Sperm DNA damage and its relation with leukocyte DNA damage

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1. Introduction

Infertility is classically defined as a state in which a couple desiring a child is unable to conceive following 12 months of unprotected intercourse. Up to one in four couples experience difficulty in conceiving and, in half of these cases, the problem has been attributed to male infertility [1,2]. Infertility is classically defined as a state in which a couple desiring a child is unable to conceive following 12 months of unprotected intercourse. Up to one in four couples experience difficulty in conceiving and, in half of these cases, the problem has been attributed to male infertility [1,2].

One of the etiologies that has been identified as a potential cause of male infertility is sperm DNA damage [3–5]. Sperm DNA damage may provide a better evaluation of sperm function as opposed to the standard semen analysis. For instance, some infertile men with idiopathic factors have normal semen parameters with increased DNA damage [6–8]. Intra-cytoplasmic sperm insemination (ICSI) has been considered as the first line of treatment in male infertility [1,2]. During the course of ICSI, sperm directly inseminates into the cytoplasm of a mature oocyte thereby all barriers to fertilization, which prevents penetration of damaged, immature and abnormal sperm, will be bypassed. Therefore, considering the fact that insemination of sperm with damaged DNA during ICSI may not preclude fertilization and pronucleus formation [9], thus much concern remains regarding insemination of sperm with damaged DNA. In addition, evidence suggests that the sperm DNA disturbance has a negative correlation with the fertility potential of spermatozoa, either in vivo or in vitro [10,11].

DNA damage may be induced by exogenous and endogenous factors. Endogenous factors include: abnormal chromatin packaging, oxidative stress, and abortive apoptosis [8,12,13], while exogenous factors include physical, environmental, behavioral and socioeconomic factors. These factors may induce DNA damage through the production of reactive oxygen species (ROS) [14]. Therefore, many chemical and physical agents found in the workplace and environment could be considered as reproductive toxins.

One of the procedures widely used for assessment of exogenous DNA damage is the blood Comet assay. This genotoxicity assay is increasingly used for biomonitoring in occupational and environmental studies. Elevated levels of DNA damage have been observed in leukocytes of persons in putatively high-exposure circumstances.

There are several types of scoring systems in the Comet assay, including continuous (e.g. percent of DNA in the tail (% T) and tail length) and categorical (visual scoring in arbitrary units) measurements [15]. Furthermore, it has been shown that visual score and % T have linear dose–response relationships with known strand breaking agents over a wide dose range and they can be compared

Abstract

DNA fragmentation in human sperm has been related to endogenous and exogenous factors. Exogenous factors can also affect leukocyte DNA integrity. This study evaluated the relation between sperm DNA damage and leukocyte DNA integrity, as a predictor of exogenous factors. DNA damage in the sperm and leukocytes of 41 individuals undergoing ICSI were measured by Comet assay. In addition, sperm chromatin dispersion (SCD) was carried out on semen samples. A positive correlation was observed between the DNA integrity of sperm with leukocytes. When patients were divided into low and high DNA exposure groups, sperm DNA fragmentation was significantly different between the two groups. Cleavage rate and embryo quality showed significant correlation with leukocyte DNA integrity. The results showed that leukocyte DNA integrity could be used to identify individuals at high risk in order to reduce the extent of DNA damage in patients before ICSI in order to improve the subsequent outcome of this procedure.
between laboratories. In addition, there is a reasonably good linearity between the visual score and % T.

In line with these studies, the cut off value for leukocytes and sperm DNA migration in healthy humans has been defined as 10% T (or 10 arbitrary units) and 25%, respectively [15,16]. Therefore, the aim of this study was to evaluate the relation between sperm and leukocyte DNA damage as well as their relation with fertilization rate and embryo development in ICSI patients.

2. Materials and methods

The study received the approval of the Institutional Review Board of Isfahan Fertility and Infertility Center and Royan Institute. All chemicals were obtained from Merck (Germany), unless otherwise stated.

