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Cytochemical evaluation of sperm chromatin and DNA integrity in couples with unexplained recurrent spontaneous abortions

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Summary

The aim of this study was to examine the possible relationship between sperm DNA integrity and chromatin packaging evaluated by cytochemical assays, traditional sperm parameters and recurrent spontaneous abortion (RSA) of unknown origin. In this cohort study, 40 couples with a history of RSA and 40 couples with proven fertility were considered as case and control groups respectively. The semen samples of all husbands were analysed for sperm parameters and also sperm chromatin and DNA integrity assessed using cytochemical tests including aniline blue (AB), chromomycin A3 (CMA3), toluidine blue (TB), acridine orange (AOT) and nuclear chromatin stability assay. Among different sperm parameters, only slow motility was significantly different between the two groups. In sperm chromatin evaluations, there were significant differences between the two groups in all of the tests. In addition, the majority of semen samples in RSA patients exhibited upper percentages of abnormal spermatozoa than the cut-off values regarding different cytochemical assays. Our study showed that in the cases of RSA, slow motility had a significant reduction in comparison with controls and also spermatozoa of men from RSA group had less chromatin condensation and poorer DNA integrity than spermatozoa that obtained from fertile men with no history of RSA.

Introduction

'Recurrent spontaneous abortion' (RSA) or 'recurrent pregnancy loss' is expressed as three or more continuous terminated pregnancies before 20 weeks of gestation, which affects about 1% of couples trying to have a baby (Rai & Regan, 2006). It is shown that the frequency of RSA is about 24% following two clinical pregnancy losses, 30% after three and 40% after four habitual spontaneous abortions (Regan *et al.*, 1989). Although recurrent abortions may be associated with anatomical, genetic, endocrine, psychological, thrombotic, infectious and immunological causes, more than half of the cases remain unexplained, even following extensive evaluations (Andrew & Claire, 2005).

Male gamete supplies fifty per cent of the embryonic genome, so it is supposed that recurrent pregnancy losses may have a paternal effect (Sutovsky & Schatten, 2000; Gil-Villa *et al.*, 2009). 'Paternal effect' is a new term which indicates cases where normal pre-implantation embryos are formed, but they fail to implant or are lost soon after clinical pregnancy. There is an obvious link between sperm DNA damage and reproductive outcome (reviewed by Tarozzi *et al.*, 2007). Recent evidence shows that male partners of couples with idiopathic RSA have poorer sperm functional capabilities, and in cases of fertilisation, the developing embryos may have such (Gil-Villa *et al.*, 2009) anomalies resulting in early pregnancy loss (Saxena *et al.*, 2008). While the relation between standard semen parameters and recurrent pregnancy loss

is still controversial (Sbracia *et al.*, 1996; Gopalkrishnan *et al.*, 2000; Sexena *et al.*, 2008), several studies have indicated an increase in rates of sperm chromosomal aneuploidy, sperm DNA and chromatin condensation anomalies, male germ cell apoptosis and decreased invitro sperm chromatin decondensation rate in semen samples of male partners from couples with RSA in comparison with fertile controls (Evenson *et al.*, 1999; Carrell *et al.*, 2003a,b; Bernardini *et al.*, 2004). In addition, more than half of spontaneous pregnancy losses show aneuploidies, genetic and epigenetic anomalies (Philipp & Kalousek, 2002).

Several studies confirmed that in cases of natural pregnancy miscarriages, sperm DNA integrity has diminished in comparison with fertile couples (Evenson *et al.*, 1999; Carrell & Liu, 2001; Loft *et al.*, 2003; Bhattacharya, 2008). Although the results of a meta-analysis study have shown a well association between sperm DNA integrity and successful natural pregnancies (Evenson & Wixon, 2008), a more recent study indicates that sperm DNA damages do not relate to unexplained RSA (Bellver *et al.*, 2010). It seems that the extent of sperm DNA damage is also important in terms of fertilisation rate and pregnancy achievement. Nijs *et al.* (2009) showed that the threshold value of [high DNA stainability in sperm chromatin structural assay (SCSA)] about 15% can predict good fertilisation rate and reaching pregnancy in IVF cycles.

