



ORIGINAL ARTICLE

An additional marker for sperm DNA quality evaluation in spermatozoa of male partners of couples undergoing assisted reproduction technique (IVF/ICSI): Protamine ratio

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Abstract

The aim of this study was to evaluate the relationship between the protamine ratio (P1/P2), DNA fragmentation of spermatozoa and protamine deficiency. Patients were grouped into fertile (G1; $n = 151$) and sub-fertile (G2; $n = 121$). DNA fragmentation in spermatozoa was analysed by a TUNEL assay (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling), and the protamination was determined by CMA3 staining, while Western blot was used to measure protamine P1 and P2. While sperm DNA fragmentation (SDF) and protamine ratio were significantly elevated in G2 compared with G1 ($12.31 \pm 7.01\%$ vs. $17.5 \pm 9.5\%$; $p = .001$) and (0.91 ± 0.43 vs. 0.75 ± 0.42 ; $p = .003$); respectively, the CMA3 positive showed no difference at all between G1 and G2. In G1, the CMA3 positive correlated negatively with the P1/P2 ratio and SDF ($r = -.586$, $r = -.297$; $p = .001$ respectively). In contrast, the protamine ratio correlated positively with SDF ($r = .356$; $p = .001$). In G2, no correlation was observed between CMA3 positive, SDF and the P1/P2 ratio but the P1/P2 ratio showed a positive correlation with SDF ($r = .479$; $p = .001$). In conclusion, the spermatozoa DNA deterioration was closely associated with abnormal protamination but showed an association with the protamine ratio, more than with CMA3 positive. Therefore, for the evaluation of DNA damage in spermatozoa, the P1/P2 ratio might act as an additional biomarker.

KEYWORDS

DNA fragmentation, male infertility, protamine deficiency, protamine ratio, sperm DNA

1 | INTRODUCTION

According to the recommendations of the World Health Organization (WHO), analysis of semen is the basis of diagnosis and evaluation of male fertility (Cooper et al., 2010).

Infertility is defined as the inability to conceive after 12 months of regular and unprotected intercourse by a healthy couple (Skakkebaek et al., 2006).

However, the limits of semen study have been more broadened by investigations demonstrating that 15% of men, considered normal according to the WHO 2010 guidelines, are infertile and other men with aberrant sperm parameters are concerned their spouses might become pregnant naturally (Agarwal & Prabakaran, 2005; Nallella, Sharma, Aziz, & Agarwal, 2006).

It is therefore insufficient to evaluate and predict male fertility potential, based primarily on routine semen analysis, assessing

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sperm count, motility and morphology (WHO, 2010). Consequently, science is now shifting towards analysing the molecular aspects of spermatozoa.

The role of spermatozoa is to deliver the paternal genetic information to the oocyte containing the maternal genetic information. Before this can occur, a remarkable arrangement takes place during the post-meiotic phases of spermatogenesis (Rathke, Baarends, Awe, & Renkawitz-Pohl, 2014).

Spermatids go through major morphological changes, involving specific chromatin compaction and reorganisation. As a result, the structure of the nucleosome, based on the histones, is almost completely changed in structure due to protamine. Histone variants, namely post-translational adjustment of histones, mediate this change and effect breaks in and rearrangements of DNA strands (Rathke et al., 2014).

Therefore, during the process of the chromatin remodelling of spermatozoa, many anomalies may occur in each step, especially at the level of the organisation of the DNA, during histone–protamine exchange and disulphide bond formation. However, the sperm chromatin conformation and the DNA integrity are both important for fecundity (Sakkas, Seli, Bizzaro, Tarozzi, & Manicardi, 2003; Spano, Seli, Bizzaro, Manicardi, & Sakkas, 2005) and fertility (Spano et al., 2005). In addition, the sperm DNA plays a crucial role in the conservation of the genetic information for future offspring.

For the last two decades, sperm chromatin aberration has been widely studied (Agarwal & Said, 2003). Anomalies in spermatozoa's chromatin structure, damaged DNA and unremodelled chromatin of maturing sperm could be indicators of infertility in humans, regardless of the value of the other settings of the normal semen parameters (Sakkas & Tomlinson, 2000). Moreover, the sperm quality of the chromatin is in correlation to the gestation outcomes of in vitro fertilisation (IVF; Duran, Morshedi, Taylor, & Oehninger, 2002; Hammadeh, Strehler, Zeginiadou, Rosenbaum, & Schmidt, 2001).

In the last two decades, there have been attempts to suggest other methods for exploring sperm DNA stability and integrity in order to overcome fertility problems and to ameliorate the semen parameter analysis for procreation in vitro and in vivo.

It is now obvious that there is a close association between impaired spermiogenesis and sperm DNA damage, causing aberrant chromatin remodelling. Aitken and De Iuliis (2011) proposed that the latter was linked to the creation of vulnerable spermatozoa that readily default to an apoptotic pathway, recognised by the generation by reactive oxygen species (ROS) by mitochondria and the creation of oxidative DNA adducts, leading to the formation of strand breaks. Oxidative stress is accordingly regarded as the main cause of sperm DNA damage. Increased ROS production has been associated mainly with the products of mitochondrial respiration (cellular metabolism). In fact, mitochondria are believed to be the first targets of oxidative damage. ROS can also be produced in response to different environmental and lifestyle factors (Cui, Kong, & Zhang, 2012).

Smoking has been shown to negatively affect semen quality and to increase sperm DNA fragmentation (SDF) and

aberrant protamination (Hammadeh, Hamad, Montenarh, & Fischer-Hammadeh, 2010; Taha, Ez-Aldin, Sayed, Ghandour, & Mostafa, 2012). Gundersen et al. (2015) found that marijuana use of more than one time per week reduced sperm concentration by 28%.

Firns et al. (2015) showed that alcohol consumption reduces semen volume, sperm concentration, motility and morphology. However, the claim that semen quality is likely to be influenced by how frequently people drink remains a controversial issue.

Diets rich in soya have a negative effect on sperm concentration; this could be explained by the high levels of isoflavones in soya that may disrupt hormone levels (Chavarro, Toth, Sadio, & Hauser, 2008).

Obesity is related to the development of metabolic syndrome, which has been shown to have a deleterious effect on the sperm parameters (Gorbachinsky, Akpinar, & Assimos, 2010; Kasturi, Tannir, & Brannigan, 2008).

In addition, different studies have examined the correlation between increasing age and the semen parameters and found a decreasing volume, motility and morphology associated with an increase in DNA fragmentation (Belloc et al., 2009; Johnson, Dunleavy, Gemmell, & Nakagawa, 2015; Vagnini et al., 2007).

