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Research article

De novo cytogenetic alterations in spermatozoa of subfertile males might be due to genome instability associated with idiopathic male infertility: Experimental evidences and Review of the literature

Running title: Sperm cytogenetic alterations and male infertility

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Abstract: Male infertility is caused by many factors including genetics. Although part of genetic damages are inherited and could be traced in blood leukocytes, but those de novo alterations induced in spermatogenesis are not part of diagnostic work up. De novo alterations might be the cause of many idiopathic conditions of male infertility. The aim of this study was to evaluate DNA damage, sex chromosomal aneuploidy and *DAZ* microdeletion in sperms of subfertile males in comparison with normal healthy individuals. Whole blood and semen samples were obtained from 75 subfertile and 45 normal men. Semen samples from karyotypically normal subfertile and normal individuals were used for DNA fragmentation, sex chromosome aneuploidy and *DAZ* microdeletion analysis. Sperm DNA damage was assessed by alkaline comet assay, chromosome aneuploidy and *DAZ* microdeletion was assessed using a combined primed in situ labeling and fluorescent in situ hybridization (PRINS-FISH) method. A significantly high percentage of DNA fragmentation was observed in subfertile patients compared to control. Similar observation was observed for sex chromosome aneuploidy and *DAZ* microdeletion ($p < 0.01$). A relatively small interindividual difference was seen in all three assays performed. However *DAZ* microdeletion was observed as mosaic form in Y bearing sperms. Results indicate that subfertile males experience higher genome instability in spermatogenesis expressed as DNA damage and consequently sperm chromosomal

aneuploidy or microdeletions. Occurrence of de novo genetic alterations caused by environmental chemico-physical genotoxic agents during spermatogenesis might be one of the causes of idiopathic male infertility.

Keywords: Subfertile male; idiopathic male infertility; genome instability; DNA damage; *DAZ* microdeletion

1. Introduction

Scientific reports published during the past years clearly suggest that sperm counts in man are not only declining but also suggesting that it could be continuing [1,2]. Apparently these changes have occurred across the world and getting progressively worse. Therefore, it has been suggested that they might be reflection of adverse effects of lifestyle or environmental factors on the male reproductive system. It is estimated that one in 20 males may suffer from some levels of infertility [3], but for a majority of these patients the etiology of their condition is unknown. Labeling males with descriptive terms such as oligozoospermia, oligoasthenozoospermia or teratozoospermia, fail to lead us any closer to an understanding of why such patients are infertile. We do not know which the most important factors are yet but the most likely could be environmental physico-chemical exposures and diet. Studies report a decrease in sperm concentration in industrialized countries [4] suggesting possible adverse effects of environmental genotoxic exposures on reproductive outcomes [5].

In modern life, human is under constant exposure to toxic chemical substances, air pollutions and natural or manmade sources of non-ionizing radiations (microwaves, radiowaves, mobile, etc.) and ionizing radiation used for medical or industrial purposes. These agents are mostly potent inducers of oxidative stress and reactive oxygen species (ROS) [6]. ROS are a group of highly reactive molecules implicated in the oxidative damage of biological structures. Therefore, after numerous studies concern has been raised that the reactivity of ROS to DNA nucleotides might induce genetic damages that may evolve to cancer if somatic cells; or birth defects if germ cells are damaged [7,8]. Likewise, ROS might also play a critical role in induction of DNA damage at any stages of spermatogenesis or spermatogenesis leading to sperm DNA damage and the etiology of defective sperm function and male infertility [9-13]. Sperm DNA damage might be expressed as disruption of the meiotic chromosome segregation (aneuploidy), premature chromosome condensation (PCC), fragmentation of the DNA, individual genetic mutations, disruption of the DNA structure (chromatin integrity), and production of DNA adducts.

Sperm cells carry a background level of aneuploidy and chromosome breakage [10,14-17]; however, this baseline might be increased due to a number of risk factors. Aneuploidy and structural chromosomal aberrations are major contributors to fertilization failure, preimplantation failure, spontaneous abortions, still births, physical birth defects, and mental and behavioral dysfunction [18-21]. In addition, sperm chromosome abnormalities might have a negative impact on the success rate of assisted reproduction technology (ART) program. For subfertile individuals who needed intra-cytoplasmic sperm injection (ICSI), a higher rate of sex chromosome aneuploidy has been reported [16,22,23] compared with those men requiring conventional in vitro fertilization (IVF) [24]. A number of studies have shown that men with increased frequencies of sperm chromosome abnormalities have produced chromosomally abnormal fetuses and children [25-29]. Thus, the increased frequency of

chromosomal abnormalities in sperm might be used to predict a real risk for abnormal offspring.

