Chromatin status in human ejaculated spermatozoa from infertile patients and relationship to seminal parameters

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The aim of this study was to evaluate the chromatin status in different groups of patients. Five groups of men were selected: pre-vasectomy; male factor infertility; varicocele; immunological male infertility; and idiopathic infertility. Chromatin status was evaluated using flow cytometry after staining the DNA with the fluorochrome propidium iodide. Differences were observed in the state of sperm chromatin between the male factor and varicocele groups with respect to the others. These two groups presented poorer quality chromatin, as evidenced fundamentally by a lower degree of condensation. These deficiencies in chromatin status were usually accompanied by alterations in the other standard parameters of semen analysis. Individuals who are infertile due to male factor and those presenting varicocele have spermatozoa with less condensed chromatin which might, in part, explain their sterility.

Key words: chromatin/flow cytometry/propidium iodide/spermatozoa

Introduction

During spermatogenesis, histones are replaced by small proteins of highly basic character—known as protamines—that are rich in cysteine residues. During spermatogenesis and epididymal maturation, disulphide bridges form between these residues, providing the chromatin with the added stability that is necessary to ensure the transport and integrity of the male genetic material. This state of chromatin condensation may be altered by various factors, such as a shortage of zinc from the prostate, or alterations in protamines, which affects the fertilizing capacity of the spermatozoon (Kvist, 1980; Kvist and Eliasson, 1980; Balhorn et al., 1988; Kvist et al., 1988; Björndahl and Kvist, 1990; Belokopytova et al., 1993; De Yebra et al., 1993; Bench et al., 1996; Kramer and Krawetz, 1997).

The assessment of chromatin status is very important when evaluating the ability of spermatozoa to fertilize. Many techniques have been described for the evaluation of chromatin status, such as optical microscopy (Krzanowska, 1982; Huret, 1984; Rosenborg et al., 1990), electron microscopy (Jamil, 1984; Lipitz et al., 1992) and flow cytometry (Engh et al., 1992; Zucker et al., 1992; Molina et al., 1995; Samocha-Bone et al., 1998; Evenson et al., 1999; Spanò et al., 1999). Sperm chromatin defects have been correlated with the reduced ability of spermatozoa to fertilize both in the context of assisted reproduction techniques (Bianchi et al., 1996; Hoshi et al., 1996; Sakkas et al., 1996; Hammadeh et al., 1998; Lopes et al., 1998; Filatov et al., 1999; Gopalkrishnam et al., 1999) and in the general population (Evenson et al., 1999; Hacker-Klom et al., 1999; Spanò et al., 2000). Moreover, patients with fertility problems have often been characterized by an increased frequency of spermatozoa with abnormal chromatin (Spanò et al., 1984; Engh et al., 1992; Foresta et al., 1992; Kosower et al., 1992; Liu and Baker, 1992; Hughes et al., 1996).

In this study, different groups of patients were examined, both fertile and infertile, in order to analyse the standard parameters of semen analysis and those that depend on the state of sperm chromatin. Thus, it was possible to observe differences between patient groups, using the technique of flow cytometry after DNA staining with the fluorochrome, propidium iodide (PI).

Materials and methods

In order to study sperm chromatin status, semen samples from 182 males attending the Human Reproduction Unit of the 'Virgen de las Nieves' Hospital in Granada were analysed. These patients were classified into five groups: (i) pre-vasectomy but of proven fertility within the past 2 years (n = 44); (ii) male factor infertility with one or various abnormal semen parameters according to the recommendations of the World Health Organization (WHO, 1992) (n = 84); (iii) infertile patients with varicocele (n = 18); (iv) males who were infertile due to an immunological factor (Direct Mixed Agglutination Reaction Test >20% motile spermatozoa with latex particles attached) (n = 12); and (v) men whose sterility was due to unexplained factors and whose semen analyses were normal according to WHO criteria (n = 24) (WHO, 1992). The standardized investigation of infertile couples (WHO, 1993) was normal in all those diagnosed with idiopathic infertility.