Many techniques, such as aniline blue, CMA3 assay and TUNEL (Aoki, Emery, Liu, & Carrell, 2006; Hammadeh et al., 2010; Manicardi et al., 1995), have been used to detect a correlation between protamine deficiency, alterations in DNA and the absence of sperm vitality. Lately, there have been a number of techniques, such as terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL), a sperm chromatin structure assay (SCSA), a sperm chromatin dispersion (SCD) assay and a comet assay (Agarwal et al., 2016) to evaluate DNA fragmentation.

However, most of the techniques used provide limited information about the nature of the DNA lesions evidenced and do not allow the exact pathogenesis of the disrupted sperm DNA to be highlighted (Agarwal & Said, 2004; Bungum, 2012).

Less expensive methods to assess the sperm chromatin structure use chromatin structural probes or dyes. For example, acridine orange is used to measure the susceptibility to conformational changes and toluidine blue is used to stain phosphate residues of fragmented DNA. There are also two indirect staining agents that are simple and provide results describing the general deficits in the sperm chromatin; aniline blue (AB) is used to stain selectively lysine-rich histone proteins, thereby revealing the loosely condensed chromatin (Auger, Mesbah, Huber, & Dadoune, 1990; Hammadeh et al., 2001), and chromomycin A3 is a guanine-cytosine-specific fluorochrome that competes with protamine for binding to specific regions (minor grooves) in DNA, revealing protamination defects in spermatozoa (Bianchi, Manicardi, Bizzaro, Bianchi, & Sakkas, 1993; Sakkas et al., 1996).

Although a DNA integrity test provides complementary information about the sperm's role in the pregnancy development, but up until now, there has been no approval to utilise DNA integrity tests in reproduction laboratories' routine work (Bach & Schlegel, 2016).

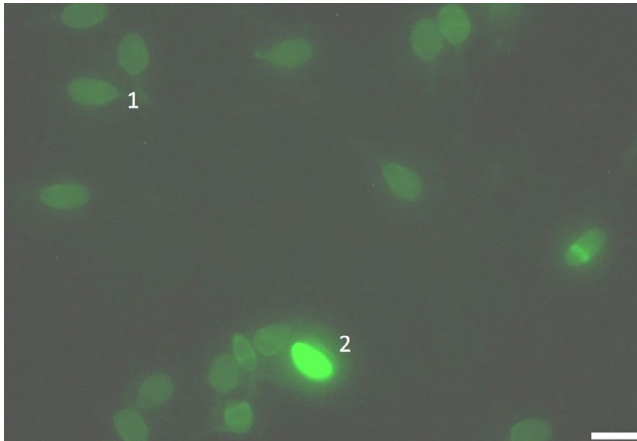


FIGURE 1 Illustrative human sperm cells stained with CMA₃. Normal condensed sperm chromatin (CMA₃ negative) fluorescent dull green (1), whereas noncondensed sperm chromatin (CMA₃ positive) fluorescent bright green (2) (GrX100)

The aims of the current study were first to discover the protamine deficiency in spermatozoa and to assess spermatozoa's DNA injury and, second, to quantify the concentration of protamines (P1 and P2) and their ratio (P1/P2 ratio) and to find out whether these investigated parameters correlated with standard sperm parameters in order to reveal whether the P1/P2 ratio can be applied as a biomarker for the evaluation of sperm function and their fertilisation potential in fertile and sub-fertile patients undergoing ICSI therapy.

2 | MATERIALS AND METHODS

2.1 | Study population

In the present study, 272 sperm samples were gathered from patients undergoing IVF/ICSI therapy at the Department of Obstetrics, Gynaecology and Reproductive Medicine, Women's Clinic, Saarland, Germany.

The exclusion criteria for patients in this study were the following: diabetes, alcohol consumption, testes injury, the existence of anti-sperm antibodies, genital tract infection, varicocele, chronic male diseases such as tuberculosis and genetic disorders such as Klinefelter's syndrome, Y chromosome micro-deletions and hormonal disorders. In addition, patients who had been exposed to environmental factors, such as toxins, chemicals and heat, were excluded too.

All the participants included in this study were of reproductive age, the range being between 25 and 50 years old.

After semen collection and liquification at 37 degrees for 20–30 min, the sperm parameters of each sample were analysed according to WHO (2010) guidelines. Five smears were taken from each sample for later use in chromomycin (CMA3), TUNEL test and sperm morphology evaluation. For chromomycin (CMA3), smears were air-dried and fixed with methanol and acetic acid (3:1 volume|volume).

For sperm morphology, slides were stained with papanicolaou, and then, 100 spermatozoa were evaluated.

Samples were prepared for insemination or injection, using the Pure Sperm technique (Nidacon International AB). The rest of the sperm was kept for later at -80°C .

2.2 | Sperm chromatin condensation assay (chromomycin A3 [CMA3])

A chromomycin A3 assay, as described by Manicardi et al. (1995) but with some changes, was used for the sperm chromatin condensation assessment.

Chromomycin is a fluorochrome that binds to the guanine-cytosine dinucleotide region of the DNA in a competitive way with protamines binding to the same region, so that spermatozoa having an elevated concentration of protamine will theoretically have a small amount of chromomycin fluorescence (Bianchi et al., 1993).

To each slide, 25 μl of CMA3 stain solution was added and the slide then kept in darkness for 30 min at 25°C . After the slides had been washed with phosphate buffer saline (PBS), they were mounted and preserved overnight at 4°C . On each slide, 200 spermatozoa were evaluated using a fluorescence microscope (Olympus). A bright green spermatozoon indicated protamine deficiency (CMA3 positive), and dull green spermatozoa were CMA3 negative (Figure 1).

2.3 | Sperm DNA fragmentation assessment (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling [TUNEL])

A TUNEL assay was used to determine the DNA injury in spermatozoa, as previously described by Borini et al. (2006).

The principal aim of the assay test is to quantify the incorporation of dUTP at single- and double-stranded DNA breaks, catalysed by the terminal deoxynucleotidyl transferase (TdT) enzyme, provided in the in situ cell death detection kit fluorescein (Roche Diagnostics).

After smear fixation with 4% paraformaldehyde (Sigma-Aldrich) for 2 hr at 25°C , the slides were incubated with 0.1% Triton for 15 min at 25°C for permeabilisation. To each slide, 25 μl of TdT-labelled nucleotide mix was added, and the slides were then preserved overnight at 37°C in a humidified chamber. After washing the slides with PBS, 25 μl of 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) was added to each slide. On each slide, 200 spermatozoa were evaluated, using a fluorescence microscope (Olympus): stained green spermatozoa were TUNEL-positive but blue-stained spermatozoa were TUNEL-negative (Figure 2).

