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Sperm DNA fragmentation in Italian couples with recurrent pregnancy loss

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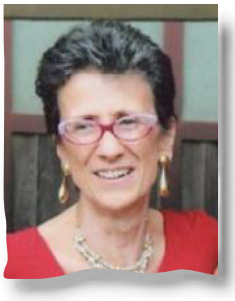

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KEY MESSAGE

Recurrent pregnancy loss (RPL) patients presented excellent semen parameters, but their sperm DNA fragmentation (SDF) values were much higher than those observed in fertile men. Although the high SDF of the RLP patients suggests involvement of a male factor in the pathogenesis of RPL, it cannot be considered a predictive factor for the risk of RPL.

A B S T R A C T

The aetiopathogenesis of recurrent pregnancy loss (RPL) is heterogeneous. The aim of this study was to investigate the male factor in Italian couples experiencing RPL following natural conception. The study investigated 112 men from RPL couples and two control groups: 114 infertile men with one or more impaired semen parameters and 114 fertile men with high-quality semen parameters. Semen parameters were examined according to WHO criteria. Sperm DNA fragmentation (SDF) was evaluated using TdT-mediated dUDP nick-end labelling (TUNEL) assay. With the exception of ejaculate volume, the seminal profile of patients with RPL was similar to that of fertile patients and better than the infertile ones. Despite good spermatogenesis, however, sperm DNA integrity was impaired in the RPL group, with SDF values significantly higher than in fertile controls (18.8 ± 7.0 versus 12.8 ± 5.3, $P < 0.001$) and similar to those of infertile patients. SDF also showed a positive correlation with the age of patients with RPL and number of miscarriages. The results suggest a correlation between increased SDF and impaired reproductive capacity in terms of both fertilization and pregnancies carried to term, but high SDF cannot yet be considered a predictive factor for the risk of RPL.

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Introduction

Miscarriage is the most common obstetric complication, occurring in 15% of all clinically recognized pregnancies. This figure rises to about 50% for preclinical miscarriages (Chard, 1991; Wilcox et al., 1998). It has been estimated that just one-third of conceptions lead to the birth of a baby (Wang et al., 2003; Zinaman et al., 1996). Recurrent pregnancy loss (RPL) is defined as two or more consecutive miscarriages diagnosed before the 14th week (ASRM, 2013). It affects about 1% of couples attempting to have a child (Porter and Scott, 2005). Its aetiopathogenesis is heterogeneous and multiple factors may be involved, complicating the identification of predisposing factors.

Despite thorough investigation of the female partner of RPL couples, it is estimated that the cause is never found in 50% of cases (Lee and Silver, 2000). It is therefore plausible to suppose that in some of these so-called idiopathic cases, the cause may be due to the male partner, which to date has been little investigated. Studies of the correlation between sperm quality and RPL have produced conflicting results (Gopalkrishnan et al., 2000; Saxena et al., 2008; Sbracia et al., 1996). Impaired semen parameters have been associated with infertility and reduced reproductive capacity, with failed fertilization and embryonal division following IVF (Oehninger, 2011). Although it is unclear as to what extent the male factor is involved in RPL, a high percentage of morphological sperm abnormalities has been associated with an increased risk of miscarriage in couples undergoing assisted reproduction treatments (Kobayashi et al., 1991). Patients with karyotype 46XY, who present impaired semen parameters, also have a high percentage of spermatozoa with aneuploidies (Vicari et al., 2003); although these are capable of fertilizing the oocyte, they give rise to an embryo with chromosome damage, which may result in spontaneous abortion.

