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Global sperm DNA methylation and intra cytoplasmic sperm injection outcomes

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Abstract

Routine semen analysis is a poor predictor of pregnancy rates after intra-cytoplasmic sperm injection (ICSI). There is an assumption that embryos' quality and fertilization rates could be influenced by epigenetic factors. We aimed at comparing global sperm DNA methylation level (GSDML) between normal and abnormal semen, investigating its relationship with sperm parameters and assessing its effect on ICSI outcomes; fertilization, good embryo and pregnancy rates. Ejaculates were obtained from 104 infertile and 60 fertile males undergoing ICSI at Faculty of Medicine, Alexandria, Egypt. Data was analyzed using IBM SPSS software package, 20. Inter-group differences in semen parameters were assessed by t-test. GSDML, measured by ELISA, showed significant positive correlation with sperm count, concentration and motility. It correlated positively but insignificantly with morphology and fertilization rate. High levels were significantly associated with embryos having good quality and positive pregnancy rates. GSDML could predict good embryo rate and pregnancy occurrence after ICSI. *(Afr J Reprod Health 2020; 24[4]: 94-100).*

Keywords: Sperm, Methylation, Infertility, ICSI

Résumé

L'analyse systématique du sperme est un mauvais prédicteur des taux de grossesse après injection intra-cytoplasmique de sperme (ICSI). On suppose que la qualité des embryons et les taux de fécondation pourraient être influencés par des facteurs épigénétiques. Nous visions à comparer le niveau global de méthylation de l'ADN du sperme (GSDML) entre le sperme normal et anormal, en étudiant sa relation avec les paramètres du sperme et en évaluant son effet sur les résultats de l'ICSI; fécondation, bons taux d'embryons et de grossesses. Des éjaculats ont été obtenus à partir de 104 hommes stériles et 60 fertiles subissant une ICSI à la Faculté de médecine d'Alexandrie, en Egypte. Les données ont été analysées à l'aide du progiciel IBM SPSS, 20. Les différences inter-groupes dans les paramètres du sperme ont été évaluées par test t. Le GSDML, mesuré par ELISA, a montré une corrélation positive significative avec le nombre de spermatozoïdes, la concentration et la motilité. Il était en corrélation positive mais insignifiante avec la morphologie et le taux de fécondation. Des niveaux élevés étaient significativement associés à des embryons de bonne qualité et des taux de grossesse positifs. Le GSDML pourrait prédire un bon taux d'embryons et la survenue de grossesses après ICSI. *(Afr J Reprod Health 2020; 24[4]: 94-100).*

Mots-clés: Sperme, méthylation, infertilité, ICSI

Introduction

Up till now, male fertility evaluation is still based on semen analysis according to World Health Organization (WHO) standards, mainly: total sperm number, concentration, morphology and motility. However, many of male infertility causes are due to sperm DNA defects, which could not be detected by routine semen analysis[1]. Although genomic DNA contributes to the majority of the inheritance, it is now clear that epigenetic information (information beyond the underlying DNA sequence) is also passed on to future generations. Paternal exposure to environmental stressors, such as psychological stress, diet and toxicants, can influence offspring phenotypes, and sperm epigenome plays a pivotal role in the transmission of such phenotypes[2].

Sperm DNA damage and epigenetic or methylation anomalies are believed to be potential candidates responsible for infertility. These genetic defects may interfere with the development of the male reproductive system and urogenital tract, arrest germ cell production and maturation, leading to the production of non-functional spermatozoa[3]. The rate of damage in sperm DNA has been shown to be higher in infertile males compared to fertile ones. The association between the outcome of assisted reproductive technology (ART) and sperm DNA damage has been under debate[4]. Epigenetics include different mechanisms for regulation of gene expression[5]. DNA methylation disorders have been associated with various human disorders, such as: fertilization failure, embryogenesis dysfunction, perinatal mortality, and babies born with low birth weight[6].

In male factor infertility, epigenetic modifications play an important role in regulation of male germ cell maintenance and development. Spermatogonia stage was associated with many DNA methylation markers: therefore, infertile males, whose DNA methylation patterns showed abnormality, may have abnormal methylation maintenance in spermatocytes or mature sperm cells and failure of re-methylation in spermatogonia. Also, abnormal DNA methylation may be linked with the abnormal activation of DNA methyl-transferases (DNMTs)[7]. Additionally, paternal obesity may alter the molecular composition of spermatozoa, specifically, spermatozoa epigenetic components, such as DNA methylation, chromatin structure and noncoding RNAs (ncRNAs)[8]. So, changes in the epigenetic profiles of infertile males may be the cause for ART complications, as premature births, congenital abnormalities, low birth weight, increased perinatal mortality rate and pregnancy complications[9]. As the development of female and male gametes differ in the first stages post-fertilization, their methylation level could be the causative agent for some implantation failures despite apparently normal gametes. Therefore, the level of methylation of paternal genome may represent an important predictor of ICSI success[10].

