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ORIGINAL ARTICLE

Semiquantitative promoter methylation of *MLH1* and *MSH2* genes and their impact on sperm DNA fragmentation and chromatin condensation in infertile men

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Abstract

To investigate the semiquantitative methylation alterations of *MLH1* and *MSH2* and the possible association among methylation of *MLH1* and *MSH2*, sperm DNA fragmentation and sperm chromatin condensation in idiopathic oligoasthenoteratozoospermic men. Seventy-five idiopathic infertile men and 52 fertile and/or normozoospermic men were included in the study. SDF was analysed using the TUNEL assay in semen samples of 100 men. Promoter methylation of *MLH1* and *MSH2* genes was assessed by semiquantitative methylight analysis in semen samples of 39 and 40 men respectively. Sperm chromatin condensation was evaluated using aniline blue staining in 114 men. *MLH1* promoter methylation was positively correlated with the percentage of aniline blue positive spermatozoa ($r = 0.401$, $p = 0.0188$). On the other hand, *MSH2* promoter methylation was negatively correlated with sperm concentration and total sperm count ($r = -0.421$, $p = 0.0068$ and $r = 0.4408$, $p = 0.009$ respectively). The percentage of aniline blue positive spermatozoa in the control group was significantly lower than in the OAT group ($p < 0.0001$) and negatively correlated with total sperm count ($r = -0.683$, $p < 0.0001$), progressive sperm motility ($r = -0.628$, $p < 0.0001$), total motility ($r = -0.639$, $p < 0.0001$) and normal morphology ($r = -0.668$, $p < 0.0001$). Promoter methylation profile of *MLH1* and *MSH2* genes may play role on sperm DNA packaging and conventional semen parameters respectively.

KEYWORDS
chromatin condensation, idiopathic OAT, *MLH1*, *MSH2*, SDF

1 | INTRODUCTION

Male infertility has a complex aetiology involving genetic factors particularly in azoospermic and severely oligozoospermic men and epigenetic components, acquired or congenital urogenital anomalies, cancers, increased scrotal heat, urogenital infections, endocrine disorders, immunological factors and toxic factors (Gunes, Arslan, Hekim, & Asci, 2016; Gunes & Esteves, 2020; Hekim et al., 2019; Krausz & Riera-Escamilla, 2018). These aetiological factors may also implicate in sperm DNA damage including sperm DNA fragmentation (SDF) (Esteves, Agarwal, & Majzoub, 2017). Sperm DNA integrity is essential for normal sperm function, fertilisation, early embryo development and pregnancy (Aitken & De Iuliis, 2010; Gunes, Al-Sadaan, & Agarwal, 2015). In male germ cells, DNA integrity is actively maintained until spermiogenesis, a process in which the self-efficacy of DNA repair diminishes due to decreased

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cytoplasmic content of the male germ cells and increase in compact packaging of sperm DNA (Gonzalez-Marin, Gosalvez, & Roy, 2012). Clinical evidence has shown that infertile men have higher SDF than fertile men and that aberrant sperm DNA integrity negatively affects paternal reproductive potential (Giwercman et al., 2010; Ramos-Ibeas et al., 2014; Schulte, Ohl, Sigman, & Smith, 2010; Simon, Zini, Dyachenko, Ciampi, & Carrell, 2017).

DNA repair is an essential and specific cellular response to DNA damage resulting from various endogenous and/or environmental factors such as ROS, protamine transition, UV, radiation and xenobiotics (Chatterjee & Walker, 2017). Mismatch DNA repair (MMR) is one of the most important DNA repair mechanisms and a highly conserved DNA excision-resynthesis processes that keep genomic stability by principally editing misincorporation and insertions/deletions during replication (Liu et al., 2019). Heterodimers, including MutS homolog 2 (MSH2), one of the MMR proteins, is involved in DNA mismatch or damage recognition, while other MMR protein, MutL homolog 1 (MLH1) acts as an endonuclease in DNA repair (Peltomaki, 2016). MMR also plays role in homologous recombination and refunctioning of a halted replication fork and in the repair of DNA double-strand breaks (DSBs) in somatic cells (Spies & Fishel, 2015). In meiosis, homologous recombination is necessary during crossover that begins with genome-wide programmed DSBs in the early stages of the prophase (Manhart & Alani, 2016). MLH1 and MSH2 proteins are expressed in numerous tissues, especially in the testis (Fagerberg et al., 2014). Errors in MMR have been reported to be associated with subfertility as well as cancer susceptibility (Gunes et al., 2015; Paul et al., 2007; Sanderson, Hassold, & Carrell, 2008; Sun et al., 2007; Terribas et al., 2010). Indeed, infertility and microsatellite instability associated with various cancers have been observed in *Mlh1*-deficient mice (Mukherjee, Ridgeway, & Lamb, 2010). Terribas et al. studied 13 nonobstructive azoospermic or severe oligozoospermic patients with spermatogenic failure, five patients with germ cell tumours and 10 obstructive azoospermic patients as controls. The testicular tissues of the patients have been analysed and lower mRNA expression of *MLH1* and other MMR genes, including *MSH4*, *MSH5* and *MLH3*, were reported in spermatogenic failure group compared to controls (Terribas et al., 2010). In addition, another study data demonstrated that rs4647269 polymorphism in *MLH1* might be a risk factor for azoospermia or oligozoospermia in 1292 idiopathic infertile men compared with 480 fertile controls. Additionally, it has been reported that the same *MLH1* polymorphism might be associated with increased sperm DNA damage (Ji et al., 2012). All these studies suggest the critical role of *MLH1* and *MSH2* genes for male fertility.

