Article

Correlation of sperm DNA damage with protamine deficiency in Iranian subfertile men

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Abstract

To compare the extent of sperm DNA damage with the degree of protamine deficiency in spermatozoa of normal and subfertile individuals, 30 semen samples from three groups of subfertile individuals (oligozoospermic, asthenozoospermic and oligoasthenozoospermic) and 14 samples from normal individuals were collected from men referred to the Fertility and Infertility Centre of Shariati Hospital, Tehran. DNA damage was measured using the alkaline Comet assay, and protamine deficiency was measured using chromomycin A3 (CMA3) staining. Results indicated a significant difference in the extent of DNA damage in spermatozoa of subfertile patients compared with normal patients ($P < 0.01$). Spermatozoa from oligoasthenozoospermic patients showed a higher level of DNA damage compared with the other two study groups of subfertile men. The percentage of CMA3-positive spermatozoa was also higher in subfertile individuals compared with normal patients ($P < 0.01$), with the highest level occurring in oligoasthenozoospermic patients. A direct correlation between protamine deficiency and sperm DNA damage was found for all subfertile patients studied.

Keywords: *CMA3+, Comet assay, DNA damage, male subfertility, protamine deficiency, spermatozoa*

Introduction

Sperm DNA carries one-half of the genomic material of the offspring. Thus, normal sperm genetic material is required for fertilization, embryo and fetal development and post-natal child wellbeing. Abnormal DNA can lead to derangements in any of these processes. The abnormality or defect in the genomic material may take the form of condensation or nuclear maturity defects, DNA breaks or DNA integrity defects and sperm chromosomal aneuploidy. Sperm nuclear defects might be the reason for a world wide decreasing trend in male fertility in terms of average sperm count and sperm quality in developed countries (Carlsen *et al.*, 1992; Auger *et al.*, 1995). Routine semen parameters may not always reflect the quality of sperm DNA (Perreault *et al.*, 2003).

The known coexistence of different sperm cell types differing in their motility or in their morphology in sperm samples from a single ejaculate suggests that biochemical compositional differences in the sperm nuclei could also be present. Mengual *et al.* (2003) did not find a correlation between the motility and morphology of spermatozoa and the concentrations of protamines in sperm nuclei. Reports have raised concern about decreasing male fertility caused by genomic abnormalities (for review, see Speit *et al.*, 2008). The causes of sperm DNA damage, much like those of male infertility, have many factors and may be attributed to interior extra-testicular factors (e.g. drugs, chemotherapy, radiation therapy, cigarette smoking, environmental toxins, genital tract inflammation, testicular hyperthermia, varicocele, hormonal factors and so on). Sperm DNA damage is clearly associated with male infertility (and abnormal spermatogenesis), but a small percentage of spermatozoa from fertile men also possess detectable levels of DNA damage (Zini and Libman, 2006). Reactive oxygen species (ROS) might also be associated with human infertility and oxidative stress might play a critical role in the aetiology

of defective sperm function and male infertility (Gomez *et al.*, 1996; Aitken *et al.*, 2003).

The presence of damaged DNA could also arise from problems in nuclear remodelling resulting directly from defective protamine deposition during spermiogenesis (Sakkas *et al.*, 2002). During spermiogenesis, the somatic cell histones are replaced by the protamine proteins, a process that results in a highly condensed transcriptionally silent chromatin. Sperm chromatin packaging occurs in a two-step process. In the first step, the transition nuclear proteins (TP1 and TP2) replace the somatic cell histones. In the second step, during the elongating spermatid stage, the sperm protamine proteins replace the transition proteins (Oliva and Dixon, 1991). In humans, there are two protamine proteins: protamine-1 (P1) and protamine-2 (P2), which occur in a strictly regulated one-to-one ratio (Corzett *et al.*, 2002). One of the functions of protamines is that they could be involved in the protection of the genetic codes delivered by the spermatozoa (Oliva and Dixon, 1991; Mengual *et al.*, 2003). Spermatozoa with low protamine concentrations retain higher concentrations of histone 2B which may be less effective in protecting sperm DNA from damage (Aoki *et al.*, 2006; Zhang *et al.*, 2006). Incomplete protamination could render the spermatozoa more vulnerable to attack by endogenous or exogenous agents, such as nucleases (Szczygiel and Ward, 2002; Sotolongo *et al.*, 2003), free radicals (Irvine *et al.*, 2000; Alvarez *et al.*, 2002) or mutagens. Poor chromatin packaging and possible DNA damage may also contribute to failure of sperm decondensation in ooplasm and subsequently result in fertilization failure (Bianchi *et al.*, 1996). Damaged DNA has been observed in testicular epididymal and ejaculated spermatozoa. Sperm DNA first become susceptible to damage if chromatin packaging is not complete during spermatogenesis (Manicardi *et al.*, 1995; O'Brien and Zini, 2005). Sperm protamine deficiency is observed in a subset of infertile men, suggesting that the relative histone to protamine ratio may be altered in the spermatozoa of these men (Nasr-Esfahani *et al.*, 2008).