Y chromosome microdeletion has also been attributed to male infertility. In the mid 1970s, the association of the long arm of the Y chromosome with spermatogenic failure was proposed and subsequently this region on Yq11, was named the azoospermia factor or *AZF* [30]. *AZF* region was further defined at molecular level and according to the certain microdeletion, this region was divided into *AZFa*, *AZFb*, and *AZFc* subregions [31]. Amongst these regions, *AZFc* is the most commonly deleted interval in men with azoospermia or severe oligozoospermia [32,33]. There is a variety of genes in this region from which *DAZ* gene is the most susceptible candidate for deletion in oligozoospermia and azoospermia males [34]. A wide range of variability of *AZFc* microdeletion from 1% [35] to 55% [36] has been reported among infertile men when genomic DNA is analyzed. These microdeletions mainly occur in severely oligozoospermic men ranges from 5–10% [37,38] and azoospermic men ranges from 10–15% [37,38]. Although transmission of *AZF* microdeletions has been reported previously, correlating ART and the incidence of *AZF* microdeletions is still a debate and the occurrence of de novo deletions and expansion have not been explained [39].

At present, in the case of severe male infertility, the molecular genetics diagnosis of Y-chromosomal microdeletions is done by using polymerase chain reaction (PCR). Amplification of selected regions of the Y chromosome using sequence tag site (STS) primers is routinely performed as part of the infertility workup in many genetic and infertility laboratories all around the world as well as Iran, so that to give an appropriate genetic counseling and explanation for the male infertility in men with azoospermia or severe oligozoospermia [31,40-42]. However, there are evidences showing that presence of *DAZ* in somatic cells might not be indicative of its presence in germ cell lineage [43,44].

To show the involvement and correlation of DNA damage, chromosomal aneuploidy and Y-chromosome *DAZ* microdeletion in idiopathic male infertility, comet assay and PRINS-FISH techniques have been used in this study.

The alkaline comet assay known as the single cell gel electrophoresis assesses DNA strand breaks and alkaline labile sites when used under alkaline condition [9,45,46]. The comet assay detects DNA strand breaks by migration of DNA out of the cell nucleus during electrophoresis. Cells with undamaged DNA appear as intact heads without tails. Fragmented DNA will contain numerous strand breaks and will therefore migrate further than normal intact DNA after specified electrophoresis time. When visualized under fluorescent microscope, the migrating DNA resembles the tail of a comet [9]. The comet assay has been used to measure DNA damage in individual human lymphocytes using relatively low doses of ionizing radiation and chemical genotoxins [47]

Several techniques have been developed to detect individual human chromosomes in sperm nuclei. The first preparations of human sperm chromosomes were done using the capacity of human spermatozoa to penetrate zona-free hamster oocyte. This method was first demonstrated by Rudak et al. (1978) [48] and later standardized by Martin et al. (1982) [49], but because of the complexity of the technique, it was used by a few laboratories, and was never applied in a clinical setting. The use of chromosome-specific DNA probes opened the way to indirectly study the chromosome constitution of large numbers of spermatozoa by multi-color fluorescence in situ hybridization (FISH) either on sperm nuclei or on human sperm-derived pronuclear [16,17,50,51]. However, the special characteristics of the sperm nucleus give rise to a series of limitations that can only be circumvented through the use of very strict technical criteria.

As mentioned earlier, screening of the Y chromosome microdeletion in the diagnostic work-up of infertile men is mainly done using polymerase chain reaction (PCR) on blood leukocytes [42].

However, there are evidences showing that presence of *DAZ* in somatic cells might not be indicative of its presence in germ cell lineage. In this report a combined PRINS and FISH technique [44] was used to show the DAZ gene on sperm nuclei. The primed in situ labeling (PRINS) technique represents a significant improvement in the detection of specific DNA sequences in situ: it is based on the annealing of specific oligonucleotide primers to chromosomal DNA and subsequent primer extension by a suitable polymerase in the presence of labeled nucleotides [52]. In this procedure, primers specific for *DAZ* genes, in combination with direct labeled centromere probes for X and Y chromosomes were used. This combined method allows simultaneous detection of *DAZ* genes and sex chromosome aneuploidy in sperm samples.

The aim of this study was to examine the difference between percentage of DNA damage, sex chromosome disomy and frequency of *DAZ* microdeletion in sperms of normal and infertile individuals in order to show possible correlation between either genetic abnormalities in sperm nuclei.

2. Materials and Methods

2.1. Sample preparation

This experimental study was performed on whole blood and semen samples obtained from 75 subfertile and 45 normal men referred to Fertility and Infertility Center of Shariati Hospital (Tehran, Iran), candidate for ART. Demography of patients' and normal subjects is presented in Table 1. In all cases, after three days of sexual abstinence, semen samples were collected by masturbation into sterile containers and were delivered to the laboratory immediately after ejaculation. The semen was allowed to stand at 37 °C for 30 min to complete liquefaction. A semen profiles were then performed and samples were classified according to World Health Organization criteria [53] into two groups (normal, subfertile). Subfertile group included oligo, astheno, oligoastheno and severe oligozoospermia. Those samples considered as normal were obtained from men referred to IVF clinic because of their spouse infertility (female infertility). Whole blood was obtained by venopuncture in heparin containing tubes. The study was approved by the Ethical Committee of the Faculty of Medical Sciences of the Tarbiat Modares University (Tehran, Iran). Patients gave their informed written consent. All donors completed a written questionnaire to obtain information related to their life style, such as dietary habits, medical history and exposure to chemical and physical agents. Therefore, all samples had been screened to exclude radiation exposure, smoker, varicocele, genital tract infections, hepatitis, and HIV antibodies. Samples were processed by swim-up techniques from pellet as described by Aitken and Clarkson (1988) [54].