In this study, we aimed at investigating the relation between semen parameters and global sperm DNA methylation level (GSDML) as well as assessing its effect on ICSI outcomes; specifically fertilization, embryo quality and pregnancy rates.

Methods

Subjects

This prospective study was conducted on 104 infertile male patients having semen with abnormal parameters and 60 males having semen with normal parameters, with unexplained infertility, who served as a control group. 164 ICSI cycles were performed in El Shatby Maternal University Hospital, Alexandria, Egypt, during a 10 months' period, between May 2018 and February 2019. The inclusion criteria for the infertile male patients included those having their first fresh cycle of ICSI. Their female partners had normal hormonal profile [basal serum follicle stimulating hormone (FSH) <10.0IU/l], all treated with long GnRH agonist protocol, their age ranged between 18-35 years and BMI<30 Kg/m^2. The exclusion criteria included: evident causes of spermatogenic impairment (such as history of orchitis or cryptoorchidism, seminal infection, sperm autoantibodies, varicocele), history of ovarian surgery in the female partner, history of medications known to impact ovarian and testicular functions for at least 3 months. All subjects were recruited from El Shatby Maternal University Hospital and Infertility Outpatient Clinic of Alexandria Main University Hospital.

All subjects enrolled in our study signed a written informed consent before participation. All details that might disclose the identity of the patients under study were excluded. All procedures performed in this study, involving human participants, were in accordance with the ethical standards of the institutional research committee, i.e. received approval of the Medical Ethics Committee of the Faculty of Medicine, Alexandria University and the practical work was carried out in accordance with the code of Ethics of the World Medical Association (1964 Declaration of Helsinki and its later amendments).

Sperm preparation for assisted reproduction

Sperm were prepared using All Grad Gradient (Brussels, Belgium). The gradient consists of two concentrations: 90% and 45%. Firstly, 200 ul of the 90% solution, then 200 ul of the 45% solution were added. They were added slowly on the wall of the tube then 1 ml of semen was deposited. The tube was then centrifuged at 300 g for 20 min. After centrifugation, the lowermost layer, which contains motile sperm, was collected and washed with 1 ml

of Ferticult Flushing medium (FertiPro N.V., Belgium), then centrifuged at 600 g for 10 min. The pellet was then re-suspended in IVF Medium (Scandinavian IVF, Sweden)[11].

Embryo culture and classification

16 to 18 hours after microinjection, oocytes were assessed for presence of sign of fertilization which is the two-pronuclear stage (2PN). Forty-eight hours after retrieval of oocyte (day 2), the embryos were classified according to their morphology into: *Grade A*: No fragmentation, *Grade B*: 25% fragmentation. *Grade C*: between 25 and 50% fragmentation and *Grade D*: more than 50% fragmentation[12]. Embryos were cultured on sequential medium: P-1 medium (Irvine Scientific, USA) for the first 3 days and Blastocyst medium (Irvine Scientific) for the last 2 days of culture before their transfer into the uterus.

The transfer of all embryos took place at stage of blastocyst (day 5). The extra-numerary embryos were cryopreserved, if their morphological states allowed it (i.e. grade A or B). Pregnancies were confirmed by measuring serum β-HCG test, 15 days after the transfer of embryos (ET) and clinical pregnancies were confirmed by doing vaginal ultrasound 30 days after ET which showed the presence of an intrauterine gestational sac and pulsating fetal heart beats.

Sperm DNA methylation analysis

DNA was extracted from the remaining sperm after microinjection using Qiagen DNA isolation kit (QIAamp 96 DNA Kit, cat # 51161). Isolated genomic DNA was stored at -20ºC until being assayed. Global sperm DNA methylation level was determined by an ELISA kit; MethylFleshTM Methylated DNA Quantification Kit (Colorimetric), (EpiGentek ,USA,cat # P-1034). We used the 96 assay kit. DNA was bound to strip wells that have a DNA affinity. The methylated fraction of DNA was detected using capture and detection antibodies and then quantified colorimetrically by reading the absorbance on a microplate spectrophotometer. The amount of DNA methylated was proportional to the optical density (OD) measured.

