Evaluation of the Viability of Sperms with DNA Fragmentation in Infertile Men

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ABSTRACT:

BACKGROUND:

The sperm DNA integrity is a vital factor to transmit the genetic material and complete the process of the formation of new generation. Many studies tried to explain the male infertility of unknown cause as a result of the presence of high percentage of sperms with DNA damage and found its correlation with the standard seminal analysis parameters.

OBJECTIVE:

This study was conducted to find the correlation between percentage of sperm death and percentage of sperms with DNA fragmentation and to calculate the percentage of live sperms with DNA damage.

METHODS:

Thirty four infertile patients were included in this study. And twenty fertile patients were volunteered as controls. Standard clinical semen analysis was performed according to World Health Organization (1999) criteria. The stain eosin–Y was used to assess sperm viability. The Terminal deoxynucleotidyl transferase-mediated dUTP- nick end labeling assay (TUNEL) was performed to observe the sperms with DNA fragmentation.

RESULT:

The percentage of sperms with DNA damage in the seminal sample was significantly positively correlated with the percentage of dead sperms (r=0.888; *p*=0.0001) and the percentage of sperms with abnormal morphology (r=0.707; *p*=0.0001). The percentage of live sperms with DNA damage was about 7% in infertile samples and about 11% in healthy samples.

CONCLUSION:

The small percentage of live sperms with DNA fragmentation in ejaculate could not explain idiopathic male infertility. This study concluded also that sperm DNA fragmentation could be considered as an independent parameter of sperm quality to differentiate infertile from fertile semen samples.

KEYWORDS: sperm dna fragmentation, tunel assay, sperm vitality.

INTRODUCTION:

Conventional semen analyses provide information on the clinical status of male fertility [(1)]. The results of standard seminal analysis are only moderately predictive of an individual's fertility [(2)]. Since male factor infertility is solely responsible in about 20% of infertile couples and contributory in another 30%-40% [(3)], it is important to develop new methodology and assays to improve the clinical diagnosis of male infertility and sperm function.

Sperm DNA damage and sperm apoptosis have been considered as potentially useful indices of male fertility. Cellular apoptosis is a normal event that occurs both during and after embryonic development. Germ cell loss, now recognized as occurring via apoptosis, is a dominant process during spermatogenesis and is regulated by caspaces, and Fas expression levels with many alternate pathways [(4)]. The cause of human sperm DNA fragmentation and its impact on fertilization and pregnancy remain unclear. Gorczyca et al. (1993) [(5)] proposed that the presence of endogenous nicks in ejaculated human sperm is characteristic of programmed cell death, as seen in apoptosis of somatic cells. In addition, oxidative stress has been shown to affect the integrity of sperm chromatin and to cause high frequencies of DNA single and double-strand breaks. Exposing spermatozoa to artificially produced reactive oxygen species (ROS) significantly increases DNA damage [(6)]. A third possibility is based on the fact that histone

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replacement and chromatin rearrangement are related to the presence of endogenous DNA nicks in elongating spermatids [7]; ejaculated sperm with DNA fragmentation might result from failure of spermatozoa to mature normally.

The degree of sperm DNA fragmentation reflects the integrity of the genetic material of the gamete; this parameter is important since many types of DNA lesions induce mutations commonly observed in mutated oncogenes and tumor suppressor genes [8]. Transmission of damaged DNA to the offspring, particularly at levels that exceed the DNA repair capacity of the oocyte, could have serious consequences [9]. The most commonly used techniques to assess sperm DNA integrity are the TUNEL, Comet, and sperm chromatin structure assays.

It has been generally accepted that apoptosis and necrosis are two distinct modes of cell death. Apoptosis, or programmed cell death, is an active and physiological mode of cell death. Early changes during apoptosis are loss of intracellular water and increase in the concentration of ionized calcium in the cytoplasm [10]. Chromatin condensation, followed by nuclear disintegration and formation of apoptotic bodies represent other typical features of apoptosis. The integrity of the plasma membrane is preserved to the late stages of apoptosis.

