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GAMETE BIOLOGY

Association between the MTHFR-C677T isoform and structure of sperm DNA

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Abstract
Purpose The aim of this study is to evaluate whether the MTHFR contribution to male decreased fertility can be attributable to anomalies in sperm nucleus DNA structure in relation to defective methylation.
Methods The presence of MTHFR C677T, contributing at most for male infertility, was determined from a venous blood sample, using real-time polymerase chain reaction (PCR). Sperm DNA fragmentation (SDF) and sperm nucleus decondensation index (SDI) measurements were performed using acridine orange and flow cytometry. SDF and SDI of men MTHFR C677T heterozygous or homozygous were compared to a general population of hypo-fertile patients
Results SDF is not increased either in homozygous or heterozygous carriers of MTHFR C677T. In contrast, SDI is increased with a higher incidence in homozygous ($p = 0.0006$) than in heterozygous ($p = 0.029$) patients when compared with the control population. Using a critical threshold of 20% for either SDI or SDF assayed with our technique, the percentage of patients with results higher than this value is not significant with respect to fragmentation (0.128), but is significantly increased for decondensation (0.0003).
Conclusions Defective methylation linked to MTHFR may contribute to sperm pathogenesis via increased SDI. After DNA structure analysis, especially SDI, treatment with 5-methyl tetrahydrofolate (MTHF), the metabolite downstream from the action of MTHFR, should be recommended as a therapeutic approach. Patients with a high SDI should be tested for MTHFR isoforms as part of a healthcare policy.

Keywords MTHFR isoform C677T · Sperm · DNA fragmentation · Nucleus decondensation

Introduction

According to worldwide statistics, between one in four and one in five couples are affected by fertility problems. These problems are equally distributed between males and females. The role of an "isolated" male factor in a couple's

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infertility is difficult to quantify, as it can be masked by factors associated with issues related to the fertility of the female partner. Ultimately, male reproductive failure is thought to represent 50–70% of infertility cases in Western countries. Isolated male fertility problems account for one half of these and are a contributory factor in the other half [1]. Routine semen parameter analysis generally includes sperm count, concentration, motility, vitality and the percentage of abnormal forms. The advent and large-scale development of assisted reproductive technology (ART) and especially intracytoplasmic sperm injection (ICSI), which bypass natural sperm selection processes, have precipitated a shift in perspectives, with a focus on tests to determine decays in DNA structure such as DNA Fragmentation (SDF) and Sperm Nucleus Decondensation Index (SDI) [2–4]. The final structure of sperm chromatin is dependent upon two major post-translational processes, i.e. methylation and acetylation. The process of methylation affects not only the stability of the DNA structure, but also the acquisition of epigenetic/imprinting marks. DNA methylation of CpG islands, together with histone lysine methylation determines chromatin structure and status. A clear link between oxidative stress and methylation defects has been established, with a strong negative impact on sperm quality [5–7]. Several meta-analyses [8, 9] have demonstrated that MTHFR polymorphism, and especially the C677T isoform is associated with male infertility. The MTHFR gene is located on the short arm of chromosome1 (1p36.3), [10]; the enzyme converts folate to 5 Methyl THF (5MTHF), the active molecule involved in the one carbon cycle (1-CC, see Fig. 1). The 1-CC regenerates homocysteine, with formation of methionine and then S-adenosyl methionine, the universal effector of methylation. The C677T isoform has a single nucleotide substitution of thymine for cytosine at position 677 on the gene. Folic acid metabolism is mandatory for the process of methylation and for DNA stability and repair. The MTHFR 677C>T variant decreases the activity of the enzyme by one third in heterozygote carriers and by two thirds in homozygote carriers [10]. It is now evident that the prevalence of the heterozygote C677T reaches 20–25% in the general population, with race-related variation [11]. The present study investigates the effect of the C677T isoform on sperm DNA structure, i.e. SDF and SDI, in a population consulting for infertility.

Materials and methods

SDF and SDI testing Sperm samples collected by masturbation were frozen immediately post-liquefaction. All samples were transferred to the laboratory whilst still frozen, and within a week thawed and analysed by acridine orange flow cytometry according to a previously described technique [12]. The samples were washed /concentrated using a phosphate buffer containing albumin at 10mg/mL in order to reach a final concentration of at least 1×10^6 cells/mL. Albumin is necessary in order to avoid agglutination. Then the staining solution (acridine orange in phosphate buffer solution) is added. The suspension is gently shaken for 30 s in ice. Each individual sample was analyzed separately, stained and processed immediately after thawing in order to ensure complete reproducibility of results. Results were quantified by gathering sperm cell signals obtained at 525 nm for SDI (green fluorescence) and at 620 nm for the lesions of single DNA strands (SDF, red fluorescence). The SDI is the reflection of a faulty compaction, i.e. abnormal tertiary structure, and is generally associated with immaturity. The number of cells analysed is roughly 8000. As mentioned in the reference paper, correlation with aniline blue (for SDI) and TUNEL assay (for SDF) is regularly monitored.