2.4 | Protamine extraction

Protamines (P1 and P2) were extracted from the stored sperm pellets, as described by Hammadeh et al. (2010). The pellets were cleaned with 1 ml of the washing solution 1 (1 mM of phenylmethylsulfonyl fluoride [PMSF]) and later put on centrifuge (250 g/5 min) at 25°C . Then, 100 μl of washing solution 2 (20 mM EDTA and PMSF

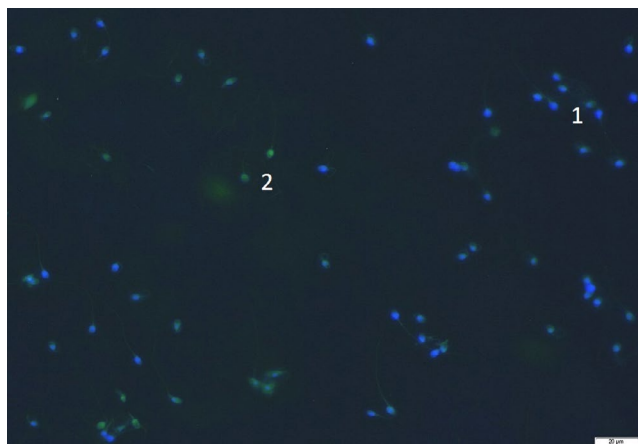


FIGURE 2 Illustrative human sperm cells stained with TUNEL (DAPI). TUNEL-negative sperm stain blue (1) and TUNEL-positive sperm stain green (2) (GrX40)

[1 mmol, pH 8.0]) was added to the pellets and mixed for 15 s and 100 μ l of decondensation solution 1 (6 M guanidine hydrochloride and 575 dithiothreitol [DTT]) was included and mixed again for a few seconds.

Thereafter, 200 μ l of decondensation solution 2 (522 mM sodium iodoacetate) was added, mixed for 30 s and incubated for half an hour at RT. After this step, 1 ml of absolute ethanol was included, mixed and preserved for 1 min at -20°C . The mix then was placed in a centrifuge (1,000 g/10 min) at 4°C . At that point, 0.8 ml of denaturing solution (0.5 M HCl) was added to the pellet and preserved at 37°C (15 min). This preparation was then placed in a centrifuge (1,000 g/10 min) at 4°C . Supernatant was exchanged to a second tube including 200 μ l of precipitating buffer (100% trichloroacetic corrosive [TCA]) and incubated in ice for 3 min followed by a centrifugation (1,000 g/10 min) at 4°C . Finally, the precipitate was cleaned with 1 ml of washing solution (1% β -mercaptoethanol in 100% acetone) and mixed roughly for a few seconds. The mix was placed in a centrifuge (1,000 g/8 min) at 4°C , and the pellet was left to dry at 4°C for the night and then preserved at -80°C .

2.5 | Preparation of the human protamine standard and control samples

Sperm samples of 30 proven fertile men were mixed and centrifuged (250 g/10 min) at RT. Then, a PBS buffer was used to wash the pellet that was later placed in a centrifuge (250 g/10 min) at RT.

Next, a mixture of 0.5 ml of the denaturing solution (0.5 M HCL) and the pellets was preserved at 37°C (15 min) and centrifuged (250 g/5 min) at 25°C . The pellets were kept and washed with 0.25 ml of washing solution 2 and re-centrifuged, as before. After that, the same procedure for protamine extraction, as described before, was performed. The RC DC protein assay kit (Bio-Rad) and the spectrophotometer (Ultrospec 2100 pro UV/Visible; Amersham Biosciences) were used for the evaluation of the protein concentration. For calculation of the protamine 1 and 2 concentrations, four

protamine standards (1.5, 1, 0.5 and 0.25 $\mu\text{g}/\mu\text{l}$) were used to draw a regression curve ($R^2 \geq .98$) for each run.

For control, 40×10^6 sperm aliquots were stored at -80°C and one aliquot was used for every run with the studied samples.

2.6 | Protamine quantification: Western blotting

The acetic acid-urea polyacrylamide gel electrophoresis (AU-PAGE) method, combined with the Western blot, was used for estimation of the extracted protamines.

Before the samples were loaded, the gel was electrophoresed (200 V, 25 mA/3 hr) with reverse polarity.

Eighty microliter of loading buffer (0.375 M potassium acetate, 15% sucrose and 0.05% methyl green [pH 4.0]) was added to previously extracted nuclear proteins, and then, 10 μ l of each sample was loaded onto the gel with the control sample and then placed in a vertical home-made gel electrophoresis system (200 V, 25 mA/3 hr).

The proteins were removed to a PVDF membrane (Roche) by using a blotting tank with 0.0009 N acetic acid as a transfer buffer (150 mA/overnight). Later, the membranes were blocked in a blocking buffer (PBS, pH 7.4, 0.1% [v/v] Tween 20 and 5% [w/v] nonfat dry milk) for 1 hr at RT with shaking. The membranes were washed with a washing buffer (1 \times PBS, pH 7.4, 0.1% [v/v] Tween 20, 1% [w/v] nonfat dry milk) three times, each for 7 min with shaking at RT. The membranes were incubated with the primary antibody diluted in the previously mentioned washing buffer (4 $^{\circ}\text{C}$ /overnight) with shaking. One membrane was incubated with a Hup 1N (anti-protamine 1)-specific primary antibody, diluted 1:100,000, and the second membrane was incubated with a Hup 2B (anti-protamine 2)-specific primary antibody, diluted 1:500,000, overnight at 4°C .

Then, the membrane was washed and re-preserved with the horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Dianova) for 1 hr with shaking at RT.

The Lumi-light chemiluminescence kit (Roche) and the enhanced chemiluminescence system (Bio-Rad) were used to measure the intensity of the protamine bands.

The standard curve was used to calculate the protamine concentrations for each sample. The P1/P2 ratio was generated using the image analysis software as follows: (P1 band-background)/(P2 band-background).

2.7 | Statistical analysis

The data were analysed at the Institute of Medical Biometry and Medical Information, Saarland University, Germany, using the Windows Software SPSS 24 (SPSS Inc.).

Based on the Shapiro test, z-value kurtosis and skewer tests, studied samples were nonnormally distributed.

A Mann-Whitney test was used to compare the means of quantitative variables and the different correlations that had been expressed, according to the Spearman correlation test.

3 | RESULTS

The semen samples included in the present study were divided into fertile ($n = 151$) and sub-fertile groups ($n = 121$).

Patients who had one child or more and had normal semen parameters (volume: 1.5 ml, sperm count: 15 million spermatozoa/ml; normal forms: 4%; vitality: 58% live; progressive motility: 32%; total [progressive + non progressive] motility: 40%, according to WHO guidelines 2010) were considered as a fertile group, and those who had failed to have children and had one sperm parameters under WHO (2010) criteria were considered as a sub-fertile group.

3.1 | The different parameters in the set of studied subjects

All parameters measured in the present study have been expressed in mean \pm standard deviation and are presented in Table 1. The sperm parameter values were $64.81 \pm 39.66 \times 10^6$ /ml for concentration, $35.90 \pm 18.64\%$ progressive motility, and the normal morphology was $29.65 \pm 23.46\%$.