In addition to the importance of DNA integrity, it is believed that sperm chromatin condensation has also a critical role in male fertility, early embryonic development and pregnancy outcome (Zini *et al.*, 2001a,b).

In the process of spermiogenesis, the degree of compaction of sperm chromatin changes considerably as histones are replaced in a step manner by testis-specific nuclear proteins, transitional proteins and finally by protamines (Aoki & Carrell, 2003; Dadoune, 2003). Any abnormality in expression of each kind of sperm-specific nucleoprotein alters sperm chromatin structure and may cause male infertility (Carrell & Liu, 2001; Oliva, 2006). The inter- and intramolecular disulphide bonds between the protamine molecules are essential for sperm nuclear compaction and stabilisation. It is generally accepted that this type of nuclear compaction protects sperm genome from external damages including oxidative stress, temperature elevation and acid-induced DNA denaturation (Kosower *et al.*, 1992; Chohan *et al.*, 2006).

The effect of altered sperm chromatin integrity on post-implantation embryonic development and also on recurrent pregnancy loss is still a matter of debate. On the other hand, simple effective markers are needed that predict pregnancy outcome and the risk of adverse reproductive incidents following sperm chromatin damage.

There are several kinds of assays for sperm chromatin/DNA evaluation which show different forms of damages and provide different results in relation to embryonic development and pregnancy loss. Chromatin structural probes using nuclear dyes with cytochemical bases are sensitive, simple and inexpensive which do not need special instrument like flow cytometry. On the other hand, there are insufficient cytochemical-based studies indicating the effects of sperm chromatin abnormalities on unexplained spontaneous recurrent abortion (Saxena *et al.*, 2008; Kazerooni *et al.*, 2009). So, the aim of this study was to examine the possible relationship between sperm DNA integrity/chromatin packaging evaluated by cytochemical assays, traditional sperm parameters and recurrent spontaneous abortion.

Materials and methods

Patient selection

Forty couples with a history of at least three consecutive pregnancy losses referring to the genetic counselling clinic were considered as case group (group A). A complete evaluation for RSA including sonography, physical examination, cytogenetic, immunological and reproductive hormonal assays was done. Those cases with the normal range of the aforementioned assays were considered as idiopathic recurrent spontaneous abortion patients. The second 40 couples with proven fertility (having a baby during last 2 years) were considered as control group (group B).This study was approved by the ethics committee at the Yazd Research and Clinical Center for Infertility and inform consent forms signed by all cases and controls.

Semen analysis

All semen samples were obtained by masturbation following 2–3 days of intercourse abstinence and then placed in 37 °C incubator for 60 min to undergo liquefaction. An aliquot of each sample was used to analyse the sperm parameters (morphology and motility) according to WHO criteria (WHO, 1999). Progressive motility including rapid 'grade a', slow 'grade b' and nonprogressive motility 'grade c' was assessed manually by counting 200 spermatozoa at the same temperature in an incubator which was provided by hot plate. Papanicolaou staining was done, and 200 spermatozoa were seen using oil immersion with magnification of ×100 under light microscope. The sperm count was assessed by Mackler chamber (Sefi Medical Co., Haifa, Israel). All analyses with duplicate counting were performed by one experienced technician blinded to the study.

Sperm chromatin/DNA evaluation

DNA integrity and chromatin condensation assessments were done by standard cytochemical techniques including acridine orange test (AOT), aniline blue (AB), toluidine blue (TB), chromomycin A3 (CMA3) and nuclear chromatin stability test (SDS test). All dyes and chemicals were purchased from Sigma Aldrich Company (St Louis, MO, USA).

