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Epidemiology of sperm DNA fragmentation in a retrospective cohort of 1191 men

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Abstract

Background: The scientific and clinical communities now recognize that sperm DNA integrity is crucial for successful fertilization, good embryo development, and offspring quality of life. Despite the apparent unanimity, this criterion is rarely evaluated in clinical practice. We evaluated the sperm DNA fragmentation index of nearly 1200 sperm samples and its connections based on the patient's age, body mass index, the season of sperm collection, geographical location, medical history, and addictive behaviors.

Methods: A cohort of 1503 patients who were referred to the Royan Institute between July 2018 and March 2020 was examined. Only 1191 patient records with demographic data, complete semen analysis, and DNA fragmentation index measurements were included in the final cohort. Documents were classified, incorporated into statistical models, and analyzed.

Results: The results confirmed previous findings that the sperm DNA fragmentation index was significantly higher in aging men. The sperm DNA fragmentation index and high DNA stainability levels were significantly higher in spring and summer samples than in those of other seasons. No correlation was found between semen DNA fragmentation index and patient body mass index, although the study cohort was significantly overweight. Contrary to what might be expected, we observed that the

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sperm DNA fragmentation index was higher in rural than in urban patients. Intriguingly, epileptic patients exhibited significantly higher sperm DNA fragmentation index levels. **Discussion and conclusion:** Age is the factor that is most strongly associated with sperm DNA fragmentation index levels. Our analysis of 1191 samples indicates that between the ages of 19 and 59, the sperm DNA fragmentation index increases by an average of 2% each year. Intriguingly, from an epidemiological perspective, the warm season (spring/summer) is associated with a higher sperm DNA fragmentation index in the study population, possibly due to the deleterious effect of temperature on sperm quality. Some neurological diseases, such as epilepsy, are associated with decreased sperm DNA integrity. This observation could be related to the iatrogenic effects of associated therapies. In the study cohort, body mass index did not appear to be correlated with the DNA fragmentation index.

KEYWORDS
age factors, DNA fragmentation, epilepsy, mumps, rural health, seasons, spermatozoa

1 | INTRODUCTION

One of the most important requirements for successful mammalian reproduction is the nuclear/DNA integrity of the spermatozoa. Over the past ten years, it has been increasingly evident that changes to the paternal genetic materials, whether they occur at the chromatin, DNA, or epigenetic mark levels, are likely to have an impact on the health of the progeny.[1] However, the quality of spermatozoa genetic material is still not taken into account in clinical practice when evaluating male patients from infertile couples. Worldwide, the WHO limits its recommendation for pre-assisted reproductive technology (pre-ART) screening of men to a simple assessment of sperm count, morphology, and motility, with the latter being considered increasingly unnecessary in view of intracytoplasmic sperm injection (ICSI).

Because of the complex and very specific nature of the organization of the mammalian spermatozoa genetic material and the incapacity of mature spermatozoa to repair itself,[2] there are many ways in which the sperm nucleus/DNA can be altered. Leaving aside the peculiar situation of chromosomal abnormalities,[3] the sperm nucleus is frequently concerned by sub-optimal condensation that could be due to defective protamine-mediated compaction or excessive sperm DNA fragmentation (SDF), whether it is single or/and double DNA breaks.[4] SDF has multiple origins which are not mutually exclusive. During spermatogenesis, it can arise from unrepaired meiotic breaks, non-evacuated apoptotic germ cells, or mechanical shearing upon protamination of the sperm nucleus at the final spermiogenesis stage. SDF may also result from oxidative insults during spermatogenesis, especially in inflammatory situations associated with oxidative bursts, such as varicocele and orchitis. More frequently, oxidative alterations of the sperm nucleus, eventually leading to SDF, occur during the post-testicular life of the sub-mature sperm cell during epididymal maturation and storage, as well as when emitted at contact with sub-optimal seminal fluid. Two main reasons explain the susceptibility of post-testicular spermatozoa to oxidative insults that may lead to nuclear damage and SDF. First, as mentioned above, mature spermatozoa leaving the testis are transcriptionally silent and therefore unable to activate gene responses to stressful situations. In addition, and particularly important in the context of sperm DNA damage, they are also unable to activate DNA repair pathways such as the base excision repair pathway.[2] The near absence of cytosol in the sperm cell also explains its poor ability to protect itself by cytosolic protecting players, whether enzymes, blocking peptides, or small metabolites. Second, while the seminiferous tubule is a well-protected epithelium from systemic influences, the epithelia of the accessory organs (starting with the epididymis) are largely more permeant, exposing maturing spermatozoa to potential aggressors that may generate DNA damage.[4,5] Consequently, any environmental stressors leading to a rise in systemic inflammatory status will result in post-testicular sperm cell alterations, including DNA damage and SDF.[6]