Given the critical roles of MLH1 and MSH2 proteins in DNA repair and meiosis, we aimed to analyse the impact of *MLH1* and *MSH2* genes on sperm DNA in greater detail. In this respect, the relationship among the immature histone-rich spermatozoa, SDF and promoter methylation of *MLH1* and *MSH2* genes were investigated. Besides, the findings were correlated with semen parameters and the smoking status of the participants.

2 | MATERIALS AND METHODS

2.1 | Subjects

Infertile men who have a wife with normal obstetric evaluation and failure to achieve a clinical pregnancy despite having at least 1 year of unprotected intercourse and diagnosed with oligoasthenoteratozoospermia (OAT) based on two or more semen analyses were included in the study. Infertile patients diagnosed with idiopathic OAT attending the Urology Clinic of Ondokuz Mayis University (OMU), and fertile and/or normozoospermic men between 2018 and 2019 were enrolled. Seventy-five idiopathic infertile patients with OAT aged between 18 and 50 have been included in the study group. Patients genetically diagnosed with karyotype abnormalities and Y-chromosome microdeletions were excluded from the study.

The institutional review board (IRB) approved the study protocol (IRB No. KAEK 2016/185). All participants provided an informed consent and filled out a detailed questionnaire about age, occupation and smoking status.

The control group consisted of 52 men aged between 24 and 50 (20 normozoospermic, 27 fertile and 5 proven fertile men). Semen samples from both patients and controls were obtained by masturbation after 2–5 days of sexual abstinence. After incubating the samples at 37°C for 20–30 min, liquefied semen samples were analysed in the Andrology laboratory according to the World Health Organization (WHO) 2010 guidelines (Cooper et al., 2010). OAT was diagnosed with at least two semen analyses (Jungwirth et al., 2012). Then, the semen samples were prepared for the evaluation of SDF and chromatin condensation using the TUNEL assay and the aniline blue staining respectively. Additionally, the remaining semen was separated for further use in DNA isolation for the assessment of promoter methylation status of *MLH1* and *MSH2*.

2.2 | TUNEL assay

Semen samples of 56 out of 75 infertile men and 46 out of 52 controls were analysed for SDF using TUNEL assay (Kabartan, Gunes, Arslan, & Asci, 2019). Before starting the TUNEL analysis, the seminal plasma of fresh semen samples was removed by centrifugation at 500 × *g* for 7 min. The pellet was then washed once in 1 ml of phosphate-buffered saline (PBS) (Gibco). Afterwards, sperm samples were fixed until further use by adding 3.6% paraformaldehyde (PFA) (Merck KGaA) to pellet and kept at 4°C up to 2 weeks. Then, the fixed samples were washed twice with PBS and the supernatant was discarded. The pellet was dropped on poly-L-lysine-coated slides with phosphate-buffered (PB) sucrose and stored overnight at + 4°C in a dark and humid chamber. The next day, sperm samples were permeabilised with 0.1% sodium citrate and 0.1% Triton X-100 permeabilisation solution. The in situ Cell Death Detection kit (Roche Diagnostics GMbH) was used according to the instructions of the manufacturer. Briefly, label and enzyme solutions were