Aniline blue (AB) staining
Aniline blue selectively stains lysine-rich histones and has been used for the determination of those sperm chromatin condensation anomalies that are related to residual histones (Auger *et al.*, 1990). To do this staining, air-dried smears were prepared from washed semen samples and then fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 min at room temperature. Each smear was stained with 5% aqueous AB stain in 4% acetic acid (pH 3.5) for 7 min. In light microscopic evaluation, 200 spermatozoa were counted in different areas of each slide using ×100 eyepiece magnification (Talebi *et al.*, 2008).

Chromomycin A3 staining
Chromomycin A3 is fluorochrome specific for guanosine cytosine-rich sequences and is used for evaluation of the degree of protamination of sperm chromatin (Lolis *et al.*, 1996; Manicardi *et al.*, 1995). For this purpose, the smears were dried first and then fixed in Carnoy's solution (methanol/glacial acetic acid, 3 : 1) at 4 °C for 10 min. The slide was treated with 100 μl of CMA3 (0.25 mg ml^{-1}) in McIlvain buffer for 20 min. After staining, the slides were washed in buffer and mounted with buffered glycerol. In each sample, at least 200 spermatozoa were counted under axiplane fluorescent microscope with a 460-nm filter and ×100 eyepiece magnification (Talebi *et al.*, 2008).

Toluidine blue (TB) staining
Toluidine blue is a metachromatic dye which determines both the quality and the quantity of sperm nuclear chromatin condensation/DNA fragmentation via binding to phosphate groups of DNA strands (Rosenborg *et al.*, 1990). This test was performed as described earlier by Erenpreisa *et al.* (2003). Briefly, air-dried sperm smears were fixed in fresh 96% ethanol–acetone (1 : 1) at 4 °C for 30 min and then hydrolysed in 0.1 N HCl at 4 °C for 5 min. Thereafter, the slides were rinsed 3 times in distilled water for 2 min and finally stained with 0.05% TB in 50% citrate phosphate for 10 min at room temperature. In each sample, at least 200 spermatozoa were counted under light microscopy using ×100 eyepiece magnification.

Acridine orange test (AOT)
Acridine orange is a metachromatic fluorescence probe for the measurement of sperm nuclear DNA susceptibility to *in situ* acid-induced denaturation by distinguishing between double-stranded DNA (green fluorescent) and single-stranded DNA (red fluorescent) (Hoshi *et al.*, 1996). This staining was performed as described in detail by Tejada *et al.* (1984). Briefly, the air-dried smears were fixed overnight in Carnoy's solution. Each sample was stained by freshly prepared AO (0.19 mg ml^{-1}) in McIlvain phosphate–citrate buffer (pH 4) for 10 min. Smears were assessed on the same day using fluorescent microscope (Zeiss Co., Jena, Germany) with a 460-nm filter.

Nuclear chromatin stability assay

Sperm chromatin decondensation assay is a method by which the structural quality and the stability of sperm chromatin can be assessed. In mammals, the stability of sperm nuclear chromatin is mostly established by S–S cross-links formed between thiol groups of adjacent protamines (Saowaros & Panyin, 1976). Decondensation of the nuclear chromatin can be induced by treatment with sodium dodecyl sulphate (SDS) which exclusively cleaves S–S bonds (Gonzales *et al.*, 1998). To do this assay, 50 μl of each semen sample was mixed with 350 μl of 1% SDS in 0.05 M borate buffer. Following 120 min of incubation at 37 °C, the reaction was stopped by adding an equal volume of 2.5% glutaraldehyde in 0.05 M borate buffer (Rosenborg *et al.*, 1990).

Statistical analysis

Statistical analysis was performed using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA). Student's *t*-test was applied to compare the groups, and the term 'statistically significant' was used to denote a two-sided P value < 0.05 for sperm parameters and cytochemical tests.

