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Sperm Epigenome Characteristics Related to Folic Acid: A Randomized Controlled Trial

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ABSTRACT

Aims: Folic acid is a prominent intermediary in transferring the methyl groups in DNA synthesis, stability, and integrity.
Material and methods: In this study, we divided one hundred subjects into three groups as the controls, as well as two randomly intervention groups such as first treatment (vitamin E 400 IU and selenium 200 µg per day) and second treatment (selenium 200 µg, folic acid 5 mg daily, & vitamin E 400 IU). The DNA methylation percentages, the sperm chromatin/DNA quality, and DNMTs expressions profiles were evaluated before and after each intervention using ELISA, cytochemical tests, and qRT-PCR, respectively.
Results: Sperm parameters were considerably enhanced in comparison to the three-drug group. Moreover, sperm decondensation and fragmentation of sperm DNA in the three-drug group were significantly lower than in the two-drug group. The global methylation of sperm DNA in the three-drug group was significantly reduced compared to pre-intervention.
Conclusion: The increased global methylation has an association with the declined sperm parameters as well as chromatin integrity. The expression of DNMTs was not affected by the intervention. The utilization of folic¬ acid could ameliorate sperm quality and epigenetic features in terms of balancing the status of global methylation, increasing DNA integrity and chromatin, and also improving the sperm parameters.

Keywords: Methyltransferases; Gene Expression; Spermatozoa, Global Methylation; Chromatin; Folic Acid.

Abbreviations:

DNMTs: DNA Methyltransferases
qRT-PCR: Quantitative real time polymerase chain reactions.
ELISA: Enzyme-Linked Immunosorbent Assay
TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling.
SAM: S-Adenosyl-L-Methionine

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Introduction

Infertility is the commonest health problem that affects an equal proportion of men and women [1]. In sperm chromatin improvement, already supplements have been used as a complementary in spermatogenesis in men who have a sperm quality defect [2]. For example, the role of vitamin E with its antioxidant effects on the development of normal spermatogenesis is important in normal sperm function [3]. Improving the performance of the sperm and accessory glands makes it reasonable to use this vitamin or other antioxidants such as selenium [4]. The antioxidant activity of glutathione peroxidase has a strong dependence on the presence of selenium in the enzymes' active site. This enzyme is effective in preventing sperm membrane lipid peroxidation and ultimately affects sperm function by improving sperm motility [5]. From another glance, in male infertility epigenetic changes can play a part in modulating and maintaining males' germ cells. Therefore, epigenetic disorders are associated with disorders of regulation of DNA methylation in sperm cells [6,7]. Remarkable modifications in the methylation levels of the sperm specimens in infertile men lead to abortion in about 20% of the cases; the result that confirms this issue is that epigenetic disorders can be a reason for the increased occurrence of abortion in humans [8]. Sperms with proper quality are likely to have a stable genetic and epigenetic status and provide sufficient epigenetic information for the growth and development of the embryo [9]. One of the families of DNA methyl-transferases (DNMTs) catalyzes DNA methylation. DNMT1 is one of the most abundant DNMTs in somatic cells and has a functional priority in semi methylated DNA strand. Therefore, it is thought of as the main enzyme responsible for copying and maintaining the pattern of methylation after DNA replication [10]. Moreover, DNMT3B and DNMT3A are both excellent candidates for the formation of new methylation patterns [11]. One of the important intermediates in the transfer of methyl groups is folic acid, which contributes to DNA synthesis, stability, integrity as the main methyl donor in methylation and macromolecule production [12]. Folic acid converts to 5-methyltetrahydrofolate, which acts in the transformation of homocysteine to methionine. Then, methionine is metabolized to s-adenosyl-methionine, which is one of the methyl donors in methylation and macromolecular synthesis [13]. In a study of the impact of zinc sulfate as well as folic acid on sperm quality, researchers evaluated the content of protamine and acrosome integrity after varicocelectomy, both supplements together as oxidizing free radicals had the highest improvement on the variables [14]. In the males who showed higher folate intake, the decreased frequency of different kinds of sperm aneuploidy such as Disomy 21 and X was reported [15]. In an animal model, receiving a folic acid-free diet for 36 weeks resulted in irreversible changes, suggesting that the long-term shortage of diet caused enduring epigenetic and genetic modifications in the liver, which could not be compensated from modification of the natural diet [16]. Given that folic acid consumption in spermatogenesis disorders is prescribed for antioxidant purposes, administration of this supplement has not been investigated with the approach to improve the status of sperm DNA methylation in cases suffering from spermatogenesis disorders. Therefore, the present research intended to assess improvement of the sperm parameters as well as chromatin followed by the balance of methylation levels of the sperm genome by improving the global DNA methylation and transcription of methyltransferase genes like DNMT3A, DNMT3B, and DNMT1 after use of folic acid in men with severe spermatogenesis dysfunction.