2.2. Karyotype analysis

Whole blood was cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), antibiotics (penicillin (100 U/mL)/streptomycin (100 µg/mL), and 0.1 ml phytoheamaglutinin (all materials from Gibco BRL). One hour before harvesting, cells were treated with colcemid at final concentration of 0.4 µg/mL. Cells were harvested at 72 h after culture initiation. Harvesting, slide preparation and G-banding were performed according to routine standard procedure. Those patients with normal karyotype were enrolled for sperm analysis.

2.3. Sperm comet assay

The alkaline single-cell gel electrophoresis (comet) assay was performed based on existing methods described by Singh (1996) [55] with minor modifications as described in our previously published paper [9]. Briefly, 10 µL of spermatozoa suspension was mixed with 75 µL of 0.75% low melting point agarose (Sigma, USA) at 37 °C and was rapidly transferred on the coated slide with 0.1% normal agarose, covered with a coverslip and left at 4 °C for 5 min. The slides were immersed in a Coplin jar containing freshly prepared cold lysis solution (2.5 M NaCl, 100 mM ethylene diamine-tetraacetic acid (EDTA), 10 mM Tris; pH = 10, with 1% Triton X-100 (all chemicals from Sigma, USA) added just before use) for 1 h at 4 °C. Slides were then incubated for 30 min at 4 °C with 10 mM dithiothreitol (Sigma, USA) followed by 90 min incubation at 20 °C with 4 mM lithium diiodosalicyclate (LIS; Sigma, St. Louis, MO, USA). The slides were then placed into a horizontal gel electrophoresis tank filled with fresh alkaline electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH = 13; Sigma, USA) at 4 °C. The slides were left in this high pH buffer for 20 min at 4 °C to allow the sperm DNA to unwind, and then subjected to electrophoresis for 10 min at 25 volts (0.7 V/cm) adjusted to 300 mA. After electrophoresis the slides were drained, and flooded with neutralization buffer (0.4 M Tris; pH = 7.5; Sigma) for 5 min. Cells were stained with 20 µL ethidium bromide (2 µg/mL, Merck, Germany) under a coverslip. Observations were made at a magnification of 200× using a Nikon E800 epifluorescence microscope equipped with 546–516 wave length band and a 590 nm barrier filter. The comets were analyzed by visual classification and for each sample 1000 cells were scored. Damage was assigned to five classes (0–4) based on the visual aspect of the comets, considering the extent of DNA migration according to the established criteria [9]. Damage scores were calculated based on the following equation (1) adopted from Jaloszynski et al. (1997) [56] that ranged from 0 to 400 arbitrary units, corresponding to situations ranging from no damaged comets to extremely damaged based on equation (1):

$$DD\ (au) = (0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4) / (\Sigma n /100) \quad (1)$$

where; DD (au): arbitrary unit DNA damage score, n_0–n_4: number of Class 0–4 comets, Σn: total number of scored comets. Coefficients 0–4 are weighting factors for each class of comet. An example of sperm nuclei with various degree of DNA damage observed as comet is shown in Figure 1A.

2.4. Disomy and DAZ microdeletion assessment of sperms

Semen samples were washed in PBS (2×) and fixed in Carnoys' fixative (methanol: acetic acid; 3:1 v/v). Slides were made for each sample and air dried.

PRINS-FISH technique was performed on the prepared slides as described previously [44]. Briefly, the air dried slides were immersed in a 0.5M NaOH solution at room temperature for 5 min to allow the simultaneous decondensation and denaturation of sperm nuclei [57], washed in PBS for 2 min, dehydrated in ethanol series (70%, 85% and 100%) for 2 min each and air dried. Slides were then put in a 70% formamide in 2× SSC at 73 °C for 3 min for further denaturation of sperm DNA. Slides were dehydrated again through ice cold ethanol series. A reaction mixture was prepared in a final volume of 50 µL containing: 10× PCR buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.2 mM dATP, dCTP, and dGTP, 0.02 mM dTTP, 0.02 mM fluorescent labeled dUTP, 0.01% BSA, 2.5U of Taq DNA polymerase (all reagents from Roche Diagnostics, USA) and

200 pmol of each oligonucleotide primer for *DAZ*
(5'-CTCTGCCTCTGGCTTTAC CA-3',
5'-GAGGAGGCATCTGGAAATCATT-3',
5'-GGAAGCTGCTTTGGTAGATAC-3',
5'-TAGGTTTCAGTGTTTGGATTCCG-3') for each slide [44,58,59]. The four forward primer sets used for *DAZ* microdeletion detection were previously validated by Kadandale et al. (2002) [58] and Gao et al. (2011) [59] who studied DAZ situation on metaphase chromosomes prepared from lymphocytes. Therefore the primers used in this study clearly show *DAZ* gene status on sperm nuclei as has been previously shown by Mozdarani and Ghoraeian (2012) [44]. Moreover, a blast search of the primer sequences showed that they were specific for the intended targets. The prepared slide was then placed on a PCR thermal block (Eppendrof) and heated for 3 min at 76 °C, then hold at 60 °C. PRINS reaction mixture was transferred on the slide, covered with a coverslip and sealed with rubber glue. The slide was left at 60 °C for at least 1 min to allow the temperature of reaction mixture reach to annealing temperature. The PRINS was then performed by incubating the slide at 55 °C for 20 min to allow primers to anneal, and then at 72 °C for 40 min for primer extension.

