

Quantitation by image analysis of global DNA methylation in human spermatozoa and its prognostic value in in vitro fertilization: a preliminary study

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Objective: To determine the relationship between sperm DNA methylation level and sperm characteristics and pregnancy rates.

Design: Prospective study. Quantitation by image analysis of DNA methylation in sperm nucleus.

Setting: Department of Reproduction Biology, Edouard Herriot Hospital, Lyon, France.

Patient(s): Infertile couples undergoing IVF-ET.

Intervention(s): The immunostaining of 5 methyl-cytosine was performed on the spare sperm suspension that was used for an assisted reproduction technology procedure.

Main Outcome Measure(s): Sperm characteristics according to World Health Organization criteria, sperm motility parameters with computer-assisted semen analysis, sperm DNA methylation level, and heterogeneity index (HI).

Result(s): Sperm DNA methylation level and HI are correlated with sperm DNA characteristics. HI is negatively correlated with fertilization rate; sperm DNA methylation level is correlated with pregnancy rate.

Conclusion(s): The DNA methylation level in human spermatozoa could be a new approach to evaluating the ability of spermatozoa to fertilize and lead to normal embryo development. (Fertil Steril® 2003;80:947–53. © 2003 by American Society for Reproductive Medicine.)

Key Words: Human sperm, DNA methylation quantitation, image analysis, CASA, fertilization

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Differential gene expression in germ cells is regulated at the transcriptional level by several mechanisms, including specific protein-DNA interactions and changes in chromatin structure. One change in chromatin structure that often precedes the activation of tissue-specific gene expression is demethylation (1). During the later stages of spermatogenesis and epididymal maturation, sperm chromatin undergoes a gradual condensation in preparation for transport to the egg (2). Consistent with this, dynamic series of changes in DNA methylation have been observed during spermatogenesis in the mouse (3).

Studies examining spermatogenesis-specific genes have shown that the acquisition of the appropriate pattern of DNA methylation by the sperm genome may represent a critical facet of sperm maturation (4, 5). This DNA

methylation occurs during spermatogenesis but also during epididymal transit (5). Indeed, mammalian sperm DNA is reported to have a 5-methyl-cytosine (5MC) content lower than that in somatic cells from the same species (6), but higher than that in premeiotic germ cells (7).

The importance of DNA methylation to male germ cell development is demonstrated by the effect of 5-azacytidine on spermatogenesis. This drug is incorporated into DNA during replication and blocks DNA methylation (8), resulting in lowered numbers of condensed spermatids and spermatozoa in the testes and epididymis and abnormal preimplantation development (9). Moreover, in cloned embryos, it has been suggested that phenotype abnormalities could be related to defects in the global DNA methylation (10).

Sperm DNA integrity is essential for accurate transmission of genetic material to offspring. Male factors are involved in about 60% of infertility cases, alone or in conjunction with female factors. The maturation status of spermatozoa can be defined by the motility (flagellum maturity), the acquisition of zona-binding receptors (membrane maturity), the acrosome maturation, the protamine content (nuclear maturity), and other factors—mainly nuclear—that need to be more precisely defined with regard to sperm fertilizing capacity.

The aim of this study was to assess the impact of sperm methylation status in IVF success in terms of both fertilization and implantation rates. This novel procedure involves immunostaining of 5MC, which can be quantified by image analysis and thus provides an objective estimation of global DNA methylation.

MATERIALS AND METHODS

Patients

Semen samples ($n = 23$) were obtained from men undergoing IVF at the Laboratory of Reproduction Biology, Edouard Herriot Hospital, between October 1997 and April 1998. All patients had previously given their informed consent for the study. Embryos generated by intracytoplasmic sperm injection (ICSI; four cases) and conventional IVF (c-IVF; 19 cases) were included in the study. Semen samples were collected by masturbation after 3 days of sexual abstinence. Spermatozoa were selected on a discontinuous three-layer Percoll gradient as described elsewhere (11).

IVF Procedures

All men were the partners of women with fallopian alterations only. Semen characteristics were classified according to World Health Organization criteria (12). Ovarian stimulation was carried out using a GnRH agonist in a long protocol and human gonadotropins (recombinant FSH).

Ovum retrieval was carried out 36 hours after injection of 10,000 IU hCG. The ETs were performed 48 hours after ovum retrieval if at least one embryo was obtained.