A simple sperm nuclear condensation assay could greatly increase the rapid evaluation of the patient's sperm nuclear integrity. In addition, clinicians now have access to several assays which directly or indirectly address SDF. These include the TUNEL assay, the Comet assay, the sperm chromatin dispersion assay, and the Sperm Chromatin Structure Assay (SCSA). Although there is no consensus as to which of these tests is the most relevant, reliable, and cost-effective, as well as the one with the best prognostic value, the SCSA has undoubtedly gained a lot of credence because of its extensive evaluation in very large cohorts.[7] Threshold values for the DNA fragmentation index (DFI) obtained via SCSA assessments are now in effect and it is well accepted that when the DFI is greater than 25%–30%, ART reproductive failure is to be expected.[8,9]

Studies addressing the impact of various stressors on the level of SDF already exist. For example, positive correlations were reported with biological factors (age, infection, presence of varicocele, obesity), lifestyle choices (smoking, drugs, medication) and environmental

factors (pollutants, heat, exposure to ionizing or/and electromagnetic radiations), as reported in several reviews in the last few years.[10–15] However, the reported studies were often underpowered, mainly because of small cohort sizes.

In the present work, we have addressed the question of SCSA-assessed SDF measurements on a large cohort of nearly 1200 patients. In that cohort, we have worked out the correlation we could make with various parameters, including sperm classical structural and functional parameters, as recommended by the World Health Organization (WHO, 2010),[16] patient age, body mass index (BMI), lifestyle, occupational status, season of spermatozoa sample collection, geographical origin, and disease trajectory.

2 | MATERIALS AND METHODS

2.1 | Patients and study design

The Royan Institute ethical committee approved the study under the accession number (IR.ACECR.ROYAN.REC.1400.012). In the first screen, from July 2018 to March 2020, 1503 men referred to the Royan Institute (andrology laboratory) for SDF testing as part of the routine pre-ART examination were included in the study. Patient records were included in the final cohort only if they provided all of the necessary information, ranging from complete seminal evaluation, age, BMI, history of disease and surgery, current medical treatment, exercise, diet, and addictive behaviors (such as smoking, drinking, recreative drug use, …), occupational hazards and geographical origin. Patients with heavy medical treatments such as radio- and chemotherapies were excluded from the final cohort. In addition, patients for whom incomplete data were available were excluded. Following these selection criteria, the final cohort consisted of 1191 men.

2.2 | Semen analysis

Semen samples were collected via masturbation (abstinence time 3–5 days) according to WHO guidelines (WHO, 2010).[16] After semen liquefaction, sperm concentration, and motility were assessed via a computer-assisted sperm analyzer (CASA, SCA, Microptic Co., Spain). Sperm morphology was evaluated via Papanicolaou staining. Leukocyte concentration was determined after peroxidase staining and counting under 400 × magnification, as described by Endtz.[17]

2.3 | Sperm chromatin structure assay

Fresh samples were evaluated for DNA fragmentation. The sperm chromatin structure assay (SCSA) was carried out by adding 200 μl of acid detergent solution (0.1% Triton X-100, 0.15 mol/l NaCl and 0.08 N HCl, pH 1.2; Sigma-Aldrich Chemical Co., Germany) to the diluted spermatozoa for 30 s. Afterward, the samples were mixed with 1.2 ml acridine orange (AO) stain solution containing 6 μg AO/ml in a buffer consisting of: 0.037 M citric acid, 0.126 M Na_2HPO_4, 0.0011 M EDTA (di-sodium) and 0.15 M NaCl, pH 6.0 (Sigma-Aldrich Chemical Co., Germany) as reported previously.[16] Sperm cells (50,000) were then analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an air-cooled argon-ion laser. AO intercalated in double-stranded DNA emits green fluorescence, whereas AO associated with single-stranded DNA emits red fluorescence. The SCSA data were converted into the DFI following SCSA software treatment and were expressed as total DFI as consensually admitted in worldwide infertility clinics. Total DFI corresponds to the total percentage of AO-positive sperm cells. It is consensually admitted that when DFI reaches values around and above 25% it is considered pathological. The flow cytometer provides a second interesting parameter to monitor: the percentage of cells showing a high level of fluorescence (High DNA stainability [HDS]), which is classically admitted to corresponding to spermatozoa with an immature level of nuclear condensation.[18]

2.4 | Statistical analysis

Statistical analysis was performed using SPSS software (version 22; Inc., Chicago, IL, USA). The normality of data was assessed by the Shapiro-Wilk test. Parameters were compared using an analysis of variance followed by simultaneous Post hoc Tukey tests to analyze continuous variables.