After the completion of PRINS reaction, the coverslip was removed and slide was washed in 0.4× SSC, at 72 °C for 2 min, left in dark to air dry. The slide was put on the thermal block holding at 72 °C, mixture of direct labeled centromeric probes for X and Y chromosomes (10 µL) (Cytocell, UK) placed on the slide, covered with a coverslip and sealed with a rubber glue. The sample DNA and probes were co-denaturatured at 73 °C for 5 min then placed in a humidified chamber at 37 °C overnight. The following day, slide was washed in 0.4× SSC at 72 °C for 2 min followed by a further wash in 0.4× SSC containing 0.05% Tween 20 (AMRESCO Inc., USA). Slides were stained with DAPI counterstain (0.3 µL/mL) containing Vectashield mounting media (Vector Laboratories, Inc, Burlingame, Calif.), covered with a coverslip, and stored at 4 °C in a light-free slide box. The slides were examined under the epifluorescence microscope (E800, Nikon, Tokyo, Japan), using a triple band-pass filter, and confirming the coloration of the fluorescent spot with single band-pass (FITC or Rhodamine) filters. Figure 2A shows sample visualization of X and Y centromere and Figure 3A shows visualization of Y centromere and DAZ gene on sperm nuclei.

2.5. Statistical analysis

Results were analyzed using SPSS statistical software (version 16.0, SPSS Inc., Chicago, IL, USA). The Student's T-*test* was used to compare differences between data obtained for subfertile and normal groups. Sigma plot 2004 for Windows, version 10.0 was used to draw figures. $p < 0.05$ was considered as statistically significant.

3. Results

Patients' demography and data obtained in the study is summarized in Table 1. Figure 1B shows the results obtained after DNA fragmentation analysis of studied samples. For DNA fragmentation analysis 1000 cells were scored for each sample. As seen, mean percentage of DNA damage in normal samples was about 22% which increased to about 50% for samples from infertile groups. The result obtained for infertile patients was significantly different from normal control ($p < 0.01$). Box plots in Figure 1B also shows small inter-individual variation especially for normal individuals, however high upper limit for infertile patients indicates higher degree of DNA damage in sperm

samples of individuals affected with more severe infertility problems such as oligoasthenozoospermia or severe oligozoospermia.

Table 1. Demographic characteristics of subjects screened for DNA damage, sex chromosomal aneuploidy and percentage of *DAZ* microdeletion in studied groups.

Study groups	Normal individuals	Subfertile individuals
Number of subjects	45	75
Mean age ± SD (Years)	33.5 ± 5.2	35 ± 5.7
Mean period of Infertility ± SD (Years)	6.1 ± 5	6.75 ± 4.1
Mean sperm count ± SD ($\times 10^6$/mL)	69.8 ± 19.2	14.83 ± 6.95
Percent DNA damage	22.8 ± 4.1	48.1 ± 10.4
Percent Y bearing Sperms with *DAZ* microdeletion	0.325 ± 0.187	38.13 ± 14.6
Sperm with XX chromosome	0.095 ± 0.055	0.193 ± 0.25
Sperm with YY chromosome	0.078 ± 0.057	0.178 ± 0.26
Sperm with XY chromosome	0.16 ± 0.09	0.49 ± 0.69

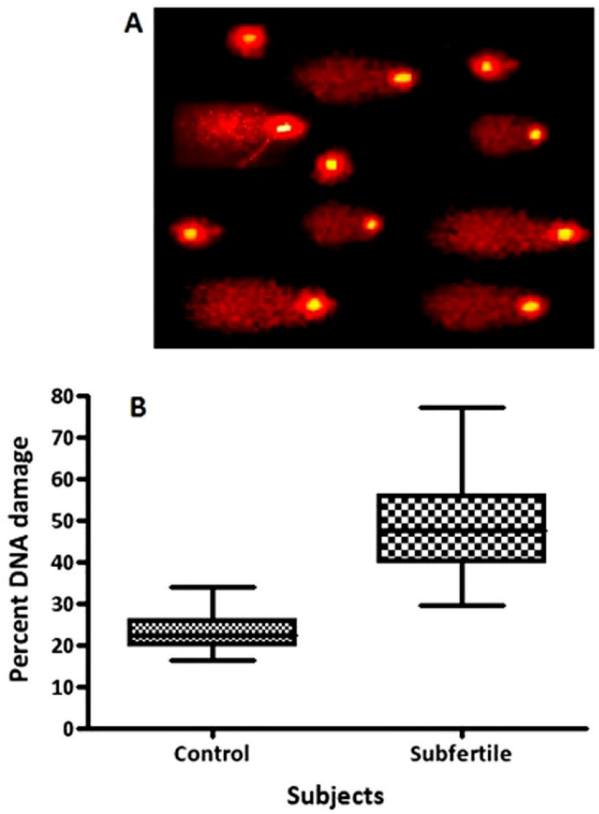

Figure 1. Panel (A), is showing a typical photomicrograph of a sperm with heavily damaged DNA producing a comet with long tail (Magnification ×1000). Panel (B), box plots showing baseline DNA damage for controls and subfertile patients measured by alkaline comet assay. The boxes extend from the 25th percentile to the 75th percentile, with a horizontal line at the median (50th). The whiskers present the 10th and the 90th of the data.