Materials and methods

Selecting the Patients

Based on our randomized control trial study, 100 men who have been referred to the andrology laboratory at Yazd Research and Clinical Center for Infertility (Yazd, Iran) were our population. This investigation was registered in Iran Clinical Trials registry with ID number IRCT201507285261N2. The infertile men who were defined as oligoasthenoteratozoospermia (OAT) (n=50) according to WHO 2010 criteria (17,18) and fertile men who had normal semen parameters were considered as the controls (n=50) were included. We divided the cases randomly into 2 groups. Twenty-five OAT infertile men receiving three drugs such as vitamin E (400 IU), and folic acid (5 mg) and selenium (200 µg) daily were administered and for 25 OAT infertile men undergoing two drugs were administered selenium (200 µg) and vitamin E (400 IU) daily. The prescription was done for three months by the urologist. Patients' inclusion criteria were age 25-40, no use of phenytoin and phenobarbital, sperm concentrations 7 to 14 million ml-1, normal morphology <4%, and total motility <40%. The informed consent form was signed by each participant and the use of all samples was approved by the legal standards in the Ethics Committee of Shahid Sadoughi Hospital with ethical number IR.SSU.MEDICINE.REC.1396.163 that it conforms to the provisions of the Declaration of Helsinki (as revised in Tokyo 2004). All participants signed the informed consent for this research.

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Sperm parameters

According to the research design, we collected the semen specimens through masturbation following the 2-5 days of sexual abstinence. Then, all the samples underwent liquefaction and were assessed in terms of sperm concentration, morphology, and motility, based on the World Health Organization [17] criteria. In order to assess the morphological abnormality, we examined not less than 200 sperms per slide [19] and used the Makler counting chamber to assess the sperm concentration. Also, in order to assess its' viability, we performed eosin-nigrosin staining and mixed 10 µl of the procured sperm with 10 µl of eosin-nigrosin stain onto a glass slid. After that the light microscope was used to assay it for determining the live sperm percentage. Each analysis was done by one professional laboratory technician who was blinded to this research.

Sperm DNA integrity tests

Sperm DNA integrity was assessed by tests like TUNEL assay for sperm DNA fragmentation detection, aniline blue staining to detect the excess histones in chromatin, and toluidine blue for the sperm chromatin de-condensation status [20].

TUNEL assay

In this step, TUNEL assay with In-Situ Cell Death Detection Kit (Roche Diagnostics GmbH; Mannheim: Germany) was utilized to determine the percentage of apoptotic spermatozoa in all samples with the use of the fluorescent microscopy that normal DNA detected as the Light green and the damaged DNA was observed as the Bright green[21].

Aniline blue and toluidine blue staining

Like our earlier research, aniline blue staining was carried out [22] and then we fixed the air-dried smears in 3% buffered glutaraldehyde in 0.2 m phosphate buffer at a pH of 7.2 for thirty minutes at room temperature. After that, all smears were stained with 5% aqueous Aniline blue stain in 4% acetic acid at a pH of 3.5 for five minutes. Results showed the un-stained or pale blue stained spermatozoa (Aniline blue$^-$) showed to be normal and dark blue stained spermatozoa (Aniline blue$^+$) was considered as ab-normal.

