Does advanced paternal age affect global DNA methylation of human spermatozoa and intracytoplasmic sperm injection outcome?

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Abstract

Objective: This study was performed to (I) evaluate the potential effect of advanced paternal age on global DNA methylation in spermatozoa, and (II) to investigate the association between the outcome of intracytoplasmic sperm injection (ICSI), semen parameters, and advanced paternal age.

Material and Methods: This study comprised 230 semen samples collected from males with a mean age of 38.2±8.5 years. Medical records were used to gather clinical information related to the female partner. The participants were divided into three groups depending on age: age <30 years; age 30-40 years; and age >40 years. The DNA was extracted from purified spermatozoa. Then the sperm global DNA methylation, sperm DNA fragmentation, and chromatin decondensation were evaluated by an ELISA, TUNEL, and Chromomycin A3 staining, respectively.

Results: The sample counts were n=50 (21.8%), n=90 (39.1%) and n=90 (39.1%) for the <30, 30-40 and >40 year age-groups, respectively. A significant variation was found in the age of males included in this study ($p<0.001$). There was a significant reduction in sperm count, total motility, and non-progressive motility in the older group compared to the younger group ($p<0.001$). There was also a significant elevation in chromatin decondensation, DNA fragmentation, and global DNA methylation of spermatozoa in the older age group ($p<0.001$). Finally, there was a significant positive correlation between the percentage of non-motile sperm, sperm chromatin decondensation, DNA fragmentation, global DNA methylation status, and paternal age ($p<0.001$).

Conclusion: These results suggest that advanced paternal age increased the DNA fragmentation, chromatin decondensation, and global DNA methylation level in human spermatozoa, which negatively affects the ICSI outcomes in couples undergoing ICSI cycles. (J Turk Ger Gynecol Assoc 2023; 24: 18-27)

Keywords: Global methylation, ICSI outcomes, paternal age, spermatozoa

Received: 26 May, 2022 **Accepted:** 09 November, 2022

Introduction

In assisted reproductive technologies (ART), maternal age plays a critical role in determining the success rate of the intracytoplasmic sperm injection (ICSI) process. Several studies have shown that paternal aging can directly damage sperm DNA, increase the level of sperm DNA methylation (1,2), and increase the rate of sperm damage through the production of excessive reactive oxygen species (3,4). Other studies reported that human spermatozoa have a very distinct pattern of age-associated alteration (5,6), whereas one study observed an increase in global spermatozoa DNA methylation and a strong bias toward regional loss of methylation at sites known to be impacted by aging (7). It has been reported that male age was associated with alterations in sperm DNA methylation levels at 1,698 CpGs and 1,146 regions, which were associated

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Journal of the Turkish-German Gynecological Association published by Galenos Publishing House.
DOI: 10.4274/jtgga.galenos.2022.2022-4-8

with more than 750 genes enriched in embryonic development, behavior, and neurodevelopment (6). Additionally, the DNA methylation profiles in sperm of young men (≤35 years) and older men (≥50 years) were compared and the authors identified around 49,792 differentially methylated CpG sites related to neurodevelopmental relevant diseases (8). Another study observed that there are more hypermethylated (62%) than hypomethylated (38%) CpG sites in sperm from older aged men and the distribution of age-related hyper- and hypomethylated CpGs in sperm is not random. The CpG sites that were hypermethylated with advanced age were frequently located in the distal region to genes, whereas hypomethylated sites were near gene transcription start sites. Consequently, the effect on gene function is potentially related to diseases in offspring (9). Despite these recent advances, data about age-associated sperm DNA methylation is still limited.

An association was reported between the reduction in the rates of pregnancy and advancing paternal age (10). Furthermore, several studies have observed a negative correlation between sperm quality and paternal age, which negatively influences embryo cleavage and in vitro fertilization (IVF) clinical outcomes (11-13). In contrast, other studies have shown that paternal age has no influence on the fertilization rate, embryo quality (14), and the rate of pregnancy during conventional IVF techniques (15). Nevertheless, there is currently a lack of consensus concerning the contribution of paternal aging to sperm parameters, sperm DNA integrity, and clinical ICSI outcomes (16-18). Human and animal studies have reported that ART is associated with epigenetic changes in embryonic and extra-embryonic tissues (19,20). Additionally, epigenetic events may impair key steps during fertilization, implantation, embryo development, and sperm maturation (21,22). Epigenetics is defined as alterations in gene expression without changing the DNA sequence (23). Well-known epigenetic regulation mechanisms include DNA methylation, histone modifications, and non-coding RNAs (ncRNAs) (24).