The embryos were classified according to their morphology. Classification was as follows, grade A: no fragment and four regular cells; grade B: less than 25% of fragments; grade C: between 25% and 50% of fragments; and grade D: more than 50% of fragments (13). The grade A and grade B embryos were pooled and classified as "good embryos."

Analysis of Sperm Motility

Motility analysis was performed with the ATS 20 computer-assisted semen analysis (CASA) system (J.C. Diffusion International, Granville, France). A 6- μ L sperm suspension was placed in a semen analysis chamber (m-cell; Fertility Technology, L'Aigle, France). The following motility parameters were measured: curvilinear velocity (VCL),

straight-line velocity (VSL), linearity (LIN), and amplitude of lateral head displacement (ALH).

Immunostaining for DNA Methylation Analysis

Spermatozoa were cytospun. Cytoцентрифугed slides were prepared using a Cytospin (Shandon-Southern Product, Runcorn, UK) at 750 rpm for 8 minutes, air-dried for 12 hours, and frozen at -20°C until use. Several experiments were performed to test successive steps of the staining procedure.

For fixation, methanol/acetic acid (3/1) (Merck, Darmstadt, Germany) at $+4^{\circ}\text{C}$ for 20 minutes was used. Slides were washed twice in phosphate-buffered saline with Tween 0.5% (Sigma, St. Louis, MO) (PBS-T) for 5 minutes at room temperature (RT). For sperm DNA decondensation, slides were incubated at RT in 1 M hydrochloride acid (HCl), Tris buffer, pH 9.5 (Merck), containing 25 mM dithiothreitol (Sigma) for 20 minutes. The slides were then washed twice in PBS-T for 5 minutes.

To ensure methylated DNA was accessible to the methylated base, the sperm DNA was denatured with HCl (6N) for 15 minutes. The slides were washed with Borax (3.8 g/100 mL) (Sigma) for 10 minutes and with PBS-T for 5 minutes. Slides were then incubated with mouse anti-5MC antibody (14) diluted 1:10 in PBS-T for 20 minutes at RT and washed twice in PBS-T for 5 minutes. Controls consisted of slides incubated with buffer instead of the primary antibody.

Slides were further incubated with an anti-mouse biotinylated antibody for 10 minutes, washed twice in PBS-T, and incubated with streptavidin conjugated to horse radish peroxidase for 10 minutes (UltraVision Kit, Lab Vision, Fremont, CA). They were then washed twice in PBS-T. Immunostaining was visualized by diaminobenzidine (DAB), with an incubation time of 10 minutes. Slides were mounted in anhydrous glycerol.

Digital Image Analysis of 5MC Staining

Image analysis was performed with a Quantimet 600 (QTM 600) (Leica, Cambridge, UK) fitted to a color tri-CCD camera (DX930P; Sony, Tokyo, Japan). This camera possesses a minutely controlled electronic shutter speed manual speed selection (1H or 1 frame step). The charged coupled device (CCD) was a high-density three 1/2-inch IT Hyper HAD sensor that allowed a high horizontal resolution of 720TV lines. The lux sensitivity went from 5.0 to 2,000 lux. The signal-to-noise ratio was 56dB.

Immunostained slides were observed under a microscope (DMRXA, Leica) using a $40\times$ plan achromat objective (numerical aperture 1.3). Microscope stage and focus were motorized and controlled by software. In this way, the fields of views for which measurements were taken were systematically sampled.

For each field, the focus was adjusted to visualize the head and tail of spermatozoa and glare correction was done.

Segmentation was done on the color image, and quantitation of immunostaining was done through the blue channel of the color camera for 5MC staining. This allowed us to obtain the same images as would otherwise be acquired using a specific filter.

Correction for uneven illumination was performed with the shading correction procedure included in the QTM 600 software. For this we used a blank image corresponding to a region of the slide with an empty field of view. The system was calibrated using neutral-density filters of known absorbencies. The gray levels were converted automatically to optical density (OD). After image acquisition and correction, a threshold level of gray was established to detect sperm cells.

At least 200 spermatozoa were analyzed for each slide. Artifacts and disrupted nuclei were rejected by setting conservative limits on the object area and shape. Overlapping cells were separated using a QTM 600 segmentation algorithm based on morphological filters. An interactive step was included to improve automatic segmentation results.

Total 5MC staining was evaluated by mean optical density (MOD) and integrated optical density (IOD) in arbitrary units (a.u.). The MOD reflected the concentration of 5MC, and the IOD reflected the total quantity of 5MC and was thus called the DNA methylation level. As the sperm population was heterogeneous, the coefficient of variation (CV) of DNA methylation level was calculated as a percentage and called the heterogeneity index (HI).