As both the spermatozoa and the oocyte contribute equally to the genetic makeup of the embryonic DNA, it is reasonable to presume that genetic and epigenetic sperm damage may compromise the development of the embryo and placenta and thus cause miscarriage. There has been great interest in the study of sperm DNA fragmentation in recent years, as the integrity of sperm DNA is crucial for the accurate transmission of genetic information to the embryo. A meta-analysis conducted by Evenson and Wixon (2008) found that the lower the sperm DNA damage, the greater the successful natural pregnancy rate, while major sperm chromatin damage increases the risk of congenital abnormalities (Kumar et al., 2012) and predisposition to childhood cancers in the offspring (Aitken and Krausz, 2001). Various studies in the literature have investigated the relationship between sperm DNA fragmentation and RPL. Systematic review and meta-analysis (Robinson et al., 2012; Zini et al., 2008) found that sperm DNA damage is significantly correlated with an increased risk of miscarriage following IVF and intracytoplasmic sperm injection. Some studies have found high sperm DNA fragmentation in the male partners of RPL couples following natural conception (Brahem et al., 2011; Carrell et al., 2003; Imam et al., 2011; Kumar et al., 2012), while others (Coughlan et al., 2015; Gil-Villa et al., 2010) contradict the theory that sperm DNA damage is one of the factors involved in RPL.

Given the growing interest in the study of sperm DNA fragmentation, the aim of this study was to investigate the male factor in Italian couples experiencing RPL following natural conception. It focused on quality of spermatogenesis and sperm chromatin integrity, to establish any paternal contribution to the aetiopathogenesis of RPL.

Materials and methods

Ethics statement

The study was approved by our University Hospital's institutional review board (number 182/11, 18 February 2011). Written informed consent was obtained from all study participants.

Patients

The study recruited 112 men from Caucasian couples reporting two or more spontaneous abortions (**Figure 1**) attending the RPL Sterility Unit at ASL Roma C who underwent a semen examination at the Seminology Laboratory–Sperm Bank ('La Sapienza' University of Rome, Department of Experimental Medicine). The miscarriage was diagnosed before the 14th week but fetal heartbeat was documented between the eighth and 12th week.

Patients with an abnormal chromosome number or structure, blood relationship with their partner or miscarriage after exposure to radiotherapy or chemotherapeutic treatments were excluded. Both male and female partners had normal karyotypes.

A full screening of the RPL women was carried out, including physical examination, testing for immunological, acquired or inherited thrombophilia and reproductive hormonal assays. The presence of any infectious or parasitic disease was excluded. The female partners (<38 years old) presented a normal uterus, as confirmed by vaginal ultrasound, and showed an endometrial thickness consistent with the cycle phase. None of the female partners presented pre-eclampsia or intrauterine growth retardation. None of the couples had undergone assisted reproduction treatments. The female partners did not have any risk factors for RPL.

Two control groups of the same ethnic origin as the study group were recruited based on their semen quality. CTRL 1 consisted of 114 patients with one or more impaired semen parameters who were attending our department due to infertility of at least 2 years duration, which was not associated with any female factor. None of the couples had undergone assisted reproduction treatments.

CTRL 2 consisted of 114 fertile men with high-quality semen parameters who had undergone an andrological check-up in the same

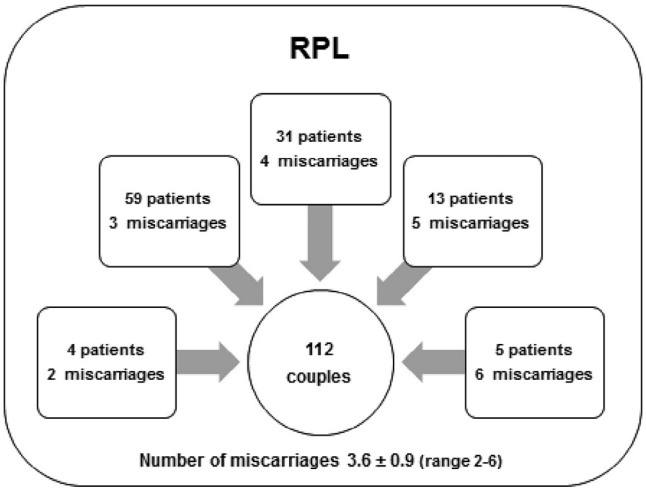


Figure 1 – RPL couples categorized by number of miscarriages. RPL = men from couples with recurrent pregnancy loss.

department. The ages of the patients in both control groups were similar to those of the study group. The exclusion criteria for the control groups were as follows: cancer, cancer treatments and history of spontaneous abortion in female partner. The patients had not been medically or surgically treated in the 3 months before the study and did not have any conditions (fever, recent sudden stress) that might interfere with the semen analysis.