Also, we performed Toluidine blue staining according to the directions in our earlier research [23]. We regarded the pale-blue sperm cells as the normal ones (Toluidine blue$^-$) and the dark-blue or violet and purple spermatozoa were grouped into ab-normal cells (Toluidine blue$^+$). It is notable that for the two experiments, we examined not less than 200 spermatozoa in all slides, and thus normal as well as abnormal spermatozoa were presented as a percentage.

Preparing the sperm via discontinuous density gradient

Sperm preparation for all semen samples was performed by the standard-gradient isolation conducted by the WHO [17,18] in order to isolate and purification of sperm samples. We procured a density gradient medium (*In vitro*, Denmark) in a 15 mL test-tube via layering 1 mL of 40% (v/v) density gradient medium on one mL of 80% (v/v) density-gradient medium. Approximately, 1 mL of each specimen, which has been completely mixed, was put on the top of the density-gradient media. Afterward, centrifugation was done at 0.4 RCF for fifteen min. After washings, samples were collected in sterile vessels, stored at -80 ºC for further analysis.

Global DNA methylation analysis

According to the research design, 5-mC DNA ELISA-Kit Catalog Nos was utilized to conduct the global DNA methylation analysis. D5326 (ZYMO RESEARCH CORP., USA). Then, 100 ng of the isolated denatured (single-stranded) sperm DNA from the chosen specimens was dissolved with a 5-mC Coating Buffer and incubation was done at 37 ºC for one hour. Following the removal and washing of all wells in triplicate with 200 µl of 5-mC ELISA Buffer, incubation of the specimens was done at 37 ºC for thirty minutes. Then, anti-5-methyl-cytosine, as well as secondary antibody in 5-mC ELISA Buffer, were poured and incubation was done at 37 ºC for one hour. Following the wells' washing, we added the HRP Developer into all wells and allowed the color for developing for twenty minutes at room temperature. In this step, we measured absorbance at 450 nm with the use of an ELISA plate-reader (star fax-2100; Awareness Technology: USA). Then, the standard curve was created with the use of the positive as well as negative internal controls on a similar plate through various dilutions. After that, the negative control was unmethylated DNA consisting of 0% of 5 methyl-cytosine and positive internal controls were methylated DNA consisting of 5% of 5-methyl-cytosine. Finally, global methylation of all samples was performed two times and the resulting average value was obtained.

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cDNA synthesis and RNA extraction

The RNeasy Plus Universal Mini Kit (Qiagen) was utilized to extract Total RNA from all of the specimens according to the company's directions. The RNA concentration was determined by a NanoDrop spectrophotometry (Thermo scientific, 2000c). The adjusted concentration of 100 ng/µl of purified total RNAs was utilized for cDNA synthesis using RevertAid First-Strand cDNA Synthesis kit; Thermo Scientific based on the company's guide

Real Time Polymerase *Chain Reaction*

We applied the quantitative RT-PCR for evaluation of the gene expression with the use of the Fast SYBR®Green Master Mix, Applied Biosystems. All evaluated genes (*DNMT1*, *DNMT3A*, and *DNMT3B*) and the internal control gene beta-2-microglobulin (*B2M*) primers were presented in table 1.

Table 1: Gene's primer sequence, sequence amplified and product size

Gene	Primer Sequence (5'-3')	Sequence Amplified	Product Size
DNMT1	F: TGGACGACCCTGACCTCAAAT R: GCTTACAGTACACACTGAAGCA	NM_001318731.1	168 bp
DNMT3A	F: TATTGATGAGCGCACAAGAGAG R: GGGTGTTCCAGGGTAACATTGAG	NM_001320893.1	111 bp
DNMT3B	F: GGCAAGTTCTCCGAGGTCTCTG R: TGGTACATGGCTTTTCGATAGGA	NM_001207056.1	113 bp
B2M	F: AGATGAGTATGCCTGCCGTG R: TGCGGCATCTTCAAACCTC	NM_004048.2	106 bp