Protamine deficiency was measured by CMA3 staining, and sperm DNA fragmentation (SDF) evaluated via a TUNEL test was $34.30 \pm 15.97\%$ and $14.60 \pm 8.58\%$ respectively.

P1 concentration was 432.35 ± 124.14 (ng/ 10^6 spermatozoa), P2 concentration was 397.85 ± 125.19 (ng/ 10^6 spermatozoa), and the protamine ratio was 0.83 ± 0.49 .

The various correlations are presented in Table 2a. A significant negative correlation between age and these investigated parameters was found as follows: sperm count ($r = -.144$; $p = .018$), progressive motility ($r = -.129$; $p = .034$) and normal morphologically spermatozoa ($r = -.248$; $p = .001$). Besides, CMA3 positive (protamine deficiency) increased with the increasing age of patients.

Moreover, a correlation was noticed between age and SDF ($r = .199$; $p = .001$). Similarly, a positive correlation was observed between the age of patients and the protamine ratio ($r = .234$; $p = .001$) that had been registered.

In addition, the concentration of spermatozoa correlated negatively with SDF ($r = -.20$; $p = .001$), CMA3 positive ($r = -.172$;

$p = .005$) and (P1/P2) ratio ($r = -.208$; $p = .001$) but correlated positively with morphologically normal spermatozoa ($r = .261$; $p = .007$) and with the progressive motility ($r = .342$; $p = .0001$).

In contrast to the age and the concentration, the progressive motility correlated positively with protamine 1 ($r = .171$; $p = .05$) and protamine 2 ($r = .239$; $p = .001$) but negatively with the SDF ($r = -.334$; $p = .001$).

In addition, the mean percentage of normal morphology spermatozoa demonstrated a significant positive correlation with the CMA3 positive ($r = .216$; $p = .001$) but correlated negatively ($p < .01$) with protamine 1, protamine 2 and protamine ratio ($r = -.271$; $r = -.259$; $r = -.323$ respectively).

Sperm DNA fragmentation presented a highly significant positive correlation with the protamine ratio ($r = .433$; $p = .001$) and negative, insignificant correlations with protamine 1 and protamine 2 and the CMA3 positive ($r = -.022$; $r = -.102$; $r = -.093$ respectively; Table 2b).

CMA3 positive was negatively correlated with protamine 1 ($r = -.154$; $p = .011$) and with the protamine ratio ($r = -.349$; $p = .001$; Table 2b).

Protamines 1 and 2 showed a positive correlation ($r = .796$; $p = .001$). The protamine ratio correlated positively with P1 ($r = .269$; $p = .001$), unlike P2 (Table 2b).

3.2 | Comparison between fertile and sub-fertile patients

By comparing the sperm parameters, expressed as the mean \pm standard deviation (Table 1), between the two groups of fertile and sub-fertile men, it was found that there was no significant difference regarding sperm morphology and the CMA3 positive despite the two parameters being lower in the men who were sub-fertile.

The age and protamine ratio (P1/P2) were higher in the sub-fertile group ($p < .01$), and the other parameters were lower (Table 1).

In the fertile group, the correlations between CMA3 positive, SDF and protamines P1, P2 and their ratio are listed in Table 3.

TABLE 1 Comparison of studied parameters between fertile and sub-fertile groups

Parameters	All patients ($n = 272$; $M \pm SD$)	Fertile ($n = 151$; $M \pm SD$)	Sub-fertile ($n = 121$; $M \pm SD$)	p-value
Sperm concentration ($\times 10^6$ spz/ml)	64.81 ± 39.66	78.45 ± 38.84	47.90 ± 33.85	.001**
Progressive motility (%)	35.90 ± 18.64	47.39 ± 15.04	21.56 ± 11.32	.001**
Morphologically normal spermatozoa (%)	29.65 ± 23.46	31.36 ± 22.06	27.52 ± 25.01	.180
Sperm DNA fragmentation (SDF; %)	14.60 ± 8.58	12.31 ± 7.01	17.50 ± 9.50	.001**
Protamine deficiency (CMA3 positive; %)	34.30 ± 15.97	32.94 ± 15.12	35.99 ± 16.89	.118
Protamine 1 (ng/ 10^6 spz)	432.35 ± 124.14	447.29 ± 120.85	413.69 ± 126.15	.026*
Protamine 2 (ng/ 10^6 spz)	397.85 ± 125.19	416.90 ± 120.14	374.08 ± 127.77	.005**
P1/P2 ratio	0.83 ± 0.49	0.75 ± 0.42	0.91 ± 0.43	.003**

Abbreviations: M, mean; SD, standard deviation; Spz, spermatozoa.

*Correlation is significant at the .05 level.

**Correlation is significant at the .01 level.

TABLE 2 (a) Correlations between various parameters for all investigated patients (N = 272). (b) Correlations between different measured parameters for all patients (N = 272)