All partners in the study group and the control groups had the same ethnic origin.

Semen analysis

All patients in the three study groups underwent semen examination to evaluate spermatogenesis. Semen samples were collected by masturbation directly into a sterile plastic container after 3–5 days of sexual abstinence. They were examined by optical microscope according to World Health Organization (WHO, 2010) criteria. The following variables were taken into consideration: sperm concentration (10^6/ml), total sperm number (10^6/ejaculate), progressive motility (%) and morphology (% abnormal forms).

Sperm DNA fragmentation

Sperm DNA fragmentation (SDF) was evaluated using the TdT-mediated dUDP nick-end labelling (TUNEL) assay (*In situ* Cell Death Detection Kit, Fluorescein; Roche, Basel, Switzerland). After cytological and morphological examination of the semen parameters, the samples were washed twice in phosphate-buffered saline and then cytocentrifuged (Cytospin 3; Shandon Inc., Pittsburgh, PA, USA). The method used was as described in Gandini et al. (2000). The samples were then analysed using a fluorescence microscope (Leica DMR; Leica, Wetzlar, Germany), counting at least 500 cells.

Statistical analysis

As not all the semen variables showed a normal distribution on the Kolmogorov–Smirnov test, non-parametric tests were used (Mann–Whitney test, one-way ANOVA–Kruskal–Wallis test with Dunn post-test, Spearman correlation test). The patients in the three groups were subdivided according to the cut-off SDF ≥12.8% and SDF <12.8%, which corresponded to the mean value for CTRL 2, and were analysed by the chi-squared test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

The data for age, semen parameters and SDF of the three groups are reported in **Table 1**. For semen parameters, there were statistically significant differences between RPL and CTRL 2 for ejaculate volume and percentage of abnormal forms ($P < 0.05$ and $P < 0.001$, respectively) but no significant differences for any of the other semen parameters. There were statistically significant differences between RPL and CTRL 1 and between CTRL 1 and CTRL 2 in all semen parameters ($P < 0.001$) except ejaculate volume. There was no statistically significant difference in SDF between RPL and CTRL 1 (18.8 ± 7.0 versus 20.8 ± 8.9 respectively), but both of these groups had higher SDF ($P < 0.001$) than observed for CTRL 2.

Table 1 – Age, semen parameters and SDF for the three study groups.

Patients	Age (years)	Volume (ml)	Sperm concentration (10^6/ml)	Total sperm number (10^6/ejaculate)	Progressive motility (%)	Abnormal forms (%)	SDF (%)
RPL (112 patients)	38.3 ± 4.6 (39.0)	3.0 ± 1.3 (3.0)	93.6 ± 68.4 (80.0)	257.5 ± 170.2 (220.0)	50 ± 10.2 (50.0)	76.2 ± 5.6 (76.5)	18.8 ± 7.0 (17.1)
CTRL 1 (114 patients)	37.5 ± 4.7 (38.0)	3.3 ± 1.5 (3.0)	19.4 ± 19.6 (13.4)	62.2 ± 68.2 (35.0)	20.1 ± 7.5 (20.0)	88.1 ± 6.8 (88.0)	20.8 ± 8.9 (19.7)
CTRL 2 (114 patients)	37.4 ± 4.6 (38.0)	3.5 ± 1.5 (3.2)	93.9 ± 62.9 (79.0)	304.5 ± 178.8 (255.6)	53.8 ± 4.6 (50.0)	80.1 ± 3.2 (80.0)	12.8 ± 5.3 (12.2)
RPL versus CTRL 1	NS	NS	**	**	**	**	NS
RPL versus CTRL 2	NS	*	NS	NS	NS	**	**
CTRL 1 versus CTRL 2	NS	NS	**	**	**	**	**

Values shown as mean ± SD (median). One-way ANOVA–Kruskal–Wallis test with Dunn post-test.
* $P < 0.05$.
** $P < 0.001$.
CTRL 1 = men from infertile couples with one or more impaired semen parameters; CTRL 2 = fertile men with high-quality semen parameters; NS = not significant; RPL = men from couples with recurrent pregnancy loss; SDF = sperm DNA fragmentation.