All the steps were performed according to the manufacturer's instructions. Briefly, genomic DNA samples were adjusted to the same concentration by using the provided DNA binding buffer. Along with a positive control (methylated DNA control, ME4) and a negative control (DNA binding buffer alone, ME3). Then genomic DNA samples were put into assay wells for 90–120 min at 37°C. Capture (ME5) and detection (ME6) antibodies were added correctly to the wells after washing steps. A developing solution (ME8) and a stop solution (ME9) were added to the wells after washing. Absorbance was read on a microplate reader at 450 nm within 2 to 15 minutes.

A standard curve was generated where the OD values were plotted versus the amount of ME4 at each concentration point. Then the slope of the standard curve was detected using Linear Regression and the most linear part of the curve was considered for optimal slope calculation.

GSDML of each genomic sample was calculated by normalizing samples against the positive and negative controls provided in the kit as follows:

5-mC Calculation: We calculated the slope and then calculated the level of methylated DNA by the following formulas: 5mC (ng) = Sample OD – ME3 OD / Slope x 2

GSDML= 5mC % = 5mC amount (ng) / S X 100%

Where: *S= the amount of input sample DNA in ng*

2= a factor to normalize 5mC in the positive control to 100%

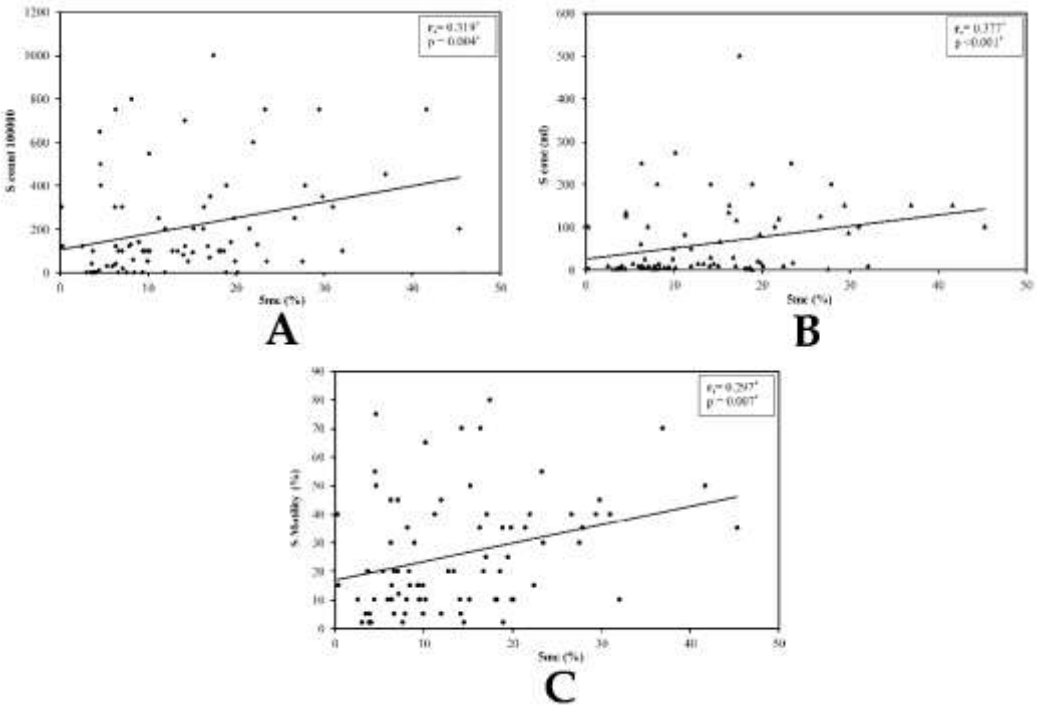
Statistical analysis

Data was analyzed using IBM SPSS software package version 20. Results were expressed as mean ± SD. Inter-group differences in semen parameters (e.g., between oligoasthenozoospermic and normozoospermic men) were assessed by t-test. The X^2-test was used to analyze the qualitative parameters. Spearman coefficient was calculated to assess the correlation between DNA methylation and different parameters. All hypothesis testing was two-sided with a probability value of 0.05 defined as significant[13].