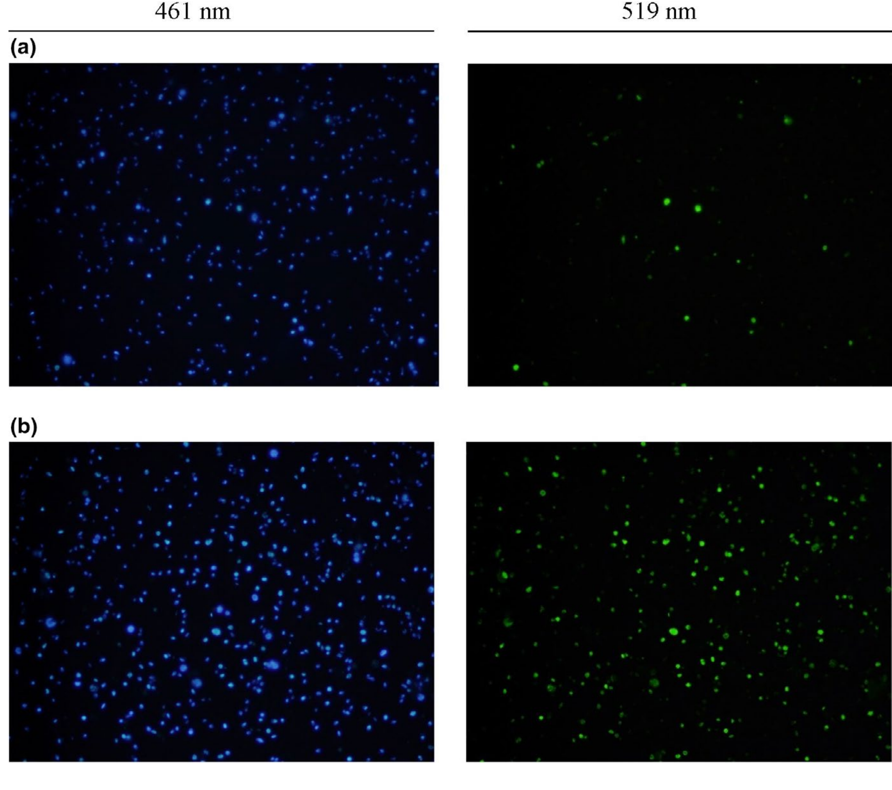

FIGURE 1 DAPI (461 nm) and FITC (519 nm) images of samples with low DNA (a) and high (b) DNA fragmentation (40× magnification)

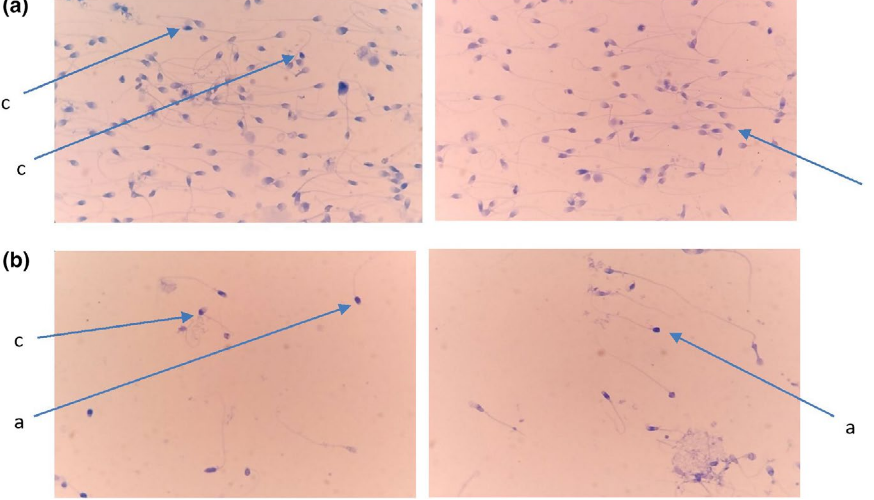

FIGURE 2 Images of aniline blue staining from control (A) and OAT (B) group (a) Aniline blue positive spermatozoa (b) Aniline blue negative spermatozoa (c) Intermediate-stained spermatozoa

mixed and added on the slides. Then, the slides were incubated for 1 hr at 37°C in a dark and humid chamber. At the end of the incubation, slides were washed with PBS and analysed using a fluorescence microscope (BX51, Olympus Life and Material Sciences) under 40X magnification at 461 nm and 519 nm for DAPI and FITC respectively (Figure 1). All cells were labelled as DAPI-positive, while the spermatozoa with strong FITC-fluorescence were evaluated as TUNEL-positive. An average of 500 spermatozoa and at least 3 separate areas were photographed for each sample. Then, spermatozoa from these images were separately counted with DAPI and FITC signals and analysed using the ImageJ program (LOCI, University of Wisconsin). The DNA fragmentation index (DFI) was calculated by a formula of FITC-positive spermatozoa/number of DAPI-positive spermatozoa × 100.

2.3 | Aniline blue staining

Chromatin condensation was evaluated using the aniline blue staining in 65 men with OAT and 49 controls. In brief, the pellets of fresh semen samples were washed twice with PBS, and then, 10–15 µl of the pellets were spread on clean slides and dried at room temperature (Pourmasumi et al., 2019). Dried slides were fixed with 3% glutaraldehyde (Sigma-Aldrich, Merck KGaA) for 30 min at room

temperature. Afterwards, the slides were stained in acidic (pH 3.5) 5% aniline blue solution for 15 min. Then, the slides were rinsed thoroughly with tap water and stored at + 4°C in a humid container until they were examined. Spermatozoa were evaluated under the light microscope (CX31, Olympus Life and Material Sciences) at 100 × magnifications. Immature spermatozoa were characterised in dark blue compared with mature spermatozoa packaged with protamines that appears in pale blue colour (Mostafa et al., 2018). Fully or partially stained spermatozoa were evaluated as aniline blue positive (rich in histone). Unstained or pale blue spermatozoa were assessed as aniline blue negative (rich in protamine) (Figure 2). Average of 200 spermatozoa was evaluated for each sample, and the percentage of aniline blue positive spermatozoa was calculated.