The RPL couples were then subgrouped according to number of miscarriages, with 63 couples having had two to three miscarriages and 49 couples four to six miscarriages. There was a statistically significant difference in age of male partner between these two subgroups (37.7 ± 4.5 versus 39.2 ± 4.7 respectively, $P < 0.05$), but no statistically significant difference in semen parameters or SDF (**Table 2**).

The SDF distribution of CTRL 1, CTRL 2 and RPL is shown in **Figure 2**. The three groups were then subdivided at the cut-off of SDF 12.8%, which corresponded to the mean value for CTRL 2, i.e. the fertile men with high-quality semen parameters. This revealed that 81.3% of RPL patients, 81.6% of CTRL 1 patients and 44.7% of CTRL 2 patients had SDF ≥12.8%. The breakdown by cut-off was therefore similar for RPL and CTRL 1, and both these groups showed a statistically significant difference in comparison with CTRL 2 ($P < 0.001$) (**Figure 3**).

SDF showed an inverse correlation with progressive motility ($r = -0.41$, $P < 0.001$), but there was no statistically significant correlation with total sperm number or percentage of abnormal forms. SDF also showed a positive correlation with the age of the RPL patients ($r = 0.28$, $P < 0.01$) and number of miscarriages ($r = 0.20$, $P < 0.05$) (**Figure 4**).

Discussion

RPL affects about 1% of couples attempting to conceive. Given the close tie between mother and fetus, the female partner has been studied intensively, but the role of the male partner remains largely unknown and is rarely discussed. Investigations of a possible male factor have assessed spermatogenesis and sperm chromatin integrity in the male partners of RPL couples.

Hill et al. (1994) studied 98 men whose partners had had three or more miscarriages, finding no differences in ejaculate volume, sperm concentration or percentage of abnormal forms in comparison with 17 fertile men.

Sbracia et al. (1996) compared the semen parameters at the time of recruitment and 3 years later of 120 men from RPL couples against a control group consisting of 30 healthy male partners of couples experiencing no reproductive difficulties (infertility or recurrent miscarriage). The patients with RPL were subdivided into three groups based on reproductive outcome: 48 couples who had had a child, 39 with further miscarriages and 33 who had become infertile. The authors did not find any statistically significant differences in the semen parameters of the cases and controls and excluded the involvement of sperm morphology in determining RPL. However, they suggested that impaired semen parameters might be involved in the infertility of the RPL couples.

A study by Gopalkrishnan et al. (2000) compared the semen parameters of the men from 32 RPL couples against 51 men whose partners were in the first trimester of pregnancy, finding normal semen parameters in both cases and controls. However, they found statistically significant differences in the percentage of abnormalities in the sperm head and a lower sperm decondensation capacity in the cases than in the controls, as evaluated by the nuclear chromatin decondensation test.

As spermatozoon nuclear integrity is crucial for the accurate transmission of genetic information to the embryo, the study of sperm DNA integrity may be the key to understanding the complexities of RPL. Carrell et al. (2003), followed by Brahem et al. (2011), used TUNEL

Table 2 – Age, semen parameters and SDF for the RPL subgroups categorized by number of miscarriages (two to three or four to six) (Mann–Whitney test).

RPL group	Age (years)	Volume (ml)	Sperm concentration (10^6/ml)	Total sperm number (10^6/ejaculate)	Progressive motility (%)	Abnormal forms (%)	SDF (%)
2–3 miscarriages (63 patients)	37.7 ± 4.5 (38.0)	3.1 ± 1.2 (3.0)	93.6 ± 63.2 (80.0)	269.9 ± 166.6 (225.0)	51.7 ± 7.5 (55.0)	76.1 ± 5.0 (77.0)	17.7 ± 6.6 (16.6)
4–6 miscarriages (49 patients)	39.2 ± 4.7 (40.0)	3.0 ± 1.5 (2.9)	93.7 ± 75.3 (80.0)	241.5 ± 175.2 (200.0)	47.7 ± 12.6 (50.0)	76.4 ± 6.4 (76.0)	20.3 ± 7.4 (18.2)
P-value	<0.05	NS	NS	NS	NS	NS	NS

Values shown as mean ± SD (median).
RPL = men from couples with recurrent pregnancy loss; SDF = sperm DNA fragmentation.
Mann–Whitney test: NS = not significant.

