Original Investigation

Can Advanced paternal age affect global DNA methylation of human spermatozoa and ICSI outcome?

Laqqan and Yassin. Advanced paternal age and global sperm DNA methylation

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Abstract

Objective: This study was performed to (I) evaluate the potential effect of advanced paternal age on global DNA methylation on spermatozoa, and (II) to investigate the association between the intracytoplasmic sperm injection (ICSI) outcomes, 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. The medical records were used to gather the medical information related to the female partner. The participants were divided into three groups depending on males' age: (age < 30; n = 50 "21.8%", age = 30-40; n = 90 "39.1%", and age > 40; n = 90 "39.1%"). 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: A significant variation has been found in the age of males included in this study ($P < 0.001$). A significant reduction has been observed in sperm count, total motility, and non-progressive motility in the older group compared to the younger group ($P < 0.001$). Additionally, a significant elevation in chromatin decondensation level, DNA fragmentation level, and global DNA methylation of spermatozoa in the older age group ($P < 0.001$) has been found. A significant positive correlation has been detected between the percentage of non-motile sperm, sperm chromatin decondensation, DNA fragmentation, global DNA methylation status, and paternal age ($P < 0.001$).

Conclusion: This study pointed out that advanced paternal age increases the DNA fragmentation, chromatin decondensation, and global DNA methylation level in human spermatozoa which negatively affects the ICSI outcomes in couples undergoing ICSI cycles.

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

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Introduction

In assisted reproductive technologies (ART), maternal age plays a vital role in determining the success rate of the intracytoplasmic sperm injection (ICSI) process. Several studies showed 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 the human spermatozoa have a very distinct pattern of age-associated alteration (5,6), whereas a study observed an increase in the global spermatozoa DNA methylation and a strong bias toward regional loss of methylation at the sites known to be impacted by aging (7). A study showed that male age was associated with alterations in sperm DNA methylation levels at 1698 CpGs and 1146 regions, which were associated with more than 750 genes enriched in embryonic development, behavior, and neurodevelopment (6). Additionally, another study measured the DNA methylation profiles in sperm of young men ($\leq$ 35 years) and older men ($\geq$ 50 years) and 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 of aged men and the distribution of age-related hyper- and hypomethylated CpGs in sperm is not random, whereas 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 (TSS). Consequently, the effect on genes is potentially related to diseases in offspring (9). Despite those recent advances, the information on 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). Besides, several studies observed a negative correlation between sperm quality and paternal age which negatively influences embryo cleavage and IVF clinical outcomes (11-13). Conversely, other studies showed that paternal age has no influence on the fertilization rate, embryo quality (14), and the rate of pregnancy during conventional IVF techniques (15). Nevertheless, until now there is a lack of consensus concerning paternal aging's contribution to sperm parameters, sperm DNA integrity, and clinical ICSI outcomes (16-18). On the other hand, human and animal studies reported that ART is associated with epigenetic changes in embryonic and extra-embryonic tissues (19, 20). Additionally, epigenetic events may impair key steps of fertilization, implantation, embryo development, and sperm maturation (21, 22). Epigenetics is defined as alterations in gene expression without changing the DNA sequence (23). The most recognized epigenetic regulations are DNA methylation, histone modifications, and non-coding RNAs (ncRNAs) (24).

DNA methylation is defined as the addition of a methyl group (CH_3) 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 characterized by less methylation than non-CpG islands (27). It is worth mentioning 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 the 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 development potential of embryos (33). Several studies pointed out that the increased paternal age influences testicular function (34), sperm parameters (35), sperm DNA integrity (1), and epigenetics (36). Until now, there remains no consensus around the influence of paternal age on the 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 the ICSI outcomes, semen parameters, and paternal age.

Materials and methods

Study population

This prospective study comprised two hundred and thirty couples with a mean age for males 38.2 ± 8.5 years old and for females 36.1 ± 5.9 years old. This study was conducted between May 2010 and September 2013. All cases underwent the first ICSI cycles at Al-Basma Fertility Center, Palestinian Territories. All participants women were selected according to the following inclusion criteria: first ICSI cycle, embryo transfer after 3 days from the injection, women undergoing gonadotropin-releasing hormone (GnRH) antagonist stimulation protocols, normal body mass index, and women who have a regular menstrual cycle. On the other hand, the exclusion criteria included tobacco smoking (cigarette or water pipe), alcohol drinkers, diabetes mellitus, women using an oral contraceptive, women suffering from endocrine abnormality, and endocrine disorders (polycystic ovarian syndrome, history of ovarian surgery, and endometrioma). However, the male partner exclusion criteria were diabetes mellitus, alcohol drinkers, smokers, the presence of anti-sperm antibodies, varicocele, Y chromosome microdeletions, Karyotype abnormalities, males subjected to surgical operation in the reproductive system, abnormal hormonal parameters, and abnormal body mass index. The medical records were used by the researcher 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-hCG. The participants were divided into three groups depending on males' age: (age < 30; n = 50 "21.8%", age = 30-40; n = 90 "39.1%", and age > 40; n = 90 "39.1%").