(a)					
	Age (Year)	Concentration (×10 ⁶ /ml)	Progressive motility (%)	Normal morphology (%)	
Age (year)	–	<i>r</i> = −.144* <i>p</i> = .018	<i>r</i> = −.129* <i>p</i> = .034	<i>r</i> = −.248** <i>p</i> = .001	
Sperm concentration (×10 ⁶ spz/ml)	<i>r</i> = −.144* <i>p</i> = .018	–	<i>r</i> = .342** <i>p</i> = .001	<i>r</i> = .164** <i>p</i> = .007	
Progressive motility (%)	<i>r</i> = −.129* <i>p</i> = .034	<i>r</i> = .342** <i>p</i> = .001	–	<i>r</i> = −.095 <i>p</i> = .120	
Morphologically normal spermatozoa (%)	<i>r</i> = −.248** <i>p</i> = .001	<i>r</i> = .164** <i>p</i> = .007	<i>r</i> = −.095 <i>p</i> = .120	–	
Sperm DNA fragmentation index SDF (%)	<i>r</i> = .199** <i>p</i> = .001	<i>r</i> = −.201** <i>p</i> = .001	<i>r</i> = −.334** <i>p</i> = .001	<i>r</i> = −.048 <i>p</i> = .432	
Protamine deficiency (CMA3 positive; %)	<i>r</i> = −.198** <i>p</i> = .001	<i>r</i> = −.172** <i>p</i> = .005	<i>r</i> = −.110 <i>p</i> = .071	<i>r</i> = .216** <i>p</i> = .001	
Protamine 1 (ng/10 ⁶ spz)	<i>r</i> = .078 <i>p</i> = .202	<i>r</i> = .117 <i>p</i> = .054	<i>r</i> = .171** <i>p</i> = .005	<i>r</i> = −.271** <i>p</i> = .001	
Protamine 2 (ng/10 ⁶ spz)	<i>r</i> = .022 <i>p</i> = .716	<i>r</i> = .104 <i>p</i> = .087	<i>r</i> = .239** <i>p</i> = .001	<i>r</i> = −.259** <i>p</i> = .001	
(P1/P2)ratio	<i>r</i> = .234** <i>p</i> = .001	<i>r</i> = −.208** <i>p</i> = .001	<i>r</i> = −.008 <i>p</i> = .894	<i>r</i> = −.323** <i>p</i> = .001	
(b)					
	DFI (%)	CMA3 positive (%)	Protamine 1 (ng/10 ⁶ spz)	Protamine 2 (ng/10 ⁶ spz)	P1/P2 ratio
Sperm DNA fragmentation (SDF; %)	–	<i>r</i> = −.093 <i>p</i> = .125	<i>r</i> = −.022 <i>p</i> = .723	<i>r</i> = −.102 <i>p</i> = .094	<i>r</i> = .433** <i>p</i> = .001
Protamine deficiency (CMA3 positive; %)	<i>r</i> = −.093 <i>p</i> = .125	–	<i>r</i> = −.154* <i>p</i> = .011	<i>r</i> = −.081 <i>p</i> = .184	<i>r</i> = −.349** <i>p</i> = .001
Protamine 1 (ng/10 ⁶ sperm)	<i>r</i> = −.022 <i>p</i> = .723	<i>r</i> = −.154* <i>p</i> = .011	–	<i>r</i> = .796** <i>p</i> = .001	<i>r</i> = .269** <i>p</i> = .001
Protamine 2 (ng/10 ⁶ sperm)	<i>r</i> = −.102 <i>p</i> = .094	<i>r</i> = −.081 <i>p</i> = .184	<i>r</i> = .796** <i>p</i> = .001	–	<i>r</i> = .039 <i>p</i> = .524
P1/P2 ratio	<i>r</i> = .433** <i>p</i> = .001	<i>r</i> = −.349** <i>p</i> = .001	<i>r</i> = .269** <i>p</i> = .001	<i>r</i> = .039 <i>p</i> = .524	–

Abbreviation: Spz, spermatozoa.

*Correlation is significant at the .05 level.

**Correlation is significant at the .01 level.

Sperm DNA fragmentation correlated negatively with the CMA3 value ($r = -.297$; $p = .001$) and protamine ratio (P1/P2; $r = .356$; $p = .001$).

In turn, the value of CMA3 is negatively correlated ($p < .01$) with protamines P1, P2 ($r = -.249$, $r = -.212$ respectively) and their ratio ($r = -.586$; $p = .001$).

P1 had a highly significant correlation with P2 ($r = .857$; $p = .001$) and the P1/P2 ratio ($r = .329$; $p = .001$). The P1/P2 ratio had a significant positive correlation with the protamine 2 ($r = .176$; $p = .031$).

In the sub-fertile group, the CMA3 positive showed no correlations to SDF and protamines P1, P2 and their ratio (Table 4). SDF had a highly positive correlation with the protamine ratio (P1/P2; $r = .479$; $p = .001$).

Protamine 1 correlated highly positive with protamine 2 ($r = .720$; $p = .001$) and with the protamine ratio (P1/P2; $r = .255$; $p = .005$; Table 4).

3.3 | Comparison between the measured parameters in all patients according to the CMA3 test results

The sperm DNA protamination (DNA condensation) was measured by the chromomycin (CMA3) staining technique. According to the results from this test, the patients were divided into two groups,

TABLE 3 Correlations between DNA integrity parameters and protamine by fertile patients

	Sperm DNA fragmentation (SDF; %)	Protamine deficiency (CMA3 positive; %)	Protamine 1 (ng/10 ⁶ spz)	Protamine 2 (ng/10 ⁶ spz)	(P1/P2) ratio
Sperm DNA fragmentation (SDF; %)	–	$r = -.297^{**}$ $p = .001$	$r = .024$ $p = .771$	$r = -.017$ $p = .834$	$r = .356^{**}$ $p = .001$
Protamine deficiency (CMA3 positive; %)	$r = -.297^{**}$ $p = .001$	–	$r = -.249^{**}$ $p = .002$	$r = -.212^{**}$ $p = .009$	$r = -.586^{**}$ $p = .001$
Protamine 1 (ng/10 ⁶ spz)	$r = .024$ $p = .771$	$r = -.249^{**}$ $p = .002$	–	$r = .857^{**}$ $p = .0001$	$r = .329^{**}$ $p = .001$
Protamine 2 (ng/10 ⁶ spz)	$r = -.017$ $p = .834$	$r = -.212^{**}$ $p = .009$	$r = .857^{**}$ $p = .001$	–	$r = .176^{*}$ $p = .031$
P1/P2 ratio	$r = .356^{**}$ $p = .001$	$r = -.586^{**}$ $p = .001$	$r = .329^{**}$ $p = .0001$	$r = .176^{*}$ $p = .031$	–

Abbreviation: Spz, spermatozoa.

*Correlation is significant at the .05 level.

**Correlation is significant at the .01 level.

according to the value of CMA3 positive, as defined by Zandemami et al. (2012).

- Group of condensed chromatin (CMA3 positive $\leq 31\%$, $n = 133$)
- Group of noncondensed chromatin (CMA3 positive $> 31\%$, $n = 139$).

Table 5 illustrates the comparison of various studied parameters between the two investigated groups.

Progressive motility and protamine ratio were significantly lower in the group of noncondensed chromatin in comparison with the condensed chromatin group ($33.01 \pm 16.90\%$ vs. $38.92 \pm 19.92\%$; $p = .009$; 0.66 ± 0.47 vs. 1.00 ± 0.32 ; $p = .001$ respectively), whereas the morphologically normal spermatozoa and the CMA3 value were higher among the noncondensed chromatin group in comparison with the group of condensed chromatin ($35.46 \pm 24.83\%$ vs. $23.59 \pm 20.31\%$; $p = .001$; $46.36 \pm 12.95\%$ vs. $21.69 \pm 5.96\%$; $p = .0001$).

3.4 | Comparison between the measured parameters among all patients according to the results of the TUNEL test

According to the sperm DNA fragmentation (SDF), defined by Chohan, Griffin, Lafromboise, Jonge, and Carrell (2006), the following three groups were formed:

- Group with nonfragmented DNA (SDF $\leq 15\%$)
- Group with moderately fragmented DNA ($16\% < \text{SDF} < 30\%$)
- Group with fragmented DNA (SDF $\geq 30\%$).

The age, the P1/P2 ratio and the SDF were significantly higher in the group with fragmented DNA ($p = .001$), whereas the concentration, the progressive motility and the protamine 2 were significantly higher in the group with nonfragmented DNA ($p = .002$; $p = .001$ and $p = .012$ respectively; Table 6).