Of many tests available for assessment of sperm DNA damage, the alkaline Comet assay, TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labelling (TUNEL) and sperm chromatin structure assay have been used most frequently (see for review Agarwal and Said, 2004; Lewis and Aitken, 2005). The 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 (see for review Lewis and Agbaje, 2008;Speit *et al.*, 2008). The Comet assay is already recognized as being among the most sensitive methods available for measuring DNA strand breaks (Leroy et al., 1996); it has further advantages of speed (Fairbairn et al., 1995), reproducibility (Hughes et al., 1997), simplicity, and the fact that observations are made at the level of single cells. The alkaline Comet assay can detect damage equivalent to as few as 50 single-stranded breaks per cell. DNA damage can also be assessed indirectly by means of sperm chromatin integrity assays and by evaluation of nuclear protein concentrations. Sperm chromatin integrity assays include slide-based sperm nuclear protein stains (e.g. aniline or toluidine blue) which detect histones, and chromomycin A3 (CMA3) staining which detects under-protamination (Lolis *et al.*, 1996).

Therefore, to investigate the existence of a correlation between protamine deficiency and DNA integrity, this study evaluated the extent of DNA damage using the alkaline Comet assay and protamine deficiency using CMA3-positive (CMA3+) staining of spermatozoa from three subfertile groups (oligozoospermia, asthenozoospermia and oligoasthenozoospermia) and compared the results with samples obtained from normozoozoospermic individuals.

Materials and methods

Sperm preparation and morphology assessment

The study was approved by the Ethical Committee of the School 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 lifestyle, such as dietary habits, medical history and exposure to chemical and physical agents. Therefore, all samples were screened to exclude radiation exposure, smokers, varicocele, genital tract infections, hepatitis, and human immunodeficiency virus antibodies. Semen samples were obtained randomly from 44 subfertile male candidates for assisted reproduction treatment, and referred to the Fertility and Infertility Centre, Shariati Hospital (Tehran, Iran). In all cases, after 3 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 after which liquefaction was complete. Semen profiles were then performed and classified according to World Health Organization criteria (World Health Organization, 1999) and classified into four groups (normal, oligozoospermia, asthenozoospermia, and oligoasthenozoospermia). Samples were processed by swim-up techniques from a pellet, as described by Aitken and Clarkson (1988). A 200 µl volume of semen was removed for protamine deficiency assessment, and the rest was used for the alkaline Comet assay.

Sperm Comet assay

The alkaline single-cell gel electrophoresis (Comet) assay was performed based on existing methods described by McKelvey-Martin *et al.* (1993), Hughes *et al.* (1996) and Singh (1996), with minor modifications. Briefly, all procedures were carried out under yellow light to prevent possible further induction of DNA damage in spermatozoa. Semi-forested microscope slides were gently heated, covered with 100 µl 0.5% normal-melting-point agarose in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (Sigma, USA) at <45°C and immediately covered with coverslip. The slides were placed in a chilled metal tray and left at 4°C for at least 30 min to allow the agarose to solidify. The coverslips were then removed, and 10 µl spermatozoa suspension in 10 µl Biggers-Whitten-Whittingham medium was mixed with 75 µl 0.5% low-melting-point agarose (Sigma, USA) at 37°C. This suspension was rapidly pipetted on the top of the first agarose layer, then covered with a coverslip and allowed to solidify at 4°C. The cells were then lysed by removing the coverslip and immersing the slides in a Coplin jar that contained freshly prepared cold lysis solution (2.5 mol/l NaCl, 100 mmol/l EDTA, 10 mmol/l Tris, pH 10; with 1% Triton X-100 added just before use; all chemicals from Sigma,