DNA methylation is defined as the addition of a methyl group (CH3) to the fifth (C-5) position of the cytosine ring in CpG dinucleotides by DNA methyltransferase to form 5-methylcytosine (5-mC), where the S-adenosyl-methionine is used as a donor for the methyl group (25,26). The CpG dinucleotides can be found in clusters that have been termed CpG islands and are characterized by less methylation than non-CpG islands (27). It is worth noting that CpG islands are found in about 60-70% of gene promoters (28). The methylated state of CpGs has a crucial impact on gene transcription during embryonic growth, genomic imprinting, X-chromosome inactivation, and tumor development (29,30). Several studies have illustrated that changes in DNA methylation of specific genes in germ cells are associated with oligozoospermia, reduced sperm progressive motility, and abnormal sperm morphology (31,32). A previous study noted that the alteration in the DNA methylation level of male spermatozoa may influence the developmental potential of embryos (33). Several studies have highlighted that increased paternal age influences testicular function (34), sperm parameters (35), sperm DNA integrity (1), and epigenetics (36). However, there is still no consensus around the influence of paternal age on global DNA methylation of human spermatozoa and the reproductive capacity of males during ICSI cycles. Therefore, this study was designed to (I) evaluate the potential effect of advanced paternal age on global DNA methylation, DNA fragmentation, DNA condensation in human spermatozoa, and ICSI outcomes, and (II) investigate the relationship between ICSI outcomes, semen parameters, and paternal age.

Material and Methods

Study population

This prospective study comprised two hundred and thirty couples with a mean age for males of 38.2±8.5 years and for females of 36.1±5.9 years. The study was conducted between May 2010 and September 2013. All cases underwent the first ICSI cycles at Al-Basma Fertility Center, Palestinian Territories. All participant women were selected according to the following inclusion criteria: first ICSI cycle; embryo transfer after three days from the injection; undergoing gonadotropin-releasing hormone (GnRH) antagonist stimulation protocols; normal body mass index; and women who have a regular menstrual cycle. Exclusion criteria for females included: tobacco smoking (cigarette or water pipe); alcohol drinkers; diabetes mellitus; women using an oral contraceptive; women suffering from endocrine abnormality; and endocrine disorders including polycystic ovarian syndrome, history of ovarian surgery, and endometrioma. Male partner exclusion criteria were: diabetes mellitus; alcohol drinkers; smokers; the presence of anti-sperm antibodies; varicocele; Y chromosome microdeletions; karyotype abnormalities; history of surgical operation in the reproductive system; abnormal hormonal parameters; and abnormal body mass index. Medical records were used to gather general and medical information that included age, body mass index, menstrual history, the number of retrieved oocytes, mature oocytes, immature oocytes, fertilized oocytes, the number of embryos transferred, and the value of Beta-human chorionic gonadotropin (β-hCG). The participants were divided into three groups depending on the male partner's age: <30 years; 30-40 years; and >40 years.

Ethics approval and consent to participate

This study was approved by the Health Research Council, Palestinian Territories (approval number: 03/10, date: 23.03.2010), and consent was provided in accordance with the Declaration of the Helsinki Committee. Samples were analyzed according to the guidelines and standard procedures of the Al Basma Fertility Center, Palestinian Territories. All participants gave written informed consent to participate in this study.

Ovarian stimulation and embryo transfer

All women included in the present study underwent ovarian stimulation using GnRH antagonist protocols with a recombinant follicle-stimulating hormone. Briefly, ultrasonography was conducted on the third day of the menstrual cycle to evaluate the anatomical characteristics of the female reproductive system and to determine the antral follicular count. The basal levels of estradiol (E2), FSH, luteinizing hormone, prolactin, and anti-Müllerian hormone were measured by immunoassay using a Tosoh AIA-360 instrument (Tokyo, Japan). A GnRH antagonist was administered once the dominant follicle was >14 mm and continued to the day of hCG administration. When at least three follicles were ≥18 mm, ovulation was triggered with hCG. The oocyte pickup was scheduled for 33-36 hours after the administration of 5,000 to 10,000 IU of hCG (Pregnyl), depending on the age of the women and the number of oocytes. The fertilization status of oocytes was checked after 16-18 hours from ICSI. The criteria for normal fertilization were the presence of two clearly visible pronuclei. Embryo cleavage and quality were evaluated 48 hours after ICSI. For each couple, a maximum of three embryos with high quality (grade I or II) were transferred into the uterine cavity after three days from ICSI. All women received luteal support with vaginal progesterone until a pregnancy test was performed. The women were described as pregnant women when the β-hCG hormone level reached >5 mIU/mL.

