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Role of DNA Integrity of Spermatozoa in Male Infertility

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Abstract

Background Classical semen analysis gives an approximate evaluation of the functional competence of spermatozoa, but not the quality of sperm DNA. With the advent of assisted reproductive technologies (ART), the concern over using damaged DNA has become apparent.

Objective To clarify the role of DNA integrity and maturity of ejaculated spermatozoa in male infertility.

Methods A randomly selected group of 50 nonazoospermic infertile patients with a history of infertility of at least 1 year duration were included in this study. Whereas control group consisted of semen samples obtained from healthy volunteers of proven fertility (n = 27). Two main assays were studied in ejaculated spermatozoa: The green Acridine Orange (AO) fluorescence test that measures DNA integrity and the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling (TUNEL)- based ApopTag® technology that assesses DNA fragmentation as a late apoptotic marker. Results were compared with the standard sperm characteristics (concentration, motility and morphology) between infertile patients as well as some patients' subgroups against control donors.

Results Significant low levels of green AO fluorescent spermatozoa were observed in all patients and patient's subgroups with asthenoteratozoospermia and oligoasthenoteratozoospermia (P = 0.00001). High percentages of TUNEL-positive spermatozoa were significantly noticed, against control donors, in all patients as well as asthenoteratozoospermic and oligoasthenoteratozoo-spermic infertile patients. Interestingly, normozoospermic patients had a significantly low percentage of green AO fluorescent spermatozoa and high levels of TUNEL-positive spermatozoa versus control donors (P = 0.0005, P = 0.0069, respectively).

Conclusion From this study, it can be concluded that male infertility is associated with high rates of DNA damage in the spermatozoa, and that sperm DNA damage analysis could reveal a buried deformity of sperm nuclear DNA in infertile men classified as idiopathic, having apparently normal standard sperm parameters.

Keywords DNA denaturation, DNA fragmentation, acridine orange, TUNEL.

Introduction

Traditionally, the diagnosis of male infertility is based on microscopic assessment of semen sample, including the concentration, motility, and morphology of the sperm, as well as other parameters, but the results of this conventional semen analysis are insufficient as a diagnostic tool in male infertility [(1)]. New markers are needed that might be better for accurate diagnosis of infertile from fertile men and that may predict pregnancy outcome and the risk of adverse reproductive events, where conventional sperm parameters fall short. There is now some evidence to suggest that markers of sperm DNA integrity may be better measures of male fertility potential than conventional measures [(2)]. Intact human sperm DNA is an essential prerequisite for successful fertilization and embryo development.

Immature spermatozoa from infertile men have been shown to contain various nuclear alterations, including abnormal chromatin structure, microdeletions, chromosomal rearrangements, aneuploidy and DNA strand breaks [3].

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 [1]. Potential mechanisms for generating DNA damage in sperm have been proposed, and include incomplete chromatin packaging [4], abortive apoptosis [5], and oxidative stress by reactive oxygen species (ROS) [6]. Unlike other cells, spermatozoa are more vulnerable to DNA damage because they do not have the capacity for DNA repair [1]. Sperm DNA damage can be measured directly by assessment of oxidation and fragmentation using Terminal deoxynucleotidyl Transferase dUTP nick end labeling "TUNEL" assay, liquid chromatography to measure DNA oxidation levels, and by using single-cell gel electrophoresis assay or "Comet" assay [2]. DNA damage can also be assessed indirectly by means of sperm chromatin integrity assays and by evaluation of nuclear protein levels using DNA stains for detection of denatured or single-stranded DNA (e.g., acridine orange) as well as nuclear protein stains (e.g., aniline or toludine blue) for detection of histones [2].

The study of sperm DNA damage is particularly relevant in an era where advanced forms of assisted reproductive technologies are frequently used. The objectives of this study are to evaluate sperm DNA integrity in semen samples from infertile men compared to control donors; by calculating the percentage of mature spermatozoa that enclose native double-stranded and normal DNA using the acridine orange (AO) fluorescence test. In another approach, DNA fragmentation and presence of single- and double-stranded DNA breaks were measured by the TUNEL-based In Situ Apoptosis Detection Kit. Then, the relationship of the above studied markers and their correlation, if any, with conventional sperm parameters (sperm concentration, motility, and morphology) were inspected.