Table I. Analysis of the standard seminal parameters in the different patient groups

Parameter	Pre-vasectomy	Male factor	Varicocele	Idiopathic	Immunological
Concentration ($\times10^6$/ml)	112.8 ± 13.4 (27.0–356.0)	37.9 ± 3.4[a] (0.2–129.0)	46.6 ± 11.6[b] (1.5–147.0)	93.2 ± 9.1 39.0–236.0	152.1 ± 26.5 (15.0–276.0)
Motility (% grade 'a')	41.4 ± 1.9 (10–0–62.0)	15.0 ± 1.7[a] (0.0–62.0)	21.0 ± 5.0[b] (0.0–62.0)	41.6 ± 2.0 (25.0–62.0)	37.6 ± 3.6 (15.0–52.0)
Vitality (% dead)	16.5 ± 1.0 (5.0–34.0)	42.4 ± 1.7[a] (14.0–75.0)	42.6 ± 4.3[b] (12.0–68.0)	21.5 ± 0.6 (14.4–25.0)	26.0 ± 3.4 (11.0–49.0)
Morphology (% normal form)	34.4 ± 1.7 (15.0–59.0)	22.2 ± 1.1[a] (5.0–48.0)	26.7 ± 3.5[b] (6.0–48.0)	40.8 ± 2.3 (30.0–65.0)	41.6 ± 2.6 (25.0–52.0)
Head alterations (%)	50.6 ± 2.0 (26.0–80.0)	60.8 ± 1.4[a] (29.0–84.0)	55.2 ± 3.7 (32.0–76.0)	46.4 ± 2.3 (21.0–65.0)	41.5 ± 3.0 (31.0–58.0)
Volume (ml)	3.3 ± 0.1 (1.5–5.2)	2.7 ± 0.1[a] (0.8–6.5)	2.9 ± 0.2 (1.4–5.6)	2.9 ± 0.2 (2.0–6.3)	3.5 ± 0.2 (2.8–5.0)
Hypo-osmotic test (% tail swelling)	73.8 ± 1.5 (54.0–90.0)	62.5 ± 1.4[a] (31.0–86.0)	69.3 ± 3.2 (48.0–90.0)	70.7 ± 1.6 (60.0–88.0)	73.0 ± 4.0 (53.0–92.0)

Values are mean ± SEM; values in parentheses are ranges.
[a]$P < 0.05$, male factor versus pre-vasectomy group.
[b]$P < 0.05$, varicocele group versus pre-vasectomy group.

Table II. Analysis of the parameters related to the state of sperm chromatin in the different patient groups (mean channel of fluorescence)

Parameter	Pre-vasectomy ($n = 44$)	Male factor ($n = 84$)	Varicocele ($n = 18$)	Idiopathic ($n = 24$)	Immunological ($n = 12$)
Condensation	29.8 ± 0.4 (25.9–37.1)	36.3 ± 0.8[a] (27.0–58.0)	38.0 ± 1.9[b] (29.4–50.5)	29.7 ± 0.5 (25.2–35.3)	29.2 ± 0.8 (25.9–33.5)
Decondensation	37.9 ± 0.6 (29.7–49.4)	42.7 ± 0.8[a] (28.5–62.8)	48.6 ± 2.5[b] (36.2–67.3)	38.0 ± 1.0 (27.6–49.5)	39.3 ± 1.8 (35.0–52.3)
Hyperstability	28.9 ± 0.5 (24.9–39.8)	34.7 ± 0.9[a] (21.3–59.7)	37.3 ± 2.4[b] (27.0–52.6)	27.1 ± 0.6 (22.6–33.5)	26.4 ± 1.0 (23.1–33.3)

Values are mean ± SEM; values in parentheses are ranges.
[a]$P < 0.001$, male factor versus pre-vasectomy group.
[b]$P < 0.05$, varicocele group versus pre-vasectomy group.

Semen samples were obtained by masturbation after 3 days of sexual abstinence, and collected in a sterile plastic container. They were allowed to liquefy for 30 min at 37°C, after which an analysis was performed to measure the following parameters: concentration, motility, percentage of normal forms, percentage of spermatozoa with head anomalies, vitality, hypoosmotic test, mixed agglutination reaction test and volume of ejaculate (WHO, 1992).

The ejaculates were used to study sperm chromatin status by analysing the uptake of PI, using flow cytometry with a FACScan IV cytometer (Becton Dickinson, Mountain View, CA, USA). In this study, only one of the three fluorescence detectors available to the FACScan IV was used, to measure the fluorescence corresponding to the red colour of PI. PI-stained cells were analysed in a flow cytometer equipped with a 488 nm argon laser as the light source and a 560 nm optical filter. For each determination 10 000 sperm nuclei were measured.