Data were analyzed using the 2-tailed Student *t*-test for independent data, Fisher exact test, and a two-by-two table between groups, where appropriate. Univariate and multivariate linear regression analyses were used to determine associations between independent variables and DFI. Multiple linear regression analysis (multivariate analysis) was performed with the independent variables that were significant in univariate analyses at a level of 0.10. Results were expressed as odds ratios with a 95% confidence interval. A p-value < 0.05 was considered statistically significant. All data were shown as mean ± SD.

3 | RESULTS

3.1 | Characteristics of the cohort

Epidemiological characteristics (age, BMI), classical semen parameters (sperm count, progressive and total motility, morphology, and presence of white blood cells), patient's medical history, origin, environmental exposures, and reported addictions are presented in Tables 1–3. Briefly, the age of the cohort ranged from 19 to 59 years, with a mean ± SD of 37.5 ± 5.48 years (Table 1). The most represented age groups were in their 30s, with 406 patients aged 30—35 years and 402 aged 36—40 years. The 41—45 years age group was next with 214 patients, whereas the youngest (<30 years) and oldest (>45 years) were the least represented age groups with 72 and 97 patients, respectively (Table 2).

The BMI of the cohort ranged from 14.2 to 46.9, with a cohort mean ± SD of 27.5 ± 4.17, which is clearly overweight. Looking in

TABLE 1 Age, body mass index (BMI), and classic seminal parameters of the study cohort.

Model	Number	Mean	Standard deviation	Std Error	Median	Min.	Max.	Range
Age (year)	1191	37.35	5.48	0.16	37	19	59	40
BMI (kg/m^2)	1036	27.50	4.17	0.13	27.36	14.23	46.90	32.67
Sperm concentration (10^6/ml)	1191	67.34	48.98	1.40	60	1.8	310	310
Sperm motility (%)	1191	54.11	25.25	0.72	59	0.00	87	87
Progressive Motility (%)	1191	27.33	18.90	0.54	25	0.00	72	72
Morphology (%)	1191	2	1.50	0.04	2	0.00	9	9
White Blood Cells (WBC) (10^6/ml)	1191	0.12	0.38	0.01	0.00	0.00	6.50	6.50

TABLE 2 Percentage of patients in age classes, BMI classes, and with defective sperm parameters.

Model		Frequency	Percent
Sperm concentration (10^6/ml)	≤20	235	19.7
	>20	956	80.3
Sperm Motility (%)	≤45	447	37.5
	>45	744	62.5
Progressive Motility (%)	≤40	912	76.6
	>40	279	23.4
Morphology (%)	≥4	72	6
	<4	1119	94
WBC (10^6/ml)	0-1	1168	98.1
	>1	23	1.9
BMI (kg/m^2)	<18.5	12	1.2
	18.5 - <25	269	26
	25 – 30	501	48.3
	30≤	254	24.5
Age (year)	<30	72	6
	30-35	406	34.1
	36-40	402	33.8
	41-45	214	18
	> 45	97	8.1

more detail at the distribution of patient BMI, Table 2 shows that very few patients (12 of 1036 = 1.2%) were considered lean (BMI < 18.5) and only 269 of 1036 (26%) had what is considered a normal BMI (18.5 < BMI < 25). The largest group of patients (501 of 1036; 48.4%) were overweight (25 < BMI < 30), whereas the remaining patients (254 of 1036; 24.5%) were classified as obese (BM1 > 30).

For the seminal parameters monitored, sperm concentration ranged from 1.8 to 310 M/ml, with a mean ± SD of 67.34 ± 48.8 M/ml. The sperm concentration was low (< 20 M/ml by WHO 2010 standards) in 19.7% of the cohort samples. Progressive sperm motility ranged from 0% to 87% with a mean ± SD of 27.33% ± 18.9% (see Table 1).