Methods

This study included a randomly selected group of nonazoospermic Iraqi infertile patients (n = 50) attending the Higher Institute of Infertility Treatment and Assisted Reproductive Technology/ Al-Nahrain University/ Baghdad, with a history of infertility of at least 1 year duration between June and December 2008. Their age ranged from 20–50 years with a mean of 32.58 ± 6.3 years and they all had a normal physical evaluation. Their female partners had no history of untreated female–factor infertility. Written consent for use of the spermatozoa for research was obtained from the patients according to guidelines established by the local research ethics committee of Al-Nahrain College of Medicine. Freshly ejaculated semen samples were obtained by masturbation into a wide-mouthed sterile specimen container after 3–5 days of sexual abstinence. Controls consisted of samples obtained from healthy donors of proven fertility (i.e., fathered a child within the last 12 months) (n = 27). Their age ranged from 22–46 years with a mean of (32.81 ± 6.47 years). Normal control donors had an ejaculate volume of at least 2 mL and a sperm concentration of at least 20 × 10^6/ mL, of which at least 50% were motile and 30% had normal sperm morphology according to the World Health Organization classification [7]. Infertile men with azoospermia were excluded from the study.

Ejaculates were allowed to liquefy at 37^{o}C for 30 minutes before analysis. Semen profile was assessed by light microscopy according to the procedure proposed by the WHO. Following liquefaction, semen specimens were evaluated for sperm motility, concentration and morphology and scored according to WHO guidelines [7].

The AO Fluorescence of Human Spermatozoa:
Acridine Orange staining was performed according to the method of Tejada *et al* [8]. Slides were read by two independent observers blindly counting on the same day of staining, who gave results that generally agreed within 10% discrepancy. The nuclei of at least 300 spermatozoa from each individual were examined and scored as fluorescing green, red or yellow. Spermatozoa displaying green fluorescence were recorded as mature, whereas sperm heads displaying red, orange or yellow fluorescence were considered as immature; as well as those displaying green and red color simultaneously [8]. The threshold of green AO fluorescence as 50% was adopted from Hoshi *et al*. (1996) and the values <50% were considered as positive in the test [9,10].

Spermatozoa TUNEL assay:
DNA fragmentation in sperm nuclei was investigated using the TUNEL assay, which is the basis of the ApopTag® technology (ApopTag® Peroxidase In Situ Apoptosis Detection Kit, CHEMICON® International, Inc., USA & Canada). The reagents provided in the Kit are designed to label the free (3'-OH) DNA termini in situ with chemically labeled and unlabeled nucleotides. Staining was detected by brightfield microscopy (OLYMPUS® model BX41TF, Olympus Optical Co., LTD., Tokyo, Japan). At least 200 spermatozoa were counted for each sample and the percentage of spermatozoa with fragmented DNA (TUNEL-positive) was determined. Photographic records were obtained using (OLYMPUS®, Camedia C- 60 zoom) digital compact camera.

Statistical analysis:
The results were expressed as (mean ± SD). Results of the standard semen characteristics in addition to the results of the main tests employed (AO and TUNEL assays) were compared between infertile patients and fertile controls; as well as between some patients' subgroups and control donors; using unpaired Student's t- test for two samples of unequal variance. The different types of relationships and correlations accomplished in this work (between results of main tests and the basic sperm parameters) were examined using bivariate Pearson's correlation coefficient (two-tailed) test. All hypothesis testing was two-sided with a probability value of < 0.05 deemed as significant. Analyses were conducted with Microsoft Excel/ Microsoft Office XP 1985-2001 and Statistica/ version 6.0 (USA) statistical package.

Results

According to the WHO criteria of semen variables, subjects included in this study exhibited isolated asthenozoospermia (5 subjects); oligoasthenozoospermia (4 subjects); asthenoteratozoospermia (12 subjects); oligoasthenoteratozoospermia (18 subjects); normozoospermia (10 subjects) and finally; oligozoospermia (1 subject).

Conventional Semen Analysis:
Table (1) shows the results of the classical semen analysis parameters performed by light microscopy, according to the WHO criteria, for the 50 infertile men and 27 control volunteers. Compared to controls, infertile patients had significantly lower sperm concentration (25.74 ± 25.3 vs. 129.76 ± 96.18; $P = 0.00001$), reduced progressive motility (31.1 ± 20.4 vs. 62.59 ± 12.74; $P = 0.00001$) and poorer sperm morphology (32.1 ± 12.37 vs. 45.30 ± 5.62; $P = 0.00001$) (Table 1).