The flow cytometry parameters analysed were: condensation of sperm chromatin; resistance to decondensation after treatment with 1% sodium dodecyl sulphate (SDS) plus 6 mmol/l ethylenediamine-tetraacetic acid (EDTA) decondensing solution in borate buffer for 5 min; and the tendency to achieve a state of hyperstability after 6 h of incubation at 37°C and subsequent treatment with SDS–EDTA (Molina *et al.*, 1995). Three aliquots of the semen sample were taken for analysis. The first was directly stained with 50 µg/ml PI, using

the commercial Cycle test kit (Beckton Dickinson). This permitted analysis of the state of condensation of the sperm chromatin, as this is directly related to PI uptake. The second aliquot was treated with SDS–EDTA and then stained with PI, while the third aliquot was incubated for 6 h in B2 medium (Bio Mérieux, Marcy l'Etoile, France) at 37°C and 5% CO_2 and then treated with SDS–EDTA. This method was employed in order to observe the tendency of the spermatozoa to achieve a state of hyperstability. The mean channel (MCH) of fluorescence was used to analyse the accessibility and, consequently, the degree of staining ('stainability') of sperm DNA after staining with PI.

For each sample, the percentage of variation of stainability after the decondensation step under normal and hyperstability conditions was calculated as follows:

$$\text{(MCH after SDS–EDTA)} - \text{(MCH before SDS–EDTA)} / \text{(MCH before SDS–EDTA)}$$

Results were presented as the mean ± SEM. All percentage data were transformed using the formula: $\log(x + 0.5)/(100.5 - x)$.

This transformation has been suggested as a suitable method to normalize percentage data (Atkinson, 1985). Variance analysis was used to examine group differences, and comparisons were made with Bonferroni's test. Significance was defined as $P < 0.05$. Simple linear regression analysis was used to show the degree of linear association
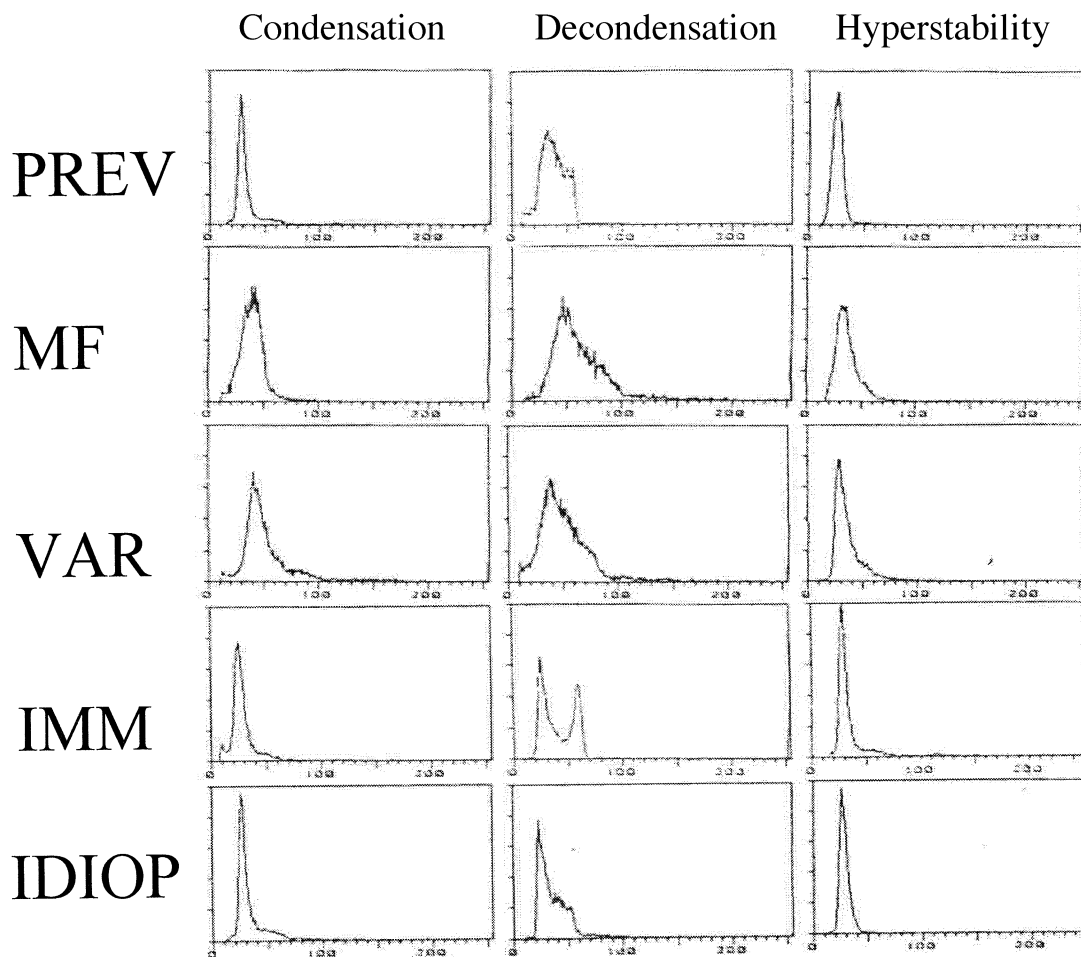
Condensation   Decondensation   Hyperstability