2.1. Study population

Semen and blood samples were obtained from 41 male partners of ICSI candidates referring to Isfahan Fertility and Infertility Center from April, 2007 to June, 2008. Azoospermic individuals and couples with less than four mature MII oocytes that survived the ICSI procedure were excluded from this study. Semen samples were divided into three portions and used for routine semen analysis according to the World Health Organization guidelines [17]. ICSI procedure, and for assessment of sperm DNA damage by the Comet assay and sperm chromatin dispersion tests.

In addition, the Comet assay was also carried out on blood leukocytes. Questionnaires mainly regarding occupation, duration of infertility, usage of drugs, alcohol and cigarettes were also completed by each couple.

2.2. Assessment of sperm DNA damage by Comet assay

One side of the common microscopic slides was precoated by dipping into 1% multipurpose agarose (Sigma). Ten μl of sperm, in PBS, were mixed with 140 μl of 0.75% low melting-point agarose (Sigma) dissolved in PBS. Then, the mixture was overlaid on the precoated slides, covered with a cover slip and allowed to solidify at 4°C for 10 min. At this point, the cover slips were removed and slides were immersed in lysing buffer (2.5 M NaCl, 100 mM ethylenediamine tetracetic acid (EDTA), 10 mM Tris (hydroxymethyl) aminomethane hydrochloride (Tris–HCl), NASLS 1% (Sigma), pH 10) for 2 h at room temperature in a dark room. The slides were removed from lysing solution and transferred into alkaline buffer: 300 mM NaOH, 1 mM EDTA, pH>13, for 40 min at 4°C, to allow the DNA to unwind. Electrophoresis was carried out for 20 min at 20 V and 300 mA at 4°C. Then slides were washed three times, each time for 5 min, in neutral buffer 0.4 M Tris–HCl (pH 7.5). The slides were then dehydrated in 96% alcohol, stained with 10 μg/ml ethidium bromide (Sigma) dissolved in distilled water and covered with cover slips for observation. The slides were viewed with a BX51 Olympus (Japan) fluorescent microscope. Visual and computerized scores were analyzed (Fig. 1A).

2.3. Assessment of leukocyte DNA damage by Comet assay

The blood Comet assay procedure is similar to the sperm Comet assay however instead of 10 μl of sperm, 10 μl of whole blood was used. The only differences were the time and type of lysing solution. The lysing buffer contained 2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, 10% dimethyl sulfoxide (DMSO) and 1% TritonX-100 (pH 10). The lysing step was carried out at 4°C for 1 h.

The analysis of Comet images were performed visually or by the automated software: Comet Assay Software Project (CASP). In visual scoring, an arbitrary score of zero to four was given to each Comet image according to the length of the visualized Comet tail. CASP was used to evaluate the percentage of Comet tail [18–20] (Fig. 1B). Percentage Comet tail was defined as the percentage of DNA in the sperm tail. The results were expressed as mean percentage tail Comet for sperm (Sperm % tail) or blood (Blood % tail).

2.4. Sperm chromatin dispersion test

Semen samples were washed with Ham’s F10 and then diluted to 5–10 million/ml. Sperm chromatin dispersion test was carried out according to Nazir-Esfahani et al. [21]. The slides were stained with Wright’s stain for light field microscopy. Sperm nuclei were considered as: intact or without fragmented DNA (large or medium-sized halos), whereas sperm showing small or no halos with solid staining of the core, or without a halo and irregular or faint staining of the core were considered as fragmented DNA or degraded. A minimum of 200 sperm per sample were scored under the 10× objective of the microscope [21].

2.5. Sperm preparation by density gradient centrifugation (DGC)

Pure Sperm gradients 40% and 80% were used for sperm preparation. All procedures were conducted under sterile conditions. Liquefied semen sample was added to the top of the upper layer (80%) and the tube was centrifuged for 20 min at 300 × g. The upper and lower layers were carefully aspirated without disturbing the pellet. The sperm pellet was washed twice and diluted with Sperm Rinse [22].