Semen collection and sperm purification

At the time of the ICSI cycle, semen samples were collected by masturbation after three days of abstinence from sexual intercourse. Semen samples were allowed to liquefy for 30 minutes at 37 °C. Then, the count of spermatozoa was evaluated immediately using a Makler counting chamber (Sefi-Medica, Haifa, Israel). Semen parameters were analyzed according to the World Health Organization guidelines (37). All samples underwent the somatic cell lysis buffer (SCLB) protocol to remove somatic cells and other debris from the sample before DNA extraction from spermatozoa. Briefly, the liquefied semen samples were loaded onto 45% over 90% discontinuous Puresperm gradients (Nidacon International AB, Sweden) and then centrifuged at 500x g for 20 minutes at 22 °C. Then the pure spermatozoa were incubated with SCLB on ice for half-hour and subsequently washed three times with phosphate-buffered saline (PBS), and centrifuged at 500+g for 10 minutes (38,39). Finally, microscopic examination was used to confirm purity of the semen samples from somatic cells and other debris.

DNA fragmentation of human spermatozoa (TUNEL assay)

The DNA fragmentation of spermatozoa (sperm apoptosis) was assessed using the terminal deoxyribonucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay. The TUNEL assay was performed using an in situ cell death detection kit following the guidelines of the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, smears were prepared using 10 μL of sperm suspension on microscope slides and allowed to air-dry and then fixed with 4% paraformaldehyde phosphate-buffered saline, pH 7.4, for two hours at room temperature, then rinsed with PBS. Smears were then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, pH 6.0, for 15 minutes at room temperature; 50 μL of the terminal deoxyribonucleotide transferase (TdT)-labelled nucleotide mixture (50 μL of enzyme solution and 450 μL of label solution) was added to each slide and incubated in a humidified chamber at 37 °C overnight in the dark. Negative controls without TdT were run in each replicate. Then, slides were rinsed twice in PBS and left to air dry, followed by adding 25 μL of 5 μg/mL DAPI stain solution to each slide as a counterstain and then coverslipped. For evaluation, a total of 500 spermatozoa were analyzed, by distinguishing spermatozoa stained bright green (TUNEL positive, fragmented DNA) from those stained dull green (TUNEL negative, with intact DNA). A Zeiss Photomicroscope III was used for the fluorochrome evaluation (Zeiss Photomicroscope III, Germany) (40).

Sperm chromatin decondensation (Chromomycin A3 staining)

Chromomycin A3 (CMA3) staining was used to evaluate chromatin non-condensation in human spermatozoa. Briefly, three semen smears were prepared from each sample and all smears were fixed using a fixative solution of methanol:glacial acetic acid, 3:1 respectively) at 4 °C for 20 minutes. Semen smears were air-dried at room temperature. After that, each smear was covered by 50 μL of staining solution (Sigma-Aldrich, St. Louis, MO, USA) and then incubated in a dark place at room temperature for 20 minutes. PBS was used to wash all slides, then the slides were mounted with 1:1 (v/v) glycerol/PBS incubated overnight at 4 °C. To estimate the results of CMA3 staining, the fluorescence microscope (Zeiss Photomicroscope III, Germany) was used to analyze 200 spermatozoa on each smear. Finally, the CMA3 staining was evaluated by differentiating the spermatozoa that stained with bright yellow (positive, bad spermatozoa) from spermatozoa that stained with dull yellow (negative, good spermatozoa) (40,41).

DNA extraction from human spermatozoa

The Isolate II DNA/RNA/Protein Kit was used to isolate DNA from human spermatozoa. At first, 600 μL of lysis buffer was added to 200 μL of pure spermatozoa. Then the mixture was vortexed for 15 seconds. After that, all the lysate was transferred to a DNA column and centrifuged for 1 minute at 14,000 g. All the procedures were carried out according to the guidelines of the manufacturer (Bioline, UK). The purity and the concentration of isolated nucleic acid were assessed using a Nanodrop spectrophotometer-2,000c (Thermo Scientific, USA), to ensure that the quantity and quality of isolated DNA were suitable and adequate for global sperm DNA methylation assay.