Results

Table 1 shows the average values and statistical analysis of the various sperm parameters in two groups. This table reveals that among different sperm parameters, only slow motility was statistically significantly different between group A and group B. Table 2 shows the analysis of sperm chromatin and DNA integrity in five different cytochemical assays. There were significant differences ($P < 0.05$) between two groups in all of the tests. Regarding AB staining, the percentages of unstained or pale blue stained (normal spermatozoa) and dark blue stained (abnormal spermatozoa) were reported. At CMA3

Table 1 The results of semen analysis in cases (group A) and controls (group B). Vertical column indicates different sperm parameters

	Case (group A)	Control (group B)	
Variables	Mean ± SD	Mean ± SD	*P*-value
Count ($\times 10^6$)	94.84 ± 52.19	108.97 ± 70.62	0.316
Rapid motility (%) (Grade a)	21.41 ± 15.02	20.05 ± 11.06	0.648
Slow motility (%) (Grade b)	33.41 ± 8.47	38.32 ± 7.49	0.008*
Progressive motility (%) (Grades a+b)	54.82 ± 15.94	58.37 ± 10.21	0.240
Non progressive motility (%) (Grade c)	13.33 ± 6.66	13.15 ± 4.22	0.884
Immotile sperm (%) (Grade d)	31.71 ± 12.39	28.72 ± 7.98	0.205
Total motility (%) (Grades a, b, c)	68.15 ± 12.45	71.52 ± 8.03	0.156
Normal morphology	37.84 ± 15.42	38.00 ± 14.35	0.398

*Statistically significant (two-tailed), *P* value <0.05.

Table 2 The results of sperm chromatin/ DNA evaluation in cases (group A) and controls (group B)

	Case (group A)			Control (group B)			
Variables	Min	Max	Mean ± SD	Min	Max	Mean ± SD	*P*-value
AB	18	79	46.84 ± 16.16	5	55	28.12 ± 12.03	<0.001*
TB	5	98	63.51 ± 22.14	8	60	28.82 ± 11.82	<0.001*
AO	20	98	64.56 ± 21.58	11	65	31.10 ± 12.28	<0.001*
SDS	72	167	124.50 ± 25.75	21	120	70.66 ± 29.66	<0.001*
CMA3	25	94	48.20 ± 12.84	7	45	24.43 ± 9.42	<0.001*

Vertical column indicates different cytochemical assays.
AB, Anline Blue; TB, Toluidine Blue; AO, Acridine Orange; SDS, Sodium Dodecyl Sulfate; CMA3, Chromomycin A3.
*Statistically significant (two-tailed), *P* value <0.001.

staining, bright yellow-stained chromomycin-reacted spermatozoa (CMA3+) were considered as abnormal form and yellowish green-stained nonreacted spermatozoa (CMA3−) were considered as normal form. In TB staining, the chromatin quality of sperm was assessed according to metachromatic staining of sperm heads in following scores: 0, light blue (good chromatin); 1, dark blue (mild abnormal chromatin); 2, violet; and 3, purple (severe chromatin abnormality) (Rosenborg *et al.*, 1990). So, the sum of spermatozoa with score 1, score 2 and score 3 was considered as TB^+ or abnormal chromatin, whereas score 0 was regarded as TB^- or spermatozoa with normal chromatin. For AOT, the percentages of green (normal double-stranded DNA) and orange/red (abnormally denatured DNA) fluorescence spermatozoa per sample were calculated. Finally, sperm nuclear swelling was assessed after TB staining in four different scores: score 0 = healthy chromatin (light blue) with lacking head swelling, score 1 = mild abnormal chromatin (dark blue) with mild sperm head swelling, score 2 = abnormal chromatin (violet) with head swelling and score 3 = severe chromatin abnormality (purple) with extremely swelling of sperm head. The following formula was applied to assess the 'total score': Total Score = $(S0 \times 0) + (S1 \times 1) + (S2 \times 2) + (S3 \times 3)$ (Talebi *et al.*, 2007).

