	85.5±14.7	< 0.001
VSL (μm/s)	25.9±2.3	31.6±3.3	< 0.001
VAP (μm/s)	32.2±2.9	43.3±6.1	< 0.001
LIN (%)	43.4±2.8	37.4±2.7	< 0.001
ALH (μm)	2.7±0.3	3.9±0.7	< 0.001
HYP (%)	0.2±0.3	1.4±1.5	0.**003**
STR (%)	80.4±3.1	73.6±3.1	< 0.001

Mean ± SD.
P values calculated by Student's t-test (two-tailed).
Total motile sperm count = volume of semen (mL) × sperm concentration (×10^6/mL) × sperm motility (%)
VCL: curvilinear velocity, VSL: velocity of straight line, VAP: average path velocity, LIN: mean linearity, ALH: amplitude of lateral head displacement, HYP: hyperactivated (VCL ≥100 μm/s, LIN <60%, ALH ≥5 μm), STR: straightness.

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product was measured with a microplate reader at 450 nm (Spectramax plus, Molecular Device, Canada). All samples were assessed in duplicate. The range of measurement was 5~5,000 pg/mL and the intra- and inter-assay CVs were <6.0% and <7.0%, respectively.

Statistical analysis was performed using MedCalc (ver. 4.15, MedCalc Software, Mariakerke, Belgium). The Student's t-test was used to compare outcomes between the subgroups. Spearman's correlation test was used to assess an association for different parameters. Results were considered statistically significant when a p-value was <0.05.

RESULTS

Basic semen characteristics of forty-five healthy donors were summarized in Table 1. They were divided into two groups according to the percentage of motile sperm as motility. Men with motility <50% had significantly lower sperm concentrations and total motile sperm counts compared to men with motility ≥50%. Interestingly, DNA fragmentation rate tended to be lower in group with motility <50%. However, TNF-α level in seminal plasma was similar between the two groups.

In this study, sperm DNA fragmentation rates were ranged between 1.9% and 53.0% (mean ± SD, 12.4± 9.6%). Univariate analysis revealed that DNA fragmentation rate was not associated with sperm concentration and motility. However, DNA fragmentation had a negative correlation with linearity (r=-0.325, p=0.03) and a positive correlation with age of donors (r=0.484, p=0.001) (Table 2, Figure 1).

Table 2. Correlation coefficients between sperm quality parameters, DNA fragmentation and TNF-α level in seminal plasma from forty-five healthy donors

	DNA fragmentation of sperm by TUNEL (%)		TNF-α level in seminal plasma (pg/mL)	
	R value	P value	R value	P value
Age of men (years)	0.484	0.001	0.071	0.696
Volume of semen (mL)	0.159	0.297	-0.191	0.287
Sperm concentration ($\times 10^6$/mL)	0.281	0.062	-0.084	0.643
Sperm motility (%)	0.201	0.186	-0.145	0.421
Total motile sperm count ($\times 10^6$)	0.257	0.088	-0.114	0.527
VCL (μm/s)	0.205	0.177	-0.087	0.632
VSL (μm/s)	0.130	0.396	-0.042	0.818
VAP (μm/s)	0.162	0.289	-0.093	0.608
LIN (%)	-0.325	0.030	0.146	0.417
ALH (μm)	0.205	0.176	-0.103	0.568
HYP (%)	0.030	0.848	-0.059	0.744
STR (%)	-0.280	0.062	0.164	0.362
TNF-α level in seminal plasma (pg/mL)	-0.059	0.745	–	–

R values calculated by Spearman's correlation test.