Evaluation of global DNA methylation in human spermatozoa

The status of global DNA methylation (5-methylcytosine) in all DNA samples was evaluated using the MethylFlash™ Methylated DNA Quantification ELISA Kit (colorimetric) according to the guidelines of the manufacturer (EpiGentek Group Inc, USA). Briefly, 100 ng of extracted DNA was incubated with the DNA binding buffer solution provided with the kit for 1.5 hours at 37 0C. During this assay a blank, a positive and a negative control were included in triplicate. After washing the microwell four times, methylated DNA capture solution was added to each well and incubated at 22 °C for one hour. After that, detection antibodies were added to each well and incubated for half an hour at room temperature. After washing three times, the developing solution was added to each well and incubated at room temperature in a dark place for six minutes, and at the end of this time, stop solution was added. A microplate ELISA reader was used to determine the absorbance at 450 nm. The global DNA methylation level was calculated using the equation: 5-mC(ng) = [(sample OD - blank OD)/100].

Statistical analysis

All the data were analyzed using IBM SPSS for Windows, version 24.0 (SPSS Inc., Chicago, IL, USA). The skewness test, kurtosis test, and Shapiro test were used to investigate the normality of data distribution. Kruskal-Wallis (H test) and Mann-Whitney (U test) were applied to compare quantitative variables between the study groups. The Spearman rank correlation coefficient was used to study the association between paternal age and other clinical parameters. A p-value of <0.05 was accepted as indicating statistical significance.

Results

Clinical parameters and ICSI outcome among different age groups

This study comprised 230 semen samples collected from males with a mean age of 38.2±8.5 years. On stratification by male partner age, the groups comprised n=50 (21.8%), n=90 (39.1%) and n=90 (39.1%) for the <30, 30-40 and >40 year age-groups, respectively. As expected there was a significant difference between the age of the males in the different groups (p<0.001) but there was no significant difference between the female partner's ages (p=0.676) (Table 1). A significant reduction was found in the sperm count, percentage of total sperm motility, progressive motility, and non-progressive motility between the different age groups (p<0.001). A significant increase was observed in semen volume and the percentage of non-motile sperm (p=0.022 and p<0.001, respectively) among the different age groups. Additionally, a significant variation was observed among the different age groups in the proportion of spermatazoa with normal and abnormal forms (p=0.013). There were also significant differences between the different age groups in terms of oocyte fertilization rate and the number of embryos transferred (p<0.001 and p=0.041, respectively). Furthermore, a significant decrease in the level of ß-hCG was noted with increasing age (p=0.009). There was a significant increase in the degree of sperm chromatin decondensation (Figure 1), sperm DNA fragmentation (Figure 2), and the global sperm DNA methylation level (Figure 3) in older males compared to younger (p<0.001).

Correlation between the paternal age and clinical parameters of the study population

There was a significant negative correlation between the sperm count (r=-0.581, p<0.001), percentage of total sperm motility (r=-0.391, p<0.001), progressive motility (r=-0.359, p<0.001), non-progressive motility (r=-0.351, p<0.001), the level of ß-hCG (r=-0.166, p=0.01), and paternal age (Table 2). In contrast, a significant positive association was found between semen sample volume (r=0.220, p<0.001), percentage of non-motile sperm (r=0.391, p<0.001), degree of sperm chromatin decondensation (r=0.423, p<0.001), sperm DNA fragmentation (r=0.391, p<0.001), degree of global DNA methylation (r=0.321, p<0.001), and increasing paternal age.

Clinical parameters and ICSI outcome in pregnant women compared to non-pregnant

There was a significant increase in the number of collected oocytes (p=0.029), mature oocytes (p=0.044), fertilized oocytes (p=0.023), embryo cleavage (p<0.001), number of embryos transferred (p<0.001), and the levels of ß-hCG (p<0.001) in pregnant compared to non-pregnant women (Table 3). Additionally, a significant decline was found in the level of sperm chromatin condensation (Figure 4), sperm DNA fragmentation (Figure 5), and degree of global DNA methylation (Figure 6) in the partners of pregnant women compared to the partners of non-pregnant women (p<0.001).