Ethics approval and consent to participate

This study was approved by the Health Research Council, Palestinian Territories (Reference. Number 03/10), and consent was provided according to the Declaration of the Helsinki Committee. The samples were analyzed according to the guidelines and standard procedures of the Al Basma Fertility Center, Palestinian Territories. All participants signed an informed approval form to participate in this study.

Ovarian stimulation and embryo transfer

All women included in the present study underwent ovarian stimulation by using GnRH antagonist protocols with a recombinant FSH (r-FSH). Briefly, the 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 (AFC). The basal levels of estradiol (E2), follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), and anti-müllerian hormone (AMH) 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 human chorionic gonadotropin (hCG) administration. When at least 3 follicles were ≥ 18 mm, ovulation was triggered with hCG. The oocyte pickup was scheduled for 33–36 hours after the administration of 5000 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 3 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 beta-hCG hormone level reaches > 5 mIU/mL.

Semen collection and sperm purification

At the time of the ICSI cycle, the semen samples were collected by masturbation after three days of abstinence from sexual intercourse. The semen samples were allowed to liquefy for 30 minutes at 37 Celsius degree. Then, the count of spermatozoa was evaluated immediately by using a Makler counting chamber (Sefi-Medica, Haifa, Israel). The semen parameters were analyzed according to the World Health Organization guidelines (37). All samples underwent the somatic cell lysis buffer (SCLB) protocol to remove the somatic cells and other debris from the sample before the 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 500×g for 20 minutes at 22 Celsius degree. After that, the pure spermatozoa were incubated with SCLB on ice for half-hour and then washed three times with phosphate-buffered saline (PBS), then centrifuged at 500×g for 10 minutes (38, 39). Finally, the microscopic examination was used to prove the 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 by using the in situ cell death detection kit following the guidelines of the manufacturer company (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 2 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 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 Celsius degree overnight in the dark. Negative controls without the TdT enzyme were run in each replicate. Then, slides were rinsed two-time in PBS and left to dry in the air followed by adding 25 µl of 5 µg/ml DAPI stain solution to each slide as a counterstain and then covered by coverslips. 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)

The chromomycin A3 (CMA3) staining was used to evaluate the chromatin non-condensation in human spermatozoa. Briefly, three semen smears were prepared from each sample and all smears were fixed by using a fixative solution (methanol-glacial acetic acid, 3:1 respectively) at 4 Celsius degrees for 20 minutes. The 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. The phosphate-buffered saline (PBS) was used to wash all the slides, then the slides were mounted with 1:1 (v/v) glycerol/PBS incubated

overnight at 4 Celsius degrees. 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 the human spermatozoa. In the first, 600 µl of lysis buffer was added to the 200 µl of pure spermatozoa; then, the mixture was vortexed for 15s. After that, all the lysate was transferred to the DNA column and centrifuged for 1 minute at 14,000 g. All the procedures were carried out according to the guidelines of the manufacturer company (Bioline, UK). The purity and the concentration of isolated nucleic acid were assessed using a Nanodrop spectrophotometer-2000c (Thermo Scientific, USA), to ensure that the quantity and quality of isolated DNA are 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 company (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 Celsius degree (the blank, a positive and negative control have been used in triplicate during this assay). After washing the microwell four times, the methylated DNA capture solution was added to each well and incubated at 22 Celsius degree for 1 hour. After that, the 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 6 minutes, and at the end of this time, the stop solution was added. The 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].

Statistics Analysis

All the data were analyzed using IBM SPSS for Windows software package version 24.0 (SPSS, Inc., Chicago, IL, USA). The skewness test, kurtosis test, and Shapiro test were used to investigate the type of data distribution. Kruskal–Wallis (H-test) and Mann-Whitney (U-test) were applied to compare the quantitative variables between the study groups. The Spearman rank correlation coefficient was used to study the association between paternal age and other clinical parameters. The results in the above-mentioned procedures were accepted as statistically significant when $P \leq 0.05$.