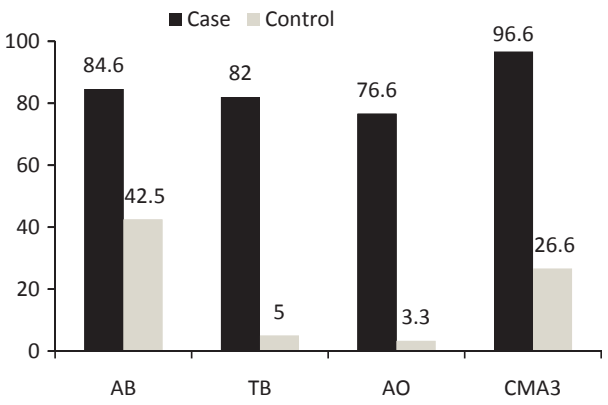

Fig. 1 Percentages of samples which were above the cut-off values in different cytochemical tests. Aniline blue (AB), toluidine blue (TB), acridine orange (AO) and chromomycin A3 (CMA3).

Figure 1 shows the percentages of samples which were above cut-off values in different cytochemical tests. From previous studies, the 'cut-off' values have suggested as 50%, 35%, 45% and 30% for acridine orange, aniline

blue, toluidine blue and chromomycin A3 respectively. The figure reveals that majority of samples in RSA patients exhibited upper percentages of abnormal spermatozoa than cut-off values regarding different cytochemical assays. For instance, in CMA3 staining, almost all samples (96.6%) of group A showed higher percentage of sperm chromatin abnormality than cut-off value, but this percentage was 26.6% in control group.

Discussion

Various factors may cause recurrent spontaneous abortion, but aetiology of more than 50% of cases remains unknown. In the present study, to investigate the possible contribution of sperm in RSA, we assessed semen quality of a group of male patients whose partners had at least three consecutive abortions in first or early second trimester of gestation following natural conception. Between different sperm parameters, slow motility had statistically significant difference between two groups. The relation between standard semen parameters and repeated pregnancy loss is still controversial (Sbracia *et al.*, 1996; Gopalkrishnan *et al.*, 2000; Sexena *et al.*, 2008). Regarding the effects of sperm motility on RSA, our results are in accordance with the data of a recent work which showed that sperm total motility was significantly lower in males of partners with unexplained Early Pregnancy Loss (EPL) than healthy controls. However, like our results, no statistically significant differences were noted in the sperm concentration, total count per ejaculate and rapid progressive motility of spermatozoa between the two groups (Bhattacharya, 2008). Although we did not find any relationship between sperm morphology and pregnancy losses, Kazerooni *et al.* (2009) demonstrated that abnormal sperm parameters including progressive motility and abnormal morphology were related to RSA. Lundin *et al.* (1997) found that regardless of a normal fertilisation rate, there was an increased abortion rate in patients with <4% morphologically normal spermatozoa. It means that the main problem with morphologically abnormal spermatozoa is not the fertilisation rate, but these abnormal forms of spermatozoa may affect embryonic development and cause abortions. In fact, there is a relationship between semen quality and embryonic development such as cleavage rate and reaching to the blastocyst stage (Jammy & Menezo, 1994).

In addition to contribution of sperm parameters in pregnancy outcomes, any alteration in sperm chromatin condensation and DNA integrity can influence male fertility, early embryonic development and pregnancy outcome (Zini *et al.*, 2001a,b). Hence, the male gamete supplies one-half of the genomic materials of the embryo, it is logical to assume that spermatozoal factors may affect embryonic development (Nanassy & Carrell, 2008; Puscheck & Jeyendran, 2007).

Regarding AB staining, we found a significant difference between two groups and sperm samples of RSA group had more spermatozoa with retained histones than control ones. On the contrary, according to our data (Fig. 1), the majority of samples in RSA patients exhibited upper percentages of abnormal spermatozoa than the cut-off value (30%) in AB test.