2.4 | Somatic cell lysis and sperm DNA isolation

Somatic cell lysis was performed to eliminate somatic cells from semen before sperm DNA isolation (Goodrich, Johnson, & Krawetz, 2007; Gunes, Agarwal, et al., 2018). Thawed semen samples were centrifugated for 8 min at 300 × *g*, and the pellets were washed with PBS. The supernatant was discarded, and the pellet was dissolved in 12 ml somatic cell lysis buffer containing 0.5% Triton X-100 and 1% sodium dodecyl sulphate and incubated on ice for 25 min and finally centrifugated for 15 min at 300 × *g*. The supernatant was discarded and the residual part was microscopically checked for the presence of somatic cells. The process was repeated until only sperm cells were observed in the suspension. After dissolving of somatic cells completely, sperm DNA was immediately isolated from the samples. The Quick-gDNA Miniprep Kit (Zymo Research) was used for the isolation of sperm DNA according to the manufacturer's instructions. The concentration of sperm DNA was measured by Multiscan Go spectrophotometer (Thermo Fisher Scientific) and adjusted to 200 ng/μl before the bisulphite conversion.

2.5 | Bisulphite conversion

Prior to the methylight analysis, isolated sperm DNA was treated with bisulphite using EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's instructions (Gunes, Agarwal, et al., 2018).

2.6 | Methylight analysis by real-time PCR

The promoter methylation status of *MLH1* and *MSH2* genes was evaluated by the semiquantitative methylight method (Eads et al., 2000). Methylight analysis was performed on the Rotor Gene-Q real-time thermal cycling (Qiagen) using the EpiTect Methylight PCR Kit (Qiagen). Promoter methylation of *MLH1* gene was evaluated in 18 controls and 21 infertile men. Promoter methylation of *MSH2* gene was analysed in 17 controls and 23 infertile men. Methylated primers and probe sequences for the methylation analysis of the *MLH1* promoter, methylation-independent primers, and probe sequences for actin beta (*ACTB*) were used before (Kahn, Ronnett, Gravitt, & Gustafson, 2008; Perez-Carbonell et al., 2010). Primers and probe sequences of *MSH2* gene promoter were designed by Beacon Designer 8.2 (Premier Biosoft). The primer and probe sequences for *MSH2* were F: 5′-GGGATTATGGCGTGTGATATTACG-3′, R: 5′-AAAATCGAAACTACGATAAACCGTAAA-3′, and probe: 6-FAM 5′-ACCACTACACTCCAACGTAAACGACAAATT-3′BHQ-1.

PCR amplification was performed in a total volume of 20 μl containing 1X EpiTect Methylight master mix, 0.4 μM forward and reverse primers, 0.2 μM probe, bisulphite converted DNA (≤100 ng) and RNase-free water for each sample. Cycling conditions were initial PCR activation at 95°C for 5 min followed by 45 cycles of denaturation at 95°C for 15 s and binding/elongation at 55°C for *MLH1* and 60°C for *MSH2* for 60 s.

2.7 | Calculation of methylation percentage

The relative quantification of methylation was calculated according to a $2^{-\Delta\Delta Ct}$ formula using threshold cycles acquired from methylated PCR assays (Livak & Schmittgen, 2001). ΔΔCT equals to $[(CT_{\text{target gene}} - CT_{\text{reference gene}})_{\text{sample}} - (CT_{\text{target gene}} - CT_{\text{reference gene}})_{\text{fully methylated DNA}}]$. Percentage of fully methylated reference (PMR) was assessed using $2^{-\Delta\Delta Ct} \times 100\%$ formula (Wu, Ding, Tan, Li, & Xiong, 2016).

2.8 | Statistical analysis

Data were analysed statistically using MedCalc Statistical Software version 19.2 (MedCalc Software Ltd). After testing data for normal distribution using the Kolmogorov–Smirnov test, either parametric or nonparametric tests were employed. The relationship between normally distributed parameters was evaluated by an independent group *t* test. Mann–Whitney *U* test was used to analyse the parameters that were not compatible with normal distribution between the OAT and control groups. Correlations between the parameters were tested by Spearman's rank correlation analysis. Spearman's rank correlation coefficient (*r*) was used to determine the strength and orientation of the relationship between two variables. Considering that some of the results due to a small sample size were borderline; therefore, retrospective sample size calculation according to Machin, Tan, and Tan (2009) and Hanley and McNeil (1982) was performed (Hanley & McNeil, 1982; Machin et al., 2009). Threshold values were determined by ROC analysis. $p < 0.05$ were considered statistically significant.

3 | RESULTS

A total of 75 infertile men with idiopathic OAT and 20 normozoospermic, 27 fertile and 5 proven fertile men as a control group were

enrolled in this study. There was a significant difference ($p = 0.0001$) between the age of the control (35.94 ± 5.25 years) and patient (31.89 ± 5.94 years) groups. Semen parameters and age of the both groups are presented in Table 1.

3.1 | Sperm DNA fragmentation index

The DFI of the control group was slightly, but not significantly, lower than of the OAT group ($p = 0.0554$) (Table 2). The difference between groups would be significant if the control group would have 96 and the patient group 113 subjects. Overall, DFI was negatively correlated with sperm concentration ($r = -0.266$, $p = 0.007$), total sperm count ($r = -0.299$, $p = 0.002$) and total progressive motile sperm count ($r = -0.253$, $p = 0.011$) (Table 3). The cut-off value of DFI between patient and control groups was calculated as 24.9% with 67.4% sensitivity and 57.4% specificity (area under the ROC curve (AUC) = 0.612, $p = 0.052$). If the control group would have consisted of 96 and the patient group 113 subjects, the AUC would be significant.