Results

Clinical parameters and ICSI outcome among different age groups

As shown in Table 1, a significant difference has been found in the age of males included in this study ($P < 0.001$). However, no significant difference has been found among the women's age ($P = 0.676$). A significant reduction was found in the sperm count, percentage of total sperm motility, progressive motility, and non-progressive motility among the different age groups ($P < 0.001$). A significant increase has been observed in the 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 percentages of sperm normal and abnormal forms (P = 0.013). The results showed significant variations among the different age groups in the 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 has been noted with increasing age (P = 0.009). On the other hand, a significant increase was found in the level 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

As indicated in Table 2 a negative significant correlation was found 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 the paternal age. Conversely, a significant positive association has been found between the semen volume (r = 0.220, P < 0.001), percentage of non-motile sperm (r = 0.391, P < 0.001), level of sperm chromatin decondensation (r = 0.423, P < 0.001), sperm DNA fragmentation (r = 0.391, P < 0.001), global DNA methylation level (r = 0.321, P < 0.001), and the advanced paternal age.

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

Table 3 revealed a significant increase was found 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 value of ß-hCG (P < 0.001) in pregnant compared to non-pregnant women. Additionally, a significant decline has been found in the level of sperm chromatin condensation (Figure 4), sperm DNA fragmentation (Figure 5), and global DNA methylation level (Figure 6) in the partners of pregnant women compared to the partners of non-pregnant women (P < 0.001).

Correlation between the paternal age and clinical parameters of the pregnant women

As illustrated in Table 4 a significant positive correlation has been found between the level 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 pregnant women group. On the other hand, No significant associations have been found between the ICSI outcomes and the male partner's age in the pregnant women group.

Discussion

Nowadays, large attention has been paid to studying the impact of paternal age on ICSI outcome and fertilization rate. Several studies reported that paternal age is contributing negatively to semen production, fertility, pregnancy outcome, and ICSI outcomes (42-44). Other studies showed that paternal age is linked to genetic and epigenetic abnormalities in spermatozoa (18 45, 46). In this study, we assessed the potential effect of paternal age on ICSI outcomes and global DNA methylation in human spermatozoa. The present study revealed a significant reduction in sperm count, sperm total motility, progressive motility, non-progressive motility, and other semen parameters among 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 match with other studies that found a significant decline in sperm motility and fecundity status in males age older than 40 years compared to other males who are 35 years old or less (50-52). In contrast, other studies showed no significant differences in semen volume, sperm concentration, sperm motility, and morphology between the different paternal age groups (35, 53). On the other hand, previous studies found no drastic effects on the semen parameters of healthy men or proven fertility men 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.

A significant increase has been found in the level of global DNA methylation, sperm chromatin decondensation, and sperm DNA fragmentation in the older man group compared to younger men. These results support the findings of other previous studies that showed an increase in the level of global DNA methylation (56) and sperm DNA fragmentation in elder males (57, 58). Other previous studies reported that paternal age is associated with hypermethylation globally (2, 59). In addition, another study showed an increase in global 5-methylcytosine levels in the spermatozoa that were obtained from men after 9–21 years from the first samples and an association between age and 5-methylcytosine in sperm (2). A study proposes 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 and his colleagues investigated the DNA methylation in the sperm of 17 men collected 9–19 years apart and found 139 regions that became significantly hypomethylated and 8 which became hypermethylated with 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 the older 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 the age group < 30 years old (1, 63,64). Other studies could 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 older men at age 41 years old 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 old (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 beta-hCG. Similar studies support these findings and suggest that paternal age impacts birth outcomes (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 matching 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 matching with the results of other studies (64, 75). This study supported the findings of a previous study that found a strong correlation between the 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 exhibited that the advancing paternal age increases the level of global DNA methylation, DNA fragmentation, and chromatin decondensation in human spermatozoa. Additionally, negative associations have been found between advancing paternal age and the basic semen parameters. Consequently, all of these findings may be negatively affect the ICSI outcomes and success rates in couples undergoing ICSI cycles.

Declarations

Funding: No funding was received for this study.

Conflict of interests: The authors have no relevant financial or non-financial interests to disclose.

Availability of data and material: The authors do not have the right to share any data information as per their institution's policies.

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

Clinical parameters	Study Population		Age < 30 (n = 50)		Age 30-40 (n = 90)		Age > 40 (n = 90)		P-Value
	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; P ≤ 0.05: Significant; P > 0.05: non-significant

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

Clinical Parameters	r	P-Value	Clinical Parameters	r	P-Value
Semen Volume (ml)	0.220	< 0.001	**Sperm DNA Fragmentation (TUNEL-positive)**	0.391	< 0.001
Sperm Count (Milli/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; P ≤ 0.05: Significant; P > 0.05: Non-significant

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-Value
	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
Oocytes 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; P ≤ 0.05: Significant; P > 0.05: Non-significant.

