Influence of global sperm DNA methylation on IVF results

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BACKGROUND: In cases of male infertility, routine analysis for sperm characteristics is a poor predictive factor for the segmentation rate and embryo development in assisted reproductive technologies. It is assumed that epigenetic factors could have an influence on the embryo's quality. The aim of this work was to determine the relationship between sperm DNA methylation level and fertilization and pregnancy rates according to the assisted reproduction technique performed. METHODS: A prospective study was undertaken. Ejaculates were obtained from men ($n = 63$) undergoing an assisted reproduction procedure. 5-Methylcytosine was immunostained with a polyclonal antibody and revealed by fluorescein isothiocyanate. The DNA methylation level was then quantified by flow cytometry. RESULTS: Sixty-three conventional IVF cycles were performed, 760 oocytes were retrieved, an average of 8.1 ± 4.8 embryos was obtained, and 2.4 embryos were transferred. Neither the fertilization rate nor the rate of good quality embryos was correlated with the DNA methylation level ($r = -0.1$ and $r = -0.08$ respectively; not significant). When sperm DNA methylation was >555 arbitrary units, the pregnancy rate was 33.3% compared with 8.3% in the lower (<555) group ($P < 0.05$). CONCLUSION: DNA methylation level in human sperm could represent a new approach to study the ability of sperm to lead to pregnancy in an assisted reproduction procedure, especially when sperm samples with normal characteristics are used.

Key words: flow cytometry/IVF/pregnancy/sperm DNA fragmentation/sperm DNA methylation

Introduction

DNA methylation is an important form of gene regulation during mammalian development and has been implicated in such diverse processes as genomic imprinting (Li *et al.*, 1993), X-inactivation (Beard *et al.*, 1995), and differential gene expression (Eden and Cedar, 1994). This DNA methylation is supported by the cytosine base to obtain the 5 methylcytosine residue (m5c) in CpG dinucleotides.

After zygote formation, the parental alleles maintain their identity so that one allele eventually becomes preferentially expressed. Methylation of CpG dinucleotides is proposed to be one mechanism for differentially marking the parental chromosomes, since methylation can be stably inherited in somatic cells yet can be removed and reset in the next generation according to the parent of origin (Razin and Cedar, 1994; Jaenisch, 1997). The developmental stages prior to blastocyst formation are of particular importance in genomic imprinting (Solter, 1998). Genome-wide, oocyte DNA tends to be hypomethylated while sperm DNA tends to be hypermethylated (Monk *et al.*, 1987). During preimplantation development, the overall level of methylation decreases. Most methylation moieties present on the original parental

chromosomes are removed from the DNA by the morula stage, giving rise to a predominantly unmethylated genome which remains this way at least through blastulation. A wave of *de novo* methylation follows, leading to an overall increase in genome methylation levels as the newly implanted embryo develops and differentiates. Disruption of global methylation patterns is lethal to mammals (Li *et al.*, 1992).

Because male and female pronuclei do not exhibit the same evolution during the first stages post-fertilization (Bouniol-Baly *et al.*, 1997; Haines *et al.*, 2001), the methylation level of male and female gametes may influence the development potential of embryos (Mayer *et al.*, 2000). Thus abnormal methylation level in one or other gamete could explain some implantation failures whereas these gametes were apparently normal. We had evoked this deleterious effect concerning abnormal methylation level in male gametes in a previous work (Benchaib *et al.*, 2003a), on a small group of patients. The influence of sperm DNA methylation on pregnancy was shown on mice by use of 5-aza-2′-deoxycytidine, which incorporated into DNA and led to decreased DNA methylation (Kelly *et al.*, 2003). Moreover,

in altered sperm the imprinting genes seemed to be more altered (Marques *et al.*, 2004).

Recently the conditions of assisted reproduction techniques were suspected of increasing the frequency of some pathologies in relation to genomic imprinting in, for example, Angelman syndrome and Beckwith–Wiedemann syndrome (Gosden *et al.*, 2003). One explanation could be the loss of gene imprinting during preimplantation development under certain culture conditions (Mann *et al.*, 2004). Thereby, the methylation status of the paternal genome may represent an important factor.