PREV

MF

VAR

IMM

IDIOP

Figure 1. Flow cytometry analysis of the parameters related to the state of sperm chromatin (condensation, resistance to decondensation and hyperstability) in the different patient groups. PREV = pre-vasectomy; MF = male factor; VAR = varicocele; IMM = immunological factor; IDIOP = idiopathic cause.

between chromatin status and seminal parameters. A stepwise multiple regression was used to predict sperm chromatin status; a variable was included if its partial regression coefficient was significant at the 0.05 level, and was eliminated if its partial regression coefficient failed to reach significance at the 0.10 level. Statistical analyses were performed using the BMDP statistical package (BMDP Statistical Software, Los Angeles, CA, USA).

Results

The standard seminal parameters (sperm concentration, motility and vitality, morphologically normal forms and head anomalies) showed significant differences between the male factor and varicocele and pre-vasectomy, idiopathic and immunological infertile groups (Table I). The hypoosmotic test showed statistically significant differences between the male factor group and the pre-vasectomy and idiopathic groups (Table I). Finally, a significant reduction was observed in the seminal volume in the ejaculate of the male factor group, compared with that of the pre-vasectomy group.

An analysis of chromatin-related parameters (condensation, resistance to decondensation and hyperstability) was carried out for the previously established patient groups. A significantly lower degree of condensation was observed between those affected by male factor sterility and varicocele groups on the one hand, and the pre-vasectomy group on the other hand (Table II; Figure 1). On measuring resistance to decondensation after treatment with SDS–EDTA, significant differences were found between the male factor and varicocele groups, and the pre-vasectomy and idiopathic groups, with a higher degree of chromatin decondensation being observed in the former two groups (Table II; Figure 1). Finally, with respect to the tendency of sperm chromatin to attain a state of hyperstability, significant differences were observed between the male factor group and the others (except the varicocele group), the former reaching a lower level of hyperstability. The same result was found in the varicocele group with respect to the others (Table II; Figure 1).

The percentage increase in stainability after the decondensation step under normal conditions was lower in the male factor group than in the pre-vasectomy group (Table III). No significant differences between the different groups were observed when the percentage of variation of stainability after the decondensation step under hyperstability conditions was analysed.

An analysis was also performed of the relationship between the state of sperm chromatin and the standard seminal para-

Table III. Percentage of variation of stainability after decondensation step in normal and hyperstability conditions (%)

	Pre-vasectomy ($n = 44$)	Male factor ($n = 84$)	Varicocele ($n = 18$)	Idiopathic ($n = 24$)	Immunological ($n = 12$)
Decondensation	28.2 ± 3.0 (1.0–90.3)	18.9 ± 1.7[a] (–13.1–53.4)	28.3 ± 3.0 (8.1–54.2)	28.1 ± 2.7 (–1.1–52.3)	34.7 ± 3.8 (18.9–56.3)
Hyperstability	–2.9 ± 1.9 (–13.8–53.6)	–5.0 ± 0.6 (–24.4–4.18)	–3.0 ± 1.4 (–9.8–7.9)	–8.7 ± 0.9 (–21.1–1.4)	–9.4 ± 1.3 (–16.3–0.29)

Values are mean ± SEM; values in parentheses are ranges.
[a]$P < 0.05$, male factor versus pre-vasectomy group.