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

Clinical Parameters	r	P-Value
Number of Collected Oocytes	-0.035	0.724
Number of Mature Oocytes	0.069	0.484
Number of Fertilized Oocytes	0.066	0.503
Oocytes Fertilization Rate	0.179	0.066
Number of Embryo Cleavage	0.041	0.679
Number of Embryo 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; P ≤ 0.05: Significant; P > 0.05: Non-significant.

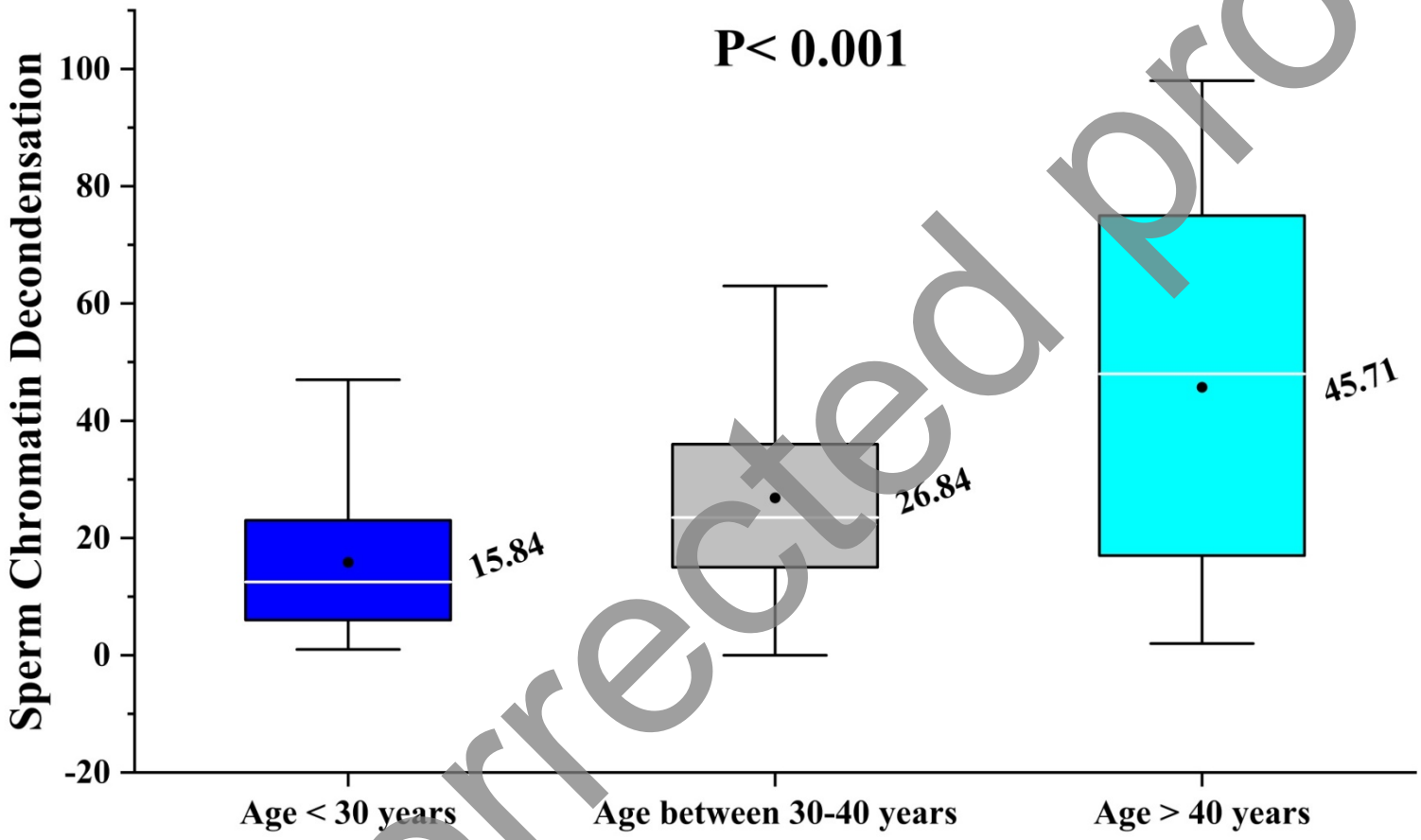

Figure 1. Sperm chromatin decondensation among the different paternal age groups; P ≤ 0.05: Significant, P > 0.05: non-significant