2.6. Intra-cytoplasmic sperm insemination procedure

All media were purchased from Vitrolife (Gothenburg, Sweden), G3 series plus, unless otherwise stated. After oocyte collection, the oocytes were treated in hyaluronidase (Hyase) in G-MOPS medium. Oocytes were then washed in fresh G-MOPS and transferred to G-oocyte under oil in a Falcon 1006 dish for microinjection. The Pure Sperm processed semen sample was also introduced into ICSI100 (a viscous sperm handling solution) in the same dish. An Eppendorf micromanipulator mounted on a Nikon inverted microscope was used to perform ICSI. Motile spermatozoa with the best morphology were selected for oocyte insemination. The injected oocyte was then washed and incubated in G1 medium.

Around 16–18 h post-ICSI, fertilization rate was assessed by the presence of pronuclei. The percentage of fertilization was calculated by the ratio of fertilized oocytes to the total number of survived injected metaphase II (MII) oocytes multiplied by 100 in both groups. The percentage of cleavage rate was calculated by the ratio of cleaved embryos to number of fertilized oocytes. Embryo quality was assessed at days 2 and 3 post-oocyte retrieval, using the 4-point score described by Giorgetti et al. [23]. All cleaved embryos were given 1 point and an additional point was added for each of the following features: absence of fragmentation (or fragmentation involving <25% of embryonic surface), absence of irregularities in blastomere size or shape, 4 cell stage for day 2 and 8 cell stage for day 3 [23].

Pregnancy rate was defined by ultrasonographic findings showing at least one fetal heart beat, 5 weeks after transfer, per embryo transfer. Implantation rate was defined by the number of fetal heart beats per transferred embryo. To reduce female factors, any patient with fewer than four mature MII oocytes that survived the ICSI procedure was excluded from this study. Furthermore, immature, deformed, and post-mature oocytes, or any oocyte with certain types of abnormality were excluded from this study [23].

2.7. Statistical analysis

The Kolmogorov–Smirnov Z test was used to assess the normal distribution of data. Coefficients of correlation and Student’s t-tests were carried out by using

Fig. 1. Show representative diagrams of DNA damage in sperm (A) and blood samples (B) measured by the Comet assay, respectively (400×).
the Statistical Package for the Social Sciences (SPSS 11.5, Chicago, IL) software to compare results between different groups.

3. Results

Table 1 shows the descriptive results for semen parameters, female and male ages, number of MII oocytes, cleavage rates, embryo quality scores on days 2 and 3, sperm and leukocyte DNA migration [expressed as Sperm % tail DNA (T%) and Blood % tail DNA (T%)], the percentage of sperm and leukocytes with a score of 0 (percentage of undamaged sperm and leukocytes assessed by visual score), and the percentage of DNA fragmentation in sperm using the sperm chromatin dispersion test.

Fig. 2A and B shows the percentage of undamaged sperm with a score of 0 or the Sperm % T in individuals with lower and higher than ten percent (Blood % T) DNA, respectively. The percentage of undamaged sperm with a score of 0 was significantly higher in individuals with lower than ten percent Blood % T DNA when compared to individuals with higher than ten percent (p = 0.027). In contrast, Sperm % T was significantly higher in individuals with higher than ten percent Blood % T as compared to individuals with lower than ten percent (p = 0.05).

The results in Table 2 show a comparison of different parameters between individuals with lower and higher than 25% Sperm % T. The percentages of leukocytes that scored 0 by visual scoring (84.83 ± 16.78 vs. 71.03 ± 20.76; p = 0.049), Blood % T (2.00 ± 1.25 vs. 6.6 ± 4.91; p = 0.002) and percentage DNA fragmentation assessed by SCD (26.00 ± 8.81 vs. 41 ± 11.41; p = 0.006), were significantly different between the two groups. In addition, the embryo quality score on day 3 was almost significantly different (6.75 ± 0.74 vs. 5.4 ± 2.41; p = 0.053). In addition the female age, male age and pregnancy rate between the two groups were compared, which was not significantly different.