The aim of the present study was to assess the impact of sperm methylation level in IVF success, in terms of both fertilization and pregnancy rates. This procedure involves immunostaining of m5c and its quantification by flow cytometry, which together provide an objective estimation of global DNA methylation.

Materials and methods

Patients

The prospective study concerned 63 cycles of conventional IVF performed during a given period (8 months: from April 2002 to November 2002) within the Department of Medicine of Reproduction in Edouard Herriot Hospital, Lyon. Assisted reproduction procedures involving cryopreserved sperm were excluded from this study. ICSI cycles were also excluded in order to avoid bias related to severe sperm abnormalities that are frequent in ICSI-treated patients.

Ovarian stimulation

After 3 weeks of desensitization by GnRH analogues (Decapeptyl®; Ipsen), ovarian stimulation was achieved by recombinant FSH (Gonal F®; Serono; or Puregon®; Organon), and monitored by endo-vaginal echography and plasma estradiol. When the follicles reached a correct diameter, 36 h before oocyte retrieval, 10 000 IU of hCG (Organon) was administered. The oocyte retrieval was carried out under general anaesthesia by a vaginal ultrasonographic-guided aspiration.

Sperm preparation for assisted reproduction

Sperm were prepared using a discontinuous PureSperm® gradient (Nicadon, Sweden). The gradient consisted of three layers of 1 ml of PureSperm: 90, 70 and 50%. On the 50% layer was deposited 1 ml of semen. The gradient was then centrifuged at 300 g for 20 min. After centrifugation, the 90% layer was collected and washed with 5 ml of Ferticult Flushing medium (FertiPro N.V., Belgium) at 600 g for 10 min. The pellet was then resuspended in IVF Medium (Scandinavian IVF, Sweden). The viability in selected sperm was >90% in all the samples.

Embryo culture and classification

Sixteen to 18 h after insemination or microinjection, the oocytes were assessed for fertilization two-pronuclear stage. Forty-eight hours after oocyte retrieval, the embryos were classified according to their morphology. Classification was as follows: grade A, no fragmentation and four regular cells; grade B, <25% fragmentation; grade C, between 25 and 50% fragmentation; grade D, >50% fragmentation (Ebner *et al.*, 2001). The transfer of the embryos took place either at 48 h, or at 72 h, or at the blastocyst stage. When the

transfer was made at 48 or 72 h, the supernumerary embryos were cryopreserved if their morphological states allowed it (grade A or B). If they were not cryopreserved, they were cultivated in sequential medium until the blastocyst stage was reached, and if one or more good quality blastocysts were obtained, those were then cryopreserved. When a transfer at the blastocyst stage was programmed, embryos were cultivated on sequential medium: P-1 Medium (Irvine Scientific, USA) for the first 2 days, Blastocyst Medium (Irvine Scientific) for the last days of culture. After the transfer, the remaining good morphology blastocysts were cryopreserved. A clinical pregnancy was assessed by the succession of three positive plasma β-hCG determinations and ultrasound detection of a fetal heart beat.

Semen sample preparation for DNA methylation study

The detection of DNA methylation level was performed on the spare sperm suspension that was used for the assisted reproduction procedure (the volume was between 50 and 60 μl of selected motile sperm suspension). All patients had previously given their informed consent for the study.

The first protocol described was changed in order to allow an immunostaining with suspension cells (Benchaib *et al.*, 2003a). For fixation, ethanol (70%) (Merck, Germany) at −20;°C for 20 min was used. Cell pellets were washed twice in phosphate-buffered saline with Tween 0.5% (Sigma, USA) (PBS-T) for 5 min at 500 g. For sperm DNA decondensation, cells were incubated at room temperature in 1 mol/l hydrochloric acid (HCl)–Tris buffer, pH 9.5 (Merck), containing 25 mmol/l dithiothreitol (DTT; Sigma) for 20 min. The cells were then washed twice in PBS-T.

To ensure that methylated DNA was accessible to anti-m5c antibody, the sperm DNA was denatured with HCl (6 N) for 15 min. The cell pellets were washed with Tris (1 mol/l, pH 9) (Sigma), then with PBS-T. Pellets were then incubated with mouse anti-m5c antibody (14) diluted 1:10 in PBS-T for 20 min at room temperature and washed twice with PBS-T. Controls consisted of cells incubated with buffer instead of the primary antibody.