USA) for 1 h at 4°C. Slides were then incubated for 30 min at 4°C with 10 mmol/l dithiothreitol (DTT; Sigma) followed by incubation for 90 min at 20°C with 4 mmol/l lithium diiodosalicylate (LIS; Sigma) in order to decondense the DNA. The slides were removed from the lysis solution and drained. A horizontal gel electrophoresis tank was filled with fresh alkaline electrophoresis solution (300 mmol/l NaOH, 1 mmol/l EDTA, pH 13; Sigma) at 4°C. The slides were placed into this tank side by side. The slides were left in this high-pH buffer for 20 min at 4°C to allow the sperm DNA to unwind. The DNA fragments were then separated by electrophoresis for 10 min at 25 V (0.7 V/cm) adjusted to 300 mA by raising or lowering the buffer concentration in the tank. After electrophoresis the slides were drained, and flooded with three changes of neutralization buffer (0.4 mol/l Tris, pH 7.5; Sigma), each for 5 min. This buffer removed any remaining alkali and detergents which would interfere with ethidium bromide staining. Cells were stained with 20 µl ethidium bromide (2 µg/ml; Merck) under a coverslip. Observations were made at a magnification of ×200 using a Nikon E800 epifluorescence microscope (Japan) equipped with a 546–516 nm wavelength band and a 590 nm barrier filter. The Comets were analysed by visual classification (Kobayashi *et al.*, 1995), 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 (Visvardis *et al.*, 1997; Dusinska *et al.*, 2002; Shahidi *et al.*, 2007). Comets with a bright head and no tail were classified as class 0 (cells with no DNA migration) and Comets with a small head and a long diffuse tail were classified as class 4 (severely damaged cells). Comets with intermediate appearance were classified into classes 1, 2 and 3. Damage scores were calculated based on the following equation adopted from Jaloszynski *et al.* (1997) that ranged from 0 to 400 arbitrary units, corresponding to situations ranging from no damaged Comets to all Comets extremely damaged:

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

Where DD (au) is DNA damage score in arbitrary units, n_0–n_4 is the number of class 0–4 Comets, and Σn is the total number of scored Comets.

Coefficients 0–4 are weighting factors for each class of Comet. One may suspect that the visual classification may be inferior to computerized analyses, such as tail moment analysis of images captured by digital camera. However, it has been clearly shown that there is no statistical difference between visual quantification and image analysis by computer for tail moment quantification (Kobayashi *et al.*, 1995; Shahidi *et al.*, 2007).

CMA3 staining

Semen smears prepared from 200 µl samples were fixed in Carnoy's solution (methanol:glacial acetic acid 3:1; Sigma) at 4°C for 5 min. For CMA3 staining, each slide was treated for 20 min with 100 µl CMA3 solution (0.25mg/ml in McIlvain buffer: 7 ml citric acid (0.1 mol/l) + 32.9 ml $Na_2HPO_4.7H_2O$ (0.2 mol/l, pH 7.0, containing 10 mmol/l $MgCl_2$); all chemicals from Sigma). The slides were then rinsed in buffer and mounted with buffered glycerol (1:1). Microscope analysis of the slides was performed using an epifluorescence Nikon E800 microscope (Japan), with an appropriate filter (460–470 nm). CMA3 staining was evaluated by distinguishing between spermatozoa with bright yellow staining (CMA3+ or protamine deficient) and spermatozoa with dull yellow staining (CMA3 negative) (Nasr-Esfahani *et al.*, 2004).