Correlative analyses were carried out to examine the factors that were associated with Sperm % T. The results revealed significant relationships with Blood % T (r = 0.314; p = 0.045), percentage of sperm DNA fragmentation assessed by SCD (r = 0.713; p < 0.001) (Fig. 3), sperm density (r = −0.472; p = 0.002) and embryo quality score on day 2 (r = −0.314; p = 0.046).

The percentage of sperm with score of 0 also showed significant correlation with embryo quality score on day 2 (r = 0.385; p = 0.014), embryo quality score on day 3 (r = 0.392; p = 0.012) as well as the percentage of sperm DNA fragmentation assessed by SCD (r = −0.538; p = 0.002). A significant relationship was also observed between Blood % T with cleavage rate (r = −0.389; p = 0.012), embryo quality score on day 2 (r = −0.328; p = 0.036) and percentage of sperm with score 0 (r = −0.362; p = 0.012). In this study individuals were divided into: non- and routine smokers and individuals at low or high risk (individuals working in polluted industry or drivers and bakers). All the aforementioned parameters were compared between the two groups. The

Table 1
Descriptive analysis of different parameters.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female age</td>
<td>21.0</td>
<td>44.0</td>
<td>31.2 ± 5.6</td>
</tr>
<tr>
<td>Male age</td>
<td>25.0</td>
<td>56.0</td>
<td>36.8 ± 6.4</td>
</tr>
<tr>
<td>Number of MII oocytes</td>
<td>1.0</td>
<td>25.0</td>
<td>8.8 ± 5.8</td>
</tr>
<tr>
<td>Density (million/ml)</td>
<td>0.1</td>
<td>86.0</td>
<td>40.1 ± 26.1</td>
</tr>
<tr>
<td>% Abnormal sperm morphology</td>
<td>54.0</td>
<td>100.0</td>
<td>81.9 ± 10.9</td>
</tr>
<tr>
<td>% Sperm motility</td>
<td>2.0</td>
<td>69.0</td>
<td>32.3 ± 15.5</td>
</tr>
<tr>
<td>Fertilization rate</td>
<td>0.0</td>
<td>100.0</td>
<td>79.5 ± 21.3</td>
</tr>
<tr>
<td>Cleavage rate</td>
<td>0.0</td>
<td>100.0</td>
<td>84.4 ± 28.8</td>
</tr>
<tr>
<td>Embryo quality score on day 2</td>
<td>0.0</td>
<td>4.0</td>
<td>2.7 ± 0.9</td>
</tr>
<tr>
<td>Embryo quality score on day 3</td>
<td>0.0</td>
<td>4.0</td>
<td>2.0 ± 0.8</td>
</tr>
<tr>
<td>Blood % T</td>
<td>0.6</td>
<td>17.5</td>
<td>5.1 ± 4.6</td>
</tr>
<tr>
<td>Sperm % T</td>
<td>6.5</td>
<td>69.4</td>
<td>30.7 ± 12.6</td>
</tr>
<tr>
<td>% Sperm with score 0</td>
<td>10.0</td>
<td>78.0</td>
<td>38.7 ± 15.6</td>
</tr>
<tr>
<td>% Blood with score 0</td>
<td>21.0</td>
<td>100.0</td>
<td>75.1 ± 20.4</td>
</tr>
<tr>
<td>% DNA fragmentation (SCD)</td>
<td>11.0</td>
<td>78.0</td>
<td>37.9 ± 12.4</td>
</tr>
</tbody>
</table>

