Relationship between sperm aneuploidy, sperm DNA integrity, chromatin packaging, traditional semen parameters, and recurrent pregnancy loss

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Objective: To study the possible relationship between sperm aneuploidy, sperm DNA integrity, chromatin packaging, traditional semen parameters, and recurrent pregnancy loss (RPL).
Design: Descriptive study.
Setting: University-affiliated tertiary teaching.
Patient(s): A total of 22 couples with history of RPL and 20 fertile men.
Intervention(s): Semen samples from case and control men were examined for differences in semen parameters, DNA fragmentation, chromatin condensation, and sperm aneuploidy.
Main Outcome Measure(s): Sperm DNA and chromatin integrity and sperm aneuploidy.
Result(s): Sperm progressive motility (30.2% vs. 51.5%) was significantly lower and abnormal morphology (74.8% vs. 54.2%) was significantly higher in the RPL group versus the control group, respectively. The percentage of fragmented DNA was significantly increased in the RPL group (17.1% vs. 10.2%) as well as the rate of spermatozoa with nuclear chromatin decondensation (23.6% vs. 11.8%). There was a significantly higher sperm aneuploidy rate among the RPL group as well.
Conclusion(s): The increase in abnormal sperm parameters, sperm DNA fragmentation, nuclear chromatin decondensation, and sperm aneuploidy suggest possible causes of unexplained RPL. (Fertil Steril® 2016;105:58–64. ©2016 by American Society for Reproductive Medicine.)
Key Words: Spermatozoa, pregnancy loss, DNA damage, aneuploidy

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Recurrent pregnancy loss (RPL) is defined as the miscarriage of two or more consecutive pregnancies in the first or early second trimester of gestation (1). It is one of the most common complications of pregnancy occurring in 0.8%–1.4% of couples trying to conceive (2). Even after a thorough evaluation, and due to the complex etiology involved in miscarriages, the potential cause remains unexplained in one third to one half of the cases (3). Etiologies of RPL are multifactorial, and available studies are usually focused on maternal factors due to the intimate maternal relationship with the developing embryo (4). Frequently studied maternal factors that may be involved in RPL can be classified as genetic or chromosomal

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causes, advanced maternal age, antiphospholipid syndrome, hormonal abnormalities, uterine abnormalities, metabolic, infectious, or immune disorders (5–8).

However, unable to find the cause of these losses, it is reasonable to suppose that factors within sperm may have an influence on these pregnancy losses (9, 10). The male factor has been less studied for many years, mainly basing the infertility diagnosis on semen parameters and, although this information is necessary, it is not always conclusive. Taking into account that sperm cells and oocytes provide half of the nuclear DNA to the embryo, it is reasonable to expect that genetic alterations of the sperm could affect embryonic development leading to pregnancy loss (4).

It has been described that male factor may be involved in RPL when higher percentage of sperm aneuploidy is found (11, 12). Some other data suggested that sperm DNA integrity may affect embryonic development and possibly increases miscarriage (13). Nuclear chromatin decondensation has also been suggested as a possible cause of RPL (14). Whether any of the semen quality parameters can predict future reproductive outcome of the couples with RPL remains unclear (15). The high proportion (almost 50%) of recurrent miscarriage remaining unexplained and the growing concern about the male factor, highlight the need for revised and updated diagnostic strategies for couples consulting for recurrent abortions (16). The present study is therefore designed to examine the possible relationship between sperm aneuploidy, sperm DNA integrity, chromatin packaging, and traditional semen parameters with RPL.

MATERIALS AND METHODS

Patients

Twenty-two couples with a history of two or more RPL referring to the genetic counseling clinic at the Department of Cytogenetic and Reproductive Biology, Farhat Hached University Teaching Hospital, Sousse, Tunisia, were considered as the case group. These couples were not attempting assisted reproduction treatments at the time they were accepted for the study. The female partners of these subjects had normal ovarian function and presented a normal uterus confirmed by saline hysterosonography and/or hysterosalpingography (HSG)/hysteroscopy, absence of acquired or inherited thrombophilia, and no hormonal disorders. Male or female partners who showed karyotyping abnormalities were excluded from the study. All men had no history of radiotherapy, chemotherapy, orchitis, toxic exposure, trauma, varicocele, testicular torsion, chronic illness, or medication.