According to WHO standard values (WHO, 2010), total sperm motility was low in 37.5% of the cohort, while progressive sperm motility was low in 76.6% of the cohort; this appears to be the second characteristic of this cohort, where the majority of samples had impaired motility. Abnormal sperm morphology (teratozoospermia) was also a rather characteristic of the semen samples analyzed affecting 94% of the cohort (see Table 2). The presence of white blood cells in the semen (leukocytospermia) was not very common in the study cohort, affecting only 1.9% of patients (see Table 2).

Table 3 also presents a variety of patient information to categorize the cohort according to environmental and behavioral criteria, including smoking, alcohol use, drug use, physical activity, current medical treatments, disease status, past surgeries, and chronic occupational exposures. Although this categorization is purely arbitrary because a sample may accumulate several situations, it illustrates the great heterogeneity of the cohort, which reflects the wide range of situations that are classically encountered in clinical practice. Table 3 also shows the season of the year in which the seminal samples were evaluated. It should be noted that after dividing the cohort on a 4-season basis, the sizes of the four sub-cohorts were not homogeneous since we observed that the highest referrals were recorded in the summer and autumn, totaling nearly 65% of the samples analyzed.

3.2 | DFI assessment

Sperm DNA fragmentation was assessed in this cohort using the SCSA (Evenson, 2022). Table 4a shows that 477 patients (40.1%) had DFI that was clinically considered pathological (DFI > 25). The mean total DFI of the entire cohort reached nearly 24% and ranged from 1% to 92.1%. SCSA flow cytometer-assisted monitoring, in addition to DFI determination, allows the evaluation of a second parameter: the high DNA stainability (HDS) which reflects the level of condensation of the sperm nucleus (Evenson, 2013). The mean HDS of the entire cohort was close to 7% ranging from 0 to 37.45% (Table 4b). When the mean DFI values were analyzed according to the different classes of patient distribution (Table 5), we observed that total DFI (DFI) was statistically significantly correlated with patient age. As can be seen in Table 5, the mean total DFI showed a linear increase with patient age classes. Interestingly, although not statistically significant, we observed a decreasing trend for the HDS parameter with increasing patient age (Table 5), a point that will be discussed later.

TABLE 3 Detailed patient characteristics.

Model		Frequency	Percent
DNA Fragmentation in different seasons	Spring	157	13.2
	Summer	292	24.5
	Autumn	389	32.7
	Winter	353	29.6
Occupational exposures	Light physical	653	55.4
	Chemical	250	21.2
	Heat	134	11.4
	Heavy physical	87	7.4
	Radiation and microwaves	54	4.6
Disease status	Inflammatory disease	265	31.9
	Varicocele	199	24
	Mumps infected	176	21.2
	Multiple	84	10.1
	Diabetes	39	4.7
	Hernia	20	2.4
	Metabolic and vascular syndrome	19	2.5
	Reproductive disease	15	1.8
	Mental illness	6	0.7
	Immunologic	6	0.7
Medical treatment	Blood pressure and diabetic agents-	46	28
	Psychotropic	31	19
	Anti-inflammatory	27	16.5
	Others	33	20.1
	Sexual	13	7.9
	Addiction	11	6.7
	Multiple	3	1.8
Surgery	Varicocele	295	60.5
	Non-reproductive	80	16.4
	Multiple	42	8.5
	Hernia	36	7.4
	Reproductive	35	7.2
Smoking	No	829	71.5
	Yes	331	28.5
Addiction	No	1060	91.4
	Yes	100	8.6
Alcohol	Non-regular consumer	1043	87.6
	Regular Consumer (one glass/week)	148	12.4

TABLE 4A Frequency and percentage of patients with normal or pathological DNA fragmentation Index (DFI).

Model		Frequency	Percent
DNA fragmentation index (DFI) (%)	<25	714	59.9
	≥25	477	40.1

Surprisingly, BMI was not found to be significantly correlated with either DFI or HDS. Interestingly, elevated HDS values were found to be significantly correlated with the season in which semen samples were processed, with spring and summer samples being associated with higher HDS values than fall and winter processed samples (Table 5).

For DFI, we observed no statistically significant correlation when examining the risks to which patients might be exposed due to their occupational and/or physical activities (Table 5). Only the sperm HDS value was found to be significantly correlated with occupational exposures, with sperm nucleus decondensation found to be higher in patients engaged in strenuous physical activities (Table 5).