DNMT; DNA Methyltransferase
B2M; Beta-2-Microglobulin

As seen, PCR mixture in all wells is 12.5 µl of SYBR®Green Master Mix, 8.5 µl dH2O, 2 µl of single-strand cDNA as well as 1 µl of each reverse as well as forward primers (10 pmol µl-1) in the resulting reaction volume equal to 25 µl. The thermal cycling plan involved the initial incubation at 95 ºC for twenty seconds, 40 cycles of 95 ºC for three seconds, and 58 ºC for thirty seconds. The resulting 58-95 ºC step was also employed for forming the melt curve. Product specificity was confirmed by curve analysis. All steps of the four genes expression process for samples were performed duplicated. In order to verify the analyses and generation of the given product, we loaded the PCR final products on the agarose gel 2%. The $2^{-\Delta CT}$ was computed for representing the genes' expression level after normalizing to that of *B2M*.

Statistical Analyses

In this stage, we utilized the nonparametric Mann-Whitney U test for analyzing differences in the sperm parameters, chromatin, global DNA methylation as well as mRNA expression between control and following the treatment gropus. Moreover, Wilcoxon signed-rank test was employed to compare before and after treatment. In addition, we applied Spearman's test for computing the correlation. Finally, PASW Statistics 18 (SPSS) was used for analyses and regarded P-value less than 0.05 to be significant statistically.

Results

In this RCT study, the demographic variables of patients in two groups of treatment with the drug were similar in terms of age (27±2 vs. 28±2), duration of infertility (4.5±0.6 vs. 4±0.5), and BMI (22.6±1.7 vs. 23.4±2) respectively. The results of comparison variables in control and treatment groups are listed in table 2, regarding sperm parameters, DNA integrity, and sperm chromatin tests results, global DNA methylation as well as DNMTs expression. Sperm concentration in both treatment groups exhibited a considerable enhancement in comparison to the pretreatment (P = 0.001 vs. P = 0.035), while it was still significantly lower in comparison with the controls. After three-drug treatment, normal morphology and progressive motility enhanced remarkably compared to pretreatment. Such improvement did not occur in the two-drug group. Sperm viability showed a significant increase after the three-drug treatment period consistently comparable with the controls (P=0.02 vs. P=0.07), while this improvement as a result of treatment was not observed in the two-drug treatment group (Table 2).

Based on the results of the Aniline blue+ results, sperm chromatin excess of histone showed a significant reduction after the three-drug treatment compared with the pretreatment and unlike the two-drug treatment group. However, we observed a higher level of dark blue stained spermatozoa in the three-drug treatment group as compared to the controls. A high level of chromatin condensation was obtained after both three-drug and two-drug treatment. The rate of fragmented DNA sperms with the TUNEL+ test decreased significantly in both treatment groups (P = 0.001), but it was still considerably greater than the controls as seen in table 2.

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Table 2: Comparison of sperm parameters, chromatin/DNA integrity between study groups

Parameter	Three-drug Treatment Group (n=25)			Two-drug Treatment Group (n=25)			Control Group (n=50)		
	Before	After	P^*	Before	After	P^*		P^{**}	P^{***}
Concentration (×106/ml)	10 (9, 12)	30 (23, 39)	0.001[a]	13 (11, 13.5)	20 (12, 24)	0.035[a]	138 (50, 150)	<0.001[a]	<0.001[a]
Progressive Motility (%)	3.5 (2, 20.7)	45 (17, 44)	0.01[a]	25 (15, 30)	33 (16, 38)	0.47	46 (42, 51.9)	0.08	0.002[a]
Normal Morphology (%)	1 (0.5, 2.1)	3 (2.1, 4.9)	0.003[a]	1 (0.3, 1.6)	1 (0.36, 1.5)	0.85	15 (12, 17.7)	<0.001[a]	<0.001[a]
Viability (%)	48 (38, 60)	60 (54.5, 67)	0.02[a]	55 (50, 62)	60 (50, 63)	0.1	70 (64, 74)	0.07	0.008[a]
Aniline Blue+ (%)	40 (37, 8.7)	35 (30.7, 39.6)	0.002[a]	45 (41, 47.5)	42 (37, 43.9)	0.16	25 (24.8, 30)	0.02[a]	<0.001[a]
Toluidine Blue + (%)	62.5 (57, 66)	60 (60.2, 63)	0.001[a]	63 (63, 70)	65 (63.7, 67)	0.001[a]	40 (37 , 42.7)	<0.001[a]	<0.001[a]
TUNEL+ (%)	18 (16, 19.8)	12 (10.4, 13.6)	0.001[a]	19 (17.5, 21)	16 (13, 16.6)	0.001[a]	10 (8, 10)	0.004[a]	<0.001[a]