3.2 | Percentage of aniline blue positive spermatozoa

The percentage of aniline blue positive spermatozoa in the control group was significantly lower than in the OAT group ($p < 0.0001$) (Table 2) and was negatively correlated with total sperm count ($r = -0.683$, $p < 0.0001$), sperm concentration ($r = -0.658$, $p < 0.0001$), percentage of progressive motile spermatozoa ($r = -0.628$, $p < 0.0001$), total motility ($r = -0.639$, $p < 0.0001$), total progressive motile spermatozoa ($r = -0.682$, $p < 0.0001$), normal morphology ($r = -0.668$, $p < 0.0001$) and positively correlated with percentage of immotile spermatozoa ($r = 0.635$, $p < 0.0001$). No correlation was found between the percentage of aniline blue positive spermatozoa and DFI ($p = 0.1257$) (Table 3). The distinction between the control and the OAT patient group by means of ROC curve analysis resulted in a cut-off value for aniline blue positive spermatozoa of 26.92% with an 87.8% sensitivity and an 82.8% specificity (AUC = 0.911, $p < 0.001$) (Figure 3).

3.3 | Promoter methylation of MLH1 and MSH2

MLH1 and *MSH2* promoter methylation profile did not differ between the OAT and control groups ($p = 0.6522$ and $p = 0.0688$) (Table 2). While the ROC curve analysis for the *MLH1* promoter methylation was clearly not significant (AUC = 0.542, $p = 0.6664$), the ROC curve for *MSH2* promoter methylation resulted in a trend for differentiating between infertile and fertile/normozoospermic individuals with an AUC of 0.670 ($p = 0.067$). With a reasonably larger sample size of a total of 92 subjects, the AUC would have reached significance. The threshold value to distinguish between the controls and the OAT patients for *MSH2* promoter methylation was 0.3621% with a 69.6%

TABLE 1 Semen parameters and age of infertile and control groups

	Control			OAT			
Parameters	*n*	Median	Average Rank	*n*	Median	Average Rank	*p*[a]
Age	52	35.5	79.86	75	30	53.0	**0.0001**
Volume (ml)	52	3.0	54.36	75	3.0	66.28	0.3656
Sperm Concentration (million/ml)	52	34.5	86.50	75	4.0	38.00	**<0.0001**
Total Sperm Count (million/ejaculate)	52	123.0	86.37	75	15.0	38.09	**<0.0001**
Total Progressive Motile Sperm Count (million)	52	48.0	86.46	75	1.0	38.03	**<0.0001**
Progressive Motile Spermatozoa (A) %	52	47.0	85.66	75	10.0	38.71	**<0.0001**
NonProgressive Motile Spermatozoa (B) %	52	5.0	59.52	75	5.0	61.31	0.2076
Immotile Spermatozoa (C) %	52	48.0	27.37	75	85.0	89.27	**<0.0001**
Motility (A + B) %	52	52.0	85.63	75	15.0	38.73	**<0.0001**
Normal Morphology %	52	7.0	86.50	75	2.0	38.00	**<0.0001**

Note: Statistically significant results are indicated in bold font.

[a] Mann–Whitney test, A: Progressive Motile Spermatozoa, B: NonProgressive Motile Spermatozoa, C: Immotile Spermatozoa.

TABLE 2 Comparisons of promoter methylation of *MLH1* and *MSH2*, DFI and percentage of aniline blue positive spermatozoa between OAT and control groups

Parameters	*MLH1* PMR		*MSH2* PMR		DFI		Aniline blue positive spermatozoa %	
	Control	OAT	Control	OAT	Control	OAT	Control	OAT
n	18	21	17	23	46	54	49	65
Median	0.00481	0.00964	0.09902	0.613	21.68	25.88	14.98	41.8
95% CI for the median	0.0004962–0.02215	0.002473–0.01836	0.04397–0.3499	0.3227–1.0542	18.0266–23.8791	22.4501–30.5011	10.8515–18.0444	39.4292–49.8669
Interquartile range	0.0004600–0.02294	0.001690–0.01915	0.04209–0.3644	0.1464–1.3754	15.14–31.84	19.82–34.09	9.2975–22.4975	28.2925–55.6950
Hodges–Lehmann median difference	0.0005		0.3364		4.62		26.41	
%95 CI	−0.006873 to 0.009651		−0.005339 to 0.7587		−0.15 to 9.62		20.26–32.49	
Average rank of control group	19.111		165.882		44.478		30.674	
Average rank of OAT group	20.762		233.913		55.630		77.723	
Mann–Whitney U	173		129		965		278	
Two-tailed probability	0.6522		0.0688		0.0554		**<0.0001**	

Note: Statistically significant results are indicated in bold font.

Abbreviations: DFI, DNA fragmentation index; PMR, Percentage of methylated reference.