Statistics

Statistics were compiled using Unistat software (Unistat, London). To take account of the small sample size, nonparametric tests were used. Data are presented as medians. Correlations were calculated by the use of Spearman's correlation coefficient; the χ^2 or Fisher's exact probability test were used to compare the percentages; and the Mann-Whitney test was used to compare the variables in different groups. $P < .05$ was considered statistically significant. The statistics tests were used only when the size of the group was sufficient to provide consistent data.

RESULTS

The determination of the suitable dilutions of spermatozoa and the use of cytospin allowed us to solve the problem of overlapping. Sperm dispersion on the slide, with the use of the motorized stage, made fast image quantitation possible.

Impact of HCl Exposure

To study the impact of time of HCl exposure, various incubation times were tested: 15, 30, 45, and 60 minutes. The MOD was measured at different times and repeated three times. The MOD reflects the amount of 5MC in the nucleus independent of the area. A high molarity (6N) of

TABLE 1

MOD (mean \pm SD) and CV (%) of 5 methyl-cytosine staining according to various HCl exposure times.

	15 minutes	30 minutes	45 minutes	60 minutes
MOD (a.u.)	0.70 \pm 0.12	0.70 \pm 0.13	0.68 \pm 0.17	0.69 \pm 0.14
CV (%)	17.1	18.6	25	20.3
P	NS	<.001	<.001	NS

Note: For 15 and 60 minutes, there was no difference according to the repetition of the experiments. MOD = mean optical density; a.u. = arbitrary unit; CV = coefficient of variation; NS = not significant.

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HCl was necessary, since with lower concentrations, no staining was obtained.

Using these protocols, all samples showed a normal sperm morphology (head and tail). Without dithiothreitol, no staining was obtained no matter what the time of HCl exposure was.

The measurements were reproducible for 15 and 60 minutes of HCl exposure ($P=NS$). For the other times, the reproducibility was not obtained ($P<.001$) (Table 1). To appreciate the accuracy of the measurements, the CV was calculated. The accuracy of the MOD measurements, illustrated by the CV, increased with time of exposure (Table 1). After 30 minutes of HCl exposure, the depurination was too great and a decrease of DNA methylation appeared (Fig. 1). The best result was obtained with 15 minutes of HCl exposure, where the MOD had the greater value (0.7) and the CV (17%) had the lowest value.

Impact of DAB Time Incubation

To obtain precise measurements, it is necessary to saturate the DAB reaction. Various incubation times (10, 15, 30 minutes) were therefore tested to obtain the time for allowing maximum measurement accuracy. For the MOD value, there is no difference among the various times of DAB incubation.