TABLE 4 Correlations between DNA integrity parameters and protamine in sub-fertile patients

	Sperm DNA fragmentation (SDF; %)	Protamine deficiency (CMA3 positive; %)	Protamine 1 (ng/10 ⁶ spz)	Protamine 2 (ng/10 ⁶ spz)	(P1/P2) ratio
Sperm DNA fragmentation (SDF; %)	–	$r = .136$ $p = .136$	$r = .028$ $p = .757$	$r = -.083$ $p = .368$	$r = .479^{**}$ $p = .001$
Protamine deficiency (CMA3 positive; %)	$r = .136$ $p = .136$	–	$r = .008$ $p = .927$	$r = .090$ $p = .327$	$r = -.057$ $p = .533$
Protamine 1 (ng/10 ⁶ spz)	$r = .028$ $p = .757$	$r = .008$ $p = .927$	–	$r = .720^{**}$ $p = .001$	$r = .255^{**}$ $p = .005$
Protamine 2 (ng/10 ⁶ spz)	$r = -.083$ $p = .368$	$r = .090$ $p = .327$	$r = .720^{**}$ $p = .001$	–	$r = -.072$ $p = .432$
(P1/P2) ratio	$r = .479^{**}$ $p = .001$	$r = -.057$ $p = .533$	$r = .255^{**}$ $p = .005$	$r = -.072$ $p = .432$	–

Abbreviation: Spz, spermatozoa.

**Correlation is significant at the .01 level.

Parameters	Condensed chromatin (n = 133; M ± SD)	Noncondensed chromatin (n = 139; M ± SD)	p-value
Age (years)	34.85 ± 7.07	32.26 ± 7.43	.004**
Sperm concentration (10 ⁶ sperm/ml)	68.38 ± 39.65	61.37 ± 39.52	.146
Progressive motility (%)	38.92 ± 19.92	33.01 ± 16.90	.009**
Normal morphology (%)	23.59 ± 20.31	35.46 ± 24.83	.001**
Sperm DNA fragmentation (SDF; %)	14.55 ± 7.04	14.66 ± 9.86	.918
Protamine deficiency (CMA3) positive (%)	21.69 ± 5.96	46.36 ± 12.95	.0001**
Protamine 1 (ng/10 ⁶ spz)	449.80 ± 123.97	415.66 ± 122.44	.023
Protamine 2 (ng/10 ⁶ spz)	405.00 ± 125.90	391.03 ± 124.60	.359
P1/P2 ratio	1.00 ± 0.32	0.66 ± 0.47	.001**

Abbreviations: M, mean; SD, standard deviation; Spz, spermatozoa.

**Correlation is significant at the .01 level.

TABLE 5 Comparison of studied parameters between the two groups condensed chromatin and not condensed chromatin (CMA3 test)

4 | DISCUSSION

A number of methods used to quantify protamination and DNA packaging, DNA fragmentation, chromosome aneuploidy and molecular karyotyping have been applied in the evaluation of male infertility problems (Ferlin & Foresta, 2014; Patassini et al., 2013; Tsuribe et al., 2016).

The present study was conducted to determine the sperm chromatin condensation (CMA3), the DNA integrity by a TUNEL test, the protamine (P1 and P2) concentrations and their ratio in spermatozoa from fertile and sub-fertile male patients using electrophoresis and to find out if there was a correlation between protamine deficiency, sperm DNA injury and the P1/P2 ratio. A final aim was to investigate whether the protamine ratio could be effectively used as an additional biomarker test to predict the quality of sperm at the level of molecular biology.

The anomalies of spermatozoa protamination make the sperm DNA sensitive to oxidative stress (Ozmen, Koutlaki, Youssry, Diedrich, & Al-Hasani, 2007), suggesting that any default in protamination can induce an injury in the DNA (Aoki, Emery, et al., 2006; Aoki, Liu, & Carrell, 2006; Carrell, Emery, & Hammoud, 2007; Nasr-Esfahani, Razavi, Mozdarani, Mardani, & Azvagi, 2004; Tarozzi et al., 2009; Torregrosa et al., 2006). Consequently, the deficiency in protamine occurs during the last phases of spermatogenesis, making the sperm DNA susceptible to injury and fragmentation and thus lead to male infertility (Jodar & Oliva, 2014).

Sperm DNA first becomes susceptible to damage when the chromatin packing is not fully completed during spermatogenesis (protamine replacement is occurring in elongating spermatids). Temporary nicks, linked to the topoisomerase's activity, facilitate histone-protamine replacement but if these nicks are not repaired, the DNA of mature spermatozoa will be fragmented (Smith & Haaf, 1998).

Single-stranded breaks are produced mainly due to reactive oxygen species (ROS; Agarwal & Prabakaran, 2005; Enciso, Sarasa,

Agarwal, Fernández, & Gosálvez, 2009; Ribas-Maynou et al., 2012), which may come from exogenous sources, such as environmental toxicants, smoking, alcohol, diet, radiation and/or from endogenous sources, such as an increase in leucocytes, the presence of varicocele or even ROS generated by mitochondria for the movement of sperm cells (Agarwal, Virk, Ong, & Plessis, 2014; Aitken & De Iuliis, 2009; Sakkas & Alvarez, 2010).

In addition, the sperm DNA of males experiencing fertility problems can arise through an abortive apoptotic pathway. In this case, spermatozoa will lose their capacity to undergo programmed cell death in the form of apoptosis because they are transcriptionally and translationally inert. But it is thought that this will lead to DNA fragmentation in the nucleus of spermatids, which retains the ability to metamorphose into mature spermatozoa that probably still have the ability to fertilise the oocyte (Sakkas et al., 2004).

Approximately 60% of sperm DNA alterations were clarified by the quality of the transition histone-protamine mechanism (Aitken & De Iuliis, 2007). The mean percentage of premature chromatin condensation in the sperm DNA was demonstrated to be greater in the samples presenting a strong protamine deficiency, compared to samples with low CMA3-positive values (Nasr-Esfahani, Salehi, et al., 2004).

In addition, the packaging of the sperm chromatin correlated with the presence of alterations at the DNA level, a decrease in the capability of spermatozoa to penetrate the oocyte and chromatin decondensation after fertilisation (Esterhuizen et al., 2002; Razavi, Nasr-Esfahani, Mardani, Mafi, & Moghdam, 2003).