Table 2
A comparison of the mean number of different parameters between individuals with Sperm % T higher and lower than 25%.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sperm % T &lt;25%</th>
<th>Sperm % T &gt;25%</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male age</td>
<td>37.07</td>
<td>36.78</td>
<td>0.89</td>
</tr>
<tr>
<td>Female age</td>
<td>31.41</td>
<td>31.11</td>
<td>0.87</td>
</tr>
<tr>
<td>% Blood with score 0</td>
<td>84.83 ± 16.78</td>
<td>71.03 ± 20.76</td>
<td>0.049</td>
</tr>
<tr>
<td>Blood % T</td>
<td>2.00 ± 1.25</td>
<td>6.6 ± 4.91</td>
<td>0.002</td>
</tr>
<tr>
<td>DNA fragmentation (SCD)</td>
<td>26.00 ± 8.81</td>
<td>41 ± 11.41</td>
<td>0.006</td>
</tr>
<tr>
<td>Embryo quality score day 3</td>
<td>6.75 ± 0.74</td>
<td>5.4 ± 2.41</td>
<td>0.053</td>
</tr>
<tr>
<td>Pregnancy rate</td>
<td>20%</td>
<td>22%</td>
<td>0.807</td>
</tr>
</tbody>
</table>

Fig. 2. (A and B) Comparison of the mean percentage of sperm with intact DNA (score 0) and mean percentage of DNA in sperm tail measured (Sperm % tail) in individuals with less than or greater than 10 percentage DNA in blood tail (Blood % tail) as measured by the Comet assay. The differences for both parameters were significant at p < 0.05.
only parameter which was almost significant was sperm with a Comet score of 0 when compared between non-smokers and routine smokers (41.31 ± 16.18 vs. 49.2 ± 13.69; p = 0.053).

In this study, the exposure group consisted of 20 out of 41 individuals. Out of these 20 individuals, 8 individuals were farmers and 4 were workers who have been exposed to chemical substances, the remaining 8 individuals were either professional drivers or exposed to heat stress. Thirteen out of 41 individuals were smokers. Analysis of results revealed a significant difference in the percentage of individuals exposed (occupations and smokers) in Blood % T greater than 10% (83.3%) compared to those with Blood % T lower than 10% (37.1%) with p < 0.001. Similar results were obtained when the percentage of individuals exposed were compared in individuals with a Sperm % T higher than 25% (64.2%) to those with a Sperm % T lower than 25% (23.1%), with p < 0.001.

4. Discussion

Alkaline Comet assay, originally known as the Single Cell Gel Electrophoresis assay, assesses actual DNA strand breaks and alkaline labile sites when used under alkaline conditions. This test has been shown to have a prognostic value for male infertility and assisted reproductive technology outcomes.

One area of research that has been studied as a cause of male infertility is the integrity of sperm DNA which is affected by exogenous and endogenous factors. Indeed, it has been estimated that 10,000 oxidation hits occur to the DNA of each cell per day within the human body and more than 35 different forms of oxidized bases are found in DNA in vitro [24,25]. Most of these damages are repaired by effective DNA-repair enzymes, but some escape from repair causing permanent damage. Elevated levels of DNA damage have been observed in the leukocytes of persons in putatively high-exposure circumstances either due to occupation or treatment with antineoplastic agents, although data from occupational studies are conflicting [26].

Determination of exogenous factors and their impact on germ cells is difficult because of different lifestyles, working conditions and duration of exposure. Therefore, this study evaluates the correlation between leukocytes and sperm DNA fragmentation in patients undergoing ICSI and effects of DNA damage on ICSI outcome. Thus, we use the cut off values of 10% leukocyte tail DNA, as stated in the literature, and divided individuals into high and low exposure groups. This study also has shown that when individuals were grouped for Blood % T at values lower and higher than 10%, the percentage of individuals exposed were significantly different between the two groups.

The results of Fig. 2A and B show that, in the low exogenous exposure group, the percentage of sperm with a score of 0 are significantly higher and Sperm % T is lower than the high exposure group. In addition, it has been shown that sperm concentration and total sperm count decline in workers exposed to different classes of toxic agents, such as dibromochloropropane and lead [27–29].