AO Fluorescent Test:
As compared to healthy donors, the percentage of green AO fluorescent sperms (enclosing native normal double-stranded DNA) was significantly lower in all infertile patients (39.52 ± 16.09 vs. 68.15 ± 8.52; $P = 0.00001$) (Table 1).

Figure 1 shows representative patterns of AO staining of spermatozoa. Normal sperm heads revealed a visible green color (Figure 1A), which can be differentiated from the red color of some immature sperm cells. Other sperm heads had colors that range from orange to yellow, or mixture of red and green (Figure 1B).

Table 1. Comparison of conventional semen characteristics and the results of AO, and TUNEL assays between infertile patients and healthy fertile volunteers

Spermatozoa	Patients (n = 50)	Control donors (n = 27)
Concentration **(M/ml)** ***Progressive motility (%)***	25.74 ± 25.3*	129.76 ± 96.18
(grades a and b)	31.1 ± 20.4*	62.59 ± 12.74
Normal morphology *** (%)**	32.1 ± 12.37*	45.30 ± 5.62
Green AO fluorescence **(%)**	39.52 ± 16.09*	68.15 ± 8.52
TUNEL positive cells (DNA fragmentation rate) **(%)**	44.56 ± 9.41*	8.37 24.47

* = <0.00001, According to WHO criteria1999.

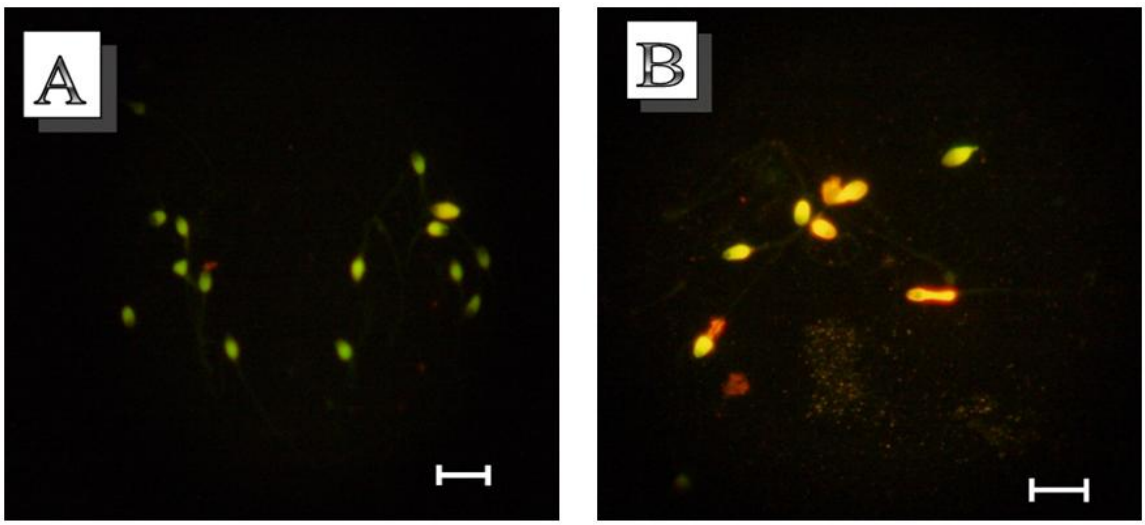

Figure 1. *A*: Representative patterns of sperm cells stained with the Acridine Orange fluorescence stain, demonstrates normal mature spermatozoa with native double-stranded DNA from fertile control donors. *B*: The green tinge which is quite discernible from the yellow-orange tinge of immature spermatozoa from infertile patients. Scale bar = 10 µ

Concerning the three major patient's subgroups, significant low levels of green AO fluorescent sperms were seen in asthenoteratozoospermic and oligoasthenoteratozoospermic patients with more lower percentages in the latter group (51.85± 22.50 and 41.49± 24.43; *P* = 0.0309 and 0.0003; respectively, vs. control donors) (Table 2). Interestingly, normozoospermic patients had also a significantly low percentage of green AO fluorescent sperms (52.08 ± 9.97; *P* = 0.0005) compared to control donors (Table 2).