In addition, 20 men (control group) with normal karyotype, normal semen profiles, no family history of diseases, and whose unique partners had at least one previous term pregnancy without complications conceived with their sperm samples within the past year were included as the control group. This protocol was approved by the medical ethics committees of Farhat Hached University Hospital and all patients and control subjects gave informed consent for the study.

Semen Analysis

All semen samples were obtained by masturbation after 3–5 days of intercourse abstinence. After complete liquefaction of the sample, semen analysis was performed according to World Health Organization guidelines (17). Semen volume, pH, sperm concentration, sperm motility, and sperm morphology were included in the semen analysis. Motility was assessed manually. Sperm count was performed in a Thoma counting chamber Assistent (Glaswarenfabrik Karl Hecht). For morphological evaluations, seminal smears were fixed on a prestained slides (test simplets) and a minimum of 100 spermatozoa were seen using oil immersion with magnification of $\times 100$ under light microscope according to David's modified classification (18). All analyses were performed by one experienced technician blinded to the study.

Sperm DNA Evaluation: TUNEL Test

TUNEL assay was performed on sperm suspension using a commercial kit (the ApopTag Kit; Qbiogene) and following the manufacturer's recommendations. Briefly, semen suspension was washed twice by centrifugation at $600 \times g$ for 10 minutes in 5–10 mL of phosphate-buffered saline (PBS, pH 7.4). The final pellets were centrifuged again with 7 mL of acetic acid/methanol mixture and the newly obtained pellet was maintained at $-20^{\circ}C$ until use. Aliquots of the resulting suspension of nuclei were smeared onto microscope slides. For cell permeabilization, the slides were incubated in PBS with a solution of 1% Triton X100. Permeabilized cells were then washed twice in PBS 1X, equilibrated with the equilibration buffer at room temperature for 10 seconds, and incubated in a dark, moist chamber at $37^{\circ}C$ for 60 minutes with the terminal deoxynucleotidyl transferase solution to allow DNA elongation. After stopping the enzymatic reaction, the slides were washed twice in PBS and the DNA elongation was identified by incubation of the cells with antidigoxigenin antibody coupled to peroxidase for 30 minutes in a dark, moist chamber. The peroxidase was revealed with diaminobenzidine (DAB). Slides were then counterstained with Harris' hematoxylin and finally mounted using Faramount mounting. A minimum of 200 spermatozoa were seen using oil immersion with magnification of $\times 100$ under light microscope. Spermatozoa with fragmented DNA had brown-colored nuclei, whereas the other cells were blue-gray (counter coloration with Harris's hematoxylin). On each slide, cells were counted, and the percentage of TUNEL-positive sperm was calculated. The cutoff of 20% TUNEL-positive sperm was used (19, 20).

Sperm Chromatin Evaluation: Aniline Blue 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 (21). To do this staining, the semen was washed twice in 5 mL of PBS at pH 7.4 and centrifuged at $600 \times g$ for 10 minutes. Aliquots of the resulting suspension of nuclei were smeared on the glass slides and allowed to air dry. All the smears were fixed in 3% buffered glutaraldehyde in 0.2 M

phosphate buffer (pH 7.2) for 30 minutes at room temperature. Each smear was stained with 5% aqueous aniline blue and mixed with 4% acetic acid (Ph 2.5–3) for 5 minutes (21). On light microscopic evaluation, a minimum of 200 spermatozoa were counted in different areas of each slide with ×100 magnification under oil immersion. Spermatozoa with residual histone had blue-colored nuclei, whereas the other cells were gray. Unstained or pale blue were considered as normal spermatozoa, whereas dark blue stained were rated as abnormal spermatozoa (22). On each slide, cells were counted, and the percentage of spermatozoa with nuclear chromatin decondensation was calculated. An ejaculate with a rate of blue-stained sperm <20% was considered normal (21, 22).