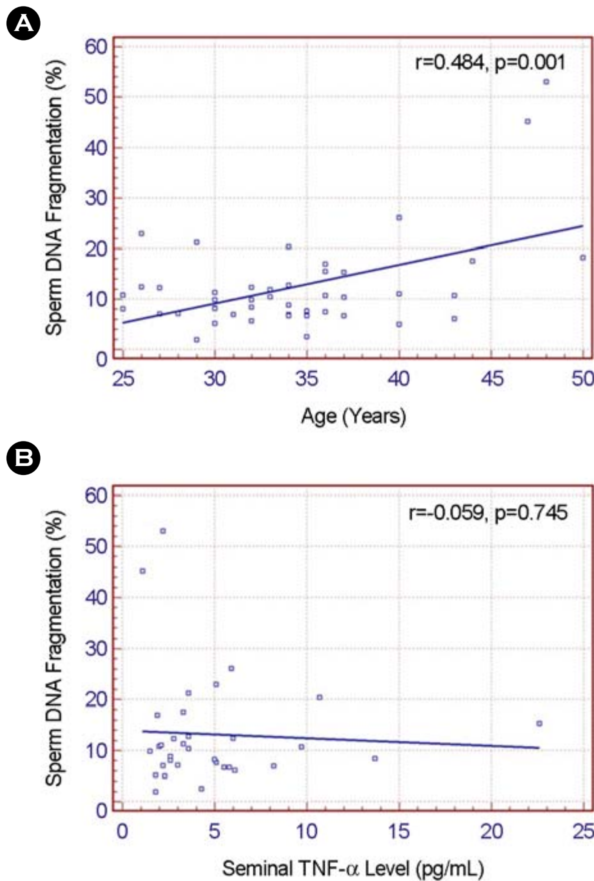

Figure 1. (A) There was a positive correlation between male age and sperm nuclear DNA fragmentation assessed by TUNEL assay **(B)** no correlation was noted between seminal TNF-α levels and sperm nuclear DNA fragmentation.

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Seminal TNF-α was not detectable in 12 subjects (i.e. <1.0 pg/mL). The mean seminal concentration of TNF-α from remaining 33 subjects was 4.9 pg/mL with a range from 1.1 to 22.6 pg/mL. Basic semen characteristics including DNA fragmentation did not differ between detectable and non-detectable TNF-α group as shown in Table 3. The TNF-α concentration had no significant correlation with clinically relevant parameters of sperm quality or nuclear DNA fragmentation rate.

DISCUSSION

The present study demonstrated that the seminal TNF-α concentration has no correlation with clinically relevant parameters of sperm quality or nuclear DNA fragmentation in asymptomatic healthy men. Although it was previously reported that supraphysiologic level of TNF-α could compromise sperm motility and induce nuclear DNA fragmentation in an *in vitro* study,[12,13] our result suggests that sperm nuclear DNA fragmentation may not be related to level of seminal TNF-α in asymptomatic men.

Disorders of the male reproductive system have become an important health issue as they can cause infertility, abortion and abnormal outcomes in the offspring.[19] Poor sperm quality is represented by abnormal sperm parameters, including low sperm concentration, poor sperm motility, and abnormal sperm morphology.[20] In addition to the morphologic and cytokinetic properties, sperm chromatin/DNA integrity might be an essential part for the accurate transmission of paternal genetic information.[21]

There have been several reports that sperm DNA fragmentation might affect the outcome of assisted reproduction. Some investigators have used the sperm DNA fragmentation rate as a marker to differentiate fertile from infertile men[6] and to predict the outcome of intrauterine insemination.[4] Pathologically increased sperm DNA fragmentation might be one of the main paternal-derived causes of repeated assisted reproduction failures in the ICSI.[22] The use of ICSI has heightened the risk that spermatozoa containing damaged DNA may participate in the development of an infant. A significant negative association was reported between the percentage of spermatozoa with DNA fragmentation and the fertilization,[6,23] embryo cleavage rate,[8,24~26] and even pregnancy rate[2,7] in assisted reproduction.

Table 3. Basic semen characteristics in forty-five healthy donors according to TNF-α

	Absence of TNF-α (n=12)	Presence of TNF-α (n=33)	P
Age of men (years)	34.3±5.7	34.2±6.3	NS
Volume of semen (mL)	2.5±0.7	2.9±1.1	NS
Sperm concentration ($\times 10^6$/mL)	59.4±17.0	66.8±30.2	NS
Total motile sperm count ($\times 10^6$)	87.2±66.1	122.0±107.5	NS
DNA fragmentation by TUNEL (%)	10.3±3.8	13.2±10.9	NS
VCL (μm/s)	73.0±1.6	76.9±1.8	NS
VSL (μm/s)	28.7±4.3	29.7±4.0	NS
VAP (μm/s)	38.0±7.2	39.5±7.6	NS
LIN (%)	39.9±2.6	39.6±4.4	NS
ALH (μm)	3.3±0.8	3.5±0.9	NS
HYP (%)	0.8±1.3	1.0±1.3	NS
STR (%)	76.1±3.1	76.2±5.0	NS

Mean ± SD.
P values calculated by Student's t-test (two-tailed).
Total motile sperm count = volume of semen (mL) × sperm concentration ($\times 10^6$/mL) × sperm motility (%)
VCL: curvilinear velocity, VSL: velocity of straight line, VAP: average path velocity, LIN: mean linearity, ALH: amplitude of lateral head displacement, HYP: hyperactivated (VCL ≥100 μm/s, LIN <60%, ALH ≥5 μm), STR: straightness.