3.1. Analysis of chromosomal disomy

For sex chromosomal disomy in sperm samples of normal or subfertile individuals, a minimum of 5000 sperm nuclei per chromosome were analyzed. The data are summarized and presented in Table 1. The frequencies of X and Y bearing spermatozoa did not differ from the expected one to one ratio in normal donors. For chromosomes X and Y, the mean disomy rates were 0.095% for X, 0.078% for Y, and 0.16% for XY respectively. There was no significant difference ($p > 0.5$) between XX and YY disomy but however, the rate of XY disomy almost two times higher, was significantly different with either XX or YY disomy ($p < 0.01$) (Table 1).

The mean frequency of disomic sperm nuclei in the infertile group was 0.193% for X, 0.178% for Y and 0.49% for XY. Results obtained for X and Y disomy for this group of patients was not significantly different ($p > 0.05$). However, the rate of XY disomy, more than twice, was significantly different with either X or Y disomy ($p < 0.01$) (Table 1).

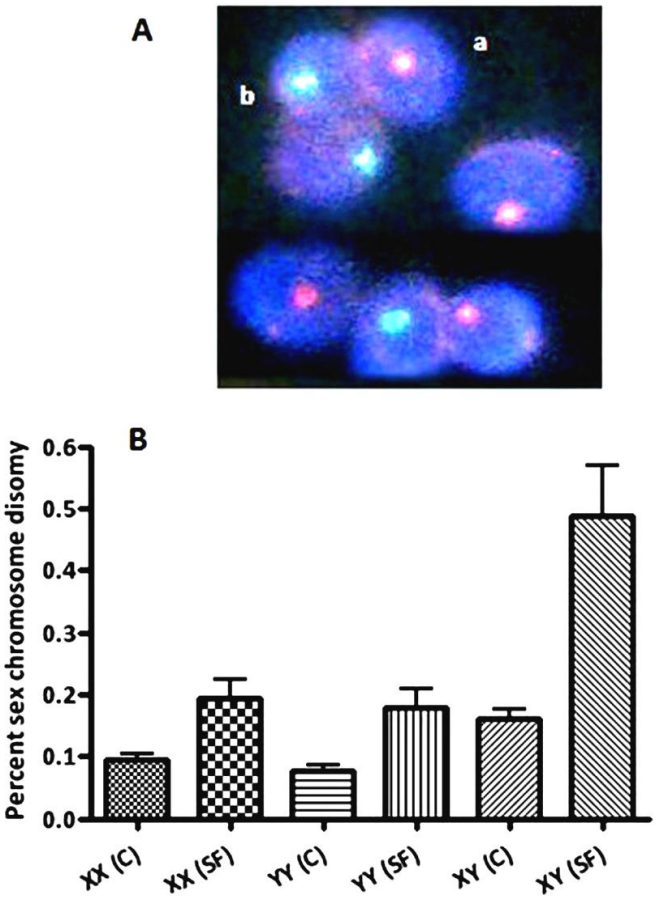

Figure 2. Panel (A), is a photomicrograph of sperm nuclei counterstained with DAPI and showing fluorescent signals either red for Y (a) or green for X (b) chromosome (Magnification ×1000). Panel (B), represents data for sex chromosome disomy observed in control (C) or subfertile (SF) groups. Errors are indicating standard deviation of mean values.

3.2. Assessment of DAZ microdeletion in sperm nuclei

Thousand sperm nuclei were screened for the presence or absence of *DAZ* gene. As seen in Figure 3B there is relatively no *DAZ* microdeletion seen in normal samples. However, in subfertile samples, high frequency of *DAZ* microdeletion is seen compared to normal, significantly different ($p < 0.01$). This figure shows the percentage of *DAZ* microdeletion in total sperm numbers and in the population of Y-bearing sperms. However, it should be noted that not all sperm nuclei showed *DAZ* microdeletion in a sample; rather it was a type of mosaic form. The frequency of *DAZ* microdeletion differed in different samples. Higher frequency of *DAZ* microdeletion was seen in samples from rare/ severe oligozoospermia patients.