Table 1. Clinical parameters and ICSI outcome among different paternal age groups (n=230)

Clinical parameters	Study population		Age <30 years, (n=50)		Age 30-40 years, (n=90)		Age >40 years, (n=90)		p
	Median	SD	Median	SD	Median	SD	Median	SD	
Paternal age (years)	38.0	8.5	27.0	1.6	35.0	3.3	48.0	2.6	<0.001
Semen volume (mL)	3.50	2.38	3.50	1.66	3.50	2.33	3.90	2.67	0.022
Sperm count (milli/mL)	58.00	63.06	144.00	63.94	60.85	63.50	31.65	29.86	<0.001
Percentage of total sperm motility	46.00	23.81	55.00	22.20	50.00	17.98	22.50	24.55	<0.001
Percentage of progressive motility	26.00	20.36	38.50	18.62	27.00	16.21	9.50	22.34	<0.001
Percentage of non-progressive motility	15.00	10.42	18.50	10.17	20.00	10.16	9.50	9.35	<0.001
Percentage of non-motile sperm	54.50	23.94	45.00	22.27	50.00	18.53	77.50	24.55	<0.001
Percentage of sperm normal form	8.50	13.26	7.00	8.02	14.50	14.54	7.00	13.47	0.013
Percentage of sperm abnormal form	91.50	13.26	93.00	8.02	85.50	14.54	93.00	13.47	0.013
Maternal age (years)	37.0	5.9	36.0	5.5	37.0	6.6	37.5	5.4	0.676
Number of collected oocytes	8.00	7.13	8.00	6.27	10.00	8.17	7.00	6.19	0.060
Number of mature oocytes	6.00	5.38	6.00	5.30	6.50	5.86	5.00	4.89	0.407
Number of fertilized oocytes	5.00	4.31	4.50	4.89	5.00	4.24	4.00	4.08	0.865
Oocytes fertilization rate	61.11	24.07	58.33	24.25	50.00	22.41	66.67	23.12	<0.001
Number of embryo cleavage	4.00	4.21	4.00	6.19	3.00	3.38	4.00	3.51	0.271
Number of embryo transferred at day 3	3.00	1.18	3.00	0.77	2.00	1.33	3.00	1.19	0.041
ß-hCG level	4.50	40.15	68.00	43.24	4.20	39.69	3.80	36.43	0.009

SD: Standard deviation, ß-hCG: Beta human chorionic gonadotropin

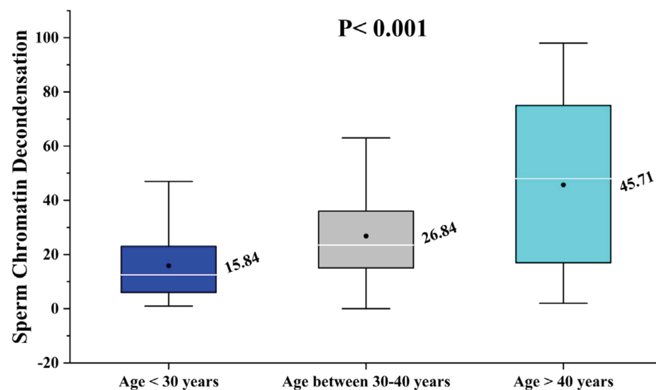

Figure 1. Sperm chromatin decondensation compared between the different paternal age groups

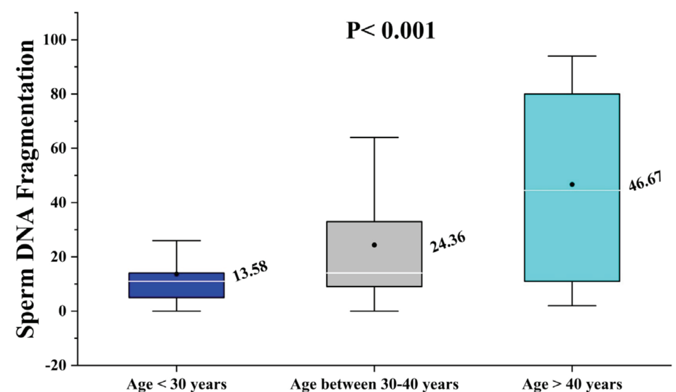

Figure 2. DNA fragmentation in human spermatozoa compared between the different paternal age groups

Correlation between paternal age and clinical parameters of women who became pregnant.