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Nonetheless, the causes of sperm DNA damage still remain unclear and appear to be multifactorial.[5] Oxidative stress has been shown to affect the integrity of sperm chromatin.[27] The pathogenic effects of ROS occur when they are produced in excess of the antioxidant capabilities of the male reproductive tract or seminal plasma. Morphologically abnormal spermatozoa might be the main source of excess ROS generation in semen.[28]

Sperm DNA damage can be measured directly (fragmentation, oxidation) or indirectly (sperm chromatin compaction). Direct assessment of DNA damage can be obtained by means of single-cell gel electrophoresis assay or Comet assay, TUNEL assay and liquid chromatography to measure DNA oxidation levels.[3,29] DNA damage can also be assessed indirectly by means of sperm chromatin integrity assays (e.g., sperm chromatin structure assay, SCSA) and by evaluation of nuclear protein levels.[30] Although sperm DNA damage may contribute to poor reproductive performance and the assessment of sperm DNA damage could be a good predictor of possible miscarriages,[21] the Practice Committee of the ASRM[31] declared that current methods for evaluating sperm DNA integrity do not reliably predict treatment outcomes, and no treatment for abnormal DNA integrity has proven clinical value.

We included forty-five healthy donors with normal sperm count in this study. However, 38% of the subjects showed subnormal sperm motility (<50%); this subgroup had relatively low sperm concentration and rather low sperm nuclear DNA fragmentation. This finding suggests that sperm motility may have an inverse correlation with sperm DNA integrity. However, univariate analysis failed

to reveal such a negative correlation, as presented in Table 3. Nevertheless, it is unlikely that subnormal sperm motility always indicate higher sperm DNA damage.

In our study, sperm DNA fragmentation had a positive correlation with male age. This finding is consistent with previous report.[32] Several studies have demonstrated an age-related effect in sperm double-stranded DNA breaks or poor chromatin packaging. Age provides increased opportunities to suffer damage in male reproductive tract from exogenous exposure or disease.[33~35] This age-related effect may happen as older men produce more sperms with DNA fragmentation due to higher exposures of oxidative stress in their reproductive tracts.[36] As reported previously, oxidative stress can damage nuclear membranes as well as sperm DNA.[37] Alternatively, in older men, anti-apoptotic functions during spermatogenesis are probably less effective, resulting in the production of more spermatozoa with fragmented DNA.[38]

Recently, a normal reference value for seminal TNF-α was established in healthy fertile men.[39] In that report, the mean seminal concentration of TNF-α was 1.5 pg/mL (upper range 40.3 pg/mL), which is somewhat lower than in our result. However, the TNF-α was detected in only 20 persons among 59 subjects. The reference value in our study cannot be directly compared with previous result since the assay kit for TNF-α, the characteristics of the study subjects and the detection rate were quite different.

In conclusion, our results indicated that sperm nuclear DNA fragmentation is not associated with seminal TNF-α level or sperm quality in asymptomatic men. The causes of sperm nuclear DNA fragmentation in asymptomatic men are still uncertain but at least, this may not be caused by seminal TNF-α.

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= **Abstract** =

Objectives: Seminal concentration of tumor necrosis factor-alpha (TNF-α) relevant to sperm nuclear DNA integrity has not been studied. The present study aimed to evaluate seminal concentration of TNF-α in correlation with sperm parameters and nuclear DNA integrity in asymptomatic healthy donors.
Methods: Semen samples were obtained by masturbation from forty-five healthy donors.
Results: Sperm quality was assessed by computer-assisted semen analysis and nuclear DNA integrity measured by the TUNEL assay in raw semen. TNF-α concentrations were measured by ELISA in frozen-thawed seminal plasmas. Sperm DNA fragmentation rates were ranged between 1.9% and 53.0% (mean±SD, 12.4±9.6%). Univariate analysis revealed that DNA fragmentation rate was not associated with sperm concentration or motility but had a correlation with linearity negatively ($r=-0.325$, $p=0.03$) and age positively ($r=0.484$, $p=0.001$). The mean seminal concentration of TNF-α was 4.9 pg/mL with a range from 1.1 to 22.6 pg/mL. The TNF-α concentration had no correlation with clinically relevant parameters of sperm quality or nuclear DNA fragmentation rate.
Conclusion: Our results indicate that sperm nuclear DNA fragmentation may be not associated with seminal TNF-α level or sperm quality in asymptomatic healthy donors.

Key Words: Sperm, Nuclear integrity, DNA fragmentation, TUNEL, Tumor necrosis factor-alpha