

Presence of MTHFR Polymorphisms and the Levels of Folic Acid and Homocysteine in Women Enrolled in an Assisted Reproductive Program

Felipe Arturo Morales (✉ drfamm@yahoo.com)

Universidad Autonoma de Nuevo Leon Facultad de Medicina <https://orcid.org/0000-0003-2140-2191>

Martha Merino Ruiz

Universidad Autonoma de Nuevo Leon Facultad de Medicina

José A. Elizondo Briseño

Universidad Autonoma de Nuevo Leon Facultad de Medicina

Laura E. Martínez Garza

Universidad Autonoma de Nuevo Leon

Oscar Vidal Gutiérrez

Universidad Autonoma de Nuevo Leon Facultad de Medicina

Luis H. Sordia Hernández

Universidad Autonoma de Nuevo Leon

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Abstract

Purpose: Folic acid and homocysteine levels are influenced by the circulating levels of estradiol. Little is known about the behavior of these metabolites during an in vitro fertilization cycle, where superovulation protocols induce a major increase in the levels of estradiol and other hormones.

Methods: We performed a prospective, comparative, transversal, descriptive and observational study that includes 49 cases (group A), which were patients that entered a fertility program, and 14 controls (group B), which correspond to the donor group. In both groups, the levels of folic acid, estradiol and homocysteine as well as the MTHFR polymorphism were determined.

Results: None of the patients has subnormal levels of folic acid or homocysteine and these levels were not modified after ovarian stimulation. We observed that homocysteine concentration tended to decrease with elevated estradiol levels produced by the stimulation of the ovary. The presence of allele C677T MTHFR was high in the populations evaluated in this study, with a particular high incidence of the TT allele.

Conclusion: The levels of plasma and intracellular folate, and homocysteine do not change during hormonal stimulation.

Background

Infertility affects approximately 10% of couples in reproductive age [1] and is considered as a major cause of stress in life [2]. One of the B vitamins, Folic acid, was proposed to be related to human infertility [3] and is important for egg quality, maturation as well as for implantation and pregnancy [4]. Folate is important role for DNA synthesis and epigenetic modification, as well as cell proliferation and its deficiency affect cells with high levels of proliferation (e.g. neural tube cells in the developing fetus), thus increasing the risk of neural tube defects as well as other birth defects [5–8]. Supplementation increase folate concentrations and decrease concentrations of plasmatic homocysteine. Although folic acid supplementation is often used by infertile women, the effect on pregnancy outcome in individuals that were diagnosed with unexplained infertility has not been fully investigated.

Homocysteine is a non-proteinogenic amino acid that is biosynthesized from methionine which can be also be recycled into methionine or converted into cysteine with the help of certain B-vitamins. Hyperhomocysteinemia can alter the reproductive potential of men and women. Several reports have associated the high levels of homocysteine with low egg quality, male infertility, congenital malformations, miscarriage and low pregnancy rates [9–11]. The enzyme Methylene tetrahydrofolate reductase (MTHFR) encoded by the *mthfr* gene catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a cosubstrate for homocysteine remethylation to methionine. There are genetic polymorphisms associated with this gene and one of the most investigated is C677T, where the nucleotide at position 677 can be either a cytosine or thymine. Those with 677TT

(T/T) have lower MTHFR activity than CC or CT and as a result, tend to display a mild hyperhomocysteinemia.

It is not clear if estrogens alters the levels of folic acid or homocysteine either directly or through intermediates and cofactors [12–14]. The administration of exogenous estrogens in women affect several endocrine and metabolic pathways, including homocysteine metabolism. For example, estrogen administration in post-menopausal women significantly decrease the total homocysteine levels [15]. Another example is that the levels of homocysteine are significantly lower in the luteal phase than in the follicular phase of the ovulatory cycle [16]. In addition, circulating levels of homocysteine decrease during pregnancy [17–19]. Previous reports have studied the relationship between folic acid and homocysteine with steroids, in particular with estradiol. However, most of the studies were focused on studying these compounds during pregnancy or menopause where there is a substantial alteration in the circulating levels of estrogens. On the contrary, very little is known about the behavior of folic acid and homocysteine during an *in vitro* fertilization cycle, where superovulation protocols induce a major increased in the levels of estradiol and other hormones.