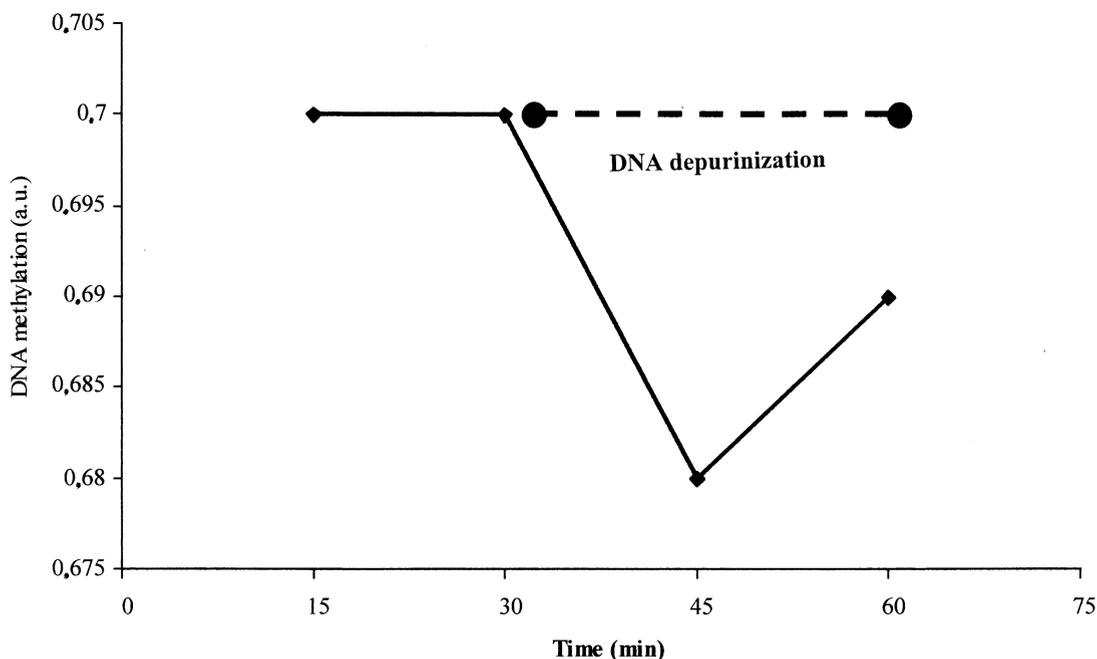
Overall Data

The medians for paternal and maternal ages were 35 and 34 years old, respectively. From the 23 couples involved in the study, the median number of oocytes retrieved and inseminated was 10 (range, 3–32). The median fertilization rate was 50% (range, 0%–100%). Fertilization rates $>50\%$ were achieved in 13 cases (56.5%), fertilization rates $<50\%$ were recorded in 6 cases (26.1%), and in 4 cases no eggs were fertilized (17.4%).

A total of 117 embryos were obtained and 43 were transferred. In five cycles (21.7%) (four due to the absence of fertilization and one because of abnormal embryos) ET could not be done, whereas in the majority of cases, 2 or 3 embryos were transferred (39.1% and 34.8%, respectively).

FIGURE 1

Evolution of DNA methylation according to the time of HCl exposure. For 15 and 30 minutes, the measurements are stable; from 45 minutes depurination of DNA appeared.



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A pregnancy was obtained in 26.1% (6/23) of cycles, which led to five singleton deliveries and one miscarriage.

Sperm DNA Methylation Level According to Semen Characteristics

The median DNA methylation level was equal to 108.2 a.u. (range 62.8–181.6 a.u.). The global DNA methylation level was decreased in the asthenospermia and teratospermia group (102.6 vs. 120.1 a.u.; $P=NS$; 98.9 a.u. vs. 121.1 a.u.; $P<.05$, respectively).

In the oligospermia group, the global DNA methylation level was 116.4 a.u. vs. 108.1 a.u. for normal group (NS).

The HI was always higher in the group with an alteration, but the difference was statistically significant only in the asthenospermia group (Table 2).

For the parameters obtained with CASA, no significant correlation was found with DNA methylation level ($r = -0.03$ for VCL, $r = -0.13$ for VSL, $r = -0.13$ for LIN, and $r = -0.11$ for ALH) and with heterogeneity ($r = -0.18$ for VCL, $r = -0.04$ for VSL, $r = -0.0$ for LIN, and $r = 0.21$ for ALH).

Sperm DNA Methylation and IVF Results

No correlation was found between sperm DNA methylation level and paternal age ($r = 0.28$, NS). IVF data were

TABLE 2

DNA methylation level (median) according to the sperm characteristics.

	Sperm		Oligospermia		Asthenospermia		Teratospermia	
	Normal (n = 10)	Altered (n = 13)	No (n = 17)	Yes (n = 6)	No (n = 13)	Yes (n = 10)	No (n = 18)	Yes (n = 5)
DNA methylation level (a.u.)	121.1	106.3	108.1	116.4	120.1	102.6	121.1 ^a	98.9 ^a
DNA methylation HI (%)	29.9 ^a	37.8 ^a	32.8	34.9	30.6 ^a	38.5 ^a	33.5	39.0

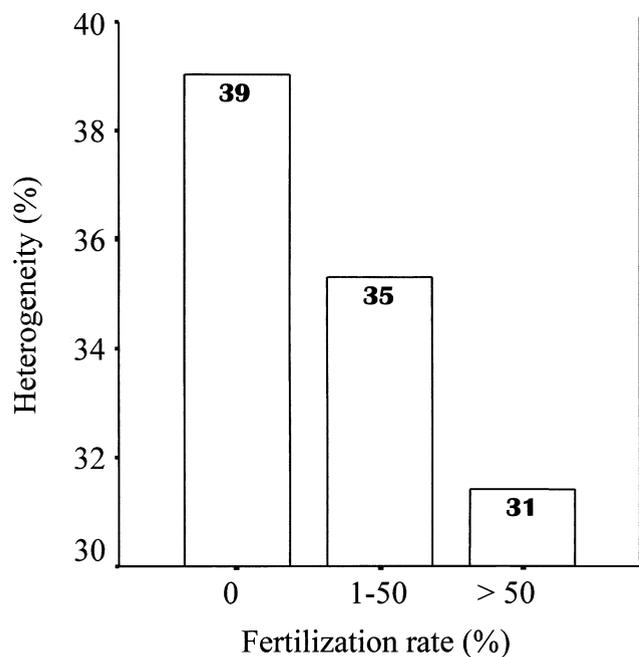
Note: a.u. = arbitrary unit; HI = heterogeneity index.