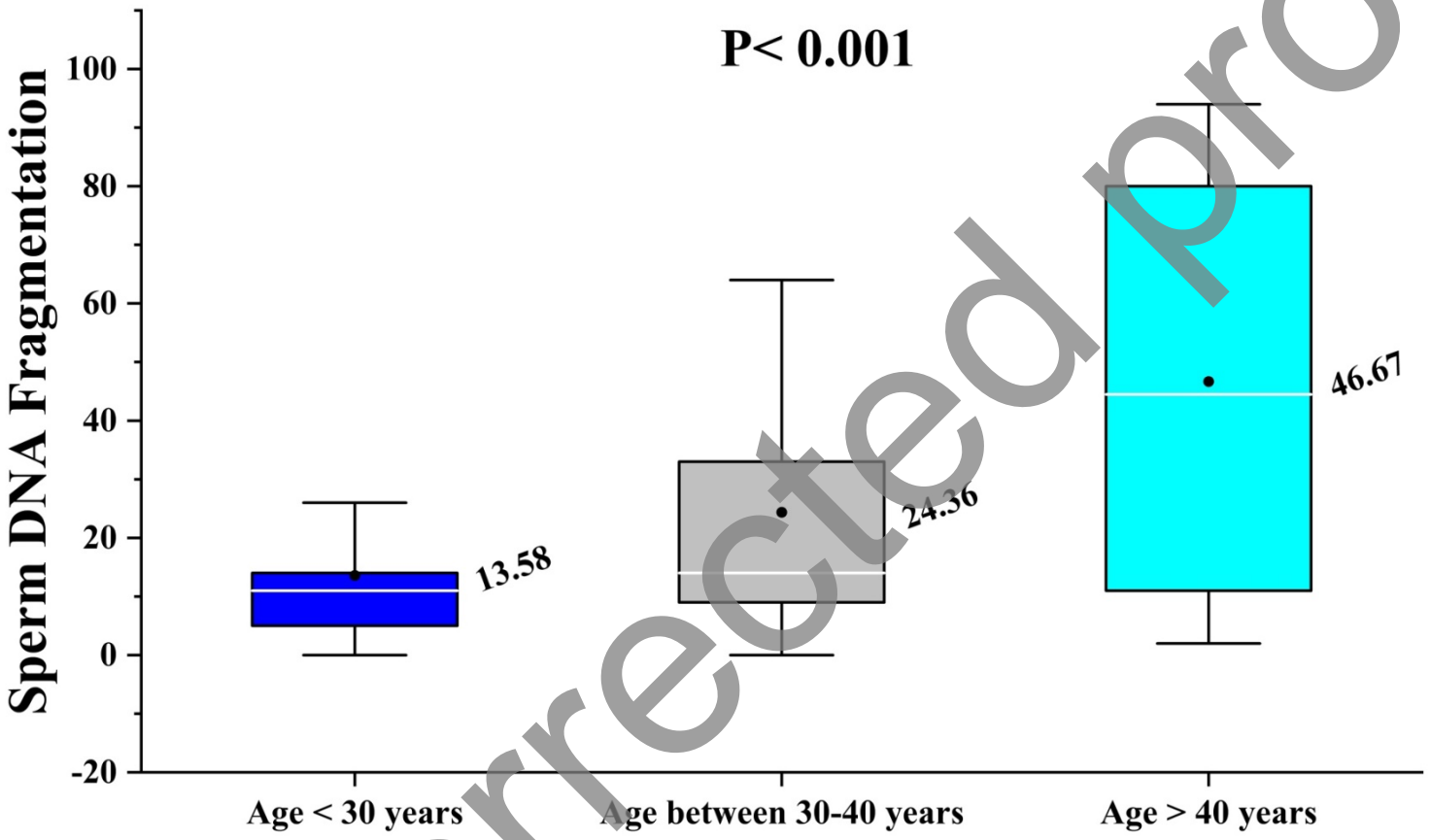

Figure 2. DNA fragmentation in human spermatozoa among the different paternal age groups, P ≤ 0.05: Significant, P > 0.05: non-significant