Although the results of AB staining have shown a clear relationship between abnormal sperm chromatin condensation and male infertility (Carrell & Liu, 2001; Foresta *et al.*, 1992), the correlation between the percentage of AB-reacted spermatozoa and other sperm parameters remains controversial (Agarwal *et al.*, 2009). An excess of sperm nuclear histones (>15%) causes inappropriate chromatin packaging and a subsequent increased susceptibility to external stresses (Kosower *et al.*, 1992). In human and mouse spermatozoa, a correlation between aberrant chromatin packaging and incidence of DNA nicks has been also reported (Sakkas *et al.*, 1995). To compare our findings with others, just one study conducted by Kazerooni *et al.* (2009) was found. They revealed that the mean percentage of AB-positive spermatozoa from men with RSA was significantly higher than that of the controls.

In CMA3 staining, we found a prominent difference between the two groups in that spermatozoa of couples with idiopathic recurrent abortions had serious protamine deficiency. In agreement with the present study but with different data, Kazerooni *et al.* (2009) demonstrated that semen samples of couples with RSA had higher rates of CMA3-positive sperm cells than those of the controls. The ratio of P1/P2 is evidently critical to male fertility, more specifically to the sperm fertilisation ability ('Aoki *et al.*, 2006; Carrell *et al.*, 2007). In humans, any increase in the P1/P2 ratio links with decreased fertilisation capacity but no change in embryo quality, whereas a decrease in this ratio is correlated with low fertilisation rate and poor pre-implantation embryo morphology (Aoki *et al.*, 2005). According to the study by Depa-Martyno'w *et al.* (2007), there is a direct correlation between protamine-1 and protamine-2 mRNA levels and the quality of pre-implantation human embryos, suggesting that protamines not only contribute to successful fertilisation but may also have an adverse effect on the pre-implantation embryos development. One of the interesting findings of our study was that almost all samples (96.6%) of group A showed higher percentage of CMA3-positive spermatozoa than cut-off value (i.e. 30%). It means that protamine deficiency is a common abnormality in sperm of RSA group, and CMA3 is an effective test for evaluation of spermatozoa in couples with consecutive unexplained pregnancy losses.

In TB test, we demonstrated that 82% of patients in group A had higher percentage of spermatozoa with fragmented DNA than cut-off value. To our knowledge, this is the first report on using TB in evaluation of sperm chromatin abnormality in RSA patients. Tsarev *et al.* (2009) showed that the threshold for this staining is 45% which provides 92% specificity and 42% sensitivity. This cut-off value was considered as a predictor for male infertility and provides additional prognostic information to that obtained by semen analysis. So, the TB test may in future be applied clinically as a simple functional test for the assessment of male fertility potential and also in prediction of repeated miscarriages. Toluidine blue test is suggested to be a simple alternative for other sperm chromatin assessment tests like TUNEL and sperm chromatin structural assay. It is reported that SCSA and TB staining using image cytometry are equally effective tests for sperm immaturity measurement in fertile men, and the results of these two tests are related to each other (Erenpreiss *et al.*, 2004). The results of a recent study conducted by Zini *et al.* (2005) also showed that during Intracytoplasmic Sperm Injection (ICSI) procedures, a high percentage of DNA-damaged sperm cells (>30%) was associated with an increase in occurrence of multinucleated blastomeres, proposing that high levels of sperm DNA damage can affect the quality of pre-implantation embryos and also cause spontaneous abortions.