This finding is consistent with our results that indicate a significant correlation between mean sperm density with Sperm % T. Therefore, it suggests that the exogenous factors affect leukocyte and germ cell DNA integrity concomitantly. In addition, when individuals are grouped according to Sperm % T lower and higher than 25%; it is discovered that the percentage of individuals exposed is significantly different between the two groups. Therefore, a similar phenomenon is observed when patients are divided according to low and high sperm DNA damage. Individuals with higher than 25% Sperm % T have more leukocyte DNA fragmentation than individuals with lower than 25% (Fig. 2) thus indicating that the exogenous factors involved in sperm DNA damage are also involved in leukocyte DNA damage. This fact is further confirmed by the significant correlation observed between blood and measured sperm Comet parameter, further suggesting that the individuals with low leukocyte DNA damage have a high percentage of sperm with intact DNA or vice versa. Oxidative damage is thought to accumulate by age [30–32]. Therefore, in this study we also evaluated the age of individuals in the two groups. No significant difference was observed between the two groups. Therefore, the above results could not be due to age differences. In addition, no significant difference was observed between the two groups in terms of pregnancy rate. Our finding is supported by Migliore et al. who obtained a positive correlation between genotoxic damage detected in somatic and germ cells during a biomonitoring study in a group of male workers exposed to styrene [32].

It is interesting to note that our analysis did not show any correlation between DNA fragmentations in the leukocyte with DNA fragmentations assessed by the sperm chromatin dispersion test. On the other hand, a significant negative correlation was observed between the percentage of sperm with a score of 0 and the percentage of sperm DNA fragmentation assessed by SCD or Sperm % T with the percentage of sperm DNA fragmentation assessed by SCD. This difference may point to the fact that Comet assays even measure low levels of strand break with high sensitivity, whereas SCD measures global DNA fragmentation. Thus, this could explain the insignificant correlation observed between SCD and leukocyte DNA damage.

In this study we also divided individuals into non-smokers and routine smokers and we compared different parameters between the two groups. The only difference which was almost significant was the percentage of intact sperm with a Comet score of 0. Since this difference was not observed for blood Comet, this might suggest that cigarette smoking has a more direct effect on sperm DNA integrity than the blood. Indeed there are different studies which propose the deleterious effect of cigarette derived toxin on male reproductive potential [33–34]. A similar comparison was carried out for individuals at risk of low and high exposure. No significant difference was observed between the two groups. This observation can be explained by different lifestyles which are difficult to identify diverse exogenous factors and label individuals to either the low or high risk groups. In addition, different duration and intensity of exposure to exogenous factors should be considered.

During this study a significant negative correlation was observed between Blood % T with cleavage rate and embryo quality score on day 2 suggesting that, in patients possibly exposed to DNA damaging exogenous agents, the likelihood of embryos arresting at pronuclear stage increases and the quality of embryos decreases. Thus, exposure to exogenous agents may increase the mean DNA fragmentation in germ cells; and if the extent of DNA damage is high, the oocytes may not have the ability for repair, resulting in pronuclear arrest. However, it is important to note that the ability of DNA to cope with the extent of DNA damage depends on the oocyte quality and female age.

The results of this study also reveal a significant correlation between the percentage of sperm with a score of 0 and embryo quality scores on days 2 and 3. This suggests that the percentage of intact sperm in a sample influences embryo quality, which has an important impact on ART outcome. Furthermore, determination and overcoming factors which influence sperm DNA integrity could have important consequences on ART outcomes.

5. Conclusion

Therefore, considering the effect of exogenous factors on both somatic and germ cells, the importance of assessing genotoxicity in human somatic and germ cells will continue to have an important role in the future of male infertility management, particularly with concerns about the detrimental effects on reproduction in terms of male infertility and sperm count. Thus, by using a simple test such
as the blood Comet assay, it is possible to identify individuals possibly exposed to exogenous genotoxic agents. By identification and prevention of exposure, and the use of appropriate sperm selection procedures [35], it might be possible to increase the number of intact sperm available for insemination, especially in couples undergoing ICSI in order to improve the chance of fertilization and subsequent development.

Conflict of interest

No competing interests.

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References