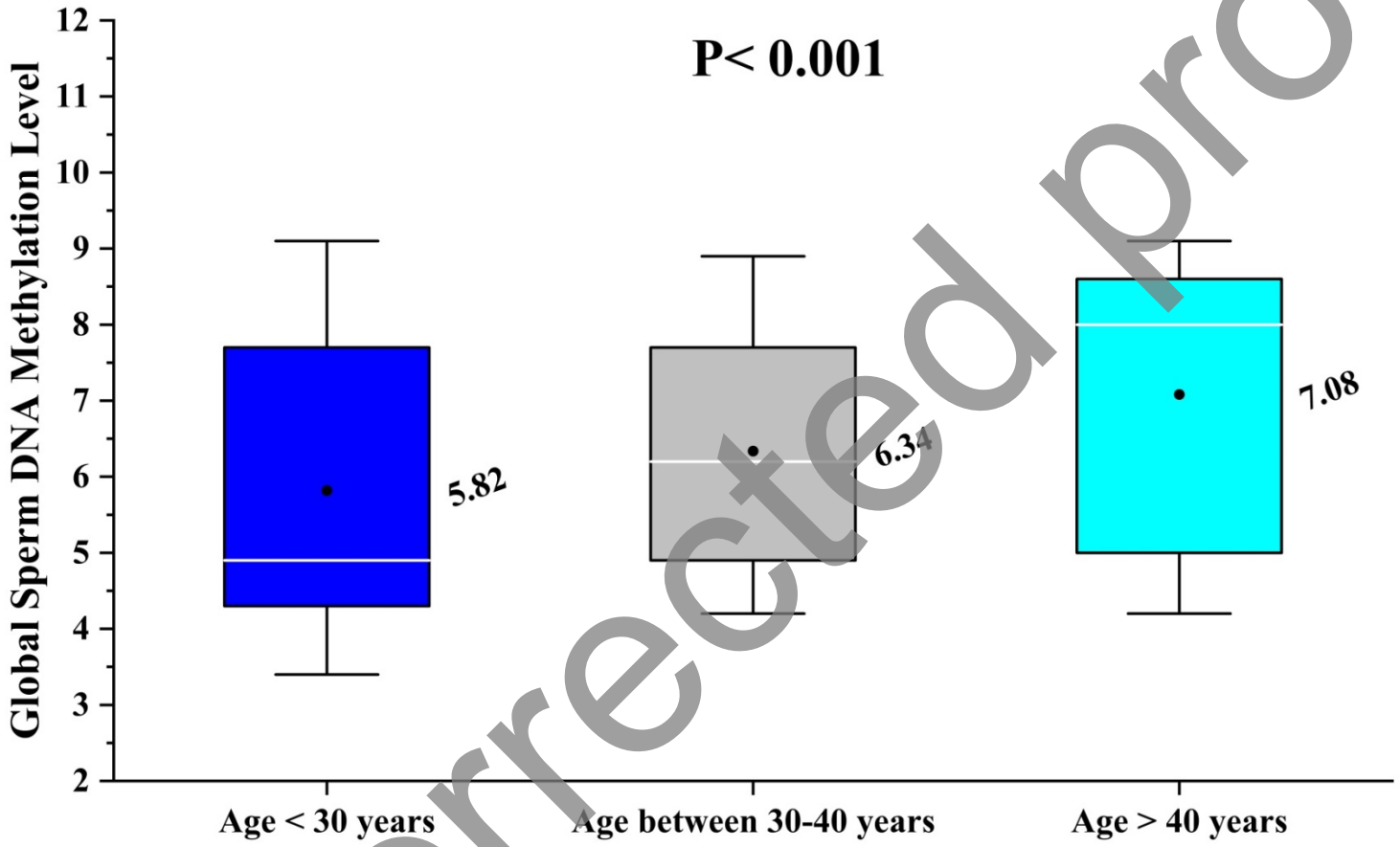

Figure 3. Global DNA methylation level at human spermatozoa among the different paternal age groups, $P \leq 0.05$: Significant; $P > 0.05$: non-significant