^a $P<.05$ (unilateral test).

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FIGURE 2

Difference in DNA heterogeneity of sperm samples according to the fertilization rate.



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divided into three groups: group 1: samples with >50% fertilization; group 2: samples with <50%; and group 3: samples with no fertilization at all.

The sperm DNA methylation levels were 123.3, 103.6, and 106.3 a.u. for groups 1, 2, and 3, respectively. The difference was not significant. The proportion of good-quality embryos was higher when the sperm DNA methylation level was higher than 90 a.u. (76% vs. 56%), but the difference was not significant.

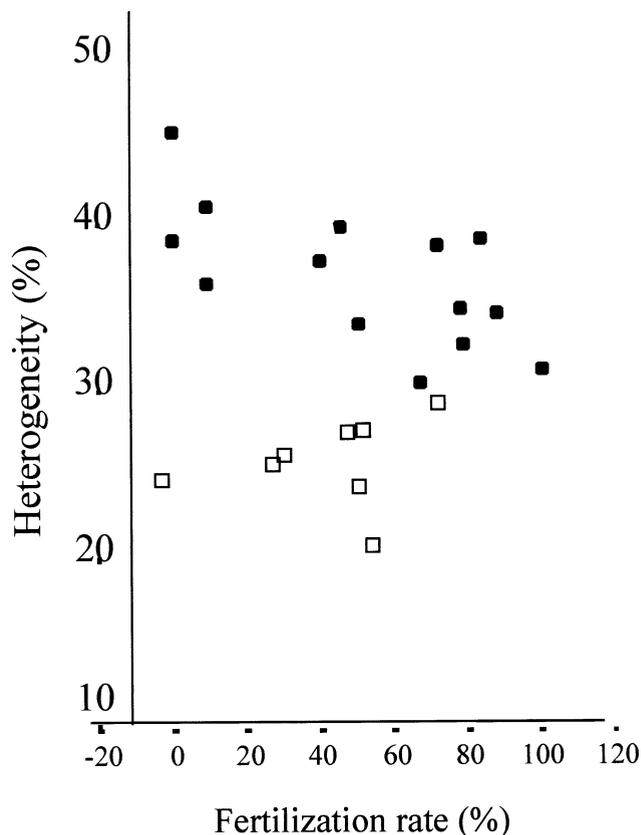
Pregnancy data were divided into three groups: group 1: pregnancy; group 2: no pregnancy after ET; group 3: no pregnancy because no embryo to transfer. The sperm DNA methylation levels were 138.7, 108.1, and 108.2 a.u. for groups 1, 2, and 3, respectively.

The HI decreased as the fertilization rate increased and was equal to 31%, 35%, and 39% in groups 1, 2, and 3, respectively (Fig. 2). When the sample was separated into two groups according to a cutoff of the HI of 30%, 8 patients exhibited an HI smaller than 30% and 15 patients exhibited an HI greater than 30%. For this last group only, there was a significant negative correlation between HI and fertilization rate ($r = -0.55$, $P < .05$) (Fig. 3).

Because of the small size of the sample, no statistic on pregnancies was available. When no transfer could be performed, the median DNA methylation level was equal to

FIGURE 3

Dispersion of DNA heterogeneity according to a cutoff of 30% compared with the fertilization rate (■ >30%, □ <30%).



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108.2 a.u. for this study, it was equal to 108.1 a.u. when no pregnancy was obtained and 138.7 a.u. when pregnancy was obtained (Fig. 4). However, these values were not significantly different. For the HI, when a pregnancy was obtained, the median was low (39.2%) and higher when no pregnancy or no transfer occurred (41.1% and 45.5%, respectively).