TABLE 3 Correlations among promoter methylation of *MLH1* and *MSH2*, DFI and percentage of aniline blue positive spermatozoa

Parameters		Sperm Concentration	Total Sperm Count	Total Progressive Motile Sperm Count	Progressive Motile Spermatozoa	Nonprogressive Motile Spermatozoa	Immotile Spermatozoa	Motility	Normal Morphology	Vitality	DFI	Aniline blue positive spermatozoa %
MLH1 PMR	*r*	0.024	0.018	−0.084	−0.129	−0.008	0.126	−0.126	0.021	−0.124	−0.145	0.401
	p	0.8841	0.9132	0.6102	0.4346	0.9597	0.4458	0.4458	0.8974	0.5911	0.4065	**0.0188**
	n	39	39	39	39	39	39	39	39	21	35	34
MSH2 PMR	r	−0.421	−0.408	−0.277	−0.201	−0.199	0.247	−0.247	−0.293	0.062	0.042	0.088
	p	**0.0068**	**0.009**	0.0831	0.2141	0.2176	0.1248	0.1248	0.0661	0.7773	0.8063	0.6154
	n	40	40	40	40	40	40	40	40	23	36	35
Aniline blue positive spermatozoa %	*r*	−0.658	−0.683	−0.682	−0.628	−0.108	0.635	−0.639	−0.668	−0.0206	0.164	
	p	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.251	**<0.0001**	**<0.0001**	**<0.0001**	0.8704	0.1257	
	n	114	114	114	114	114	114	114	114	65	88	
DFI	*r*	−0.266	−0.299	−0.253	−0.183	0.044	0.18	−0.185	−0.195	0.014		
	p	**0.007**	**0.002**	**0.011**	0.069	0.663	0.073	0.066	0.052	0.918		
	n	100	100	100	100	100	100	100	100	54		

Note: Statistically significant results are indicated in bold font.

Abbreviations: DFI, DNA fragmentation index; PMR, Percentage of methylated reference; r_s: Spearman's coefficient of rank correlation.

sensitivity and a 76.5% specificity. While no correlation was observed between *MLH1* and *MSH2* methylation and DFI ($p = 0.4065$ and $p = 0.8063$ respectively) (Table 3), *MLH1* promoter methylation was positively correlated with the percentage of aniline blue positive spermatozoa ($r = 0.401$, $p = 0.0188$). On the other hand, *MSH2* promoter methylation was not associated ($r = 0.0880$; $p = 0.6154$) with sperm chromatin condensation as evaluated by the aniline blue staining. However, *MSH2* promoter methylation was negatively correlated with sperm concentration and total sperm count ($r = -0.421$, $p = 0.0068$ and $r = 0.4408$, $p = 0.009$ respectively). In addition, the required sample size for a significant correlation between *MSH2* PMR and normal morphology and between *MSH2* PMR and total progressive motile sperm count was calculated as 89 and 100 respectively.

There was no effect of ageing on *MLH1* and *MSH2* promoter methylation and DFI ($p = 0.348$, $p = 0.4518$, $p = 0.9352$ respectively). The percentage of aniline blue positive spermatozoa, however, was significantly negatively correlated with age ($r = -0.294$, $p = 0.0015$) (Table 4). No significant difference with regard to the smoking habit was found between the OAT and control groups ($p = 0.357$). There were no differences between smokers and nonsmokers in terms of age, semen parameters, DFI, aniline blue positive spermatozoa, and the distribution of *MLH1* and *MSH2* methylation (not shown).