As illustrated in Table 4, there was a significant positive correlation between the degree of sperm chromatin decondensation (r=0.309, p<0.001), sperm DNA fragmentation (r=0.244, p=0.01), global sperm DNA methylation (r=0.269, p=0.01), and the male partner's age in the group of women who became pregnant. In contrast, no significant associations were identified between ICSI outcome and male partner's age in the pregnant women group.

Discussion

Currently, much attention has been paid to studying the impact of paternal age on ICSI outcome and fertilization rate. Several studies have reported that older paternal age contributes negatively to semen production, fertility, pregnancy outcome, and ICSI outcomes (42-44). Other studies have shown that increasing paternal age is linked to genetic and epigenetic abnormalities in spermatozoa (18,45,46). In this study, we assessed the potential effect of differences in paternal age on ICSI outcomes and global DNA methylation in human

spermatozoa. The present study identified a significant reduction in sperm count, sperm total motility, progressive motility, non-progressive motility, and other semen parameters between the different paternal age groups. These findings are in agreement with other studies that showed that sperm counts and other semen parameters decrease with increasing paternal age (47-50). Additionally, these findings are in agreement with other studies that found a significant decline in sperm motility and fecundity status in males aged older than 40 years compared to males aged 35 years old or less (50-52). However, some other studies have shown no significant differences in semen volume, sperm concentration, sperm motility, and morphology between different paternal age groups (35,53). Other earlier reported no drastic effects on semen parameters of healthy men or men with proven fertility with age (54,55). This inconsistency in the findings might result from the lack of control for some confounding factors, such as the duration of abstinence time and the method used for semen collection.

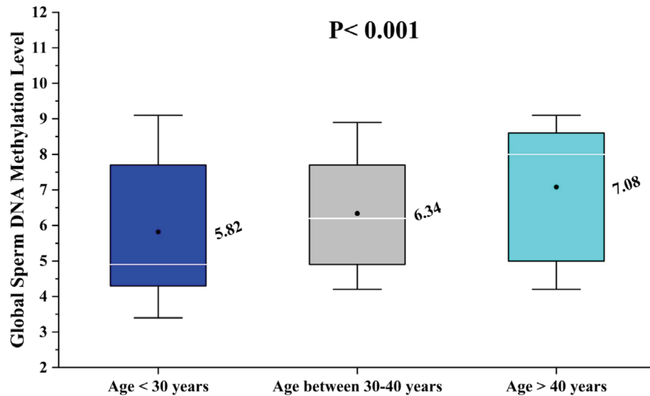

Figure 3. Global DNA methylation level in human spermatozoa compared between the different paternal age groups

A significant increase was found in the level of global DNA methylation, sperm chromatin decondensation, and sperm DNA fragmentation in the older male group compared to younger men. These results support the findings of previous studies that showed an increase in the level of global DNA methylation (56) and sperm DNA fragmentation in older males (57,58). Other previous studies have reported that paternal age is associated with hypermethylation globally (2,59). In addition, another study showed an increase in global 5-methylcytosine

Table 2. Correlation between the paternal age and clinical parameters of the study population (n=230)

Clinical parameters	r	p	Clinical parameters	r	p
Semen volume (mL)	0.220	<0.001	Sperm DNA fragmentation (TUNEL-positive)	0.391	<0.001
Sperm count (million/mL)	-0.581	<0.001	Global sperm DNA methylation level (ng/µL)	0.321	<0.001
Percentage of total sperm motility	-0.391	<0.001	Number of fertilized oocytes	0.03	0.65
Percentage of progressive motility	-0.359	<0.001	Oocytes fertilization rate	0.201	<0.001
Percentage of non-progressive motility	-0.351	<0.001	Number of embryo cleavage	0.04	0.56
Percentage of non-motile sperm	0.391	<0.001	Number of embryo transferred at day 3	-0.03	0.67
Percentage of sperm normal form	-0.04	0.58	ß-hCG level	-0.166	0.01
Sperm chromatin decondensation (CMA3-positive)	0.423	<0.001			

Spearman rank correlation coefficient, r: Correlation coefficient, ß-hCG: Beta human chorionic gonadotropin

Table 3. Clinical parameters and ICSI outcome in pregnant women compared to non-pregnant (n=230)