A correlation between protamine deficiency, alterations in DNA and the absence of sperm vitality was detected with the help of many techniques. To assess the sperm chromatin structure, chromatin structural probes or dyes were used such as chromomycin α (competing with protamine binding to DNA, it reveals protamination defects on spermatozoa), the acridine orange (measures the susceptibility to conformational changes), the toluidine blue (that stains

TABLE 6 Comparison of studied parameters between the three groups obtained following the results of the TUNEL test

Parameters	Nonfragmented DNA (<i>n</i> = 133; <i>M</i> ± <i>SD</i>)	Moderate fragmented DNA (<i>n</i> = 19; <i>M</i> ± <i>SD</i>)	Fragmented DNA (<i>n</i> = 93; <i>M</i> ± <i>SD</i>)	<i>p</i> -value
Age (year)	32.55 ± 7.05	34.14 ± 6.81	38.37 ± 10.19	.003**
Sperm concentration (×10 ⁶ spz/ml)	70.12 ± 40.51	61.59 ± 37.29	37.42 ± 32.12	.002**
Progressive motility (%)	40.85 ± 19.14	29.96 ± 15.49	25.58 ± 16.75	.001**
Normal morphology (%)	28.35 ± 20.60	29.80 ± 24.74	39.58 ± 35.31	.143
Sperm DNA fragmentation (SDF; %)	8.77 ± 3.31	20.01 ± 3.33	34.88 ± 8.25	.001**
Protamine deficiency (CMA3 positive; %)	34.11 ± 16.177	33.62 ± 15.191	39.32 ± 18.160	.356
Protamine 1 (ng/10 ⁶ spz)	443.69 ± 122.04	408.67 ± 124.66	460.09 ± 126.19	.055
Protamine 2 (ng/10 ⁶ spz)	415.66 ± 118.74	367.92 ± 128.55	404.45 ± 137.04	.012*
(P1/P2) ratio	0.69 ± 0.43	0.97 ± 0.39	1.16 ± 0.23	.001**

Abbreviations: *M*, Mean; *SD*, standard deviation; Spz, spermatozoa.

*Correlation is significant at the .05 level.

**Correlation is significant at the .01 level.

phosphate residues of fragmented DNA) and the aniline blue (that stains loosely condensed chromatin; Evenson & Wixon, 2006).

Moreover, different techniques are used to assess the DNA fragmentation. The commonly used techniques are terminal deoxynucleotidyl transferase mediated dUDP nick-end labelling (TUNEL), the sperm chromatin dispersion (SCD) assay and the comet assay (Agarwal et al., 2016). Each assay purportedly measures different forms of DNA damage.

Unfortunately, most of the available techniques provide limited information regarding the nature of the DNA lesions and do not allow the exact pathogenesis of disrupted sperm DNA to be highlighted (Agarwal & Said, 2004; Bungum, 2012). For example, chromomycin A3 (CMA3), a guanine-cytosine-specific fluorochrome competes with protamine for binding to the minor groove of DNA (Bianchi et al., 1993; Sakkas et al., 1996). Since the protamines are not directly examined, this approach still does not fully answer the question, namely: What the real cause of increased staining may be? However, CMA3 can be used as a feasible indicator and the increased stainability presents a general description about the anomalies in the chromatin packaging of spermatozoa, leading to DNA damage or other problems in the spermatozoa.

The literature concerning sperm DNA decays and sperm parameters and/or clinical outcomes is controversial. There is no clear correlation between sperm morphology assisted by strict criteria, sperm count and chromatin condensation (Berkovitz et al., 2005; Hazout, Dumont-Hassan, Junca, Bacrie, & Tesarik, 2006). A prospective analysis study of semen parameters and sperm chromatin structure assay, conducted by Sills, Fryman, Perloe, Michels, and Tucker (2004), demonstrated a nonsignificant correlation between DNA fragmentation and sperm concentration. Furthermore, the sperm DNA apoptosis rates were significantly higher in patients with abnormal sperm parameters compared to patients with normal spermatozoa (Huang et al., 2005).

In patients with unexplained recurrent pregnancy loss, aneuploidy, abnormal morphology and the apoptosis rate were significantly correlated (Carrell et al., 2003).

Cohen-Bacrie et al. (2009) examined in a study of more than 1,600 couples to evaluate the correlation between the semen parameters, including CASA and the fragmentation rates (TUNEL) and stated that sperm parameters and DNA damage were complementary rather than strongly linked.

In a large meta-analysis (43 studies), researchers concluded that SDF decreases pregnancy rates when conventional IVF or ICSI are used (Simon, Zini, Dyachenko, Ciampi, & Carrell, 2017).

Moreover, a number of studies demonstrated a significant positive correlation between the sperm DNA damage and age, and suggested that men under 35 years old had a lower DNA fragmentation rate (Belloc et al., 2009; Vagnini et al., 2007) and that a decrease in fertilisation, embryo quality, implantation and pregnancy rates (Johnson et al., 2015). However, other studies reported contradictory findings suggesting that neither the standard semen parameters and the sperm DNA fragmentation nor the fertilisation is affected by male age (Nijs et al., 2011; Winkle, Rosenbusch, Gagsteiger, Paiss, & Zoller, 2009). Tapia et al. (2017) reported that there is no difference in fertilisation or pregnancy rates when young eggs were inseminated with an old spermatozoon.

Many studies, on the other hand, have demonstrated that sperm DNA damage correlates negatively with fertilisation, cleavage, implantation and the pregnancy rate (Agarwal & Prabakaran, 2005; Benchaib et al., 2003; Host, Lindenberg, & Smidt-jensen, 2000).

The functional implications of the sperm DNA are still not clear. This is because the fragmentation of the DNA can be the consequence of (a) intrinsic factors, such as abortive apoptosis, the anomalies of the recombination and the imbalances of the protamine (P1/P2 ratio) or oxidative stress or (b) external factors, such as the storage temperatures or the cryopreservation (González-Marín, Gosálvez, & Roy, 2012).

Reactive oxygen species and oxidative damage can be a major cause of declining sperm quality; antioxidants can be important in preventing this (Ahmadi, Bashiri, Ghadiri-Anari, & Nadjarzadeh, 2016).

Despite the high incidence of defective sperm function, very few reliable therapies are available. This could be explained by the general lack of knowledge concerning the precise biochemical nature of the cause of such sperm defects. In fact, there is little reliable information concerning the factors that are causing the fertilising capacity loss of spermatozoa.

The Cochrane review suggested that the use of antioxidants caused a 1.8- to 4.6-fold increase in the chances of achieving a natural pregnancy. However, up to a 6.5-fold increase in miscarriages might also be observed (Showell et al., 2014). In ICSI therapy, it is still not obvious if antioxidant use may be effective or not in improving pregnancy results and birth rates (Agarwal et al., 2014; Tremellen, Miari, Froiland, & Thompson, 2007).

Evaluations of abnormal chromatin condensation by CMA3 staining and of the DNA fragmentation by TUNEL have been used in this study.