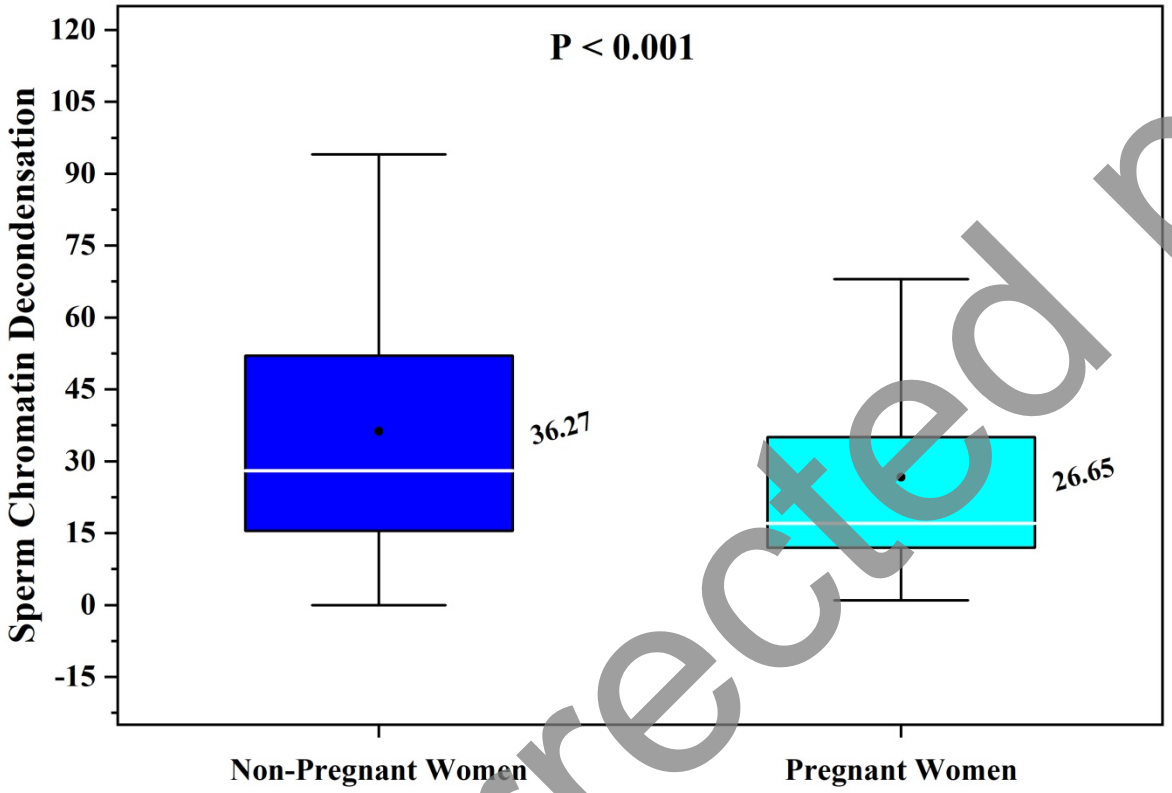

Figure 4. Sperm chromatin decondensation in male partners of pregnant women compared to the partners of non-pregnant women, P ≤ 0.05: Significant, P > 0.05: non-significant