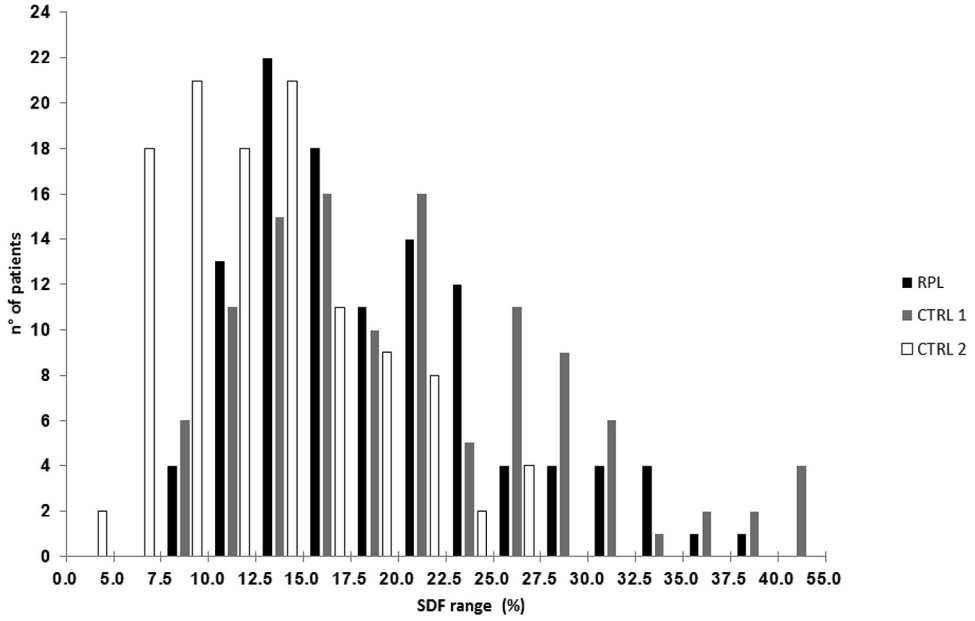
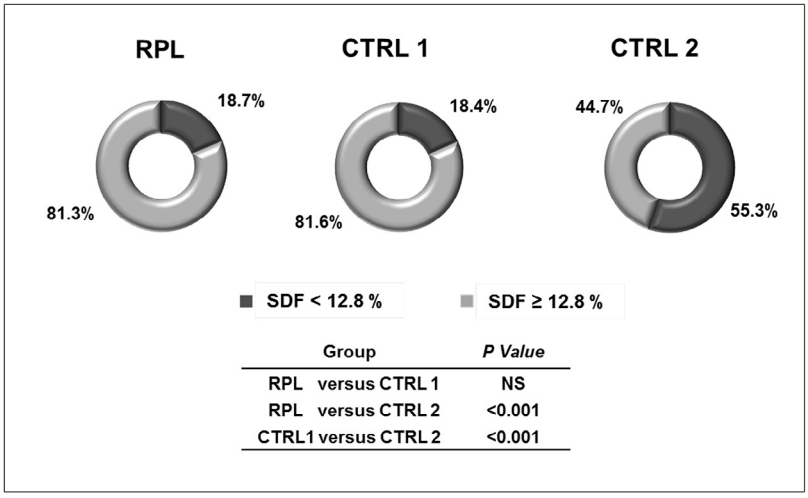


Figure 2 – Bar charts displaying distributions of the SDF in the three groups. CTRL 1 = men from infertile couples with one or more impaired semen parameters; CTRL 2 = fertile men with high quality semen parameters; RPL = men from couples with recurrent pregnancy loss; SDF = sperm DNA fragmentation.