Anti-mouse antibodies coupled with fluorescein isothiocyanate were incubated with the pellet for 30 min. The cells were then washed twice in PBS-T, and conserved at +4°C in a dark chamber until quantification with flow cytometer. The immunostaining was confirmed by the visualization of the immunofluorescence in the head of sperm with an epifluorescence microscope.

Semen sample preparation for DNA fragmentation study

For some patients ($n = 13$), both sperm DNA methylation and DNA fragmentation using TUNEL [terminal deoxynucleotidyl transferase (TdT)-mediated dUDP nick-end labelling] were measured. The two analyses were performed on two different sperm preparations, but both originating from the same ejaculate. All patients had previously given their informed consent for the study. The cells were spread out over sialinized slides. Cell fixation was carried out by a methanol/acetic acid mixture (3 volumes/1 volume) for 20 min. The cells were permeabilized with PBS with 1% of Triton ×100 (Sigma). Cells with fragmented DNA were revealed by TUNEL by use of the 'Apoptag plus' Kit (Oncor, France). The cells were all balanced with the 'balanced buffer' and then incubated in a moist chamber at 37°C, for 1 h, with the TdT solution in order to allow DNA elongation. The elongation was revealed by incubation of the cells with anti-digoxigenin antibody coupled to peroxidase, for 30 min, in a dark, moist chamber. The peroxidase was revealed with diaminobenzidine. Counterstaining of the sperm nucleus was performed with Harris's haematoxylin. A positive control was made on positive slides furnished in the Oncor Kit. The cells were observed under a transmission microscope (Zeiss, Germany) with a ×100 oil

objective. The sperm with fragmented DNA had their nuclei stained brown, whereas the other cell nuclei were blue-grey. On each slide, ~500 cells were counted; the percentage of sperm with fragmented DNA were thus determined and the result referred to as the DNA fragmentation index (DFI).

Flow cytometry

The samples were analysed by FACSCalibur (Becton Dickinson, USA). The flow cytometer was equipped with a 488 nm excitation filter and a 530 nm emission filter (the fluorescence 1: FL1) and a 585 nm emission filter (the fluorescence 2: FL2). The values of photomultiplier were linearly set. Green fluorescence (FL1) represents the sperm DNA methylation level, expressed in arbitrary units (AU). As a negative control, sperm were immunostained without the first antibody. Sample tubes were mixed well before commencing acquisition by gently flicking the tube to ensure sample homogeneity with a low level of micro air bubbles. All sample tubes were capped to prevent evaporation. Instrument settings were adjusted so that all events (cells and debris) were observed in the dot-plot diagram. Resolution was of 1024 channels. Forward scatter channel (FSC) detector and side scatter channel (SSC) photodiode were set to linear. Some cellular and non-cellular debris were excluded by adjusting the threshold of the SSC parameter. An area delimiting the region containing the cell population to be sorted was drawn on the dot-plot diagram with the use of the polygonal region

tool of the WinMDI 2.8 software (Trotter, 2000). A total of 20 000 events was analysed for each sample.

Statistical evaluation

Statistical analysis was performed with SPSS for Windows software package version 11.5 (SPSS Inc., USA). The χ^2-test was used to analyse the qualitative parameters. For the comparison of quantitative parameters, Student's *t*-test (with Levene's test) was used. Spearman's correlation coefficients were also calculated.

The optimal threshold value for the methylation level was determined as the value that permitted maximization of the relative risk (RR) of obtaining a pregnancy. The association between this threshold value and IVF parameters was then assessed. Stepwise logistic regression was performed to calculate the significant odds ratio (OR) equivalent to the RR, in order to identify the prognostic parameters in a multivariate analysis. Statistical differences were considered significant at $P < 0.05$.