Results

GSDML in normal and abnormal semen

Semen samples were considered normal if sperm concentration was ≥ 15 million/ml, count ≥ 39



S: Sperm 5mc: 5 methyl cytosine
r: correlation coefficient p: statistically significant < 0.05

Figure 1: Relationship between global sperm DNA methylation level (5mC %) and sperm characteristics: A) count (x10^6/ejaculate), B) concentration (x10^6/ml) and C) motility (%)

Table 1: Correlation between global sperm DNA methylation level (GDML), semen parameters and ICSI outcomes (n = 164)

	5mC (%)	
	r_s	**P**
Sperm Count (x10^6/ ejaculate)	0.319	0.004*
Sperm Concentration (x10^6/ml)	0.377	<0.001*
Sperm Motility (%)	0.297	0.007*
Morphology Index (%)	0.041	0.713
Fertilization (%)	0.151	0.176
	U	**P**
Positive Pregnancy	493.5	0.002*

r_s: Spearman coefficient
U, p: U and p values for Mann Whitney test for comparing between the two groups
*: Statistically significant at p ≤ 0.05

million/ejaculate, total motility ≥ 40% (with ≥ 32% progressive motility) and morphology index ≥ 4% normal forms (WHO strict criteria). Failure to achieve any of the fore-mentioned criteria was considered as an abnormal semen sample. GSDML in males with abnormal semen parameters ranged from 0.36 to 32.07% with a mean ± SD of 11.13 ± 6.92% and a median of 9.4, while in males with normal semen parameters, it ranged from 0.3 to 45.33 % with a mean ± SD of 18.23 ± 11.52 % and a median of 16.78. Comparing both groups, GSDML was significantly higher in normal semen (p=0.005).

GSDML and semen parameters

A significant positive correlation between GSDML and sperm count, concentration and motility was found (r=0.319, p=0.004 and r=0.377, p<0.001 and r=0.297, p=0.007 respectively), *(*Figure *1 A, B &C).* There was also a positive correlation with morphology index (r=0.041) but was found to be statistically insignificant (p=0.713), as shown in Table 1.

GSDML and fertilization rate

Fertilization Rate (FR) was calculated as the percentage of transformation of microinjected oocytes into two pronuclei (2PN). FR in males with abnormal semen parameters ranged from 43 to 100 % with a mean ± SD of 71.81± 16.9% and a median of 80. FR in males with normal semen parameters ranged from 38 to 100% with a mean ± SD of 75.67 ± 19.2 % and a median of 81.5.

No statistical significant difference in FR between those having normal semen parameters and those having abnormal parameters (p=0.136). Moreover, there was a positive correlation between GSDML and fertilization rate (r=0.151) but was found to be statistically insignificant (p=0.176), table 1.

GSDML and embryo quality

Embryo quality was classified into 4 grades A, B, C and D assessed on day 2 (48 hours after oocyte retrieval). In males having abnormal semen parameters, 16.2 % of embryos were grade A, 43.8 % grade B, 35.2% grade C and 4.8% grade D, while in males with normal semen parameters, 42.6 % of embryos were grade A, 46.3% grade B, 11.1% grade C and none was grade D. The difference in embryo quality, between both groups, was statistically significant (p<0.001).

GSDML in semen samples that showed good quality embryos (i.e. grades A&B) ranged between 0.30 to 45.33% with a mean ± SD of 15.56 ± 9.37% and a median of 14.91, while that in samples that yielded bad quality embryos (i.e. grades C&D) ranged from 0.30 to 26.67% with a mean ± SD of 11.0 ± 6.61 % and a median of 9.42. GSDML was significantly higher in samples yielding good quality embryos, p= 0.04.

GSDML and pregnancy rate

Pregnancy rate was calculated as the percentage of pregnant females in the total number of females participating in the study. 36.5 % (38/104) of female partners to males having abnormal semen parameters became pregnant, while 60% (36/60) of female partners to males with normal semen parameters achieved pregnancy. Pregnancy rates between both groups showed statistical significant difference (p=0.044).

Among the 74 couples who achieved pregnancy, GSDML ranged from 2.59 – 45.33% with a mean ± SD of 17.43 ± 10.5 and a median of 16.39. However, among the 90 couples who failed to become pregnant, GSDML ranged from 0.3 to 31.02% with a mean ± SD of 10.67 ± 7.31 and a median of 8.2. GSDML was statistically significantly higher in couples achieving pregnancy (p=0.002), table1.

Discussion

In the present study profiling whole genome methylation (global methylation) was carried out using ELISA technique. A significant positive correlation was found between GSDML and sperm count, concentration and motility. These results suggest that GSDML could be used as a marker of testicular function and spermatogenesis; high methylation levels could be associated with normal spermatogenesis, while low methylation levels suggest defective spermatogenesis.