Necrosis, in contrast, is a passive, catabolic and degenerative process. It generally represented cells response to gross injury and could be induced by an overdose of a cytotoxic agent. The early events of necrosis include swelling of mitochondria as well as swelling of the whole cell, combined with marginal chromatin condensation, followed by rupture of the plasma membrane and release of cytoplasmic constituents [11]. DNA degradation is not so extensive during necrosis as in the case of apoptosis.

Sperm death could be due to primary necrosis that resulted from infection with subsequent inflammation either during passage and storage in the epididymis or originating from the testis [12].

The presence of apoptosis has been reported in human spermatozoa [5]. The characteristics of apoptotic spermatozoa are consistent regardless of the pathology. However, the prevalence of apoptosis in spermatozoa varies in various pathologies: it is seen at a rate of 0.1% in fertile controls up to 10% in varicocele, infection (including AIDS) and globozoospermia patients; 20% in cryptorchid men; 25% in unexplained infertility, and 50% in testicular seminoma carriers [13]. Although the apoptotic spermatozoa can fertilize oocytes at the same rate as intact spermatozoa, the development of the resultant embryos to blastocyst and to term is very much related to the integrity of the DNA [14]. However, the oocyte has the ability to repair the DNA damage as oocytes fertilized by DNA-damaged spermatozoa did not develop further in vitro when they were cultured in the presence of inhibitors to DNA repair [9]. Oocyte DNA repair capacity is limited and is related to the degree of sperm DNA damage. Damage beyond the capacity to repair will result in fragmentation, which finally leads to degeneration of the embryo. This may be one of the causes of fragmentation in human embryos seen in clinical IVF programmes [6].

The aim of this study was to find correlation between sperm DNA fragmentation and percentage of dead sperms in seminal samples and also to find explanations of male infertility on bases of findings of sperm DNA damage percentage.

PATIENTS AND METHODS:

Thirty four infertile patients were included in this study. And twenty fertile patients were volunteered as controls. Semen samples were collected by masturbation, after a period of at least 72 hours of sexual abstinence, into sterile polypropylene containers at the seminal fluid analysis department in Kamal Al-Sameree' hospital for gynecology and infertility treatment, Baghdad. Samples were taken in a period of about 4 months (from June 2006 to November 2006). After a full history and general physical examination (performed by a specialist) including imaging and endocrine investigations (for both male and female partners). Infertile patients were diagnosed as infertility due to male factors or unknown cause. Standard clinical semen analysis was performed according to World Health Organization (1999) criteria. All the semen samples used for analysis had to contain motile sperm, sperm count above 5 million /ml, and with non significant leukocytospermia, as per World Health Organization (1999) guidelines and most of the subjects included in this study were non smokers (data not mentioned). To evaluate sperm viability, sperm samples were stained with the vital stain eosin–Y (C.I.45380) and incubated for 30 s. The specimens were then smeared on a glass slide,

air dried and observed with a 100x oil-immersion objective. For each smear, at least 200 spermatozoa were examined and the percentage of unstained spermatozoa was recorded as the percentage of viable spermatozoa. The TUNEL assay was performed using the In-Situ Cell Death Detection Kit following the manufacturer's guidelines (BioAssay™, Catalog No. T9162-80, United States Biological, Swampscott, Massachusetts, USA). Slides were observed under fluorescent microscope with a 100x oil-immersion objective. A total of 200 sperm per individual were evaluated. The intact sperm DNA stained with DAPI will appear blue while the cells with green fluorescence are the "TUNEL positive" {Figure (1)}. The percentage of the damaged DNA should be calculated from the total sample count.