Aneuploidy Analysis: Fluorescence in Situ Hybridization

Sperm samples were fixed in methanol-to-acetic acid (3:1) and processed for fluorescence in situ hybridization (FISH) analysis for each patient and control, using alpha centromeric probes for chromosomes 18, X, and Y. Briefly, semen suspension was washed twice by centrifugation at 600 × *g* for 10 minutes in 5–10 mL of PBS at pH 7.4. The final pellets were centrifuged again with 7 mL of acetic acid/methanol mixture and the newly obtained pellet was maintained at −20°C until use. Aliquots of the resulting suspension of nuclei were smeared onto microscope slides. Sperm nuclei were decondensed by incubating the slide in NaOH 1 N, at room temperature for 2 minutes. The slides were distilled-water washed, dehydrated through an ethanol series (70%–90%–100%) and air dried. Multicolor FISH was performed. Centromeric DNA probes for chromosome Y (probe DYZ3, spectrum green; Abbott), chromosome X (probe DXZ1, spectrum orange; Abbott), and chromosome 18 (D18Z1, spectrum aqua; Abbott) were used for the triple-color FISH analysis. The use of an autosomal probe, in addition to X and Y probes, allowed the distinction between disomy and diploidy. For hybridization, slides were incubated in a denaturation solution of 70% formamide, 20× standard saline citrate (pH 5.3) and distilled water at 72°C for 2 minutes. Slides were snap-cooled in 70% ethanol at −20°C for 2 minutes and then dehydrated through an ethanol series (90%–100%) at room temperature. The probes, precipitated and denatured at 72°C for 8 minutes, were applied directly to the slides, which were then covered with a coverslip and sealed with rubber cement. Slides were hybridized for 2 hours in a dark humidified chamber at 37°C. Finally slides were washed in 1× standard saline citrate, counterstained with 4′,6-diamidino-2-pheneylindole (DAPI) and stored in the dark at 4°C before carrying out microscopic observation. Analysis was done using an Axioplan epifluorescence microscope equipped with a single-band pass filter for DAPI/rhodamine/fluorescein isothiocyanate (FITC). For each probe 2,000 spermatozoa were counted per patient. Only intact spermatozoa bearing a similar degree of decondensation and clear hybridization signals were scored. Disrupted or overlapping spermatozoa were excluded from analysis.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences, version 16 (SPSS). All variables were initially tested to determine data normality by the Kolmogorov-Smirnov test. All hypotheses testing two-sided with the *P* value of <.05 were considered statistically significant.

RESULTS

Patient Characteristics

Maternal and paternal ages varied from 22–40 years (33.1 ± 5.1 years) and from 24–50 years (37.1 ± 5.4 years), respectively. Age of the men in control group varied from 27–49 years (36.9 ± 5.73 years), which did not differ significantly compared with the RPL group (*P*>.05). The mean number of miscarriages was 2.9 ± 0.8.

Semen Parameters

Table 1 shows the average values and statistical analysis of the various sperm parameters in the two groups. When comparing the routine semen analysis in men of the RPL group with the control group, semen volume, pH, and sperm concentration were within normal range in both groups. However, the percentage of total and progressive sperm motility were significantly lower in patients with spontaneous RPL versus control group (41.8 ± 5 vs. 51.5 ± 5.9; *P*<.001) and (30.2 ± 5.6 vs. 51.5 ± 5.9; *P*<.001), respectively. In the same way men in case group had significantly higher percentage of morphological defects (74.8 ± 13.7 vs. 54.2 ± 10.9; *P*<.001), especially head defects (82.5 ± 21.9 vs. 52.3 ± 18.4; *P*<.001).

Evaluation of DNA Damage

In our study, 10 patients (45%) presented with >20% TUNEL-positive sperm (range, 4%–41%) in the RPL group compared with 3 (15%) in the control group (range, 4%–21%). Evaluation of DNA fragmentation by TUNEL assay showed a higher

TABLE 1

Comparison of semen quality parameters, DNA fragementation and chromatin condensation in patients with recurrent pregnancy loss and fertile donors.

Semen parameter	RPL (mean ± SD)	Controls (mean ± SD)	*P* value
Ejaculation volume (mL)	3.1 ± 1.5	3.2 ± 1.4	.7
pH	7.6 ± 0.2	7.7 ± 0.2	.2
Vitality (%)	82.1 ± 11.2	83 ± 4.2	.7
Concentration (×10^6/mL)	166.7 ± 139.2	139.2 ± 61.4	.8
Total motility (%)	41.8 ± 5.0	51.5 ± 5.9	.001[a]
Progressive motility (a+b%)	30.2 ± 5.6	46.5 ± 5.4	.001[a]
Morphological alterations (%)	74.8 ± 13.7	54.2 ± 10.9	.001[a]
Head defects (%)	82.5 ± 21.9	52.3 ± 18.4	.001[a]
Multiple anomalies index (%)	1.48 ± 0.1	1.4 ± 0.1	.11
TUNEL positive sperm (%)	17.1 ± 11.6	10.2 ± 3.8	.016[a]
Aniline blue-positive sperm (%)	23.6 ± 9.3	11.8 ± 5.7	.001[a]

Note: RPL = recurrent pregnancy loss.
[a] Significant difference with control group (*P*< .05).