Regarding the geographic location of patients, it was counterintuitive that sperm DFI was significantly elevated in patients living in rural areas compared with those living in urban areas such as large cities and provincial state centers [state] (Table 5).

Regarding patient disease status, neither DFI nor HDS was not found to be significantly associated with any of the pathologies investigated (Table 5). DFI or HDS was also not found to correlate with the type of treatment undergone by the patients nor with any surgical procedure (Table 5).

Finally, among the five classical factors (diabetes, mumps, alcohol intake, smoking, and drug addictions) that are well known to be associated with decreased spermatogenesis efficiency and decreased sperm quality, we found that only "mumps" to be significantly associated with higher DFI, lower sperm motility and lower normal morphology (Table 6).

Uni/multivariate regression analyses confirmed that age was the only factor strongly affecting DFI and HDS (Table S1), with a beta coefficient of 1.96 for the total DFI sub-cohort (meaning that DFI increases by 1.96 units per year added) and a beta coefficient of −0.34 for HDS (meaning that HDS decreases by 0.34 units per year added). The season of sample assessments is a second variable that only affects HDS (beta coefficient: −0.47). Multiple regression results (Tables S2 and S3) showed that with each one-year increase in age, total DFI increases by approximately 1.69 units ($p = 0.000$; Table S2). Comparing semen samples based on classical semen parameters (motility and morphology), we found that when total motility increases by a single unit, total DFI decreases by 0.29 units ($p = 0.000$). When normal morphology values increase by a single unit, total DFI correspondingly decreases by approximately 1 unit ($p = 0.001$).

4 | DISCUSSION

In the majority of published studies, the correlations established between sperm DNA fragmentation and various intrinsic and extrinsic sperm factors are weakened by the fact that the cohorts studied are often not very large. The small size of cohorts and a bias in the selection of patients may influence their exploitation. With this in mind, we decided to study a large cohort of men from couples entering an assisted reproduction program at the Royan Institute in Tehran (an Iranian infertility clinic). SCSA was chosen to assess patients' SDF level because we believe that it provides a powerful, reliable,

TABLE 4B Cohort DNA fragmentation index (DFI) and high DNA stainability (HDS).

Model	Mean	Standard deviation	Std Error	Median	Min	Max	Range
Total DFI (%)	23.86	14.40	0.41	20.80	1	92.10	91.10
HDS (%)	6.69	4.83	0.13	5.41	0.00	37.45	37.45

and unbiased assessment (preventing subjective operator-based decision) performed by flow cytometry (50,000 sperm cells analyzed per sample).

Of the variables explored, we found that age was the most influential factor in SDF, as DFI increased somewhat linearly with patient age. This is unsurprising and purely confirmatory, as several anterior studies have already reported that sperm DNA integrity steadily decreases with aging.[19–23] Although not statistically significant in our cohort, we also found that HDS showed a decreasing trend with age. This may seem surprising, as one would expect aging males with higher SDF status to also exhibit nuclear decondensation, which HDS appreciates. One possible and rather logical explanation is that there is a progressive increase in systemic oxidative stress during aging which may enhance post-testicular sperm nuclear condensation via an increase in sperm protamine disulfide bridges,[24] resulting in a decrease in HDS. This phenomenon has been clearly demonstrated in animal models.[25] Decreased sperm HDS during aging in humans has also been reported recently, supporting our data.[26] Alternatively, this observation of a lower sperm HDS value with aging could be inherent in the SCSA itself and the specific thresholds that are used to consider spermatozoa as decondensed (high HDS) versus fragmented (high DFI). It is possible that in semen samples over the course of age, the fraction of fragmented cells increases at the expense of the fraction of decondensed cells, with the latter enlarging the former. More surprising thus was our finding that despite the strong overweight characteristic of our cohort (a particular feature of this cohort of Iranian men in which overweight and obese patients together represented 72.9% of the cohort) we found no significant correlation with SDF. Although high BMI has been associated with chronic low-grade systemic inflammation, oxidative stress, and DNA damage in the testis and germline,[27,28] it apparently does not consistently translate into sperm DNA damage. Similar results have been reported in previous studies.[29–33] However, many other reports instead suggest that high BMI is associated with higher risks of sperm DNA damage.[18,34–43] Given that high BMI may have several causative factors, its relationship with SDF will need to be further investigated.