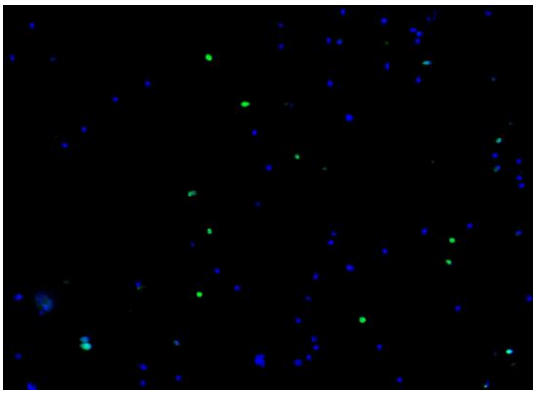

Figure 1: TUNEL assay. DNA fragmentation in human sperm assessed by TUNEL assay under fluorescent microscopy. The sperm with intact DNA stained with DAPI (blue spots) while the green fluorescence are the TUNEL positive sperms.

RESULTS:

The results of this study show that there was a significant difference in the results of standard seminal analysis including the count, abnormal morphology percentage, quality of sperm motility, and percentage of dead sperms between infertile and healthy samples. Also there were high percentages of TUNEL positive sperms in infertile samples than the healthy as shown in table 1.

Non significant correlations were obtained between the percentage of TUNEL positive sperms and the sperm count and the progressive motility (P>0.05) (figures 2 and 3). While significant positive correlations were found between sperm DNA damage and percentage of sperms with abnormal morphology and percentage of dead sperms in the seminal sample (figure 4 and 5).

The percentage of live sperms with DNA damage calculated by subtracting sperms DNA damage percentage from the percentage of dead sperms in seminal sample. This percentage in this study was about 7% in infertile samples and about 11% in healthy samples.

Table 1: Results of standard seminal analysis of infertile patients and healthy controls. Values expressed as mean± standard deviation. A probability value ≤0.05 deemed as significant.

	Infertile Patients	Fertile Controls	*p* Value
Sperm Count (10^6 sperms/ml)	32.61±21.13	57.5 ±19.89	0.0001
Abnormal Morphology (%)	46.47±20.76	22.25±10.32	0.0001
Progressive Motility (%)	37.5±16.43	59.25±16.4	0.0001
TUNEL Positive Sperms (%)	41.61±16.6	24.0±5.8	0.0001
Dead Sperms (%)	34.52±16.96	13.05±3.84	0.0001

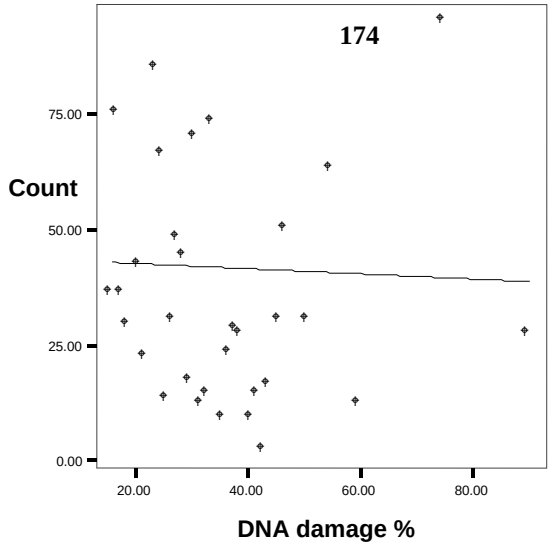

Figure 2. Correlation between sperms count and sperm DNA damage. A non significant correlation (r=-0.038; p=0.788).

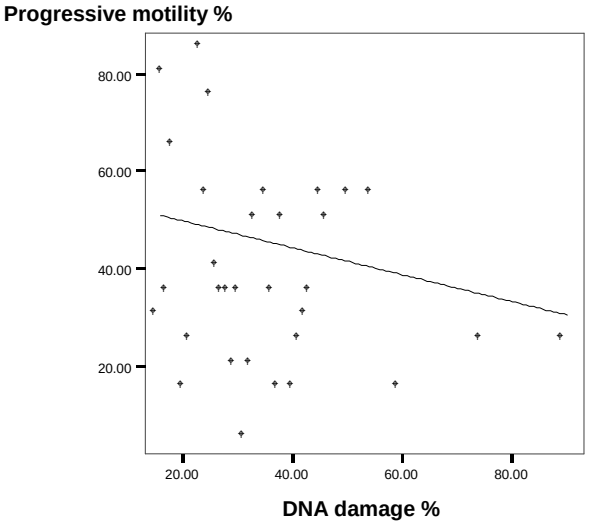

Figure 3: Correlation between sperms progressive motility and sperm DNA damage. A non significant correlation (r=-0.228; p=0.097).