Statistical analysis

Results were analysed using the Statistical Package for Social Sciences (SPSS) software version 16.0 (SPSS, USA). The non-parametric Mann–Whitney *U*-test was used to compare differences between types of infertility, and non-parametric Spearman's rho test was used for determination of the correlation coefficient. Sigma plot 2004 for Windows, version 10.0, was used to draw figures. $P < 0.05$ was considered as statistically significant.

Results

Demographic characteristics of the populations in the four study groups (normozoospermia, oligozoospermia, asthenozoospermia and oligoasthenozoospermia) as well as their relevant DNA damage and percentage of CMA3+ spermatozoa are summarized in **Table 1**. The mean (± SD) ages of all study groups were not significantly different from each other. Control samples were assigned as normal based on sperm concentration and motility. The individuals who supplied the

Table 1. Characteristics of the four groups of subfertile men and comparison of the DNA integrity and chromomycin A3 (CMA3) staining of their spermatozoa.

	Study group			
	Normal (n = *14*)	*Oligozoospermia* (n = *10*)	*Asthenozoospermia* (n = *10*)	*Oligoasthenozoospermia* (n = *10*)
Age (years)	33.3 ± 5.6	34.1 ± 7.9	34.5 ± 5.5	34.4 ± 6.9
Infertility (years)	6.6 ± 5.3	8.5 ± 6.6	5.9 ± 2.5	6.5 ± 4.4
Sperm concentration (×10^6/ml)	68.6 ± 20.4	12.4 ± 2.7	42.1 ± 18.0	9.2 ± 4.0
DNA damage (%)[a]	30.4 ± 2.1	37.7 ± 1.6	49.9 ± 4.0	64.1 ± 4.0
CMA3+ (%)[b]	21.1 ± 4.8	33 ± 6.7	45 ± 5.1	62.6 ± 7.8

Values are mean ± SD.
[a,b]There was a statistically significant difference between the normal group compared with the study groups and also between study groups themselves ($P < 0.001$).

control samples had experienced the same period of infertility as those in the study groups, but due to recurrent miscarriage rather than any male factor aetiology. Sperm counts showed a distinct difference between the study groups, with the lowest counts for oligoasthenozoospermia samples (9.2 ± 4) and the highest counts for normozoospermic individuals (68.6 ± 20.4) (**Table 1**).

As shown in **Figure 1,** a logical distribution of Comets with different degrees of damage was observed for each study group. Spermatozoa that were more severely damaged showed a higher frequency of Comets with higher scores. However, in normozoospermic samples about 30% DNA damage was observed; this percentage increased for all samples from subfertile groups, with the highest level of DNA damage (about 70%) for oligoasthenozoospermia (**Table 1**, **Figure 2**). As seen in **Figure 1**, the frequency of Comets with a higher degree (more damaged cells) increased in male factor samples, while the frequency of Comet 0 (undamaged cells) decreased. This indicates that the difference seen as percentage of DNA damage in the different study groups (**Figure 2**) was not random and is characteristic of the spermatozoa in each study group. The highest level of DNA damage was observed for oligoasthenozoospermia samples (about 70%). There was a statistically significant difference between DNA damage observed in the normal group compared with the other study groups and also between study groups themselves ($P < 0.001$). A similar trend of DNA damage was observed for CMA3+ spermatozoa in the study groups (**Figure 2**). In normal samples, about 20% of spermatozoa were seen to be CMA3+. This value increased to 50% for asthenozoospermia and to about 70% for oligoasthenozoospermia samples. The difference was statistically significant for all study groups compared with normal and between the study groups themselves (**Figure 2**, $P < 0.001$).