to demonstrate that spermatozoa from men with RPL had a statistically significantly higher DNA fragmentation than that found in spermatozoa from fertile men. Carrell et al. (2003) did not find any correlation between the percentage of sperm DNA fragmentation and phenotype in their caseload of 23 men with RPL. In contrast, Brahem et al. (2011) found a statistically significant correlation between the DNA fragmentation index (DFI) and sperm concentration ($r = -0.553$; $P = 0.001$) and percentage of abnormal forms ($r = 0.421$; $P = 0.018$) in their caseload of 31 RPL men, although there was no correlation with motility or the patients' age. Brahem et al. (2011) did not find any statistically significant differences in semen parameters between the RPL and control groups except for sperm motility, which was higher in the control group ($P < 0.001$). Bhattacharya (2008) did not find any statistically significant differences in age, sperm concentration or progressive motility between 74 RPL men and 65 fertile men. However, total number of motile spermatozoa per ejaculate, motility and DNA integrity, as assessed by the acridine orange test, were lower in the RPL group. Kazerooni et al. (2009) studied 30 RPL patients and 30 fertile controls, finding a significant reduction in sperm motility and an increase in abnormal forms in the cases in comparison with the controls, even though semen parameters were normal (WHO, 1999) in both groups. They also found a statistically significant alteration in sperm chromatin structure in RPL versus fertile controls with chromomycin A3 (CMA3) and aniline blue staining, while there was no difference with the acridine orange test. These authors also demonstrated a negative correlation between morphology and CMA3



Group	*P Value*
RPL versus CTRL 1	NS
RPL versus CTRL 2	<0.001
CTRL1 versus CTRL 2	<0.001

Figure 3 – Percentage of patients in the three groups subdivided according to the cut-off SDF ≥12.8 and analysed by the chi-squared test. CTRL 1 = men from infertile couples with one or more impaired semen parameters; CTRL 2 = fertile men with high-quality semen parameters; RPL = men from couples with recurrent pregnancy loss; SDF = sperm DNA fragmentation.