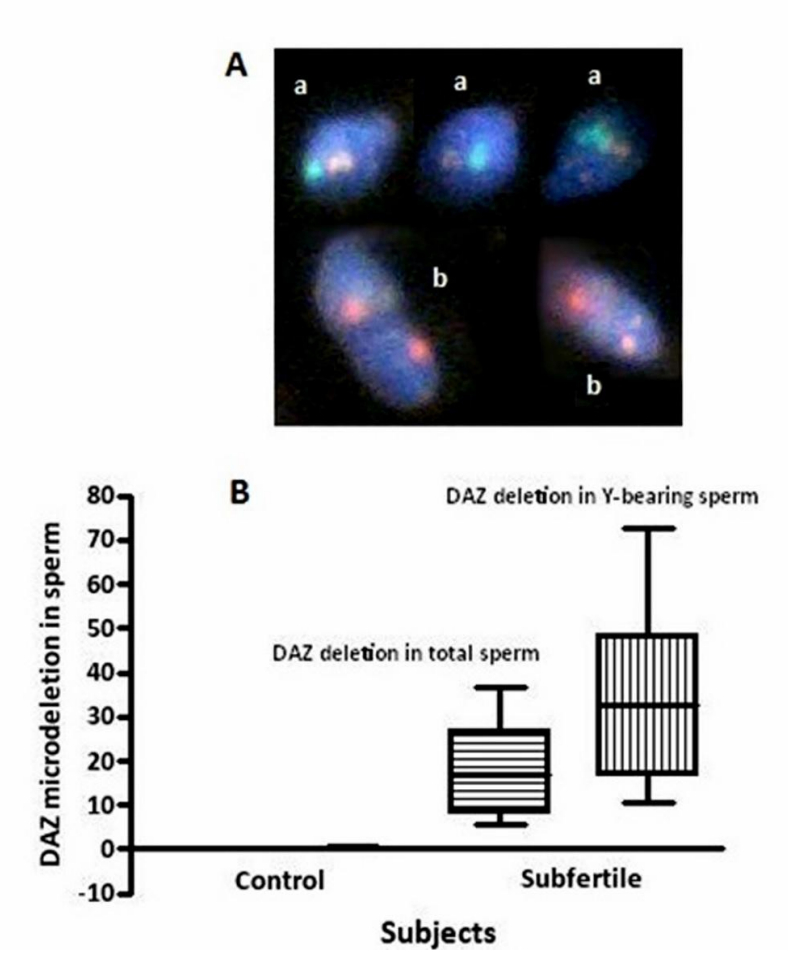

Figure 3. Panel (A), photomicrograph showing Y-bearing sperm nuclei counterstained with DAPI with normal *DAZ* (a) and with *DAZ* microdeletion (b) (Magnification ×1000). Panel B, box plots showing baseline *DAZ* microdeletion for controls and subfertile patients. The frequency of microdeletion in control group was about zero such that no box plot is formed. Box plots for subfertile group show the obtained results per total sperm (i.e., sperms with X and Y bearing chromosomes) or per only Y-bearing sperms. The boxes extend from the 25th percentile to the 75th percentile, with a horizontal line at the median (50th). The whiskers present the 10th and the 90th of the data.

4. Discussion

4.1. Sperm DNA damage

Sperm DNA damage is clearly associated with male infertility (and abnormal spermatogenesis), but a small percentage of spermatozoa from fertile men also possesses detectable levels of DNA damage [9,60].

As seen in Figure 1B, a high degree of DNA fragmentation was observed in spermatozoa from normal subjects, although the extent of damage was more than twice for subfertile group; significantly different ($p < 0.01$). There might be several factors involved in induction of DNA damage in sperms including ROS induced by various environmental factors [12,13]. Although various mechanisms of DNA damage in male germ cells and spermatozoa are proposed [61], however ROS might have an important role in both the physiology and pathology of human reproduction [62]. DNA fragmentation detected by comet assay in male germ cells clearly measure both single and double strand DNA breaks [63,64]. Sperm DNA fragmentation is a useful biomarker for male infertility diagnosis and prediction of assisted reproduction outcomes. It is associated with reduced fertilization rates, embryo quality and pregnancy rates, and higher rates of spontaneous miscarriage and childhood diseases. As sperm DNA damage is often the result of increased oxidative stress in the male reproductive tract, the potential contribution of antioxidant therapy in the clinical management of this condition is recommended [11]. The causes of sperm DNA damage, much like those of male infertility, have many factors and may be attributed to intra- or extra-testicular factors (i.e., drugs, chemotherapy, radiation therapy, cigarette smoking, environmental toxins, genital tract inflammation, testicular hyperthermia, varicocele, hormonal factors and so on). Effects of occupational radiation exposure on functional, genetic and epigenetic integrity of sperm in health workers were shown recently [65]. Spermatozoa exposed to methyl methanesulfonate (an alkylating agent) showed a significant level of DNA damage leading to abnormal embryo [66]. In a report De Iuliis and colleagues (2014) [67] have shown that mobile phone radiation induces mitochondrial ROS production and eventually DNA damage and fragmentation in human spermatozoa leading to decrease in the motility and vitality of these cells [67]. It was also shown previously that men exposed to high background natural or manmade radiation [68-72] and in vitro irradiation of leukocytes [73,74] led to genome instability in AZF region of Y chromosome especially in the *DAZ* genes.