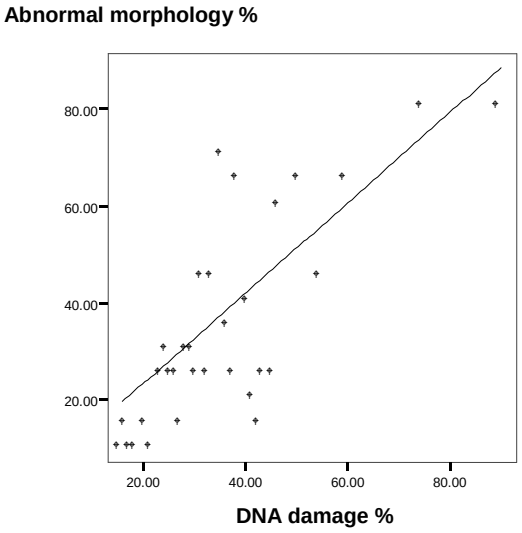

Figure 4; Correlation between sperm abnormal morphology and sperm DNA damage. A significant positive correlation (r=0.707; p=0.0001).

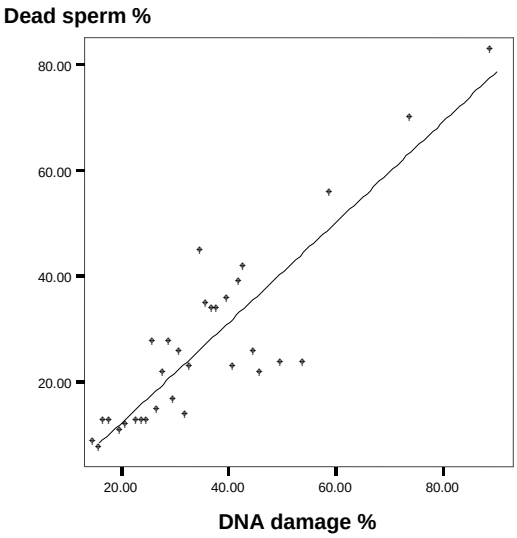

Figure 5: Correlation between dead sperms percentage and sperm DNA damage. A significant positive correlation (r=0.888; p=0.0001).

DISCUSSION:

Sperm DNA fragmentation as a prognostic factor of a man's fertility potential is a matter of debate [14]. It is now known that the populations of spermatozoa in an ejaculate are highly heterogeneous and there are always a percentage of sperms with abnormal DNA. This appears to be more evident in patients whose sperm parameters fall below normal values. Environmental stress, Smoking, Genital tract infection or even systemic infection, and many other known or inclusive factors can lead to an abnormal sperm chromatin structure that is incompatible with fertility [15].

In the present study, the sperm DNA fragmentation percentage results, as measured by TUNEL assay, was a good predictive parameter to distinguish between fertile and infertile populations. This result was in agreement with many other workers [14,16]. But found to be disagreed with some other studies [17].

The results of standard seminal analysis which is more cheap and easy to perform were in this study also highly indicative of male factor of infertility. So, studies must find the indication for sperm DNA fragmentation test which coast at least 80$ in some Europe and U.S. special labs.

Concerning the relationship between apoptosis and seminal parameters, our study noted an increase of DNA fragmentation in line with atypical forms, confirming the fact that sperm morphology is strictly correlated with sperm function. These finding were in harmony with the findings of other studies [18]. While sperm count and motility results were not correlated with sperm DNA fragmentation. And these findings were coherent with some previous studies [19].