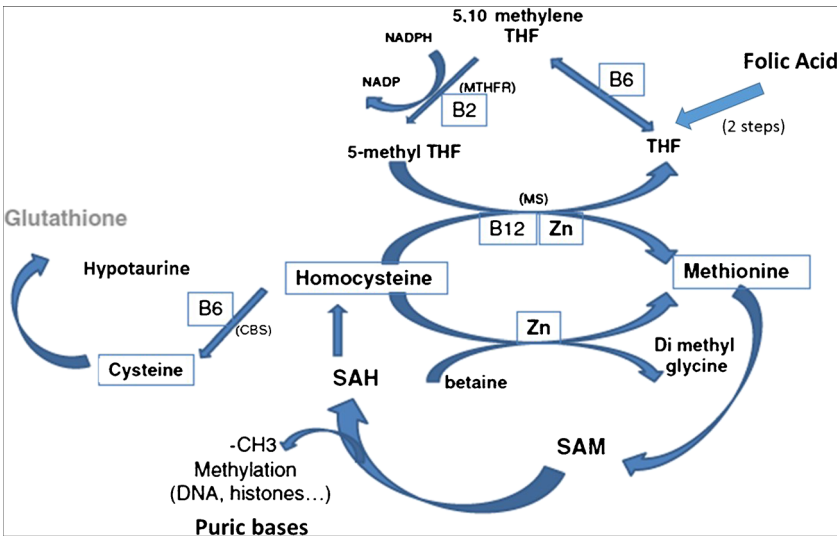

Fig. 1 Folic acid and the one carbon cycle (1-CC). MS methionine synthase, CBS cystathionine beta-synthase pathway, MTHFR methylenetetrahydrofolate reductase, THF tetrahydrofolate, SAM S-Adenosyl methionine, SAH S-Adenosyl homocysteine. The two steps transforming folic acid in THF are supported by the dihydrofolate reductase (DHFR)

Table 1 STATISTICAL ANALYSIS. Comparison of variance. The variances are not significantly different. HTZ heterozygous, HMZ homoztgous

	F	p
DNA Fragmentation		
HTZ vs HMZ	1.213	0.68
HTZ vs Control	0.88	0.48
HMZ vs Control	0.726	0.44
DECONDENSATION		
HTZ vs HMZ	1.35	0.50
HTZ vs Control	0.80	0.61
HMZ vs Control	1.08	0.62

MTHFR C677T identification The MTHFR is a 70 kDa protein resulting of the expression of a 2.2 kb in length cDNA. The presence of MTHFR C677T was determined from a venous blood sample, using real-time polymerase chain reaction (PCR) with the RealFast ™ assay (ViennaLab Diagnostic GMBH, Vienna, Austria). The two probes corresponding to the wild type (WT) and the one corresponding to the C677T isoform (MT form) are present in the reagent mixture. At the end of the process, the presence of two fluorescent peaks indicates the heterozygous (HTZ) state. The appearance of one peak indicates the homozygous (HMZ) state either (C677C × C677C normal) or (T677T × T677T). This qualitative test allows the three phenotypes to be identified: C677C (WT, normal), C677T (heterozygote) and T677T (homozygote).

Statistical analysis Due to the significant difference in population size (only 18 patients were found to be homozygous, vs 77 in the heterozygous group and 1405 in the control group), the hypothesis of variance equality was tested (*F* test, Snedecor and Cochran).

A non-parametric test (Mann Whitney) was then used to compare the different populations.

As a further (third) approach, we used Pearson's chi^2 test to compare the percentage of patients in each group who were found to have SDF and DFI > 20%.

Results

Comparison of variance allowed us to compare the three groups and showed no difference between them for either fragmentation or decondensation (Table 1). Table 2 and Fig. 2 illustrate that there was no detectable difference in SDF between homozygotes, heterozygotes and the control group. However, a significant increase in decondensation index (SDI) was observed in the presence of the MTHFR isoform: SDI is higher in heterozygotes vs control

Table 2 Comparison of SDF and DFI in the three populations Control, Heterozygous, Homozygous,

Population	Control	Heterozygote	Homozygote	Chi2 / p* (overall)
N	1405	77	18	
FRAGMENTATION (SDF)				
Mean	20.4[a]	17,3[b]	17.7[c]	
SD	16, 1	15, 1	13, 8	
Median	16, 3	14	14	
limits	[8,1;28,1]	[6,2;20,1]	[7,9;26]	
Overall				4.107, p =0.128
DECONDENSATION (SDI)				
Mean	14,4[d]	16,5[e]	21,8[f]	
SD	10, 1	10,4	9	
Median	11, 8	15	21, 8	
Limits	[7; 19,4]	[9;21]	[13;29]	
Overal				16.24, p= 0.0003
a *vs* b, p= 0.514	Population not significantly different	d *vs* e, p= 0.0291	Population significantly different	
a *vs* c, p= 0.543	id	d *vs* f, p= 0.0006	idem	
b *vs* c, p =0.78	id	e *vs* f, p=0.0126	idem	