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percentage of TUNEL-positive sperm in the RPL group compared with the control group (17.1% ± 11.6% vs. 10.2% ± 3.8%, respectively) with statistically significant differences between the two groups (P=.016).

Aniline Blue Staining

There was a higher rate of spermatozoa with nuclear chromatin decondensation from men with spontaneous recurrent abortion compared with the control group, which was statistically significant (23.6% ± 9.3% vs. 11.8% ± 5.7%; P<.001). Twelve patients (54%) presented with >20% aniline blue-stained sperm (range, 13%–44%) in the RPL group compared with 1 (0.5%) in the control group (range, 3%–21%).

FISH Analysis

Using a triple-color FISH for chromosomes X, Y, and 18, a total of 44,000 spermatozoa from 22 male partners of patients with history of RPL and 10,000 spermatozoa from 20 control men were scored. The number of spermatozoa scored, the nullisomy, disomy, and diploidy rates for patients and controls are reported in Table 2. Among the normal spermatozoa, the percentages of X- and Y-bearing spermatozoa was, respectively, 45.1% and 44.3% in RPL group versus 49.6% and 48.8% in the control group. The total rate of chromosomally abnormal spermatozoa was 10.6% in patients with RPL and 1.5% in the control group, with a statistically significant difference (P<.001). All RPL patients showed a significant increase in the rates of sex chromosomes disomy (XX, YY, XY) and nullisomy compared with the control group (P<.001). The diploidy rate was also significantly higher in patients with RPL (1.1%) compared with controls (0.1%; P<.001).

Correlation Analysis

A multiple regression analysis between the different sperm parameters and pregnancy loss was performed (Table 3). A positive correlation between acrosome defects (r = 0.68) and tail defects (r = 0.66) and pregnancy loss was found. There was also a positive correlation between TUNEL-positive sperm and pregnancy loss (r = 0.54).

DISCUSSION

Recurrent pregnancy loss is one of the most frustrating and difficult areas in reproductive medicine, because the etiology is often unknown and there are few evidence-based diagnostic and treatment strategies. The male factor contributing in the evaluation of RPL has been less investigated, it is restricted to karyotype and basic semen analysis, whereas the assessment of sperm functionality is largely ignored (23).

In the present study semen samples from the case and the control groups were analyzed according to World Health Organization guidelines. There was no significant difference in ejaculate volume, pH, and concentration of sperm in the 22 men whose partners had two or more spontaneous abortions, compared with the control group. However, a significant difference was observed in the percentage of total abnormal

TABLE 2

Results of sperm chromosome abnormalities in control and recurrent pregnancy loss groups.

Patient number	Disomy X (%)	Disomy Y (%)	Disomy XY (%)	Nullisomy sex chromosomes (%)	Disomy 18 (%)	Nullisomy 18 (%)	Diploidy (%)
P1	0.7	0.8	0.6	0.3	0.6	1.2	0.6
P2	0.8	2.0	0.9	0.7	0.9	0.5	2.6
P3	1.3	2.4	0.5	1.0	2.0	0.6	0.5
P4	2.7	3.0	0.6	1.1	2.8	0.6	1.3
P5	3.4	3.1	1.0	1.0	0.8	0.8	0.3
P6	3.3	3.4	0.7	1.1	3.5	1.1	1.7
P7	1.0	1.5	1.8	0.5	2.1	4.2	1.2
P8	1.6	2.2	0.8	1.1	2.1	0.7	1.0
P9	1.6	1.7	2.3	0.5	2.9	1.3	0.8
P10	1.9	2.4	0.7	1.2	1.7	0.5	1.4
P11	1.2	1.6	1.0	0.8	2.2	1.1	2.5
P12	0.5	0.5	0.8	0.1	0.7	0.2	0.6
P13	2.8	2.8	1.0	1.2	2.2	1.0	1.2
P14	1.5	2.2	1.9	0.4	1.9	0.9	1.3
P15	2.7	3.4	0.7	0.8	1.0	0.5	0.4
P16	2.6	2.5	1.3	0.7	4.1	0.8	0.7
P17	2.4	3.8	0.9	1.8	3.2	0.4	1.1
P18	1.4	2.1	2.4	1.2	2.2	1.0	1.3
P19	1.5	1.6	2.9	0.6	1.6	2.9	1.5
P20	1.8	1.2	1.0	0.9	1.3	3.0	0.7
P21	2.4	0.6	3.3	0	3.9	0.6	2.6
P22	2.3	0.6	3.8	0.4	1.4	1.4	0.8
Patients (mean ± SD)	1.8 ± 0.8	2.0 ± 0.9	1.4 ± 0.9	0.8 ± 0.4	2.0 ± 1.0	1.1 ± 0.9	1.1 ± 0.6
Controls (mean ± SD)	0.1 ± 0.04	0.3 ± 0.1	0.5 ± 0.1	0.1 ± 0.08	0.1 ± 0.07	0.04 ± 0.02	0.1 ± 0.08
P value	.00[a]	.00[a]	.00[a]	.00[a]	.00[a]	.00[a]	.00[a]