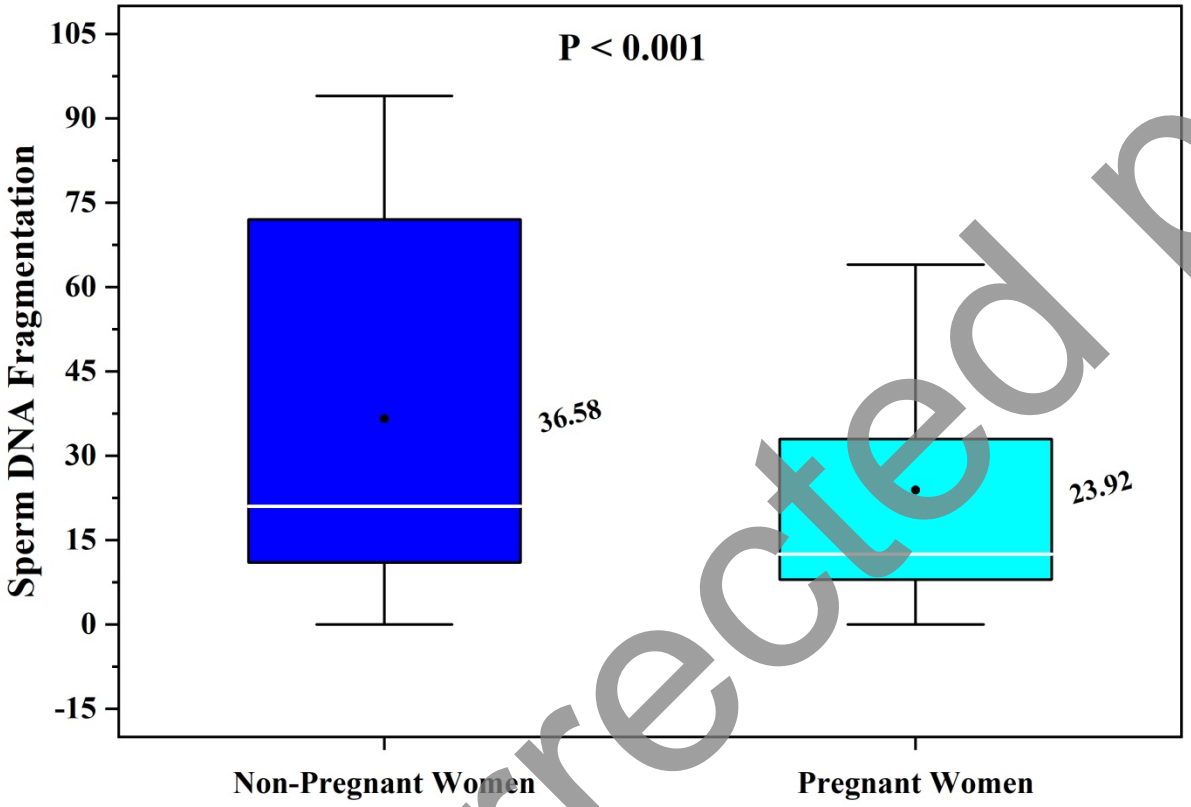

Figure 5. Sperm DNA fragmentation in male partners of pregnant women compared to the partners of non-pregnant women, $P \leq 0.05$: Significant, $P > 0.05$: non-significant

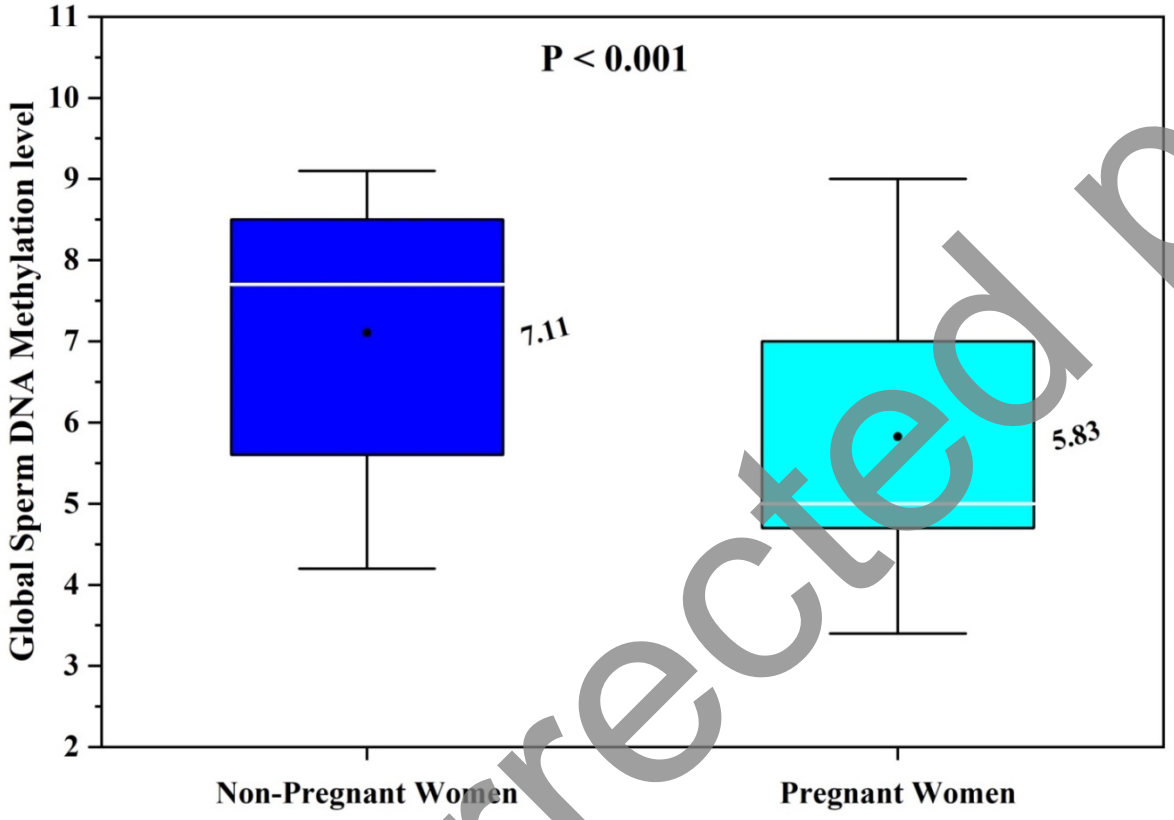

Figure 6. Global spermatozoa DNA methylation level in male partners of pregnant women compared to the partners of non-pregnant women, $P \leq 0.05$: Significant, $P > 0.05$: non-significant.