The exposure of human spermatozoa to extracellular generated ROS also induces a loss of motility that is directly correlated with the level of lipid peroxidation experienced by the spermatozoa [75]. As known, sperm DNA fragmentation is clearly associated with male infertility and abnormal spermatogenesis [60,76]. Spermatozoa are particularly susceptible to ROS-induced damage due to presence of large quantities of polyunsaturated fatty acids and low concentrations of scavenging enzymes in their plasma membranes [62]. Therefore, most of DNA damage leading to chromosomal aneuploidy might have been induced prior to chromosomal segregation. Mozdarani and his colleagues who studied preimplantation embryos produced by male mice irradiated at various stages of spermatogenesis, clearly shown that radiation induced DNA damage in spermatogenesis lead to chromosomal aneuploidy or micronuclei in preimplantation embryos [77,78]. These results might indicate the involvement of DNA damage in chromosomal segregation during meiosis leading to aneuploid sperms. Therefore it is a possibility that patients exposed to diagnostic or therapeutic doses of ionizing radiation, experience various degrees of infertility [79]. Perrin et al. (2009) have also shown that the DNA fragmentation rate depends on the presence of a chromosomal abnormality in

spermatozoa [80]. High rates of DNA fragmentation in human spermatozoa have been associated with impaired preimplantation development of the embryo, increased rates of early pregnancy loss and high rates of morbidity in the offspring including dominant genetic disease, infertility and cancer [81].

4.2. Sperm sex chromosome aneuploidy

Structural or numerical chromosomal abnormalities are relatively frequent in human germ cells causing serious reproductive problems such as spontaneous abortion and congenital defects. About 1–2% of human sperm have an abnormal number of chromosomes and approximately 10% carry structural chromosome aberrations [82,83]. Aneuploidy, i.e., abnormal chromosome number, can be detected using fluorescence *in situ* hybridization (FISH) with chromosome-specific DNA probes and PRINS technique using primers specific for centromere regions. Sex chromosomes are the chromosomes usually evaluated when assessing sperms with FISH or PRINS, as these appear to be most at risk for nondisjunction, suggesting that there is a chromosome-specific variation in nondisjunction frequencies [84]. FISH as well as PRINS has been used to assess sperm aneuploidy for lifestyle factors like tobacco, caffeine, and alcohol [85,86] and exposures to pesticides and heavy metals [87-89]; as well as paternal age, cancer chemotherapy, and radiation [85,90,91].

Valuable information on the cytogenetic of spermatozoa from normal men has been provided over the past decades using different methods. However, different values are reported depending upon the method used to estimate the mean aneuploidy frequency. At present, disomy levels for nearly all human chromosomes have been reported [92,93]. However, different research groups reported a wide variability in the frequencies of disomies for the same chromosomes. These variations might be attributed to interindividual differences. However, technical aspects such as differences in sperm decondensing protocols, number of sperms analyzed, scoring criteria, and the probes used, could be influencing factors on the results and frequencies reported [94].

In the present study, the baseline incidence of XX, YY and XY disomy was 0.066%, 0.059% and 0.184% respectively (Table 1). Our estimate for XX and YY frequency is similar to the reported rates [16,23]. However Martin et al. (1996) reported similar frequency of disomy for XX and XY but significantly higher rate for YY (0.18%) disomy [95]. A report by Kirkpatric et al. (2008) [17] indicates a significantly higher rate of disomy for sex chromosomes compared to our results (Table 1 and Figure 2B). A wide range of baseline disomy for XX (from 0.02% to 0.34%); for YY (from 0.03% to 0.27%) and for XY (from 0.06% to 0.42%) has been reported previously by several investigators [92,93]. Therefore, results presented in table 1 and shown in Figure 2B well fit in the reported ranges.

Regarding sex chromosome disomy rate in subfertile men, a significantly higher frequency of XX, YY and XY disomy for subfertile men was observed compared to normal ($p < 0.01$)). Our estimate for the rate of sex chromosome disomy (Table 1) is also different from the results reported previously [50,96].

The reported variation in sperm disomy might be attributed to the variation in meiotic recombination that has been reported among normal men [97-99]. Higher frequency of disomies has been reported by several authors with the tendency of sex chromosomes [22,93,100]. Moreover, studies on retrieved sperms from infertile men with normal 46,XY karyotypes have shown correlation of abnormal semen parameters with increased risk of sperm aneuploidy [22,50], although not supported universally [17]. The increase in sperm aneuploidy observed in infertile men is supported by the increase in de novo chromosomal abnormalities of paternal origin after ICSI [28,29].

However, increased rate of sex chromosome disomy in subfertile group might suggest that both

sex and some autosome chromosomes may have an increased susceptibility for segregation error in spermatogenesis, or that sperm maturation is more tolerant of errors in segregation involving sex chromosomes. This observation might be in contrast to the arguments of Kirkpatric et al. (2008) [17] who believe sex chromosomes are more tolerant of errors in segregation than autosomes.

Defective recombinations as well as pairing of meiotic chromosomes have been shown in infertile men with normal karyotype [51,101]. In the present study, elevated frequency of sperm aneuploidy observed for sex chromosomes might be due to defects in meiotic recombination. Furthermore, a high frequency of sex chromosomes lacking recombination might increase the risk of sex chromosome aneuploidy in the sperm.

An increased frequency of sperm aneuploidy has been reported for patients with poor semen quality [15,102,103]. The influences of both severe oligozoospermia and teratozoospermia on sperm aneuploidy are generally accepted [104-106]. It was also shown that sex chromosome disomy may have a negative impact on the success rate of assisted reproduction technology (ART) program [107].

Nondisjunctional events during meiosis with unknown causes might be the cause of sperm numerical chromosomal aberrations. However, there are reports indicating that sperm DNA damage correlate well with chromosomal aneuploidy [10,61].