[a] Significant difference with control group (P< .05).

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TABLE 3

Correlation between analyzed sperm parameters and recurrent pregnancy loss.

Variable	Correlation coefficient (*r*)	*P* value
Progressive motility	−0.13	.61
Sperm concentration	−0.31	.21
Morphological alterations	0.42	.08
Head defects	0.35	.16
Acrosome defects	0.68	.002[a]
Tail defects	0.66	.003[a]
TUNEL-positive sperm	0.54	.024[a]
Aniline blue	−0.22	.39
Total aneuploidy	−0.22	.37

[a] $P < .05$.

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sperm and sperm progressive motility ($P < .05$). When we compared our results with those in the literature, we found that the relationship between standard semen parameters and recurrent miscarriage has been a controversial subject (24, 25). Some studies have shown no significant correlation between sperm concentration (26) or abnormal sperm morphology (27) with recurrent spontaneous abortion. Carrell et al. (28) did not observe a significant difference between the RPL group and the control group for all the standard semen parameters.

In contrast to the previous data, Gil-Villa et al. (11) showed that men from the control group had a higher percentage of normal sperm morphology, concentration, and progressive motility compared with men from the RPL group. Another recent study by Absalan and Ghannadi (14) reported significant difference in sperm morphology and motility between RPL and control groups. Kobayashi et al. (29) reported that in IVF cycles, the increase in morphologically abnormal sperm was associated with delayed fertilization and cleavage rates and a greater risk for miscarriages even if the ET was successful, but in other studies morphology is not correlated with RPL (24, 30). However, when sperm quality of the donor men was compared with that of the RSA group, Bellver et al. (31) showed a significant difference in sperm concentration, but not in other parameters. But they also reported that, in both sperm donors and RPL patients, sperm concentration was still within the World Health Organization normal range. In addition and to our knowledge, this is the first study analyzing the correlation between specific morphology defects in the spermatozoa with the number of RPL, and we found a positive correlation between acrosome and tail defects and pregnancy loss. However, it is believed that semen analysis, which is traditionally used as the first step to evaluate the male factor, is insufficient to determine the fertility in vivo or in vitro (12). A failure of the conventional semen parameters to predict reproductive outcomes indicates that hidden anomalies lie at the sperm DNA or at the chromatin level (11). In fact, an increased incidence of sperm DNA damage has been associated with higher unexplained recurrent abortion rate in previous studies (28, 32). Therefore, using a TUNEL assay, we analyzed the semen samples for our patients and control group. Our data show that the percentage of sperm DNA fragmentation is significantly higher in male partners of patients who had unexplained RPL compared with controls. A positive correlation was also found between TUNEL-positive sperm and pregnancy loss. Our results were in accordance with the majority of the studies treating this subject. Carrell et al. (28), who used the same assay (TUNEL), observed that the percentage of sperm DNA fragmentation increased in a group of individuals whose partners had a history of RPL for unexplained reasons compared with control group of donors or the general population. Shamsi et al. (33) also found the same results but with a different assay (Comet) for the detection of DNA damage. However, this notion was not substantiated by the study of Gil-Villa et al. (11) because they did not find a significant difference between the RPL group and controls in sperm DNA fragmentation, which was assessed by the sperm chromatin structure assay. The inconsistency in these results could be largely attributed to the different sensitivities of the techniques used and the aspects of DNA damage tested by these techniques. Tests, like sperm chromatin structure assay or single-cell electrophoresis comet assay at alkaline or acidic pH require a denaturalization step to detect the DNA fragments or the potential breaks in the DNA. However, TUNEL or comet assay at neutral pH do not require denaturalization and they measure real DNA breakage, either on one or both strands of the DNA (34).