Clinical parameters and ICSI outcome	Pregnant women, (n=106)			Non-pregnant women, (n=124)			p
	Mean	SD	Median	Mean	SD	Median	
Maternal age (years)	35.5	5.8	37.0	36.6	6.0	38.0	0.113
Number of collected oocytes	11.32	6.99	10.00	9.67	7.19	8.00	0.029
Number of mature oocytes	8.22	5.45	6.00	7.03	5.29	5.50	0.044
Number of fertilized oocytes	6.51	4.71	5.00	5.24	3.86	4.00	0.023
Oocyte fertilization rate	60.64	24.04	60.00	60.31	24.18	61.33	0.990
Number of embryo cleavage	6.05	5.01	4.50	4.10	3.13	3.00	<0.001
Number of embryo transferred at day 3	3.05	1.17	3.00	2.39	1.11	2.00	<0.001
ß-hCG level	78.41	20.18	84.25	2.86	1.05	2.60	<0.001

SD: Standard deviation, ß-hCG: Beta human chorionic gonadotropin, ICSI: Intracytoplasmic sperm injection

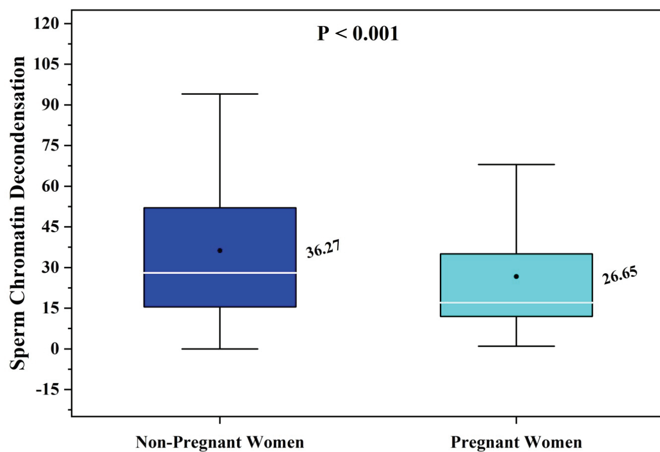

Figure 4. Sperm chromatin decondensation in male partners of pregnant women compared to the partners of non-pregnant women

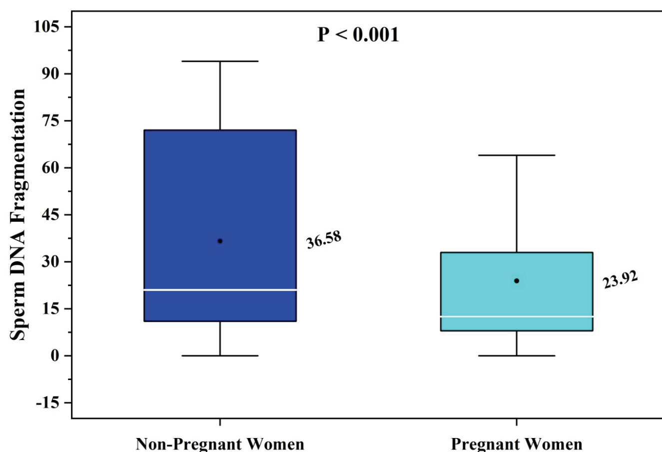

Figure 5. Sperm DNA fragmentation in male partners of pregnant women compared to the partners of non-pregnant women

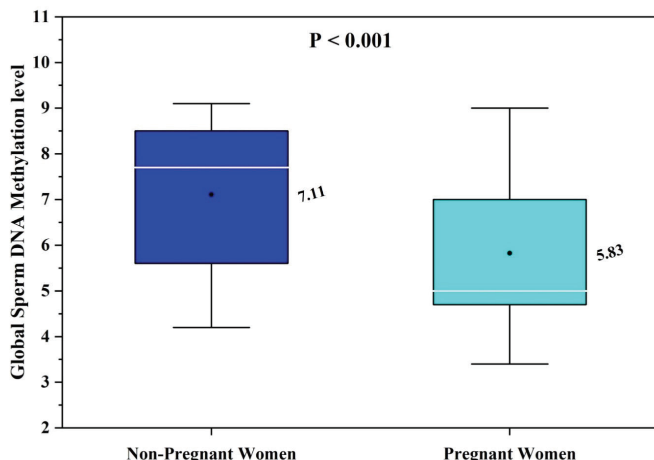

Figure 6. Global spermatozoa DNA methylation level in male partners of pregnant women compared to the partners of non-pregnant women

Table 4. Correlation between the paternal age and clinical parameters of the pregnant women (n=106)