*Kruskall-Wallis test

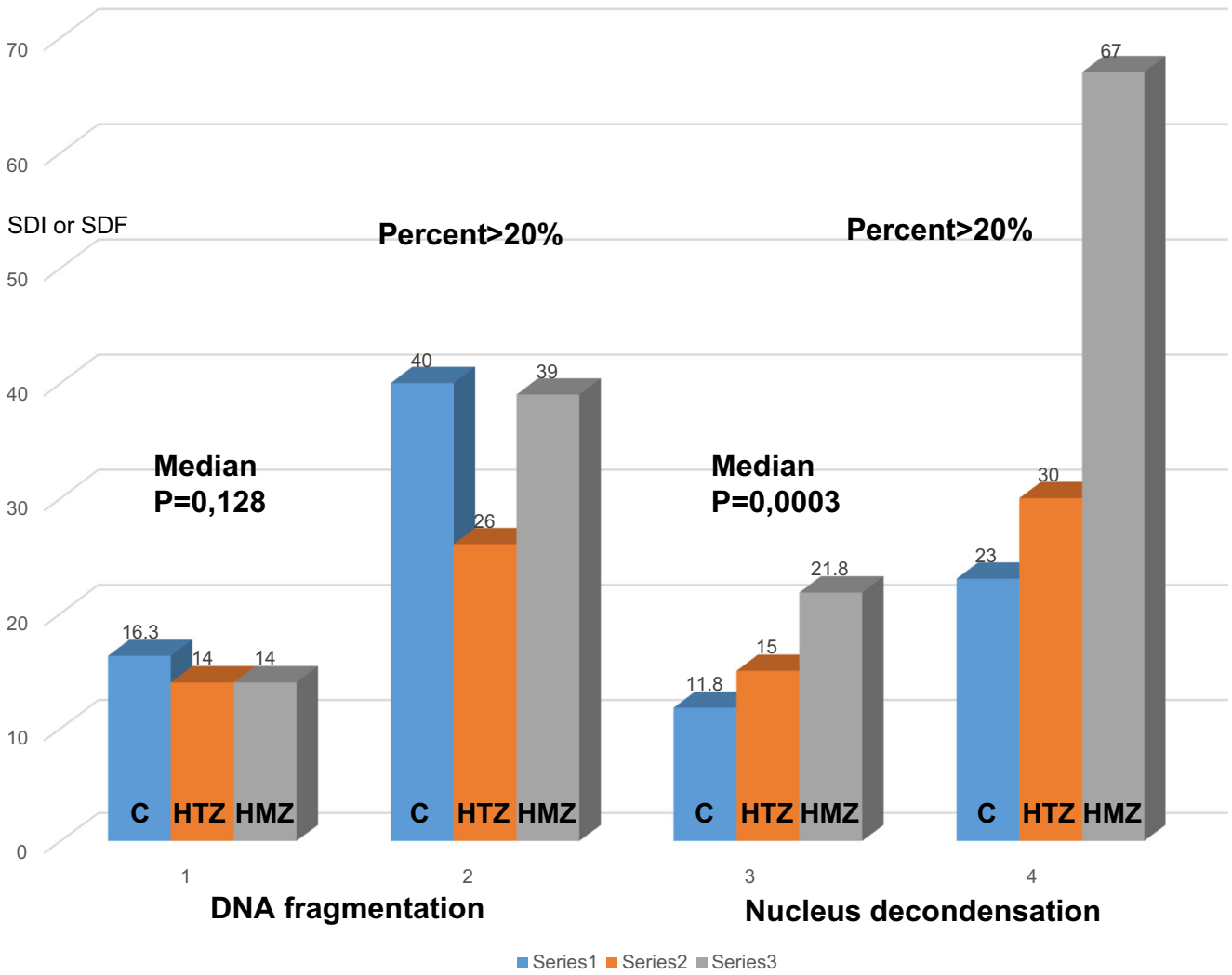

Fig. 2 Sperm DNA fragmentation (SDF) and sperm nucleus decondensation index (SDI) according to the MTHFR C677T status. And percent of the patients having a > 20% value. C control, HTZ heterozygotes, HMZ homozygotes

($p = 0.029$), and again between homozygotes vs heterozygotes ($p = 0.0126$). A similar result was observed after comparing the percentage of patients who had results higher than a threshold of 20% (Fig. 2, Table 3). The percentage of patients with SDI > a threshold of 20% is significantly higher in the homozygote group vs the heterozygote and control groups. We found a surprising significant lower SDF in the HTZ group vs the control group ($p = 0.01$).