Median (lower bound, upper bound) was measured
*Wilcoxon Signed-Rank Test
**Mann-Whitney U Test, comparing control and after three- drug treatment
***Mann-Whitney U Test, comparing control and after two- drug treatment
[a] Statistical Significant

The level of expression of DNMT3A, DNMT3B as well as DNMT1 genes after treatment in the two- and the three-drug group showed no significant difference compared with pretreatment. Global methylation decreased significantly after the three-drug treatment (P=0.002), while still a considerable difference was observed in the level of methylation in the control group. However, sperm global methylation showed no significant difference in the two-drug treatment (Table 3).

Table 3: The comparison of *DNMTs* expression and global DNA methylation between study groups.

Parameter	Three-drug Treatment Group (n=25)			Two-drug Treatment Group (n=25)			Control Group (n=50)		
	Before	After	P*	Before	After	P*		P**	P***
DNMT1 Expression	0.038 (0.02, 0.11)	0.03 (0.02, 0.25)	0.46	0.06 (0.03, 0.09)	0.04 (0.02,0.09)	0.6	0.0052 (0.16, 0.53)	0.16	0.21
DNMT3A Expression	0.049 (0.01, 0.13)	0.06 (0.02, 0.26)	0.77	0.06 (0.02, 0.21)	0.05 (0.02, 0.08)	0.19	0.004 (0.013, 0.19)	0.59	0.71
DNMT3B Expression	0.11 (0.04, 0.17)	0.1 (0.05, 0.21)	0.95	0.21 (0.11, 0.86)	0.12 (0.05, 0.19)	0.17	0.016 (0.003, 0.08)	0.27	0.42
Global DNA	3.1 (2.9, 3.8)	2.6 (2.3, 3.2)	0.002[a]	3.2 (2.7, 3.6)	3.2 (2.8, 3.6)	0.64	2 (1.7, 2.55)	0.01	<0.001[a]

Median (lower bound, upper bound) was measured
*Wilcoxon signed-rank test
**Mann-whitney U test, comparing control and after three- drug treatment
***Mann-whitney U test, comparing control and after two- drug treatment
[a] Statistical Significant

The sperm concentration was inversely correlated with *DNMT3B* transcription. No significant correlation was observed between progressive sperm motility, normal morphology, and sperm viability with expression levels of the *DNMTs* genes. The TUNEL$^+$ results were directly related to the *DNMT3A* and *DNMT3B* transcription. Global methylation level showed a negative correlation to the sperms' concentration, normal morphology as well as progressive motility (P<0.001). But, as reported by Table 4, global methylation was positively related to the results of TUNEL$^+$, Aniline blue$^+$ and Toluidine blue$^+$ results (P < 0.001).

Table 4: Correlation between sperm quality, global DNA methylation and methyltransferase expression.

Parameter	Correlations			
	DNMT1 Expression	*DNMTA3* Expression	*DNMTB3* Expression	Global DNA Methylation
Concentration (×106/ml)	-0.023 (0.829)	-0.196 (0.065)	-0.222 (0.035)[a]	-0.418 (0.000)[a]
Progressive Motility (%)	-0.158 (0.137)	-0.204 (0.054)	-0.171 (0.106)	-0.378 (0.000)[a]
Normal Morphology (%)	0.072 (0.497)	-0.053 (0.618)	-0.115 (0.279)	-0.384 (0.000)[a]
Viability (%)	0.043 (0.688)	-0.204 (0.054)	-0.161 (0.129)	-0.139 (0.192)
Global DNA Methylation (%)	0.203 (0.055)	0.207 (0.049)[a]	0.146 (0.168)	-
Aniline Blue+ Results (%)	-0.026 (0.804)	0.149 (0.162)	0.179 (0.091)	0.378 (0.000)[a]
Toluidine Blue+ Results (%)	-0.107 (0.316)	0.045 (0.676)	0.1 (0.347)	0.386 (0.000)[a]
TUNEL+ Results (%)	0.116 (0.278)	0.282 (0.007)[a]	0.303 (0.004)[a]	0.479 (0.000)[a]