Interestingly, we found a significant correlation between DFI and the season in which the SDF assessments were performed. In the spring and summer, significantly higher DFI and HDS values were recorded compared to those in the fall and winter. Higher DFI and HDS reflect sperm DNA damage and a loss of nuclear integrity. Seasonal variations in sperm quantity and quality in summer have been reported in the literature, particularly in Northern Hemisphere countries.[44–46] However, the data presented in these studies are only for sperm concentration, morphology, and motility, which were found to be lower in the summer than in the winter overall. Data on specific parameters of sperm integrity, such as the one monitored in this study (i.e., SDF), are unavailable. The fact that Iran is marked by very hot and dry spring/summer seasons could partly explain this observation, as spermatogenesis and spermatozoa are particularly sensitive to heat stress which can lead to sperm DNA damage, a likely explanation that was previously suggested.[47,48]

A rather puzzling observation from our cohort was that rural men who were referred to the Royan infertility clinic showed significantly higher DFI. This was unexpected, as we had initially hypothesized that, given the very high degree of air pollution in the Tehran area, other state and large cities, we would expect urban semen samples to be of lower integrity, as has been found elsewhere.[49] However, upon further review of the available literature, there are reports that air pollution has a weak effect on SDF.[50,51] A likely explanation for our observation could be the higher rural exposure to pesticides/herbicides and their well-known impact on spermatogenesis and semen quality.[52] Age could also contribute to this observation, as a lack of awareness of infertility problems among rural Iranians could delay the time when couples are referred to infertility centers. However, looking at our sub-cohorts, this does not appear to be the case here since rural and urban sub-cohorts showed an equivalent mean age.

Although the number of samples involved was very small ($N = 6$), men with mental illnesses were found to have significantly higher DFI. This is not a new observation, as psychiatric conditions have been suspected of having an impact on male infertility, whether mediated by the stress associated with the conditions or/and the psychotropic medications used to treat them.[53–56] Looking specifically at five conditions, two pathological (diabetes and mumps) and three behavioral (alcohol consumption, smoking, and drug abuse), that are known to impact spermatogenesis efficiency and semen quality, we found that DFI was uniquely significantly correlated with mumps. The fact that DFI is associated with mumps is not a surprise because the orchitis accompanying mumps results in a long-lasting disruption of spermatogenesis.[57–59]

In conclusion, age appears to be the most influential factor in sperm DNA fragmentation, as illustrated in this particular cohort by the fact that between the ages of 19 and 59 years, the DFI may increase on average by about 2% each year. Although this should not be automatically translated as such, it should remain present in the clinician's approach to the male partner of an infertile couple that sperm DNA fragmentation could be a valuable parameter to monitor routinely. Although BMI is widely considered a contributing factor to male infertility, our cohort does not link DFI to BMI despite its clear bias toward overweight/obesity situations. This reinforces the need for further research to understand which of the various etiologies leading to high

TABLE 5 Comparison of the DNA fragmentation index (DFI) and high DNA stainability (HDS) according to the variables monitored.