Table IV. Matrix of the significant coefficients of simple linear correlation between the different parameters studied in the different ejaculates

	Condensation	Decondensation	Hyperstability	Concentration	Volume	Motility	Vitality	Morphology	Head alteration
Condensation	–	–	–	–	–	–	–	–	–
Decondensation	0.754	–	–	–	–	–	.	–	–
Hyperstability	0.964	0.798	–	–	–	–	–	–	–
Concentration	–0.511	–0.29	–	–	–	–	–	–	–
Volume	–	–0.239	–	–	–	–	–	–	–
Motility	–0.678	–0.494	–0.643	0.542	–	–	–	–	–
Vitality	0.596	0.454	0.532	–0.487	–0.25	–0.841	–	–	–
Morphology	–0.569	–0.4	–0.542	0.517	–	0.638	–0.587	–	–
Head alteration	0.389	0.265	0.37	–0.409	–	–0.472	0.487	–0.892	–
Hypoosmotic	–	–	–	0.335	–	0.584	–0.542	0.335	–0.248

Table V. Stepwise multiple regression analysis of chromatin condensation status versus the other seminal parameters studied

Step	Variable	R	R^2	% increase R^2	F_{exp}	d.f.
1	Motility	0.678	0.460	46.04	170.67	1,200
2	Morphology	0.701	0.491	3.13	12.27	1,199
3	Concentration	0.713	0.508	1.66	6.67	1,198

d.f. = degrees of freedom.

Table VI. Stepwise multiple regression analysis of resistance to decondensation of the sperm chromatin versus the other seminal parameters studied

Step	Variable	R	R^2	% increase R^2	F_{exp}	d.f.
1	Motility	0.494	0.244	24.47	64.79	1,200
2	Volume	0.508	0.258	1.40	3.76	1,199

d.f. = degrees of freedom.

Table VII. Stepwise multiple regression analysis of the degree of hyperstability of the sperm chromatin versus the other seminal parameters studied

Step	Variable	R	R^2	% increase R^2	F_{exp}	d.f.
1	Motility	0.643	0.414	41.40	141.29	1,200
2	Morphology	0.665	0.443	2.91	10.39	1,199

d.f. = degrees of freedom.

meters. On obtaining the matrix of simple linear correlation coefficients for the different parameters studied in the ejaculates (Table IV), it was seen that the values of these coefficients were, in general, not very high. The best correlations were found between those parameters directly related to chromatin (condensation, resistance to decondensation, and hyperstability), while the other seminal parameters were less well correlated, although there was seen to be a high inverse correlation between motility and vitality (% of stained spermatozoa).

Stepwise multiple linear regression analysis indicated that chromatin condensation variability was explained by the spermatozoa motility, morphology and concentration variables (Table V). The motility and volume variables are included in the stepwise multiple regression model, which attempts to explain the variability in the resistance of sperm chromatin to decondensation after treatment with SDS–EDTA (Table VI). Finally, chromatin hyperstability was related to motility and morphology (Table VII).

Discussion

The present study was intended to evaluate chromatin status, which is of great importance when testing the fertilizing capacity of spermatozoa. On performing chromatin analysis in the different groups of patients, as described above, significant differences were found for chromatin-related parameters in the male factor and varicocele groups with respect to the pre-vasectomy group. Both groups were found to have alterations

in both spermatogenesis (seen as an alteration at the nuclear level) and standard seminal parameters.

The significant differences observed between the male factor and varicocele groups and the pre-vasectomy group have many possible explanations, including incomplete replacement of histones by protamines, aberrant ratios of protamine 1 to protamine 2, high concentrations of non-oxidized SH groups in protamine molecules, or the occurrence of DNA breaks (Balhorn *et al.*, 1988; Auger *et al.*, 1990; Belokopytova *et al.*, 1993; De Yebra *et al.*, 1993; Bench *et al.*, 1996; Aravindan *et al.*, 1997; Filatov *et al.*, 1999).