Results

5-Methylcytosine immunostaining

In order to study the impact of time of HCl exposure, various incubation times were tested: 15, 30, 45 and 60 min. A high molarity (6 N) of HCl was necessary, since with lower concentrations, no staining was obtained. Using these protocols,
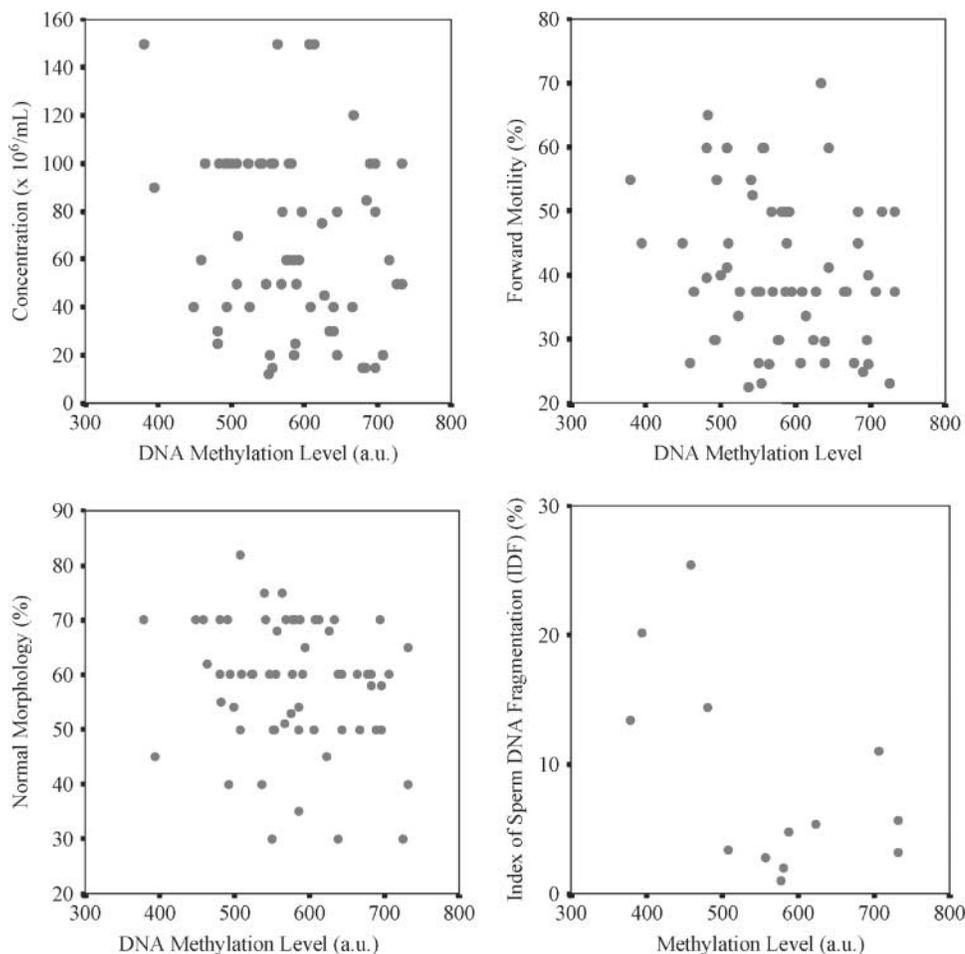
Figure 1. Relationship between sperm DNA methylation level (in arbitrary units: AU) and sperm characteristics: concentration ($\times 10^6$/ml), forward motility (%), morphology (%) and sperm DNA fragmentation index (DFI) (%).

all samples showed a normal sperm morphology (head and tail). Without DTT, no staining was obtained, whatever the length of HCl exposure.

In order to gauge the accuracy of the technique, the measurements were repeated twice for six patients and the coefficient of variation (CV) was calculated. This was $< 10\%$ for all cases.

Methylation and sperm parameters

No association was found between methylation level and sperm characteristics (concentration, motility, morphology) (Figure 1). No association was found between sperm DNA methylation level and DFI ($r = -0.45$; not significant) (Figure 1).

IVF procedure data

The average quantity of FSH administered to the patients was 2655 ± 1169 IU, the average time of stimulation was 12.2 ± 1.1 days and the plasma estradiol value at the day of the puncture was 2848 ± 1588 pg/ml. The mean numbers of oocytes, mature oocytes and embryos were 12.06 ± 6.4, 10.0 ± 5.5 and 8.1 ± 4.8 per attempt respectively. The distribution of embryos according to embryo quality was as follows: grade A, 20%; grade B, 24%; grade C, 16%; grade D, 40%.

The mean age of the women was 34.2 ± 4.3 years. Tubal alterations were found in 41.2% of the cases, and dysovulation in 17.5% of the cases; 22.2% of the women suffered from endometriosis.