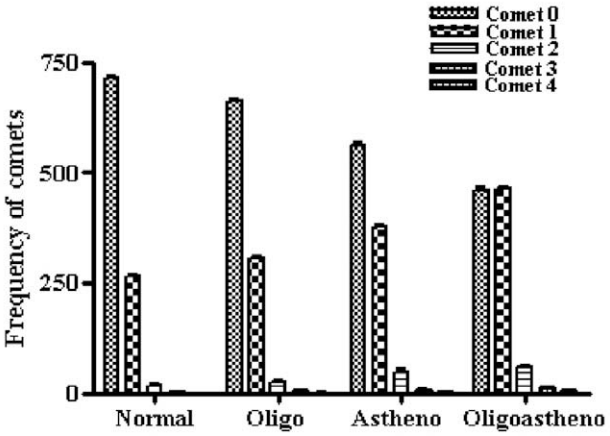

Figure 1. Frequency of Comet types (0, 1, 2, 3 and 4) in spermatozoa for the normal and subfertile groups. Comet 0 decreased as the level of DNA damage increased in each group. Distribution of various Comet types in each category is indicative of the extent of DNA damage in spermatozoa of each study group.

A very similar range of distribution was found for the results of the DNA damage and CMA3+ and is marked by the boundaries of the boxes that represent the 25th and 75th percentiles (**Figure 2**). A good correlation was found between CMA3+ samples and sperm concentration ($r = –0.665$, $P < 0.001$) and DNA damage ($r = 0.909$, $P < 0.001$) but there was no significant difference or correlation between CMA3+ samples according to age or duration of infertility (**Table 2**). As shown in **Figure 3**, there was a good linear correlation between DNA damage and protamine deficiency measured as CMA3+ spermatozoa ($r = 0.909$, $P < 0.001$).

Discussion

In this work, semen samples from normozoospermic, oligozoospermic, asthenozoospermic and oligoasthenozoospermic men were processed by the swim-up technique, and the relative DNA damage and protamine deficiency in these samples was determined. As is evident from **Table 1**, the mean age of all study groups was similar; therefore an effect of age on the results of either DNA damage or protamine deficiency was not expected; however, it has been shown previously that sperm DNA damage, as well as protamine deficiency, increase with age (Angelopoulou *et al.*, 2007; Vagnini *et al.*, 2007). The lack of existence of a correlation

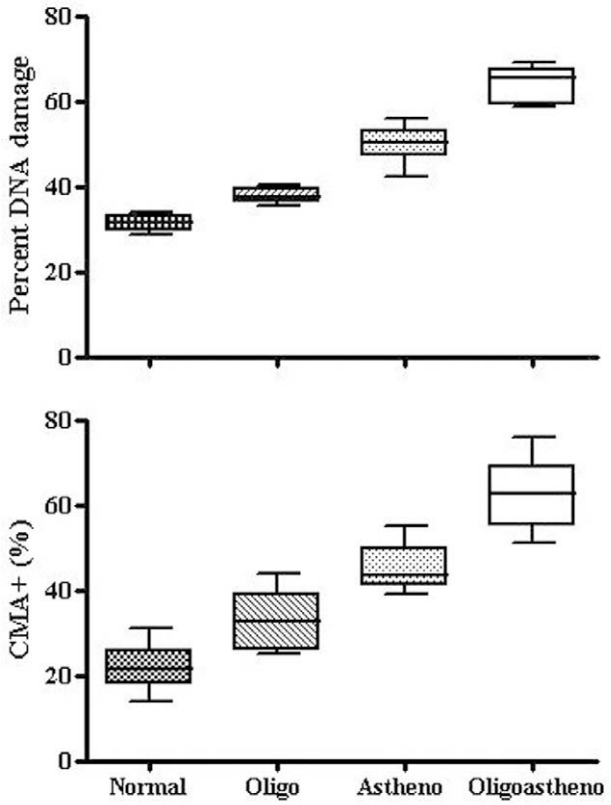

Figure 2. Box plots showing percentage of DNA damage and chromomycin A3 positive (CMA3+) for spermatozoa of normal and subfertile individuals. The boxes extend from the 25th percentile to the 75th percentile, with a horizontal line at the median (50th). The whiskers represent the 10th and 90th percentiles.

between age and protamine deficiency (**Table 2**) or DNA damage in the present study might be due to the low number of samples studied. The impaired semen quality observed in the patient population has been shown to be associated with a significantly increased rate of DNA damage (Irvine *et al.*, 2000); however, the data obtained in the present study do not support this observation because, as seen in **Table 1**, the extent of DNA damage in oligozoospermia, with mean number of 12 × 10^6 spermatozoa/ml was significantly lower than the DNA damage observed for asthenozoospermia samples, with a mean number of 42 × 10^6 spermatozoa/ml.