Table 3 Percentage of the patients having an SDF and a DFI over 20%. Test Chi^2 (Pearson)

DNA fragmentation		Chi^2	p
HTZ vs HMZ	26%/39%	1.20	0.27
HTZ vs control	26%/40.3%	6.73	0.01
HMZ vs control	39%/40.3%	0.029	0.864
Decondensation			
HTZ vs HMZ	30%/67%	8.49	0.004
HTZ vs control	30%/23%	1.911	0.167
HMZ vs control	67%/23%	18.79	0.000

Discussion

MTHFR plays a critical role in the 1-CC. The formation of 5-methyl THF (5MTHF) downstream of the enzyme is mandatory for the activity of methionine synthase, which allows recycling of homocysteine (Hcy) to methionine, and then the synthesis of two major antioxidant molecules: hypotaurine and glutathione. But in our study, DNA fragmentation does not seem to be affected by the MTHFR isoform tested, this is confirmed that the percentage of patients having an SDF > 20% is even lower in the HTZ

group than that in the control one. Homocysteine is involved in multiple pathologies (heart, brain…). Hcy disrupts the synthesis of methionine and thus has a negative effect on protein synthesis and methylation. DNA repair can also be affected due to perturbation of purine base synthesis. Moreover, Hcy can be converted to S-adenosyl Hcy, a significant inhibitor of several methyltransferases. Two thirds of the patients carrying the isoform either HTZ or HMZ have a normal homocysteine concentration in the blood below the critical threshold of 15 μmoles/L. A mild homocysteinemia (15 < Hcy < 25 μmoles/L) was observed for the rest of the patients, with the exception of three patients: a critical HCy concentration was observed in one heterozygous and two homozygous patients. This suggests that the impact on spermatogenesis is not necessarily linked to increased levels of circulating homocysteine. However, the possibility of composite heterozygote mutation (A1298C) was not tested in this study. A composite mutation may have an even greater effect on methylation processes, although the A1298C isoform does not appear to have a significant effect on fertility per se [9].

Methylation plays a key role in the testis: during neonatal and early postnatal testis development, there is a dynamic regulation of DNA methylation during spermatogonial stem cell formation and differentiation [13]. In later life, testicular spermatozoa from infertile men with abnormal spermatogenesis carry methylation defects. A strong link between the incidence of methylation/imprinting errors and defective spermatogenesis and the impact on embryonic development has now been well established [5, 17]. Defective chromatin integrity may be associated with the significant sperm DNA hypomethylation observed in carriers of chromosomal structural anomalies [18]; There is a general consensus that global sperm hypomethylation is linked to DNA instability and structural anomalies [13, 19]; however, the latter group subsequently supported the exact opposite in a later publication [20]. Methylation and acetylation are the two major post-translational effectors of correct sperm nucleus tertiary structure; methylation marks are set on DNA and on histones, both regulating imprinting/epigenetic processes. It is therefore not surprising that MTHFR isoforms have an impact on this process and a global effect on the quality of spermatogenesis. The sperm histones and DNA methylation profiles were not studied here but our results show that alteration of the 1-CC with subsequent incomplete methylation seems to affect sperm tertiary structure and nucleus condensation, in particular. Homozygous carriers of 677TT are affected to a greater extent than heterozygous patients. This confirms and possibly explains the conclusions of several meta-analyses that support a link between the MTHFR C677T allele and idiopathic male infertility. Tertiary structure of the sperm nucleus is crucial for correct timing during the process of fertilization [4, 21, 22] and during early preimplantation development [23]. However, the possibility of composite heterozygote mutation (A1298C) was not tested in this study. In terms of a healthcare policy, patients with high SDI should be tested for MTHFR isoforms, as this may reveal possible homozygous carriers and the risk of elevated serum homocysteine. For these patients, 5 methyl THF, the product of MTHFR activity, can in any case be prescribed, as it enters directly into the one carbon cycle. Preliminary data have demonstrated that 5 methyl THF regulates circulating homocysteine.

Author contributions Dominique Cornet, MD, Gynecologist; Marc Cohen MD, Gynecologist; Laetitia Fournols, MD, Endocrinologist and Edouard Amar, Urologist; have been in charge of the clinical follow-up of the patients.

Arthur Clement, MD; Patrice Clement Dr. Pharm and Yves Menezo PhD, Dr. Sci; have been in charge of the biological technical aspect of the experiments (MTHFR, SDF and SDI determination).

Paul Neveux, MD, has been in charge of all the statistical follow-up and calculation.

Yves Menezo has designed the experiment and written the paper.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

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