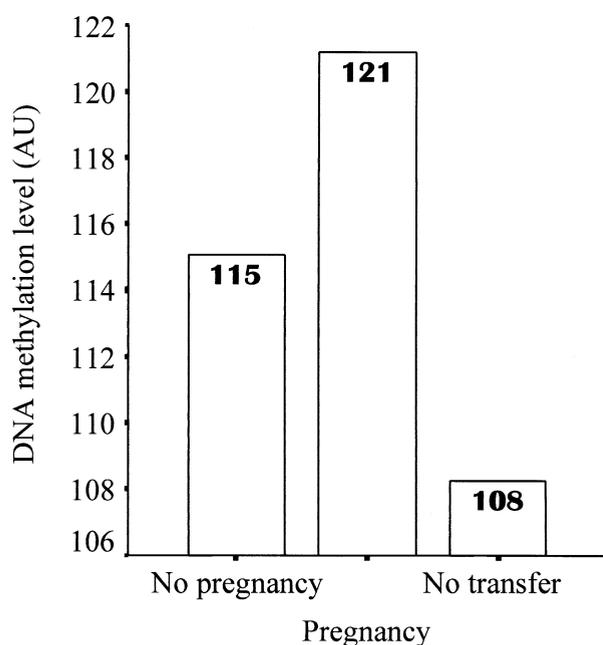
DISCUSSION

The aim of this study was to develop a technique that enables an accurate and rapid assessment of global DNA sperm methylation level to compare different sperm samples involved in an IVF program. Our results indicate that quantitation with image analysis is a useful tool in the assessment of DNA methylation and enables objective measurements to be made easily. However, before quantifying by image analysis, it is important to possess an accurate staining protocol.

We have developed a method for quantitating immunocytochemical staining of sperm cells by image analysis. This approach to quantitation of DNA methylation offers an ad-

FIGURE 4

DNA level (median) according to the IVF results.



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vantage over existing techniques, such as DNA blot and high pressure liquid chromatography, in that an assessment can be made very quickly. Another advantage of this technique is that it enables a quantitation of DNA methylation while still preserving the integrity of sperm morphology. Furthermore, this procedure does not require extra laboratory hardware other than an image analyzer, which can be used in conjunction with standard laboratory equipment.

Results show that the DNA methylation level of altered sperm samples, such as asthenozoospermia and teratozoospermia, was lower than in the normal ejaculates. This result is in contradiction with Manning et al. (15), which did not find any difference in the methylation pattern according to sperm characteristics. However, this work was done on few imprinting genes and did not concern global DNA methylation. As it was shown in the mouse that the methylation level increases during the epididymal transit (5), our data may indicate that in poor-quality sperm samples, many spermatozoa constituting the ejaculate are in some way immature. This immaturity could lead to fertilization failures if those spermatozoa are used in an IVF procedure.

In our study, when no fertilization occurred, the DNA methylation level was low, but as soon as at least one embryo was obtained, there was no difference in DNA methylation level whether the rate of fertilization was high (>50%) or low (<50%). The effect of methylation factor

was more evident if one did not consider the level of methylation by itself, but with the HI, reflecting the dispersion of the factor. A low heterogeneity was associated with a high fertilization rate, and for sperm samples exhibiting a high HI (>30%), this index was negatively correlated to the fertilization rate. The higher this index, the more heterogeneous the sperm population; therefore, the probability of the egg being fertilized by an immature sperm is increased.

A high sperm DNA methylation level seems to be related to an increase in the percentage of good embryos; however, this relationship was not statistically significant due to the lack of the power of the test in this preliminary study.

The pregnancies in this study were achieved when the DNA methylation level was high and the heterogeneity low. So the DNA methylation level and the HI seem to be the male prognostic factors important for an IVF attempt.

The influence of male factors in the process of fertilization and early development may be observed at two levels: [1] if the sperm DNA damage is too great male pronucleus formation and thereby syngamy may be impaired, leading to fertilization failure; [2] for lower levels of DNA alterations, fertilization will be possible at the first embryo divisions, since these are controlled by the maternal transcripts. However, further development of the embryo, corresponding to an activation of the paternal genome at the 6- to 8-cell stage in humans, will be impaired because of DNA damage or immaturity.

These two effects are illustrated in our study, where very abnormally low methylation levels were correlated with fertilization failure and high methylation levels, reflecting good DNA maturation, were correlated with pregnancies.

Therefore, the measurement of DNA methylation level in human spermatozoa could represent a new approach to appreciate the ability of spermatozoa to fertilize and lead to normal embryo development.

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