Others (Foresta *et al.*, 1989) have studied the different types of pathologies that damage the structure of sperm chromatin. On evaluating the percentage of sperm heads with chromatin denaturation, using acridine orange (Tejada *et al.*, 1984), these authors found that varicocele, cryptorchidia and orchitis resulting from parotiditis all result in spermatozoa having a chromatin that is less resistant to chemical denaturalization, though the incidence of this effect is not the same for all individuals and pathologies. The presence of varicocele is usually associated with abnormality in seminal parameters and in the histology of the testicle, while varicocelectomy has been shown to improve semen quality and increase the rate of pregnancies achieved (Ito *et al.*, 1986; Takihara *et al.*, 1990). DNA evaluation of testicular cells by flow cytometry has shown that spermatogenesis in males with varicocele is usually lower (Takihara *et al.*, 1990). The factors responsible for chromatin disorders in spermatozoa from the varicocele group remain unclear, though suggestions are that: (i) apoptosis is abnormally frequent in the sperm cells of the patients with varicocele, and plays a significant role in the spermatogenetic dysfunction associated with varicocele (Simsek *et al.*, 1998); or (ii) there are increasing concentrations of reactive oxygen species (Koksal *et al.*, 2000) and a reduction in antioxidant defences (Barbieri *et al.*, 1999) in higher grades of varicocele.

It should also be noted that, for those parameters that are indicative of sperm chromatin, males who are sterile due to immunological factors did not present statistically significant differences from the pre-vasectomy and 'no apparent cause' groups. This suggests that the effect of the antisperm antibodies occurs at the membrane or cytoplasm level but not at the nuclear level. It has been suggested (Naz, 1992; Naz and Menge, 1994) that there is an effect at the nuclear level in antisperm antibodies, as these could inhibit development of the pronuclei in the zygote. Furthermore, anti-DNA antibodies have been found frequently to be present in males with antisperm antibodies (D'Cruz *et al.*, 1994).

There seems to be a certain association between semen of lower quality, according to conventional measurements (WHO, 1992), and a reduction in the condensation of sperm chromatin. However, when analyses were carried out of the different flow cytometry histograms for the state of sperm chromatin in those who were infertile due to male factor, the histograms varied from being almost identical to those of normozoospermic males to showing virtually the whole sperm population as hypocondensed.

When the different seminal parameters were related, it was found that the best correlations (values of 0.7–0.9)

corresponded to parameters that are indicative of the state of sperm chromatin. This is logical, as these parameters are completely interrelated, and reflect the evolution of the sperm nucleus during spermatogenesis and subsequent maturation. However, when the correlation of sperm chromatin parameters with standard parameters for semen analysis was analysed, low values (~0.5) were found, suggesting that these reflect completely different physiological processes during spermatogenesis, and thus there is little correlation. The standard seminal parameters presented low correlation coefficients (0.4–0.6), which was in agreement with the findings of others (Wang *et al.*, 1988).

The stepwise multiple linear regression analysis performed to determine which seminal variables present the best relationship with the degree of condensation of sperm chromatin, revealed these to be motility, percentage of normal forms in the ejaculate, and the concentration of spermatozoa. Nevertheless, these parameters are only able to predict 50% of the variability observed in the condensation of sperm chromatin, as they reflect biological processes which differ from those representing nuclear quality (Spanò *et al.*, 1998; Evenson *et al.*, 1999). The origin of this weak relationship is not clear; pathological agents such as oxidative stress may affect spermatozoa at different levels at the same time, including mitochondrial function affecting motility, the acrosome and membrane functions affecting morphology and vitality (Dadoune *et al.*, 1988), and DNA affecting accessibility of PI and, consequently, chromatin-related parameters.

The parameters related to resistance to the decondensation of sperm chromatin include motility and ejaculate volume. The volume effect might be explained in individuals with a low level of prostatic secretion, as this would be manifested by a reduction in ejaculate volume, as well as a reduction in resistance to decondensation, due to the lower level of prostate-derived zinc in the ejaculate, as has been suggested by others (Kvist, 1980; Kvist and Eliasson, 1980; Kvist *et al.*, 1988; Björndahl and Kvist, 1990).

Finally, multiple linear regression analysis found the parameters most likely to predict the variability in the attainment of hyperstability by the sperm chromatin to be motility and sperm morphology, though these only predicted 45% of the hyperstability. Therefore, it is clear that there is relatively little correlation between the parameters related to sperm chromatin and the other conditions, as each is a reflection of the different processes occurring during spermatogenesis.

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