In samples from normozoospermic individuals, a relatively high percentage of DNA damage was observed (**Table 1**, **Figure 2**). Baseline DNA damage in spermatozoa in most of the published studies, although variable, is significantly higher than in somatic cells. The nature of a high background level of DNA strand breaks in spermatozoa identified with the Comet assay is still unknown. Apart from natural physiological events leading to DNA damage induction, such as production of ROS (Hauser *et al.*, 2007) and normal differentiation programme (Laberge and Boissonneault, 2005), one of the reasons for high background DNA damage associated with the Comet assay might be due to using different protocols for lysis/decondensation and different pH values during denaturation and or electrophoresis (for review see Speit *et al.*, 2008). The high background DNA damage (about 30%) observed in this study (**Table 1**, **Figure 2**) might be due to the use of DTT/LIS for decondensation with a pH of 12.5–13; this is consistent with the recent report of 32% DNA fragmentation in spermatozoa of healthy control subjects by Agbaje *et al.* (2007).

The increased level of DNA damage in spermatozoa of subfertile compared with normal individuals and also a high degree of DNA damage in oligoasthenozoospermia patients (**Figure 2**) may be due to different factors affecting spermatozoa, including ROS (Hauser *et al.*, 2007), damage mediated by heavy metals or toxins interacting with protamines (Quintanilla–Vega *et al.*, 2000), incomplete repair during meiosis (Baarends *et al.*, 2001), malfunction of topoisomerase II during spermiogenesis (Marcon and Boissonneault, 2004), incomplete removal of apoptotic cells (Sakkas *et al.*, 2002; Weng *et al.*, 2002), infection and increased oxidant action of leukocytes (Aitken *et al.*, 1995; Alvarez *et al.* 2002), increased aging and oxidation of spermatozoa during passage and storage in the male tract (Ollero *et al.*, 2001, Suganuma *et al.*, 2005), and incomplete protamination resulting in increased susceptibility of DNA (Cho *et al.*, 2001; Aoki *et al.*, 2005). As spermatozoa are particularly susceptible to ROS-induced damage due to the presence of large quantities of polyunsaturated fatty acids and low concentrations of scavenging enzymes in their plasma membranes, ROS have received special attention due to their significant role in both the physiology and pathology of human reproduction (Kodama *et al.*, 1997). However, results shown in **Figures 2** and **3** clearly support the hypothesis of aberrant chromatin packing during spermiogenesis.

Although a lower background level of CMA3+ spermatozoa (less than 10%) has been reported for healthy fertile individuals (Angelopoulou *et al.*, 2007), the background level of CMA3+ spermatozoa in samples from normozoospermic individuals was about 21% (**Table 2**). However, the percentage of DNA damage in normal spermatozoa in the present study and the percentage of DNA fragmentation in TUNEL-positive spermatozoa reported by Angelopoulou *et al.* (2007) showed good correlation with the percentage of CMA3+ spermatozoa in both studies, indicative of involvement of chromatin remodelling in DNA damage, as shown by Laberge and Boissonneault (2005).

As clearly shown in **Figure 2**, the amount of CMA3+ spermatozoa in subfertile groups was significantly different from normal ($P < 0.001$) and was found to be highest for oligoasthenozoospermic individuals with no statistically significant inter-individual differences. Nucleoprotein exchange occurring in spermatids involves the replacement of somatic histones by transition proteins and the deposition of protamines that remain present in mature spermatozoa. Sperm chromatin structure is being established during spermiogenesis and this process seems to involve the appearance of transient DNA strand breaks coincident with the chromatin remodelling steps (Laberge and Boissonneault, 2005). An alteration in the condensation state of the sperm head has been previously shown to be correlated with an increase in DNA fragmentation in the mature spermatozoa (Gorczyca *et al.*, 1993). In addition, a correlation has also been established between low concentrations of protamines and nicking of DNA (Bianchi *et al.*, 1993). Torregrosa *et al.* (2006) do not support a general correlation between DNA integrity and

Table 2. Correlation of protamine deficiency (chromomycin A3 positivity of spermatozoa) with age, sperm concentration, DNA damage and duration of infertility (Spearman's rho for correlation coefficient).