TUNEL Assay: According to table 1, the percentage of TUNEL-positive cells with fragmented DNA was significantly more in infertile patients compared to controls (44.56 ± 9.41 vs. 24.47 ± 8.37; *P* = 0.00001). Characteristic patterns of TUNEL staining of spermatozoa is shown in figure 2, with apoptotic spermatozoa stained with brown color (Figure 2A), while faint violet was the stain of normal non apoptotic cells (Figure 2B).

As for the three major patient's subgroups, high percentages of TUNEL-positive spermatozoa was noticed against control donors in asthenoteratozoospermic, in addition to oligoasthenoteratozoo-spermic infertile patients (41.77±6.90; *P* = 0.00001; and 41.21±

14.84; *P* = 0.0002; respectively). Again for the normozoospermic infertile patients (idiopathic/ unexplained infertility); significant high levels of TUNEL-positive spermatozoa were observed vs. control donors (32.72 ± 6.97; *P* = 0.0069) (Table 2).

Table 2. Comparison of the results of the standard semen parameters, AO, and TUNEL assays between three major subgroups of infertile patients and healthy fertile volunteers

Standard Semen Parameters	Normozoo-spermia (n = 10)	Asthenoteratozoo-spermia (n = 12)	Oligoasthenoteratoz-oospermia (n = 18)
***Concentrat.* (M/ml)**	94.17 ± 46.70^{ns}	49.22 ± 11. 02†	38.89± 25.09†
Progressive motility* (%) *grades (a & b)	64.00 ± 10.75^{ns}	31.67± 25.61†	37.22± 30.93†
***Normal morphology* * (%)**	46.14 ± 11.07^{ns}	20.95± 7.76‡	31.61± 14.99†
Green AO fluorescent (%)	52.08 ± 9.97†	51.85± 22.50*	41.49± 24.43†
TUNEL positive cells (%)	32.72 ± 6.97**	41.77±6.90‡	41.21± 14.84†

* = p < 0.5, ** = P < 0.1, † = P < 0.0005, ‡ = P < 0.00001, * According to WHO criteria 1999, Probability against control donors.

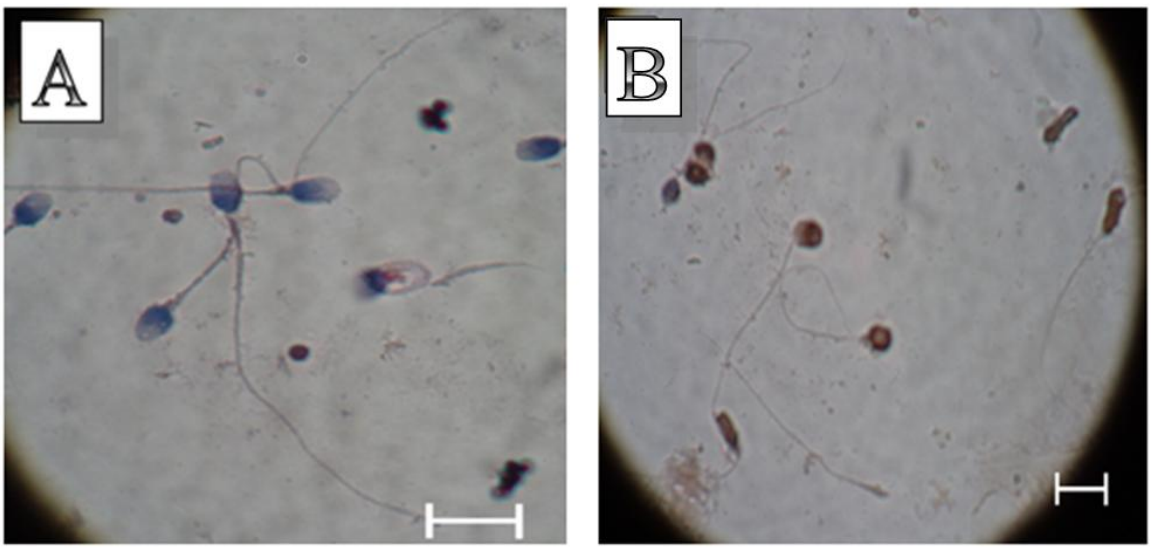

Figure 2. *A*: non apoptotic sperm cells processed using TUNEL assay with non fragmented DNA from fertile controls. Sperm heads are stained with faint violet of the counter stain Hematoxylin. *B*: TUNEL-positive apoptotic sperm cells with fragmented DNA from infertile patients stained with brown color in the head region. Scale bar = 10 µ

Correlations between Standard Semen Characteristics and the Results of AO and TUNEL Assays:

In all infertile patients, there was no significant correlation between standard semen characteristics and the results of AO and TUNEL assays. Nevertheless, there was a significant inverse correlation between the percentage of spermatozoa with normal native double-stranded DNA (green AO fluorescence) and DNA fragmentation (TUNEL-positive) (r = – 0.567, *P* = 0.0001).