Regarding AOT, we found a significant difference between the two groups and also 76.6% of patients in RSA group showed higher percentage of abnormal spermatozoa than AO cut-off value (50%) (Virant-Klun *et al.*, 2002). The results of the present study confirmed previous findings which have indicated the detrimental effects of DNA denaturation on embryo quality and pregnancy rates. Gopalkrishnan *et al.* (1999) showed that the males having female partner with repeated EPL have higher percentages of spermatozoa with damaged DNA than fertile controls. They concluded that AOT is a useful tool in evaluation of sperm nuclear chromatin integrity which is essential for normal embryonic development. Also, a more recent study using AOT has shown a statistically significant difference in the amount of DNA integrity of spermatozoa between men of couples with RSA and fertile men (Bhattacharya, 2008). Another work conducted by Virant-Klun *et al.* (2002) has indicated that sperm cells with denatured DNA detected by AOT may result in zygotes, but the occurrence of embryo fragmentation and arrested embryos with poor biological quality is very common. They also showed that persistence of spermatozoa with high levels of DNA damages is a permanent situation, and future pregnancies may be affected and lead to spontaneous abortions. In agreement with the aforementioned study, it is demonstrated that although DNA-damaged spermatozoa may fertilise an oocyte, the resulting embryo cannot complete its normal growth and leads to abortion (Tesarik *et al.*, 2002). Although AOT has some limitations like light microscopic observation and some authors questioned the using of this staining in clinical practice (Duran *et al.*, 1998), there are many clinics that evaluate male fertility by acridine orange test. Conversely, the flow cytometer is not a common instrument in all andrology laboratories.

In SDS assay, we found that the total score of *in vitro*–decondensed spermatozoa was significantly higher in case group than control one. It means that the majority of spermatozoa in RSA patients had fewer S–S bonds and following SDS treatment, and they showed larger-sized nuclei with respect to normal spermatozoa. It seems that our data did not confirm previous findings which had shown a reduction in capacity of sperm nuclear chromatin to decondense *in vitro* in patients with RSA (Gopalkrishnan *et al.*, 2000; Saxena *et al.*, 2008). We think that our results are more logical than the aforementioned findings because other cytochemical assays such as AB, AOT and CMA3 indicated the spermatozoa with less condensed chromatin in RSA group than spermatozoa from control one. Additionally, the data have shown that there is a positive relationship between the results of AB staining and SDS test (Foresta *et al.*, 1992) and also between AOT and nuclear chromatin decondensation test (Gopalkrishnan *et al.*, 1999). In fact, in the present study, the enhanced decondensation rate in the spermatozoa of RSA group which is not in keeping with other reports may be due to differences in the conformation or compaction of the sperm chromatin with a greater accessibility of SDS to the existing S-S bonds. It is also demonstrated that infertile men have higher percentage of spermatozoa with decondensed nuclei or hypostabilised chromatin than fertile ones (Zini *et al.*, 2001a,b).

Sperm DNA damage is referred a major cause of embryonic mortality (Sakkas *et al.*, 1999). To explain the relationship between sperm chromatin/DNA anomalies and RSA, it should be considered that any abnormalities in unique organisation of sperm chromatin are thought to affect the proper expression and regulation of paternal genes in early embryos (Haaf & Ward, 1995). It is generally accepted that maternal regulation is very important in the first few steps of embryonic development, whereas the paternal genes expression starts at the four-to-eight cell stage. So, at this stage, any paternal DNA anomalies may become apparent and impair embryo development. Seli *et al.*, (2004) showed that during blastocyst stage, the genome is activated and the paternal genes start to contribute significantly in embryonic function. Several reports have revealed that sperm DNA damage may be associated with decreased cleavage rates and diminished embryo quality

(Morris *et al.*, 2002; Zini *et al.*, 2005). With regard to the sperm chromatin packaging, in protamine-deficient animal models, the DNA integrity decreases during epididymal transition of spermatozoa, which in turn may affect embryonic development (Suganuma *et al.*, 2005).

In conclusion, our study showed that in the cases of RSA, among different sperm parameters only slow motility had a statistically significant reduction in comparison with controls and also we showed that spermatozoa of men from RSA group had less chromatin condensation, hypostabilised chromatin and lower DNA integrity than spermatozoa of fertile men. Also, the present study stresses the importance of cytochemical evaluation of the sperm chromatin and DNA integrity in couples with unexplained RSA, and in the cases of high sperm nuclear anomalies, these patients should be advised to change their life style and undergo to a proper treatment strategy like antioxidant therapy if they want to have a normal reproductive life.

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