Marques *et al*, assessed specific site differentially-methylated regions related to genomic imprinting using bisulfite sequencing. They found a relation between low DNA methylation levels and hypospermatogenesis. This might be explained by the fact that germ cells of men with low sperm counts demonstrate an altered expression of spermatogenesis genes and that DNA methylation is a key regulator of transcription and may contribute to the gene expression defects observed in men with poor semen parameters, specifically oligoasthenozoospermia[14]. Houshdaran *et al*.[15], performed standard semen analysis for 69 men during clinical evaluation of couples with infertility. They also measured methylation levels of DNA isolated from sperm purified from those semen samples. Methylation at numerous sequences was found to be elevated in DNA from poor quality samples. Their results suggested that the underlying mechanism for these epigenetic changes might be improper erasure of DNA methylation during epigenetic reprogramming of the male germ line.

El Hajj *et al*[16], studied the methylation levels of 2 paternally (H19 and GTL2) and 5 maternally methylated (LIT1, MEST, NESPAS, PEG3, and SNRPN) imprinted genes in 141 sperm samples, which were used for ART. Aberrant methylation imprints showed a significant association with abnormal semen parameters, but did not seem to influence ART outcome. Montjean *et al*[10], analyzed PEG1/MEST-DMR and H19-DMR methylation level in sperm, from 119 normozoospermic and 175 oligozoospermic men consulting for couple infertility. Pregnancy rate was also studied after ART treatment using sperm showing epimutations. Epimutations in H19-DMR and PEG1/MEST-DMR were found in 20% and 3% of oligozoospermic men, respectively. No correlation between ART outcome and epimutations was found.

Kobayashi *et al*[17], examined the DNA methylation status of 7 imprinted genes using a combined

bisulphite-PCR restriction analysis and sequencing technique on sperm DNA obtained from 97 infertile males. They found an abnormal paternal methylation imprint in 14 patients (14.4%) and abnormal maternal imprint in 20 patients (20.6%). The majority of these doubly defective samples were in men with moderate or severe oligozoospermia. These abnormalities were specific to imprinted loci as global DNA methylation was found to be normal in those samples. The outcome of ART with sperm shown to have an abnormal DNA methylation pattern was poor. Their data suggest that sperm from infertile patients, especially those with oligozoospermia, may carry a higher risk of transmitting incorrect primary imprints to their offspring.

Our results were opposite to Housdaran *et al*[15] study where hypermethylation was linked to poor quality sperm samples. Our results showed no significant correlation between sperm DNA methylation level and FR; which is conforming to Montjean *et al*[10], and El Hajj et al[16] that showed the same results. Benchaib *et al* [11], conducted a prospective study on 63 males undergoing ART. Ejaculates were obtained and 5-methylcytosine was immunostained with a polyclonal antibody and revealed by fluorescein isothiocyanate. The DNA methylation level was then quantified by flow cytometry. Neither the fertilization rate nor the rate of good quality embryos was correlated with DNA methylation level, however, pregnancy rate was significantly correlated ($P<0.05$).

We also proved that GSDMLs were positively and significantly correlated with good quality embryos and positive pregnancy outcome. The relationship between a low GSDML and poor quality embryos can be explained by the following: in cases of sperm DNA hypomethylation, some genes are not repressed, as they normally should be, and consequently the embryo genome expression shows some degree of assynchronism leading to arrest in its development[11]. Opposite to our results, Montjean *et al*[10] and El Hajj et al[16] suggested that DNA methylation had no role in determining pregnancy occurrence or embryo quality after ART.

Montjean D *et al* [10] found that patients with hypomethylated semen samples showed reduced fertilization rates following ICSI and all embryos obtained, following ART, showed developmental arrest. The main limitations of our study were the relatively small sample size and the lack of sufficient clinical data to correlate global sperm methylation with, for example, testicular size and hormonal profile. However, the strong points in our study were the strict exclusion and inclusion criteria adopted for the selection of cases to avoid any confounding factors that could affect the results of our study causing bias. Moreover, to the best of our knowledge, our study is the first study conducted in Egypt, to investigate GSDML in normal and abnormal semen, and its relationship with ICSI outcomes and sperm parameters.

Children conceived using ART have a higher incidence of growth and birth defects, attributable in part to epigenetic perturbations[18]. And so, we highly recommend further studies with larger sample size and with inclusion of pregnancy outcomes of the included subjects as premature births, congenital abnormalities, low birth weight, increased perinatal mortality rate and pregnancy complications.

Conclusion

Global sperm DNA methylation level could predict good embryo rate and pregnancy occurrence after intra-cytoplasmic sperm injection.

Contribution of Authors

Ossama H. Roshdy: study design
Rania E. Abdel Maksoud: selection of cases and their clinical assessment
Yasser S. El Kassar: performed ICSI
Doreen N. Younan: conception of the research idea, laboratory analysis and proof reading of the manuscript
Rana R. El Sayed: data collection, analysis and drafting the manuscript
All authors mentioned above have thoroughly read and approved the manuscript.

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