Discussion

Sperm DNA contributes one half of the genomic material to the offspring and the integrity of sperm DNA is of crucial importance

for balanced transmission of genetic information to future generations [11].

The results of this study illustrate that infertile patients and their main subgroups had significantly lower percentage of mature spermatozoa that enclose normal native double-stranded DNA i.e. higher percentage of immature spermatozoa with denatured DNA, as compared to healthy donors (Tables 1 and 2). These findings are consistent with those of several other workers who had found that infertile men had a significantly higher mean percentage of sperm with DNA denaturation than fertile men [13,12]. Sperm DNA integrity can be assayed indirectly, based on the principle that damaged DNA denatures much faster than undamaged DNA when subjected to stresses such as heat and pH changes [11]. It has been reported that cells showing a higher DNA fluorochrome stainability compared to normal spermatozoa represent sperm cells which failed to tightly condense the chromatin during spermiogenesis; cells in which histones have not been replaced by protamines during spermiogenesis; or cells which contain endogenous DNA nicks, explaining associated chromatin packaging anomalies with infertility [14-16]. The human sperm DNA integrity as assessed microscopically by AO stain, although still controversial, has been widely used for evaluation of male infertility and pregnancy outcome in ART [11].

In this study, spermatozoa DNA quality was further examined using the TUNEL assay that measures percentage of spermatozoa with DNA fragmentation. Infertile patients, whether originally or after dividing them into major subgroups, had significantly more percentage of spermatozoa with fragmented DNA compared to controls (Tables 1 and 2). These data are coherent with those of other investigators [17,18]. Presence of DNA fragmentation as a late apoptotic marker in spermatozoa from infertile patients may indicate an apoptotic mechanism that might be interrupted 'aborted' at some stage of spermatogenesis causing the seminiferous tubule release of sperms with apoptotic markers, which is coherent with a previous study [5].

Data presented in this study on the interrelations between the studied parameters, shows a significant inverse correlation between the results of TUNEL assay and DNA integrity in AO fluorescence assay ($r = -0.5667$, $P = 0.0001$), compliant with previous studies [15]. Thus, a cause– effect relationship exists between apoptosis and DNA damage (assessed indirectly by AO test). However, no significant relationship was demonstrated in this work between DNA integrity and basic sperm parameters (data not shown), probably because of the small population of the studied groups. Nevertheless, reports on correlation of nuclear integrity with semen parameters have been somewhat inconsistent [11]. In different study populations, using different assays to measure DNA damage, some investigators found associations between some semen parameters and sperm DNA integrity whereas several others did not find associations [19]. Other researchers did not observe a close relationship between sperm DNA integrity and sperm morphology, fertilization rate, embryo development or pregnancy outcome for ICSI [1].

Interestingly, normozoospermic infertile patients (idiopathic) showed a significantly lower percentages of green AO fluorescent sperm cells (enclosing native normal double-stranded DNA), i.e a significantly higher levels of denatured DNA ($P = 0.0005$) compared to controls. Moreover, patients with normozoospermia demonstrate significantly higher percentage of spermatozoa with fragmented DNA (TUNEL-positive) as compared to control donors (Table 2). In at least 30% of cases classified as unexplained or idiopathic, repeated standard semen analyses of the male partner of an infertile couple reveal normal results [20]. Men with normal spermograms may still be infertile; the cause could be related to abnormal sperm DNA [19]. Varghese *et al*., found that approximately 30% of the patients with normal sperm parameters had DNA

normality varying from 1% to 70%. They stated that these high variations in DNA normality among normozoospermic patients may account for the incidence of unexplained infertility [11]. Therefore, it is possible that sperm DNA analysis may be better at discriminating between infertile and fertile men than standard analysis. This is in agreement with other studies which have suggested that sperm DNA damage analysis is an independent test of sperm quality that may have better diagnostic and prognostic capabilities than standard sperm parameters [21,22].