			DFI (%)				HDS (%)			
				Confidence Interval				Confidence Interval		
			Mean ± SEM	Min	Max	*p*-Value	Mean ± SEM	Min	Max	*p*-Value
Age (n = 1191)	<30 years	n = 72	23.02 ± 2.03	18.84	27.08	**0.000***	7.35 ± 0.62	5.99	8.59	0.154
	30–35	n = 406	21.77 ± 0.62	20.53	23.02		7.04 ± 0.26	6.54	7.53	
	36–40	n = 402	23.69 ± 0.70	22.31	25.09		6.55 ± 0.23	6.08	7.03	
	41-45	n = 214	25.90 ± 0.96	24.01	27.79		6.42 ± 0.28	5.86	7.01	
	≥ 46	n = 97	28.83 ± 1.67	25.50	32.86		6.03 ± 0.37	5.21	6.70	
BMI (n = 1036)	<18.5 kg/m^2	n = 12	26.42 ± 5.84	13.56	39.31	0.603	7.27 ± 1.43	4.09	10.43	0.735
	18.5–24.99	n = 269	24.24 ± 0.92	22.45	26.05		6.83 ± 0.32	6.21	7.45	
	25–29.99	n = 501	23.23 ± 0.61	21.91	24.47		6.82 ± 0.22	6.41	7.25	
	≤30	n = 254	23.91 ± 0.90	22.15	25.70		6.45 ± 0.27	5.88	7.03	
Season SDF test (n = 1191)	Spring	n = 157	24.31 ± 1.09	22.15	26.51	0.386	6.43 ± 0.36	5.71	7.13	**0.000***
	Summer	n = 292	24.89 ± 0.85	23.21	26.60		8.23 ± 0.36	7.53	8.95	
	Autumn	n = 389	22.97 ± 0.72	21.60	24.45		6.19 ± 0.22	5.74	6.62	
	Winter	n = 353	23.85 ± 0.76	22.21	25.20		6.10 ± 0.21	5.69	6.50	
Occupation (n = 1178)	Heat	n = 134	24.14 ± 1.25	21.68	26.61	0.248	6.64 ± 0.41	5.84	7.44	**0.011***
	Chemical	n = 250	23.21 ± 0.91	21.41	25.00		6.49 ± 0.27	5.95	7.03	
	Radiation and waves	n = 54	27.51 ± 2.23	23.03	31.99		7.37 ± 0.71	5.94	8.80	
	Light physical	n = 653	23.37 ± 0.55	22.30	24.44		6.49 ± 0.18	6.13	6.84	
	Heavy physical	n = 87	24.55 ± 1.45	21.66	27.99		8.38 ± 0.70	6.99	9.76	
Residence (n = 1145)	State	n = 703	24.04 ± 0.53	23.07	25.09	**0.029***	6.65 ± 0.18	6.30	7.00	0.668
	City	n = 396	22.84 ± 0.70	21.45	24.22		6.82 ± 0.25	6.33	7.30	
	Village	n = 46	28.39 ± 2.47	23.40	33.37		7.72 ± 0.77	5.67	9.01	
Disease (n = 614)	Varicocele	n = 199	25.20 ± 0.93	23.35	27.05	0.382	7.48 ± 0.40	6.69	8.28	0.096
	Hernia	n = 20	23.13 ± 3.44	15.94	30.33		7.58 ± 1.16	4.77	9.53	
	Metabolic and vascular	n = 19	26.61 ± 3.14	19.99	33.22		5.05 ± 0.72	3.55	7.15	
	Immunologic	n = 6	24.85 ± 8.98	1.74	47.96		6.12 ± 1.24	2.93	9.32	
	Mental illness	n = 6	40.51 ± 6.75	23.14	57.89		5.02 ± 1.30	1.66	8.38	
	Inflammatory disease	n = 265	25.08 ± 0.98	23.07	27.01		6.10 ± 0.27	5.56	6.64	
	Reproductive disease	n = 15	25.22 ± 4.33	15.91	34.53		6.07 ± 0.98	3.96	8.18	
	Multiple	n = 84	25.40 ± 1.71	21.99	28.82		7.16 ± 0.48	6.21	8.13	
Drug (n = 164)	Psychotropes	n = 31	28.57 ± 2.93	22.57	34.57	0.918	5.27 ± 0.56	4.12	6.44	0.781
	Anti-inflammatory	n = 27	26.08 ± 2.57	20.78	31.39		6.80 ± 1.08	4.58	9.02	
	Blood pressure and diabetes	n = 46	26.89 ± 2.12	22.61	31.17		6.08 ± 0.54	4.78	7.19	
	Sexual	n = 13	25.42 ± 4.21	16.24	34.61		5.83 ± 080	4.07	7.59	
	Addiction	n = 11	28.46 ± 4.92	17.49	39.44		5.29 ± 1.09	2.86	7.74	
	Others	n = 33	27.16 ± 2.91	21.23	33.10		5.91 ± 0.65	4.58	7.26	
	Multiple	n = 3	28.63 ± 4.81	7.92	49.35		3.70 ± 076	0.41	6.99	
Surgery (n = 488)	Varicocele	n = 295	25.74 ± 0.84	24.09	27.38	0.943	7.18 ± 0.31	6.57	7.78	0.954
	Hernia	n = 36	23.50 ± 2.60	18.23	28.76		6.67 ± 0.76	5.41	8.58	
	Reproductive	n = 35	25.69 ± 2.57	20.48	31.19		6.90 ± 0.65	5.59	8.20	
	Non-reproductive	n = 80	25.65 ± 1.99	21.68	29.61		7.03 ± 0.58	5.85	8.10	
	Multiple	n = 42	25.04 ± 1.94	21.11	28.96		7.68 ± 0.85	5.96	9.41	

*$p < 0.05$ is considered significant.

TABLE 6 Comparison of DNA fragmentation index (DFI), high DNA stainability (HDS), and conventional semen parameters in five selected situations.