In the current study, the deficiency in protamine (CMA3), determined by the CMA3 test, correlated negatively with the sperm concentration ($r = -.172$, $p < .01$). However, no correlation has been found either with the motility or with the DNA fragmentation (SDF; Table 2). By dividing all the investigated samples according to the CMA3 test results ($\text{CMA3} \leq 31\%$; $\text{CMA3} > 31\%$), in the group of non-condensed DNA ($\text{CMA3} > 31\%$) the progressive motility was significantly lower ($p = .009$), compared to the group of condensed DNA ($\text{CMA3} \leq 31\%$) but there was no significant difference in the SDF, P1 and P2 values in both of the investigated groups, whereas in the noncondensed DNA group ($\text{CMA3} > 31\%$), the P1/P2 ratio was significantly lower ($p = .001$; Table 5).

These findings are partly in agreement with the results of Tarozzi et al. (2009), who found that protamine deficiency (CMA3 positive) correlated negatively ($p < .05$) with concentration, motility and normal morphology.

Moreover, Iranpour (2014) found that the CMA3 positive and the morphologically abnormal spermatozoa showed a positive correlation ($r = .461$, $p < .001$), but CMA3 positive correlated negatively with the sperm count and motility ($r = -.359$, $p < .001$; $r = -.37$, $p < .001$ respectively).

The alterations in protamination may be a possible cause of the decrease in the spermatozoa function. In fact, Carrell et al. (2007) interestingly posed two main hypotheses concerning this subject: firstly, the protamine can act as a "control" during the spermatogenesis and the aberrant expression of the protamine can increase the apoptosis level, which then causes a decrease in the quality of spermatozoa; secondly, the aberrant protamine expression can be the sign of an altered spermatogenesis during the transcription and/or translation regulation.

Moreover, it has been demonstrated that the SDF was a good predictive marker for a successful pregnancy in ART (Larson-Cook et al., 2003).

In the present study, by comparing the groups subdivided according to the SDF value ($\text{SDF} \leq 15\%$; $16\% - 30\%$; $\text{SDF} \geq 30\%$), it was shown that the age was higher ($p = .03$) in the fragmented DNA group, but the concentration and the progressive motility

were significantly greater ($p < .01$) in the nonfragmented DNA group, while morphology and CMA3 positive were similar in both groups (Table 6).

The SDF also correlated positively with the age ($r = .199$; $p = .001$) but negatively with the sperm count ($r = -.201$; $p = .001$) and the motility ($r = -.334$; $p = 0$; Table 2a).

These results from our study demonstrate the absence of any relationship between protamine deficiency in spermatozoa, and alterations of sperm DNA (Tables 4, 5 and 6) and are therefore contradictory to previous studies which demonstrated that the deficiency of protamine and the DNA fragmentation were positively correlated (García-Peiró et al., 2011; Ni et al., 2014; Nili, Mozdarani, & Aleyasin, 2009).

However, the absence of any correlation between the DNA lesions and the deficiency of protamine can be explained by the fact that several other factors, such as the oxidative stress, can lead to a fragmented DNA.

It is therefore probable that absolute or relative protamine deficiency can be a cause of defective chromatin compaction (Aravindan, Krishnamurthy, & Moudgal, 1997) and increase susceptibility to DNA damage (Aoki, Emery, et al., 2006; Aoki, Moskovtsev, et al., 2005), suggesting that sperm DNA damage may be partly due to a defect in spermiogenesis during the histone-protamine transition (Steger, Pauls, Klonisch, Franke, & Bergmann, 2000).

Abnormalities in sperm chromatin packaging are associated with the poor fertility of human spermatozoa (Aoki, Moskovtsev, et al., 2005). Protamine deficiency is associated with a decrease in the fertilising ability of spermatozoa and the quality of human embryos (Aoki, Moskovtsev, et al., 2005; Balhorn et al., 1999; Carrell & Liu, 2001; de Yebra et al., 1998).

Nevertheless, the protamine values P1 and P2 correlated significantly with the SDF in both investigated groups (Tables 3 and 4) and the protamine (P1 and P2) concentrations were significantly lower ($p < .05$) in the group of sub-fertile subjects in comparison with the fertile one whereas the protamine ratio (P1/P2) was significantly greater ($p = .003$) in the sub-fertile group (Table 1).

The protamine ratio (P1/P2), in turn, had a positive relationship with the SDF in both groups ($r = .365$; $p = .001$; and $r = .479$; $p = .001$ respectively) but correlated negatively with CMA3 positive ($r = -.586$; $p = .001$) in the fertile group (Table 3).

These findings were in accordance with various other studies confirming a correlation between the anomalies of the protamine ratio and infertility (De Mateo et al., 2009; Ni et al., 2014). Ni, Spiess, Schuppe, and Steger (2016) confirmed that the protamine ratio was lower in the fertile group in comparison with the ratio in the sub-fertile group.

In humans, P1 and P2 are expressed in nearly equal quantities, with the P1/P2 ratio close to 1, and alterations of the protamine ratio in either direction are associated with a decrease in sperm parameters (Aoki, Emery, et al., 2006).

While an altered protamine ratio has never been observed in fertile men (Carrell & Liu, 2001; Oliva, 2006), an abnormal P1/P2 ratio is associated with a low sperm count, reduced motility, abnormal head morphology, a higher frequency of DNA fragmentation and

a lower sperm penetration assay score (Aoki, Liu, & Carrell, 2005; Aoki, Moskovtsev, et al., 2005). Decreased clinical-pregnancy rates have been associated with spermatozoa having a reduced P1/P2 ratio (Aoki, Emery, et al., 2006).

In general, classic sperm parameters are not providing sufficient details about the quality and the function of sperm; this is why an assessment of the protamine ratio is useful for an assessment of spermatozoon.

It was shown that the P1 value and CMA3 positive did not differ significantly between the three groups, divided according to SDF value, whereas the P2 value was obviously greater in the nonfragmented DNA group ($SDF \leq 15\%$; $p = .012$; Table 6). Nevertheless, the protamine ratio was significantly lower in the group with nonfragmented DNA and high among the group with fragmented DNA ($p = .001$). These results are in agreement with other findings (Castillo, Simon, Mateo, Lewis, & Oliva, 2011; Hammadeh et al., 2010) that demonstrated a positive relationship between the presence of the precursors of protamine 2 or the alteration of the protamine ratio and the damaged DNA.

Ni et al. (2016) analysed 12 studies and found that protamine deficiency (CMA3 assay) correlated significantly with the DNA fragmentation of spermatozoa while the protamine ratio had no association with the DNA fragmentation.

This is the first study to demonstrate that the protamine ratio (P1/P2) correlated positively with SDF among all the investigated groups but negatively with chromatin deficiency (CMA3 positive).

5 | CONCLUSION

DNA integrity in spermatozoa is strictly linked to protamine deficiency. However, DNA fragmentation is linked to the protamine ratio instead of CMA3. This finding supports the assumption that protamines are implicated in DNA preservation. As a result, the protamine ratio (P1/P2) may indeed be used as a good biomarker for the assessment of sperm DNA.

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CONFLICT OF INTEREST

Nothing to declare.

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