Clinical parameters	r	p
Number of collected oocytes	-0.035	0.724
Number of mature oocytes	0.069	0.484
Number of fertilize oocytes	0.066	0.503
Oocyte fertilization rate	0.179	0.066
Number of embryo cleavage	0.041	0.679
Number of embryos transferred at day-3	-0.109	0.266
ß-hCG level	-0.176	0.071
Sperm chromatin decondensation (CMA3-positive)	0.309	<0.001
Sperm DNA fragmentation (TUNEL-positive)	0.244	0.01
Global sperm DNA methylation level (ng/μL)	0.269	0.01

Spearman rank correlation coefficient, r: Correlation coefficient, ß-hCG: Beta human chorionic gonadotropin

levels in spermatozoa obtained from men after 9-21 years from the first samples and an association between age and 5-methylcytosine in sperm (2). It has been suggested that increased methylation in the sperm of old males decreases the developmental potential of the resulting embryos, contributing to age-related fertility problems (60).

Recently, Jenkins et al. investigated DNA methylation in the sperm of 17 men collected 9-19 years apart and found 139 regions that became significantly hypomethylated and 8 others which became hypermethylated with increasing paternal age. Twenty-one of these sperm differentially methylated regions (DMRs) were confirmed by bisulfite sequencing (61). Another study used a genome-wide DNA methylation screen to compare sperm from young and old and revealed a significant loss of methylation in samples from older men of regions associated with transcriptional regulation (62).

The results of sperm DNA fragmentation and chromatin decondensation are in line with previous studies that found an increase in sperm DNA fragmentation in the age group ≥45 years compared to men <30 years old (1,63,64). However, other studies did not find the same variation with age (65,66). Based on the results of this study, the fragmentation of sperm DNA started to accelerate in men at age 41 years and older and this finding is in agreement with a previous study that found the acceleration point of sperm DNA fragmentation occurs at age 41.6 years (58). On the other hand, the results of DNA methylation disagree with previous studies that reported that male age is associated with a loss of sperm methylation at loci of key development genes (6,67). The difference in the findings of these studies might be due to the different techniques that were used during the evaluation of sperm DNA fragmentation or global DNA methylation, differences in the study population, inclusion criteria for participants, and sample processing.

This study showed a significant variation among the different age groups in the oocyte fertilization rate, the number of embryos transferred, and the level of β-hCG. Similar studies support these findings and suggest that paternal age impacts birth success rates (68), and leads to a reduction in the pregnancy rate from 12.3% in males aged <30 years to 9.3% in males ≥45 years old (13,69,70). A significant negative association has been found between most semen parameters, the level of ß-hCG, and paternal age. Such findings are in keeping with previous studies that showed that the reduction in sperm morphology, sperm motility, and sperm count are associated with increasing paternal age (49,52,71). Previous studies reported a negative association between sperm progressive motility and sperm normal morphology (72-74), assisted pregnancy rate (68), and paternal advancing age. In contrast, a significant positive association was observed between sperm chromatin decondensation, sperm DNA fragmentation, global DNA methylation level and paternal age. These positive correlations are in keeping with the results of earlier studies (64,75). The results of the present study support the findings of a previous study that found a strong correlation between an increase in sperm DNA methylation and advancing paternal age (76). However, the results of sperm DNA fragmentation do not match with the previous studies that found a significant negative correlation between the proportion of CMA positivity, and paternal age (76,77).

Conclusion

This study found that advancing paternal age increased the level of global DNA methylation, DNA fragmentation, and chromatin decondensation in human spermatozoa. Additionally, negative associations were identified between advancing paternal age and basic semen parameters. All of these findings may negatively affect ICSI outcomes and success rates in couples undergoing ICSI cycles.

Acknowledgments: *The authors would like to express their gratitude to all doctors and clinical staff at the Al Bassma Fertility Center, Palestinian Territories.*

Ethics Committee Approval: *This study was approved by the Health Research Council, Palestinian Territories (approval number: 03/10, date: 23.03.2010), and consent was provided in accordance with the Declaration of the Helsinki Committee.*

Informed Consent: *All participants gave written informed consent to participate in this study.*

Peer-review: *Externally peer-reviewed.*

Author Contributions: *Data Collection or Processing: L.L.M., Y.M.M.; Analysis or Interpretation: L.M.M., Y.M.M.; Literature Search: Writing: L.L.M., Y.M.M.*

Conflict of Interest: *No conflict of interest is declared by the authors.*

Financial Disclosure: *The authors declared that this study received no financial support.*

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