The mean age of men was 35.4 ± 5.5 years. In 19.1% of the cases, the sperm used for the assisted reproduction procedure presented minor anomalies according to World Health Organization (1999) standards. Moderate oligozoospermia ($15-20 \times 10^6$/ml) was found in 14.3% of cases, moderate asthenozoospermia (20–25% grade a = b motility) in 4.8% of cases, and no teratozoospermia was found. These anomalies were isolated. The mean value of sperm DNA methylation was 581 ± 83 AU.
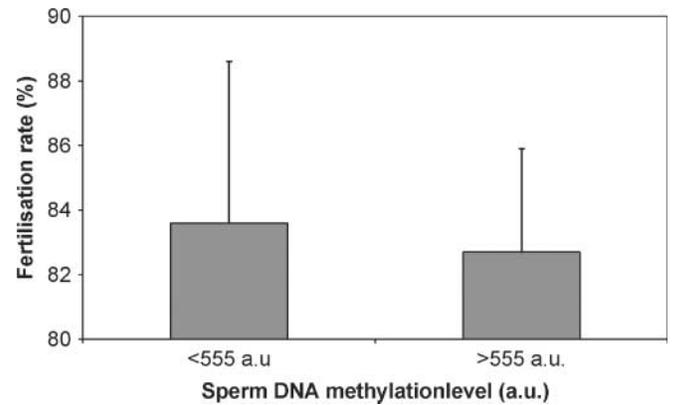
Methylation and fertilization rate

The fertilization rate was not correlated to the sperm DNA methylation level ($r = -0.1$, not significant). Moreover, this fertilization rate was similar whether DNA methylation level was below or above an arbitrary level of 555 AU threshold value (83.5 and 82.7% respectively) (Figure 2).

Methylation and embryo quality

The global rate of good quality embryos was $46.4 \pm 29.9\%$. No relationship between sperm DNA methylation rate and embryo quality was found ($r = -0.08$, not significant): the rate of good quality embryos was independent of the level of sperm DNA methylation. Its value was $46.7 \pm 30.2\%$ and $46.3 \pm 30.2\%$ for sperm DNA methylation < 555 and > 555 AU respectively.

Methylation and pregnancy

Sperm DNA methylation level was 607.7 ± 69.8 and 573.4 ± 86.8 AU for cycles with and without pregnancies

Figure 2. Distribution of fertilization rate (%) according to the threshold of the sperm DNA methylation level (in arbitrary units: AU). The data are represented as mean + SEM in percentage. The difference was not significant.
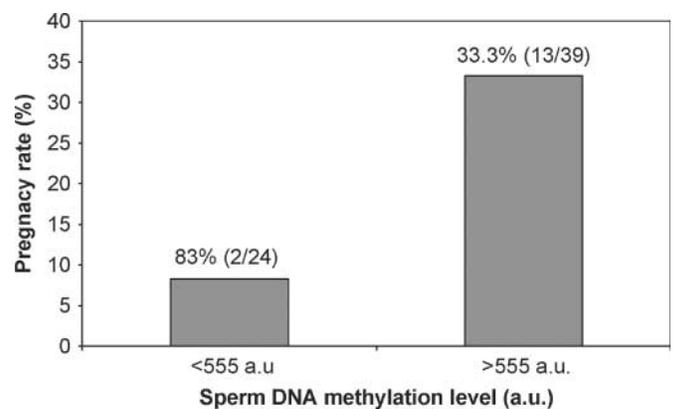
Figure 3. Distribution of pregnancy rates according to the threshold value of the sperm DNA methylation level. The difference was significant ($P < 0.05$).

following embryos transfer respectively. This difference was not statistically significant. With a threshold value equal to 555 AU, the RR of pregnancy was 1.38 ($P < 0.05$). The pregnancy rates were 8.3% (2/24) and 33.3% (13/39) for sperm DNA methylation < 555 and > 555 AU respectively ($P < 0.05$) (Figure 3).

With a stepwise logistic regression, the predictive parameters found for obtaining a pregnancy were the percentage of good quality embryos (grades A or B) (OR = 5.02, $P < 0.01$) and the sperm DNA methylation (OR = 4.55, $P < 0.05$), the threshold values being 60% and 555 AU respectively (Table I).