More recent studies suggest that idiopathic RPL is associated with a higher incidence of aberration in sperm chromatin packaging (25). Nuclear chromatin decondensation of spermatozoa and subsequent male pronucleus formation is essential for fertilization and normal embryonic development. Chromatin damage precedes the loss of fertilization potential and poor embryo quality, resulting in pregnancy loss (23). The degree of condensation can be shown with the aid of acidic aniline blue staining, which is able to discriminate between lysine-rich histones and arginine- and cysteine-rich protamines (21). Using this technique our results demonstrated that the percentages of spermatozoa with chromatin condensation disturbances increase in patients with RPL history, if compared with the control population. Our results are consistent with other studies that have also found a statistically higher rate of abnormal chromatin condensation in patients with recurrent abortions, such as Kazerooni et al. in 2009 (35), and these investigators used different techniques to assess sperm chromatin condensation. In fact, the quality of the chromatin can also be studied by methods such as aniline blue, toluidine blue, chromomycin A3, acridine orange under denaturing conditions (36). These investigators found the same results regardless of the technique used. The present study strengthens the current literature associating sperm quality with RPL, and emphasizes evaluating male factor by sperm function tests along with conventional semen parameters.

To explain the relationship between sperm chromatin/DNA anomalies and RPL, it should be considered that any abnormalities in unique organization of sperm chromatin are thought to affect the proper expression and regulation of paternal genes in early embryos (21). Ahmadi and

Ng (37) have previously reported in a mouse model system that spermatozoa with defective DNA can fertilize an oocyte and produce high-quality early-stage embryos, but then, as the extent of the DNA damage increases, the likelihood of a successful term pregnancy decreases. Seli et al. (38) have also reported that the extent of nuclear DNA damage in spermatozoa was related to embryo development to the blastocyst stage, a time when the embryonic genome is activated, transcriptional activity has begun, and the paternal genome plays a significant contributory role in embryo function. It is in fact widely assumed that the first steps of development are subjected to maternal control and that the expression of paternal genes begins at the 4- to 8-cell stage. It is therefore at this stage that the consequences of paternal DNA-induced alterations may become manifest, impairing embryo development (39). 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 (40).

Another major objective in this study was also to assess to what extent spermatozoon as the motile carrier of the paternal genome was implicated in RPL. For this purpose, we scored sperm aneuploidy rates for chromosomes X, Y, and 18 using a triple-color FISH assay. In the present work, direct analysis of decondensed sperm nuclei revealed that each chromosomal anomaly assessed appeared to be significantly increased, including nullisomy, gonosomal disomy, autosomal disomy, and diploidy. We can then consider that male partners of patients suffering from unexplained RPL are at high risk for sperm chromosomal abnormalities at least numerically. These findings suggest an implication of sperm chromosome abnormalities in recurrent pregnancy wastage. A normal constitutional male karyotype does not exclude the presence of chromosome abnormalities in spermatozoa. Such abnormalities could arise de novo in the germ cell line, and several data indicate that moderate but significant increases in a given type of disomy are related to an increase in aneuploidy in the offspring (41). Therefore, cytogenetic studies on spermatozoa are of great interest to assess their chromosomal constitution. Similar findings have been reported on the higher incidence of different chromosomal abnormalities in sperm nuclei in patients with history of RPL, specifically for sex chromosomes and also autosomes such as 1, 17, 8, 18, or 21 (15, 42, 43). The FISH assay is an accurate technique to detect the most common aneuploidies in decondensed sperm nuclei (41). If a higher incidence of specific chromosome abnormalities could be found, it would be helpful in the choice of treatment strategies.

In conclusions, although this study has a limited number of cases and controls, but the significant increase of the abnormal sperm parameters, sperm DNA fragmentation, nuclear chromatin decondensation, and also sperm aneuploidy suggest possible causes of unexplained RPL. Hence, we recommend the screening of both partners simultaneously in RPL case to orient treatment strategies and achieve a desirable outcome. In the future such studies are required with more case and control subjects to ascertain the specific sperm dysfunction, which is predominate in causing RPL.

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