Model	Diabetes (n = 1190)			Alcohol (n = 1191)			Mumps (n = 1191)			Smoking (n = 1160)			Addiction (n = 1160)		
	Yes (n = 39)	No (n = 1151)	P-value	Yes (n = 148)	No (n = 1043)	P-value	Yes (n = 176)	No (n = 1015)	P-value	No (n = 829)	Yes (n = 331)	P-value	Yes (n = 100)	No (n = 1060)	P-value
Total DFI (%)	25.94 ± 2.81	23.75 ± 0.42	0.454	23.47 ± 1.25	23.91 ± 0.43	0.717	26.07 ± 1.25	23.47 ± 0.44	**0.042***	24.17 ± 0.50	23.22 ± 0.76	0.306	25.25 ± 1.46	23.72 ± 0.44	0.318
HDS (%)	6.09 ± 0.65	6.72 ± 0.13	0.638	6.76 ± 0.39	6.69 ± 0.15	0.849	6.25 ± 0.33	6.77 ± 0.14	0.198	6.74 ± 0.17	6.67 ± 0.23	0.816	6.97 ± 0.41	6.70 ± 0.15	0.550
Sperm concentration (10^6/ml)	64.02 ± 7.03	67.44 ± 1.43	0.638	64.53 ± 4.09	67.80 ± 1.49	0.445	62.73 ± 3.33	68.14 ± 1.55	0.175	67.30 ± 1.73	67.15 ± 2.53	0.961	60.99 ± 5.00	67.87 ± 1.49	0.191
Sperm Motility (%)	55.06 ± 4.01	54.09 ± 0.73	0.794	53.64 ± 2.01	54.17 ± 0.77	0.808	49.46 ± 0.01	54.91 ± 0.78	**0.010***	53.96 ± 0.88	54.87 ± 1.37	0.575	49.60 ± 2.44	54.68 ± 0.77	0.050
Progressive Motility (%)	30.86 ± 3.18	27.20 ± 0.54	0.282	27.65 ± 1.43	27.29 ± 0.59	0.826	25.05 ± 1.55	27.73 ± 0.58	0.081	27.12 ± 0.65	27.87 ± 1.04	0.544	25.55 ± 1.77	27.50 ± 0.58	0.299
Morphology (%)	1.86 ± 0.18	2.01 ± 0.03	0.560	1.85 ± 0.10	2.02 ± 0.07	0.161	1.77 ± 0.09	2.05 ± 0.04	**0.012***	2.02 ± 0.05	1.96 ± 0.08	0.584	1.91 ± 0.15	2.01 ± 0.04	0.510
WBC (10^6/ml)	0.08 ± 0.02	0.11 ± 0.01	0.472	0.13 ± 0.03	0.11 ± 0.01	0.842	0.14 ± 0.03	0.12 ± 0.01	0.652	0.11 ± 0.01	0.13 ± 0.02	0.435	0.12 ± 0.04	0.12 ± 0.01	0.942

Abbreviations: WBC, white blood cells.
*$p < 0.05$ is considered significant.

BMI are related to sperm DNA damage. Our study confirms the existence of seasonal variations in sperm DNA damage that are likely, in our case, to be partly associated with hot summer temperatures, although this remains to be proven. In addition, the patients' habitat (urban or rural) influences SDF, probably concerning the distinct exposures to environmental toxins, they face. Considering the cumulative actions of all these different factors on SDF (individual genetics, pathological trajectory, lifestyle choices, and environmental impacts), this should argue for an assessment of sperm DNA damage in the routine examination of infertile men.

AUTHOR CONTRIBUTIONS

PY: MSc student, the main contributor to the project, survey, data collection, and drafting. **LR**: Study design, methodology, validation, investigation, drafting-reviewing, and editing of the project and final manuscript. **SV**: Methodology, data analysis, investigation, writing/revision, and editing. **EJ**: Project advisor and a primary contributor to the SCSA (DFI/HDS) assessment in the lab, data collection, writing-reviewing, and editing. **MS**, **HS,** and **AV**: Clinicians, primary contributors to participant selection, fundraising, and final manuscript review. **AS**: Project supervisor, interpretation, validation, fundraising, and review of the final manuscript. **JD**: Project advisor, methodology, interpretation, and drafting and editing of the project and final manuscript. **AA**: Project supervisor, study design, management of the project, interpretation, drafting, revision, and editing of the draft and final manuscript. All authors have read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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