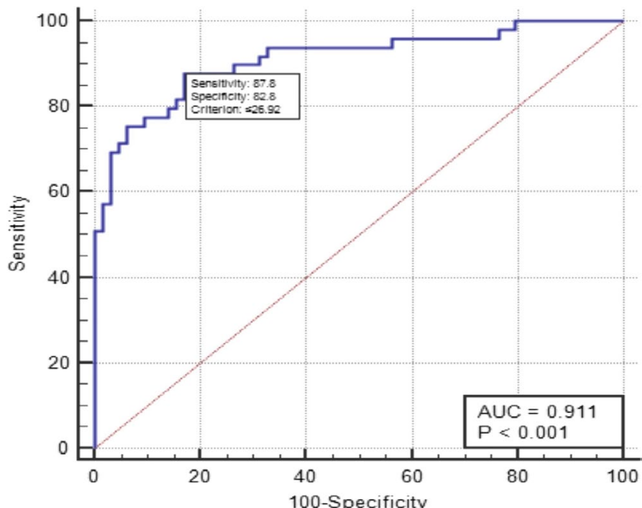

FIGURE 3 ROC curve fort the percentage of aniline blue positive spermatozoa

4 | DISCUSSION

In the present study, the association among SDF, sperm chromatin condensation and methylation status of *MLH1* and *MSH2* genes promoter have been investigated in infertile men with OAT. *MLH1* promoter methylation was found to be positively correlated with the positive aniline blue sperm percentage. Our result suggests an association between *MLH1* promoter methylation and increased histone percentage in spermatozoa. Increased histone percentage is known to cause the sperm chromatin not be packed properly and thus increase sensitivity to sperm DNA damage in spermatozoa (Zini & Libman, 2006). Additionally, the percentage of aniline blue positive spermatozoa was significantly lower in the normozoospermic/fertile controls than in the infertile men with OAT. Similarly, Pourmasumi et al. (2019) evaluated semen samples from 1,044 infertile patients with abnormal semen parameters and 342 normozoospermic controls and reported a lower percentage of aniline blue positive spermatozoa in the fertile men (Pourmasumi et al., 2019). Fertile males express sperm nuclear proteins, protamine P1 and protamine P2, in approximately the same proportions and any deviation of this ratio may cause abnormal displacements of histones with protamines (Hammoud, Liu, & Carrell, 2009). This deviation in P1/P2 ratio may lead to insufficient packaging of sperm DNA and infertility (Carrell, Emery, & Hammoud, 2008). Additionally, an increase in the percentage of histone-rich spermatozoa was also reported to be associated with early miscarriages (Jerre, Bungum, Evenson, & Giwercman, 2019).

In our study, the percentage of spermatozoa with aberrant chromatin condensation (aniline blue positive) was negatively correlated with total sperm count, sperm concentration, total and progressive motility, the total number of progressive motile sperm and normal sperm morphology. Moreover, a positive correlation was found between the percentage of spermatozoa with aberrant chromatin condensation and the percentage of immotile spermatozoa as well. The strong relationship among the changes in sperm chromatin condensation and sperm count, motility and normal morphology is also supported by other studies (Kim et al., 2013; Pourmasumi et al., 2019). Similarly with our results, Pourmasumi et al. (2019) found that percentage of aniline blue positive spermatozoa positively correlated with sperm count, progressive motility, normal morphology and negatively correlated with immotile spermatozoa in idiopathic infertile men (Pourmasumi et al., 2019).

TABLE 4 Effect of age on promoter methylation of *MLH1* and *MSH2*, DFI and percentage of aniline blue positive spermatozoa

Age	*MLH1* PMR	*MSH2* PMR	DFI	Aniline blue positive spermatozoa %
n	39	40	100	114
Spearman's coefficient of rank correlation (rho)	0.154	0.122	−0.00823	−0.294
Significance level (*p*)	0.3480	0.4518	0.9352	**0.0015**

Note: Statistically significant results are indicated in bold font.

Abbreviations: DFI, DNA fragmentation index; PMR, Percentage of methylated reference.

Likewise, Kim et al. (2013) reported a negative correlation between aniline blue sperm and normal morphology (Kim et al., 2013). Transition of histones with protamines occurs in the prolonged spermatid stage of spermiogenesis after the meiosis is completed wholly. These spermatids undergo a process that affects the motility and the fertility of mature spermatozoa during the replacement of histones by protamines (Garcia-Rodriguez, Gosalvez, Agarwal, Roy, & Johnston, 2018). Therefore, errors in during the transition of protamines are suggested to cause an increase in the histone content and may have a negative effect on semen parameters (Carrell et al., 2008).

Our results regarding *MLH1* and *MSH2* promoter methylation profile did not reveal a difference between the OAT and control groups. However, a recent study analysed the promoter methylation status of *MLH1* and *MSH2* genes in 10 severely oligozoospermic and 29 normozoospermic men. The previous study revealed that *MLH1* promoter was significantly more methylated in men with oligozoospermia compared with normozoospermic men. However, no significant difference in *MSH2* promoter methylation was reported in severely oligozoospermic men compared to normozoospermic controls (Gunes, Agarwal, et al., 2018). In the present study, *MSH2* promoter methylation was slightly, but not significantly, higher in 17 infertile men with OAT than in 23 fertile/normozoospermic men. In the previous study, promoter methylations of *MLH1* and *MSH2* were analysed by a nonquantitative method, methylation-specific PCR (MSP). However, the methylation assessment has been performed using a semiquantitative PCR in the present study. Also, in the present study, a short proximal sequence localised near to the transcription initiation site of the promoter, which has a critical impact on the loss of mRNA expression (Deng, Chen, Hong, Chae, & Kim, 1999), was selected to assess the methylation status of *MLH1* gene. Besides, the use of different techniques and promoter sequences, a slight difference in the number and the selection criteria of patient and control groups and ethnic variations might be considered as underlying causes of the difference between two studies. In the previous study, a negative association between *MLH1* and *MSH2* promoter methylations and sperm concentration have also been found. Similarly, *MSH2* promoter methylation was found to be negatively correlated with sperm concentration and total sperm count in this study. Although the studies reported that the fertility of *Msh2* knockout male mice was not affected, an increase in apoptotic germ cells and a decrease in the epididymal sperm count were observed in these animal models (Mukherjee et al., 2010; Paul et al., 2007). There was also a nonsignificant association among the *MSH2* methylation rate, normal morphology and total progressive motile sperm count.

Since this is a pilot study, not all statistical calculations were significant, but only showed a trend. To the best of our knowledge, our study is the first to investigate the relationship between *MLH1* and *MSH2* promoter methylation and DFI in male infertility. In the study, no correlation was found between DFI and promoter methylation of *MLH1* or *MSH2*.