Discussion

This study shows that the global methylation level (GML) of sperm DNA influences the pregnancy rate in IVF. The initial method based on image analysis technique was adapted in order to make measurements by flow cytometry, which allowed us simultaneously to increase the number of cells analysed and to decrease the measurement time. However, image analysis technique remains the most convenient in cases of low sperm concentrations.

771

	OR	CI	*P*
Oligozoospermia (yes/no)	3.52	0.60–20	NS
Asthenozoospermia (yes/no)	3.52	0.12–100	NS
Embryo quality: rate of (a + b) embryo grade (<60%, >60%)	6.33	1.59–25.0	<0.01
Sperm DNA methylation (<555, >555 AU)	5.49	1.01–30.30	<0.05

CI = confidence interval; NS = not significant; AU = arbitrary units.

In this study, we chose to include samples with normal or few altered sperm characteristics according to the World Health Organization standards (i.e. 40% total motility). This is why ICSI was never necessary; thus the group was relatively homogeneous regarding both sperm characteristics and the assisted reproduction technique, represented by conventional IVF. Sperm chromatin methylation analysis was completed using the same selected sperm sample that was used for IVF. No association was found between GML and sperm concentration or sperm motility, but as the sperm characteristics were normal or moderately altered, the results disagreed with the data of Marques *et al.* (2004), who found a relationship between alteration of gene imprinting and severe oligozoospermia. As sperm DNA fragmentation impacted on pregnancy in assisted reproduction treatment (Benchaib *et al.*, 2003b; Larson-Cook *et al.*, 2003), the DNA fragmentation was measured together with GML in a group of 13 patients. This comparison was important because one had to verify that methylcytosine staining did not simply reflect another estimation of DNA packaging that would decrease considerably its interest. However, the lack of correlation between DFI and methylation could be due to the low number of subjects. Figure 1 shows a tendency to negative correlation: one could hypothesize that some sperm with a hypomethylated DNA could be more sensitive to DNA fragmentation. As no relationship was found between GLM and DNA fragmentation, these indices could represent two different sperm factors. Moreover, as discussed earlier, GML is not correlated to sperm characteristics, whereas some studies have found significant correlation between sperm characteristics and DFI (Benchaib *et al.*, 2003b; Larson-Cook *et al.*, 2003). So GML seems to be an interesting factor that brings new information concerning DNA quality of sperm.

As observed in our preliminary study (Benchaib *et al.*, 2003a) this analysis could predict the pregnancy outcome: the pregnancy rate was significantly higher for GML above an arbitrary threshold value. On the contrary, sperm GML was not correlated with either the fertilization rate or with the quality of embryos. In fact, the events that are involved in the fertilization process, the embryo quality and the embryo development are all different. Normally, sperm DNA quality would not be expected to play a role in oocyte activation process or in early development, since it is assumed that in humans the first 2–3 days of development (until the 6–8-cell stage) are mainly controlled by maternal transcripts. However, this assessment can be nuanced since in some cases, sperm characteristics are so poor that one can expect

that all sperm functions are altered, leading to disturbances of both fertilization (by IVF) and embryo development (if ICSI is performed). The relationship between a low sperm GML and an abnormal embryo development is complex. It can be assumed that in the case of sperm DNA hypomethylation, some genes are not repressed, as they normally should be, thus the embryo genome expression shows some degree of assynchronism. This global hypomethylation may also alter the process of cell differentiation, as observed in neoplasic cells (Piyathilake *et al.*, 2003).

It could be argued that the zygotic paternal genome is demethylated under oocyte control (Mayer *et al.*, 2000), so the status of the male genome methylation is not as important. However, it has been recently shown that embryo development failure could be related to aberrant methylation patterns observed at the 2-cell stage and originating from gamete DNA (Shi and Haaf, 2002). Although they are not transduced, it is possible that the Alu or L1 repeated sequences, of which methylated cytosines are an important constituent (Yoder *et al.*, 1997), may play a role in the expression of genes involved in cell differentiation as postulated by Mayer (2000).

In conclusion, our data show that the global status of sperm DNA methylation does not influence the fertilization rate but does influence embryo development, which is impaired if global DNA methylation level is below a threshold value.

Acknowledgements

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