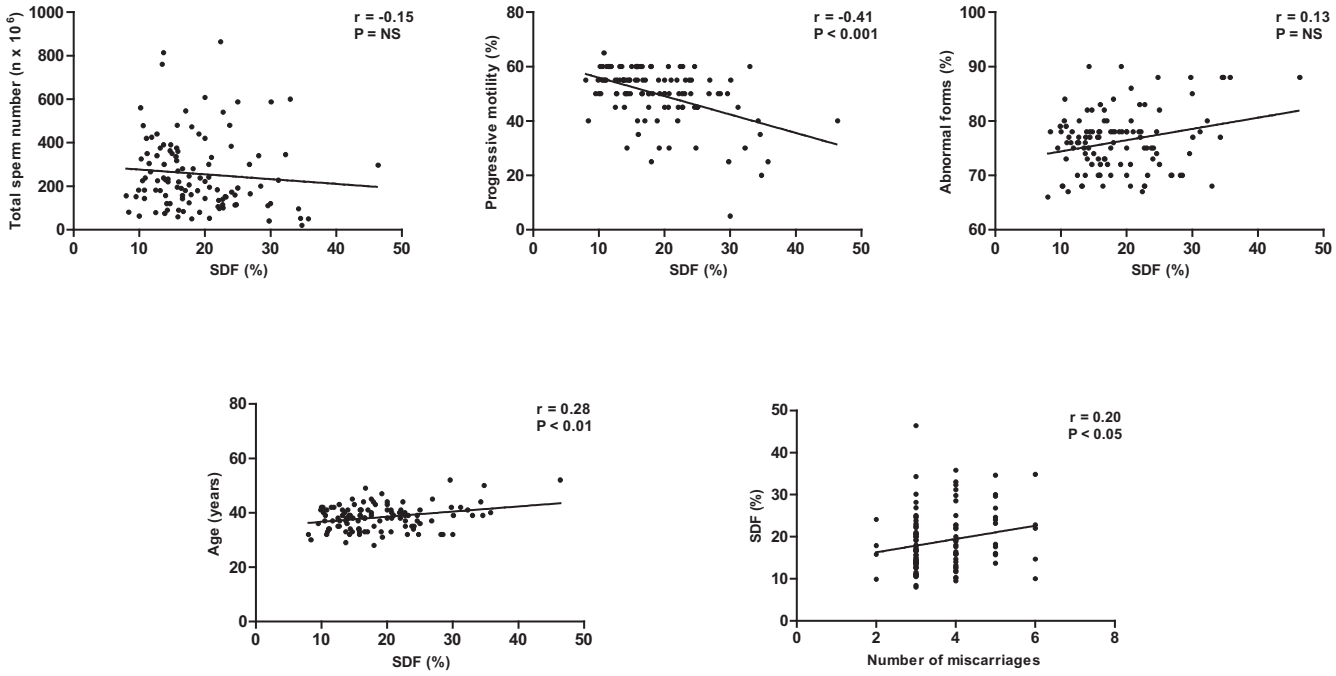


Figure 4 – Correlation between SDF and semen parameters, age and number of miscarriages in recurrent pregnancy loss patients (Spearman correlation test). SDF = sperm DNA fragmentation.

(r = –0.651, P = 0.001) and aniline blue (r = –0.572, P = 0.015) and between motility and CMA3 (r = –0.316, P = 0.043) and aniline blue (r = –0.439, P = 0.031). Bellver et al. (2010) found a significantly higher DNA fragmentation in 30 RPL men in comparison with 30 fertile men with the sperm chromatin dispersion (SCD) test. However, evaluation of the receiver operating characteristic curve showed that this index was not predictive and the authors concluded that chromatin changes are not a valid clinical tool for the investigation of RPL. In the same year, Gil-Villa et al. (2010) found impaired motility and morphology in 23 RPL men in comparison with 11 fertile controls, although there was no statistically significant difference in DFI as evaluated by the sperm chromatin structure assay (SCSA). In contrast, Venkatesh et al. (2011), Imam et al. (2011) and Kumar et al. (2012) all found an increase in DFI in RPL patients versus controls with SCSA. Absalan et al. (2012), followed by Khadem et al. (2014), both found impaired sperm motility and chromatin integrity evaluated by SCD in RPL patients in comparison with controls. Khadem et al. (2014) also found a negative correlation between sperm DNA fragmentation and progressive motility (r = –0.613; P < 0.001) and percentage of abnormal forms (r = –0.764; P < 0.001).

Zhang et al. (2012) found no significant difference between the semen parameters of 111 RPL men and 30 fertile controls, whereas sperm chromatin integrity was correlated with reproductive outcome. In fact, the partners of men with greater sperm chromatin damage were less likely to become pregnant and more likely to have RPL. Kavitha and Malini (2014) found lower values for the hypo-osmotic swelling test, nuclear chromatin decondensation test and acrosomal intactness test (P < 0.05) in 95 patients with RPL than in 37 fertile men. The authors concluded that although the semen parameters of the patients with RPL were within normal limits, the damage revealed by the functional tests might explain the aetiology of the RPL. Coughlan et al. (2015) recently investigated sperm DNA fragmentation using SCD and TUNEL in 16 men from RPL couples and seven men with children. They found no differences between the two groups and concluded that DNA fragmentation is not an important cause of RPL and that DNA integrity tests are not predictive of the risk of recurrent miscarriage. Leach et al. (2015), using SCSA, found a mean DFI of 9.5% in 108 RPL couples. A high DFI (>15%) was found in 30% of these men, which possibly contributed to the pathogenesis of repeated pregnancy loss.