Previous studies have demonstrated that spermatozoa of infertile men have higher SDF than those of fertile men (Giwercman et al., 2010; Ramos-Ibeas et al., 2014). Although our data in this pilot study failed to achieve threshold value for the significance, the difference in the DFI between the OAT and control groups shows a clear tendency to significance. Yet, sample size calculations resulted in reasonably higher sample sizes to obtain significance. DFI was also found to be negatively correlated with sperm concentration, total sperm count and total progressive motile sperm count. Corresponding to our findings, Sharma et al. (2010) analysed the DFI using the TUNEL method with flow cytometry and reported a lower SDF in 25 normozoospermic/fertile men as compared to 194 infertile men with known and unknown aetiologies of infertility (Sharma et al., 2010). In a follow-up study of the same group, a cut-off of 16.8% for DFI has been calculated with high specificity and high positive predictive value (Sharma, Ahmad, Esteves, & Agarwal, 2016). Using immunofluorescence microscopy, Kabartan et al. (2019) reported a higher DFI in the idiopathic infertile men with moderate and severe oligozoospermia compared to fertile/normozoospermic controls and found a cut-off value of 17% for DFI. Researchers also reported negative associations among DFI, sperm concentration, motility and normal morphology similar to our results (Kabartan et al., 2019). Several studies found negative correlations between SDF and semen characteristics assessed by sperm concentration, motility, morphology and vitality. Yang, Li, Jin, Guo, and Sun (2019) evaluated 2,622 assisted reproductive technology (ART) cycles and found an inverse association between SDF and sperm concentration, progressive motility and normal morphology among infertile men concordantly with our findings (Yang et al., 2019). In another study, among 272 infertile men who sought for treatment SDF evaluated by TUNEL was found to be negatively correlated with sperm concentration, progressive motility but not correlated with normal morphology (Amor, Shelko, Hamad, Zeyad, & Hammadeh, 2019). Also some studies showed a strong relationship between vitality and SDF (Aghazarian, Huf, Pfluger, & Klatte, 2019,2020). The differences in the number and features of selected patient groups may have caused this confliction between the results.

In our study, the smoking status of participants was not found to be associated with infertility. Smoking did not have an impact on the semen parameters, DFI, poor chromatin condensation and promoter methylation of *MLH1* and *MSH2*. There are conflicting results about the effects of smoking on male infertility and semen parameters (Gunes, Metin Mahmutoglu, Arslan, & Henkel, 2018). Hamad, Shelko, Kartarius, Montenarh, and Hammadeh (2014) reported that sperm count, motility and vitality decreased significantly in smokers (Hamad et al., 2014). The histone/protamine ratio in sperm samples from smokers was significantly higher than nonsmoker group. In another study conducted in infertile smokers and nonsmokers, significant differences between the two groups in terms of sperm count, progressive motility, normal morphology and sperm chromatin condensation were observed (Mostafa et al., 2018). Studies have shown that the effect of smoking on

conventional semen parameters and sperm function tests may be related to the frequency and duration of smoking (Collodel et al., 2010; Cui, Jing, Wu, Wang, & Li, 2016; Mostafa et al., 2018). Collodel et al. (2010) reported that sperm concentration varied in infertile smoker group depending on the smoking frequency. They also found that smoking did not have a critical effect on semen parameters in idiopathic infertile men (Collodel et al., 2010), a result that is corroborated by our findings. Cui et al. (2016) reported that sperm motility decreased depending on the number of cigarettes smoked, while sperm concentration, vitality and motility decreased in long-term smokers in their study group consisting of 841 smokers and 287 nonsmokers. The rate of DNA fragmentation was also reported to increase in the smoker group (Cui et al., 2016). Smoking has been shown to alter DNA methylation profile in both somatic cells and spermatozoa (Jenkins et al., 2017; Word et al., 2013). In our study, statistical analysis was assessed only as smokers and nonsmokers. We did not analyse the duration and the frequency of smoking and also the probability of smoking cessation in the infertile group before their fertility therapy. Therefore, these limitations may explain the lack of correlation with smoking and semen parameters, sperm methylation rates, DNA fragmentation and poor chromatin condensation in the study.

Studies have shown that ageing might lead to alterations in reproductive capacities in men (Gunes, Hekim, Arslan, & Asci, 2016). In our study, ageing was found to be associated with a decrease of the percentage of aniline blue positive spermatozoa and there was no relation of ageing on *MLH1* and *MSH2* promoter methylation and DFI. The small size of our groups and a higher average age of our control group might be our limitations in assessment of the possible role of age.

In conclusion, our study is the first study investigating the effects of epigenetic changes of *MLH1* and *MSH2* genes on SDF and chromatin condensation of sperm DNA in infertile men with OAT. Our data is suggesting promoter methylation profile of *MLH1* and *MSH2* genes may play a role on sperm DNA packaging and conventional semen parameters respectively. However, further studies with larger patient and control groups are necessary to validate our results.

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DATA AVAILABILITY STATEMENT

The data that support the finding of this study are available from the corresponding author [SG], upon reasonable request.

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