4.3. DAZ microdeletion

Apart from involvement of sperm DNA damage and chromosomal aneuploidy in male infertility, Y chromosome microdeletions are causally related to spermatogenesis defects and abnormal sperms. However, a significant difference exists in deletion location, frequency and its extent. *AZFc* is the most commonly deleted interval in men with severe oligozoospermia or azoospermia mainly due to intrachromosomal homologous recombination between repeated amplicons [32,33]. In this region the *DAZ* gene is located and is the most susceptible candidate for deletion [34,108]. The *DAZ* genes are expressed particularly in testicular tissue [109] therefore men with *DAZ* deletions would be incapable of producing mature sperm [110]. However, it has been shown that *DAZ* deletions occur in some men with oligozoospermia [111]. This might increases the possibility of transmission of Y chromosome microdeletions to the son [112]. That's why; microdeletion detection on blood leukocytes by the use of PCR [113] has been included in the recommended guidelines for male infertility [40]. However, there are evidences showing that the presence of de novo *DAZ* microdeletion in germ cell lineage.

For those patients requiring ICSI for infertility treatment, knowledge of the presence or absence of *DAZ* gene in sperm nuclei is of great concern. Screening for deletions involving the *DAZ* gene in severe oligozoospermia is suggested [114]. The estimated frequency of deletions involving *DAZ* locus is reported to be about 3% in severe oligozoospermia and azoospermia men when blood leukocytes is screened using PCR [114].

In this study the PRINS technique was used to detect *DAZ* microdeletion in individual sperm nucleus. The PRINS reaction which combines the high sensitivity of polymerase chain reaction (PCR) with the cytological localization of DNA sequences [115] was used to localize *DAZ* gene on sperm nuclei. The FISH method was used for detection of X and Y chromosomes on sperms based on the commercially available alpha satellite centromere probes. Combination of these two techniques not only allows detection of *DAZ* status in single cells (Figure 3a) but also makes it possible to assess sex chromosome aneuploidy in sperms (Figure 2A). As seen in Table 1, a high frequency of Y-bearing sperms in semen samples of subfertile men showed *DAZ* microdeletion compared to normal individuals. Frequency of *DAZ* microdeletion observed in total sperm retrieved from subfertile men

was significantly different compared to normal ($p < 0.01$) (Figure 3b). However, if only Y-bearing sperms are considered for calculation of *DAZ* microdeletion, the frequency dramatically increases to about 10 times more than that seen in control (Table 1, Figure 3a). It was interesting to see the *DAZ* microdeletion as mosaic form in sperm nuclei, i.e., not all sperm nuclei in a sample showed *DAZ* microdeletion but the frequency of microdeletion varied in different samples. This observation might imply that some *DAZ* microdeletion are induced de novo during spermatogenesis or even after spermiogenesis [116]. Other studies have also shown higher frequency of Yq microdeletions in sperm DNA and variation in *DAZ* copy number in infertile men as compared to DNA isolated from blood when using real time PCR [43,117,118]. There are also reports showing *DAZ* gene is vulnerable to damage by environmental physico-chemical genotoxic agents [69-73,119,120], and in particular, spermatozoa are susceptible to ROS-induced damage due to presence of large quantities of polyunsaturated fatty acids and low concentrations of free radical scavenging enzymes in their plasma membranes [62].

A high frequency of microdeletion and amplification of the *AZFc* region and *DAZ* gene microdeletion was reported for individuals exposed to natural background radiation [119]. As such many cancer patients experience different degrees of infertility following radiotherapy [79]. Moghbeli-Nejad et al. (2012) after in vitro gamma irradiated blood samples of normal and subfertile individuals showed genomic instability of *AZFc* region expressed as microdeletion and duplication [74]. These changes are certainly not detectable with somatic cell analysis if occurred during spermatogenesis. On the other hand, higher frequencies of Yq microdeletions in sperm DNA was reported as compared to DNA isolated from blood [43,117]. Therefore it would be reasonable for all candidate couples for ART to undergo Yq microdeletion screening on sperm nuclei in order to provide the most appropriate counseling and therapies [121]. Another limitation of PCR based *DAZ* analysis is the dilution effect which might not show mosaicism in a cell population. For these reasons, study of *DAZ* status by the use of PCR technique and with blood leukocytes might not be as accurate as showing *DAZ* gene on individual sperm nuclei.

There have been attempts to show *DAZ* gene by the use of FISH on sperms or metaphase lymphocytes [59,122,123] or PRINS [58,59,124] in lymphocytes either as interphase or metaphase cells. However, the PRINS-FISH method used in this study has clearly shown the status of *DAZ* gene on sperm nuclei.

5. Conclusion

The results of the present study suggest that induced DNA damage might be a prime cause of de novo *DAZ* microdeletion and induction of chromosomal aneuploidy in spermatozoa in normal and subfertile individuals. Study of *DAZ* microdeletion on DNA isolated from peripheral blood might not be representative of *DAZ* status in sperm nuclei. Therefore studying Y chromosome microdeletion and *DAZ* status using PCR in blood leukocytes might not be as accurate as showing *DAZ* gene on individual sperm nucleus.

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Conflict of interest

The authors declare there is no conflict of interest.

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