The Spearman correlation test was done.
Data was shown as R (P-Value).
[a] Statistical Significant

Discussion

In our study, for the first time, folic acid was administered with its epigenetic application regarding the expression of methyltransferases, global DNA methylation, and chromatin in human sperm. We divided the number of 50 infertile OAT patients randomly into 2 treatment groups. No complications were reported at the time of taking the drugs. Another research reported by Wong et al. showed folate concentration in the blood and seminal fluid was within the normal range and similar in the fertile and infertile groups before the onset of drug intervention [24]. For this reason, we did not examine folate concentrations in this study.

Treatment groups and quality of sperm, sperm chromatin and DNA integrity

Significant improvements were seen in the three-drug treatment group after treatment with respect to sperm parameters. Sperm parameters such as concentration, normal morphology, sperm viability as well as progressive motility were considerably enhanced in the three-drug treatment. In line with this finding, a study investigated the effect of 5 mg folic acid as well as 66 mg zinc sulfate on the semen parameters in the infertile males for 26 weeks, reporting a significant increase in the total number of sperms [24]. The results of the Toluidine blue and TUNEL tests showed significant improvement in both treatment groups compared with pretreatment. In addition, the Aniline blue+ results showed a remarkable decrease in the amount of excess histone in the three-drug treatment group in comparison to the pretreatment. In this regard, Amar et al. also found that treatment with antioxidants and B vitamins

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including folic acid, needed for one-carbon cycles in metabolism, improved sperm chromatin more than treatment with only antioxidants by decreasing sperm DNA fragmentation level and Aniline blue+ results [25]. Moreover, in the epigenetic role, folate can lead to fertility through its function in DNA synthesis because of its important role in the biosynthesis of purine, pyrimidine, and certain amino acids [26].

Treatment groups, methyltransferases expressions, and global DNA methylation

Examining the methyltransferase transcripts showed that transcripts had an insignificant change after treatment. Put differently, global methylation of the sperm DNA in the treatment group of three drugs including folic acid was significantly reduced compared with pretreatment. But, this did not occur in the two-drug treatment group. Since there is no study on the administration of folic acid by examining epigenetic variables, it can be discussed in terms of folate poverty. Epigenetic changes in cancer caused by folate deficiency in rats significantly reduced S-adenosyl-L-methionine (SAM) concentrations in the liver tested in 9 weeks. The level of mRNA and protein of the DNMTs changed in response to the methyl deficiency after 9 months [27]. Global hypomethylation has been introduced as one of the popular epigenetic incidences in the course of the initial phases of cancer, especially in repetitive elements and oncogenes. The interesting outcome was that besides hypomethylation of DNA, deficiencies in methyl and folate may cause regional hypermethylation [28], very similar to disorders of methylation, hypermethylated and hypomethylated regions of the sperm genome in infertile men [29,30].

Association between methyltransferase expression and sperm quality

Out of the sperm parameters, just sperm concentration is considerably related to DNMT3B mRNA. Moreover, the concentration of the sperms declined by enhancing in this transcript. Even though no research has not examined methyltransferase in conjunction with the sperm analysis, it is possible that there is a correlation between the presence of poly-morphism in DNMT1 and oligoasthenospermia [31]. Investigating the mRNA contents of the imprinted genes in the epigenetic modulation in the male sub-fertility showed the presence of DNMT3A in the greater amount in the poor sperm motilities, with no considerable association with the sperms' concentrations. The obtained results show the failure of abnormal sperm for gaining a correct methylation pattern [32].