The present study is the first Italian investigation of the male factor in RPL following natural conception in a large cohort of patients. With the exception of ejaculate volume, the seminal profile of the RPL patients was similar to that of the fertile patients with high-quality semen parameters (CTRL 2) and better than that of the infertile patients (CTRL 1). There was no statistically significant difference between RPL and CTRL 2 in sperm concentration, total sperm number or progressive motility, but the percentage of abnormal forms was lower in the former (P < 0.001). The excellent semen parameters seen in the RPL patients are in line with the fact that conception does take place in their partners – the problem lies in the inability to carry the pregnancy to term. The mean SDF in the RPL patients was similar to that of the CTRL 1 group, and was higher in both these groups than in CTRL 2 (P < 0.001). Furthermore, the SDF was higher than the mean value for the CTRL 2 group (12.8%), used as the cut-off, in 81.3% of the RPL patients and 81.6% of CTRL 1 patients. Other authors also found a high percentage of sperm DNA fragmentation in RPL patients using TUNEL (Bareh et al., 2016; Brahem et al., 2011; Carrell et al., 2003; Zidi-Jrah et al., 2016) and SCSA (Imam et al., 2011; Kumar et al., 2012; Venkatesh et al., 2011), suggesting that chromatin damage may compromise the normal progression of pregnancy. Unlike the spermatozoa, which is transcriptionally inactive and has no repair mechanisms, the oocyte can repair sperm DNA damage, but only up to a certain limit (Kumar et al., 2012). The results of the present study suggest that the high SDF in RPL patients is beyond the capacity of the oocyte's repair mechanisms, and repair of the chromatin damage is thus

incomplete. This gives rise to genetic modifications, which could affect both pre- and post-implantation embryonic development, causing miscarriage. It could also be postulated that SDF causes the loss of certain paternal genes crucial for embryonic development. The high SDF values seen in the RPL and CTRL 1 patients may derive from poor DNA packaging during chromatin remodelling in spermiogenesis, making the DNA more vulnerable to oxidative stress and the action of DNA nucleases (Ribas-Maynou et al., 2012), as reported by Kazerooni et al. (2009). The values reported in the literature vary considerably, making it difficult to establish an SDF cut-off that might be predictive for RPL. This diversity may be in some cases due to the small caseloads and in others the use of methods of different sensitivities, which reveal different types of sperm DNA damage. SCD is in fact an ambiguous method providing information that is difficult to interpret, as the exact type of molecular DNA damage it has detected is unknown. In contrast, the TUNEL assay quantifies the amount of DNA with single- and double-strand breakages while SCSA provides an indirect measure of DNA damage, as it evaluates the percentage of DNA susceptible to denaturation: the most easily denatured cells are those with fragmented DNA. Finally, it should be remembered that use of a viability marker is advisable to avoid underestimating sperm DNA damage, and that Mitchell et al. (2011) report a modified step for sperm DNA decondensation, which allows DNA damage to be estimated correctly.

Advancing age can affect the fertility of men as well as women. In fact, correlation data from this study show that SDF rises with increasing paternal age. Cohen-Bacrie et al. (2009) confirmed this correlation between SDF and paternal age in a prospective study of 1633 patients using TUNEL. This progressive deterioration of chromatin integrity over the years may be due to male germ cells that divide continuously by mitosis before entering the meiotic prophase, and this high number of cell replications may cause an accumulation of DNA replication errors and, hence, fragmentation.

Our data suggest that increasing SDF is also correlated with an increasing percentage of abnormal spermatozoa, although this was not statistically significant. We found no correlation between SDF and total sperm number. However, there was an inverse correlation between SDF and progressive motility ($r = -0.41$, $P < 0.001$). One of the possible causes of hypomotility is the generation of reactive oxygen species (ROS). Excessive ROS can exceed the cell's antioxidant capacity, causing nuclear and mitochondrial DNA damage. It is possible that oxidative stress might affect membrane and sperm chromatin integrity, inducing DNA strand breaks (Cocuzza et al., 2007; Venkatesh et al., 2009) or, in the spermatid phase, cause incomplete protamination during histone replacement. Finally, there was an interesting correlation between SDF and the number of miscarriages. Increased sperm DNA damage was associated with an increase in the number of spontaneous abortions, suggesting that the greater the damage, the more likely the loss of the genes involved in normal embryonic development.

In conclusion, although the RPL patients presented excellent semen parameters, their SDF values were similar to those of the infertile men and were much higher than those observed in fertile men were. It is thus reasonable to suppose a correlation between increased sperm DNA fragmentation and impaired reproductive capacity, in terms of both fertilization and pregnancies carried to term. Although the high SDF found in the men of RPL couples suggests involvement of a male factor in the pathogenesis of RPL, yet it cannot be considered a predictive factor for the risk of RPL.

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