From the present study, it can be concluded that male infertility is associated with high rates of DNA damage in the spermatozoa, and that sperm DNA damage analysis may reveal a hidden abnormality of sperm nuclear DNA in infertile men classified as idiopathic, based on apparently normal standard sperm parameters. These findings are of great concern, particularly, in an era where advanced forms of assisted reproductive technologies are frequently used, bypassing the natural barriers to fertilization, with the inevitable risk that the use of DNA-damaged spermatozoa in ART will compromise the health of the progeny.

References

1. Chi HJ, Chung DY, Choi SY, et al. 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. *Clin Exp Reprod Med* 2011; 38(1): 10-17.
2. Kadri A, Bakry S, Mansour A, et al. ICSI Outcome after Assessment of Sperm DNA Integrity for Diagnosis of Fertility Potential. *Australian J Basic Appl Sci* 2010; 4(5): 835-843.
3. Gerardo B, Morshedi M, Sergio O. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Mol Reprod* 2000; 15: 1338– 1344.
4. Nasr-Esfahani MH, Salehi M, Razavi S, 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.
5. 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.
6. Aitken RJ, Krausz C. Oxidative stress, DNA damage and the Y chromosome. *Reproduction* 2001; 122: 497-506.
7. World Health Organization laboratory manual for examination of human semen and sperm cervical mucus interaction. 4th ed. Cambridge, UK: Cambridge University Press, 1999.
8. Tejada RI, Mitchell JC, Norman A, et al. A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertil Steril* 1984; 42: 87-91.
9. Shibahara H, Onagawa T, Jorsaraei AS, et al. Clinical significance of the Acridine Orange test performed as a routine examination: comparison with the CASA estimates and strict criteria. *Int J Androl* 2003; 26: 236-241.
10. Katayose H, Yanagida K, Hashimoto S, et al. Use of diamide–acridine orange fluorescence staining to detect aberrant protamination of human-ejaculated sperm nuclei. *Fertil Steril* 2003; 79: 670-676.
11. Varghese AC, Bragais FM, Mukhopadhyay D, et al. Human sperm DNA integrity in normal and abnormal semen samples and its correlation with sperm characteristics. *Andrologia* 2009, 41: 207–215.
12. Zini A, Fischer MA, Sharir S, et al. Prevalence of abnormal sperm DNA denaturation in fertile and infertile men. *Urology* 2002; 60: 1069-1072.
13. Fischer MA, Willis J, Zini A. Human sperm DNA integrity: correlation with sperm cytoplasmic droplets. *Urology* 2003; 61: 207-211.
14. Manicardi GC, Bianchi PG, Pantano S, et al. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and it relationship to chromomycin A3 accessibility. *Biol Reprod* 1995; 52: 864-867.
15. Sailer BL, Jost LK, Evenson DP. Mammalian sperm DNA susceptibility to in-situ denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. *J Androl* 1995; 16: 80-87.
16. Troiano L, Granata AR, Cossarizza A, et al. Mitochondrial membrane potential and DNA stainability in human sperm cells: a flow cytometry analysis with implications for male infertility. *Exp Cell Res* 1998; 241: 384-393.
17. Zini A, Bielcki R, Phang D, et al. Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. *Fertil Steril* 2001a; 75: 674- 677.
18. Marchetti C, Obert G, Deffosez A, et al. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. *Hum Reprod* 2002; 17: 1257-1265.

19. Sheikh, N, Amiri I, Farimani M, et al. Correlation between sperm parameters and sperm DNA fragmentation in fertile and infertile men. *Iranian J Rep Med* 2008; 6(1): 13-18.

20. Lukanov T, Lichev D, Konova E, et al. Investigation of Sperm DNA Fragmentation by Sperm DNA Integrity Assay. J IMAB - Annual Proceeding, book 2008; 1: 14-17.

21. Zini A, Kamal K, Phang D, et al. Biologic variability of sperm DNA denaturation in infertile men. *Urology* 2001b; 58: 258-261.

22. Saleh RA, Agarwal A. Oxidative stress and male infertility: from research bench to clinical practice. *J Androl* 2002; 23: 737-752.

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