Correlation of methyltransferase expression, sperm chromatin, and DNA integrity

TUNEL+ outputs showed a remarkable association to the levels of DNMT3 B mRNA and DNMT3A mRNA so that as the transcripts enhanced, fragmentation of the sperm DNA elevated. Even though the mentioned parameters and mechanisms could not be justified in earlier research, we may address a general correlation of chromatin integrity to the variables in the epigenetic regulation. Therefore, regarding the apoptotic cells of the sperms, a number of investigations revealed that early responses to oxidative stress (OS) can be caused by the increased DNA methyltransferase expression [33]. According to the outputs of the correlation of expression of both epigenetic genes *histone deacetylase 1 (HDAC1)*, *DNMT3A* as well as chromatin integrity, in case of very high expression of *HDAC1,* our assumption was that histones were not replaced by protamine, causing the immature chromatin structure sperm with the less compacted DNA. Hence, imperfect nuclear density as a result of greater expression of *DNMT3A* as well as *HDAC1* could disturb the sperms' overall maturity [33].

Correlation of global methylation and quality of sperms

Results showed the considerable association of global methylation to the concentration, non-motility, normal morphology as well as progressive motility. Moreover, there was a correlation between the greater global methylation and lower sperm parameters. However, with regard to the investigation of the oligoasthenospermic patients, about 45% with <10 million sperm in each mL exhibited defective methylation of MEST or H19 [29]. Nonetheless, Olszewska et al. (2017) addressed the examination of global methylation level in the sperms consisting of the chromosomal aneuploidy as well as normospermia with the use of immuno-fluorescence and chromatography did not show any relationship between the global methylation and quality of the sperms [32]. The difference may be caused by an uncertain environment, lifestyle, environmental interaction of the genes, or other un-measured parameters influencing the activities as well as expression of DNA methyltransferases and also availability of the methyl groups.

Correlation of global methylation, sperm chromatin, and DNA integrity

With regard to the outputs, global methylation had a significant correlation to each result of chromatin integrity tests like TUNEL, Toluidine blue as well as Aniline blue. The increases in the global methylation in the cases enhanced the chromatin and sperm DNA disintegrity. According to the outputs of greater

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fragmentation of DNA in the infertile men with greater global methylation of the sperm DNA, this status was accompanied by declining semen quality, chromatin integrity as well as sperm DNA [34]. Moreover, analyzing the association of global methylation level with chromatin-deproteinization state with the use of the Aniline blue-test referred to the positive relationship between both parameters [32]. Put differently, we observed the tendencies for the intrinsic apoptotic cascade in conjunction with the state of the global methylation of sperm DNA [35]. Some researchers have shown that a lower concentration of homocysteine, as well as folate, would disrupt the methylation cycle. In case of occurring the uracil misincorporation with the folate deficiency, we would see instability in the chromosome. Finally, supplying the methyl groups would be of high importance for DNA protection against exposure to the free radicals [36].

Clinical applications and conclusion

A major change was made in the structures of the sperm chromatin and at the increased levels in the nuclear organization immediately following the fertilization that has been considered to be prominent to initiate and modulate the activities of the paternal gene in the course of the initial phases of the embryo development. We did not address ART outcomes for the evaluation of the impacts of the amelioration of the epigenetic status accompanied by expressing methyltransferase as well as global methylation on the successfulness of IVF treatment. It can be expressed that the declined global methylation of sperm DNA in drug treatment like folic acid is capable of affecting ART outcomes because of the sperms' population with the greater epigenetic health. Moreover, there was a correlation between the global methylation of sperms and embryo quality or fertilization [35]. In conclusion can be said, during sperm abnormalities, the sperm is eliminated from the process of gene expression by increasing methylation. As long as, sufficient methyl is available, gene expression resumes the equilibrium in the genome and changes from the state of hypermethylated to the hypomethylated. This leads to improvement of sperm condition in terms of parameters and chromatin. Sperm improvement by the balanced levels in the global methylation in the 3-drug treatment for ameliorating epigenetics, quality of sperms, and chromatin in the cases is probably due to the effects of folic acid.

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Conflict of interests

It is declared that they do not have any conflicts of interest.

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