Variable	R	P-*value*
Age	0.149	NS
Sperm concentration	-0.665	<0.001
DNA damage	0.909	<0.001
Duration of infertility	0.173	NS

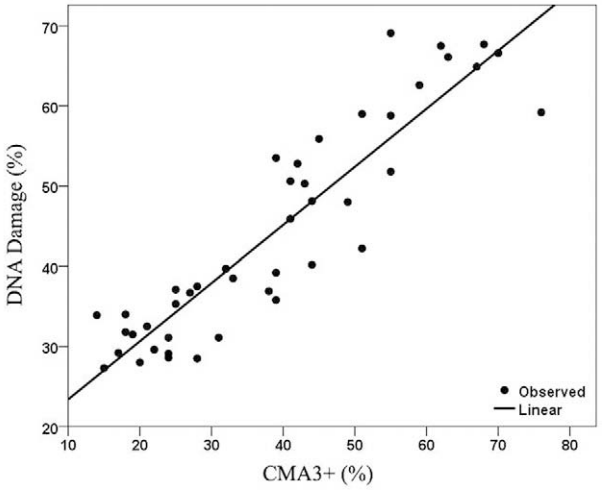

Figure 3. Linear correlation between protamine deficiency (chromomycin A3 positivity [CMA3+] of spermatozoa) and DNA damage assessed by the alkaline Comet assay ($r = 0.909$), ($P < 0.001$).

defects in protamine processing but instead, consistent with all proposed hypotheses, suggest that there are multiple independent causes for lower DNA integrity in the different patients.

A substantial variation in the percentage of CMA3-stained cells was observed in ejaculated human spermatozoa, varying between 8% and 77%. A strong negative correlation (r =–0.665, $P < 0.001$) was found between sperm count and the percentage of CMA3-stained spermatozoa (**Table 2**). No correlation was found between CMA3-stained spermatozoa and their motility, while excessive sperm morphological abnormalities were related positively to CMA3 staining. As clearly shown in **Figures 2** and **3**, there is a high correlation between CMA3+ spermatozoa and the extent of DNA damage in spermatozoa of subfertile individuals; this is in line with previous reports (Aoki *et al*., 2006; Zhang *et al*., 2006). These results demonstrate a close relationship between CMA3 staining and DNA damage, suggesting potential application of this marker for the prediction of sperm quality and fertilizing capacity (Lolis *et al*., 1996).

There now exists clinical evidence to show that sperm DNA damage is detrimental to reproductive outcomes. Morphological sperm parameters are important up to the fertilization step, while the DNA integrity becomes the most important sperm parameter related to the establishment and continuation of a pregnancy (Tomlinson *et al*., 2001). After the third stage of cleavage, the paternal genome exerts a major influence (Tesarik *et al*., 2006) and evidence of DNA damage is reflected in impaired embryonic development. It has been shown that poor chromatin packaging and possible DNA damage may also contribute to failure of sperm decondensation after intracytoplasmic sperm injection and that this may subsequently result in fertilization failure (Bianchi *et al*., 1996). A negative significant correlation between fertilization rate and CMA3 staining or P1/P2 ratio measured directly by electrophoresis, as well as embryo cleavage score with DNA fragmentation and protamine-deficient spermatozoa, has been reported (Nasr-Esfahani *et al*., 2004, 2005).

The amount of DNA damage and protamine deficiency in groups of subfertile (normal, oligozoospermia, asthenozoospermia and oligoasthenozoospermia) men is indicative that human sperm protamine content is significantly related to DNA fragmentation (r = 0.909, P < 0.001) (**Table 2**, **Figure 3**). The findings suggest that population-based measures of DNA integrity are significantly correlated with population-based measurements of protamine concentrations. Therefore, sperm protamine concentration has a negative correlation with DNA fragmentation, indicating a possible protective role of the protamine against sperm DNA damage.

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