The sperm viability was always correlated with the DNA damage percentage in the seminal samples (r=0.88; *p*=0.0001). The level of dead sperms was lower than the percentage of sperms with DNA damage. The explanation is that some sperms had damaged DNA but still alive. And many studies tried to explain male idiopathic fertility under this category [20].

This study found that the difference in TUNEL positive sperms percentage and dead sperms percentage was 7 in infertile patients and about 11 in healthy samples. Shen et al., 2002 [21] found that sperm vitality was about 77.5% (22.5% dead sperm) and DNA fragmentation was about 15% in 60 Singaporean infertile patients (so the difference is about 7.5) while the difference calculated from the findings of Al-Hashimi 2006 [22] was only less than one in 16 Iraqi infertile patients and about 3 in ten healthy fertile subjects.

This small percentage represents live sperms with DNA damage. These sperms are most likely with poor morphology (true in this study) and poor quality of motility (could not be proved in this study but proved by Nijs and coworkers 1996 [23]and unable to reach the female ovum and participate in process of fertilization [9]. This might be one of the protective mechanisms for human species because the highest quality spermatozoa from a kinetic, DNA integrity and morphological point of view will reach the ovum and only one will fertilize it [12].

The simple selection of morphologically normal shape motile spermatozoa really can eliminate most of the apoptotic spermatozoa and exclude them from a possible normal fertilizing process during intrauterine insemination (IUI) and In-vitro fertilization (IVF) [21]. However, when certain assisted reproduction techniques are employed in which the natural selection of the male gametes does not take place (e.g. intracytoplasmic sperm injection (ICSI)). Nijs and coworkers 1996 [23] found that the injection of previously immotile spermatozoa in an ovum results in either poor or no fertilization.

The higher percentage of apoptosis could lead to the risk of microinjecting a spermatozoon with fragmented DNA. In physiological conditions, such spermatozoa would be excluded from fertilization. This possibility might decrease by the swim-up procedure, followed by discontinuous density gradient centrifugation and simple sperm washing from the seminal plasma [24]. This procedure is of high efficacy in terms of elimination of non-viable and apoptotic spermatozoa. Donnenlly and coworkers [25] showed that 40% of sperm from semen and 20% of sperm from fraction (after swim up separation) with high sperm motility from infertile men had DNA fragmentation using TUNEL assay.

Bungum and coworkers 2007 [26] showed that semen samples with high rates of DNA breaks are more likely to result in pregnancy in ICSI than in IVF. The safety of ICSI has often been questioned

as the natural selection barriers during fertilization are bypassed. DNA damaged sperm in the ejaculate may be responsible for the induction of pathology such as offspring male infertility, congenital anomalies, and childhood cancer [27] and there was about 2-fold higher risk of infant malformations and the occurrence of syndromes related to errors in imprinting after ICSI [28].

The detection of fragmented DNA (TUNEL assay) may fail to discriminate the mode of sperm death: apoptosis, necrosis and autolytic cell death because the procedure of staining will stain any DNA on the slide and the DNA stained might be of live sperm or dead sperm or even leukocyte. This result was in agreement with findings of Charriaut-Mariangue and Ben-Ari 1995 [29] and Grasl-Kraupp and coworkers 1995 [30].

Based on the findings of the present study, the small percentage of live sperms with DNA fragmentation in ejaculate could not be the explanation of idiopathic male infertility.

concluded also that DNA fragmentation could be considered as independent parameters of sperm quality to differentiate infertile from fertile semen samples.

The sperm with DNA fragmentation percentage in seminal sample correlated positively with sperm abnormal morphology percentage and dead sperm percentage.

CONCLUSION:

Based on the findings of the present study, the small percentage of live sperms with DNA fragmentation in ejaculate could not be the explanation of idiopathic male infertility.

This study concluded also that DNA fragmentation could be considered as independent parameters of sperm quality to differentiate infertile from fertile semen samples.

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