We designed the present prospective study aimed at investigating whether levels of serum and red blood cell folate as well as homocysteine are influenced by the high levels of estradiol produced by super ovulation in women undergoing *in vitro* fertilization.

Methods

Subjects and samples

The study protocol was approved by the Bioethics Committee of the Hospital Universitario “Dr. José Eleuterio González”. These studies are in compliance with the Declaration of Helsinki principles. Patients were provided with written information about the study prior to giving informed consent. A total of 49 patients (Group A) consulting for infertility were included in this study (age 20-39). This group was further subdivided in two groups: patients who became pregnant after the treatment (group A1); patients that did not achieved pregnancy (group A2). Diagnosis of infertility was based on the patient’s medical history, clinical investigation, including transvaginal ultrasonography, a standard set of tests that included hormone analyses, hysterosalpingogram contrast sonography and semen analyses, as described previously [21]. A diagnosis of unexplained infertility was chosen when no explanation for infertility was found, women had normal ovarian function and normal tubal passage (demonstrated by hysterosalpingogram contrast sonography) and their partners had normal semen samples according to WHO criteria [22].

All of them were programmed for an assisted reproduction procedure (either *in vitro* fertilization or intra cytoplasmic sperm injection). In addition, 14 healthy women donors (Group B) that were recruited as part of our egg donor program were also included in this study (control group).

The following information was recorded for each patient: Age, concentration of estradiol during stimulation, duration of hormonal stimulation (days), dose of gonadotropins used in stimulation, number of follicles (>14 mm), number of eggs retrieved, number of embryos, number of transferred embryos and occurrence of biochemical pregnancy (positive of beta hCG). Each participant was subjected to 2 blood draws (10 ml each). The first one at day 3 of the cycle, prior to hormonal stimulation. The second one, the day of egg retrieval. The samples were used to quantify the concentration of estradiol, folic acid, homocysteine and 5-MTHFR polymorphism.

IVF procedure

All women started the ovarian stimulation treatment with daily injections of 300 IU recombinant follicle stimulating hormone (rFSH) s.c. on cycle day 2 (Puregon®, NV Organon, Oss, the Netherlands, or Gonal-F®, Serono Benelux BV, The Hague, the Netherlands). Administration of daily s.c. gonadotrophin releasing hormone antagonist (Orgalutran®, NV Organon, or Cetrotide®, Serono Benelux BV) was started when at least one follicle was ≥ 14 mm. To induce final oocyte maturation, a single dose of 5000 or 10 000 IU hCG s.c. (Pregnyl®, NV Organon) was administered as soon as the largest follicle reached at least 18 mm in diameter and at least one additional follicle of >15 mm was observed. Oocyte retrieval was carried out 35 h after hCG injection by transvaginal ultrasound-guided aspiration of follicles. Luteal phase supplementation of 600 mg/day micronized progesterone intravaginally was started on the evening following oocyte pick-up and continued for 12 days thereafter. On Day 3 after oocyte pick-up, a maximum of two embryos were transferred.

Determination of Folic acid, homocysteine and Estradiol

Blood samples were taken to determine plasma folate (PF) and intracellular folate (ICF). Immediately after collection, the blood samples were centrifuged at 3000 g for 10 min and the plasma stored at -20°C until analyzed. For ICF determination, 0.1 ml of blood was diluted with 2 ml of ascorbic acid in order to produce the lysis of red blood cells. Folate was measured radioimmunoassay Dualcont AF (Diagnostic Product Co, Los Angeles, CA, USA) according to the manufacturer's instructions. Blood samples showing hemolysis were excluded from the statistical analyses because of the risk of misleading values. Measuring range: IFC 175-700 ng / mL, PF 3-17 ng / mL. The intra and inter assay coefficient of variation were 5.2% and 9.2%, respectively.

For homocysteine determination, immediately after collection, the blood samples were centrifuged at 3000 g for 10 min and the plasma stored at -20°C until analyzed. Blood samples showing hemolysis were excluded from the statistical analyses because of the risk of misleading values. Homocysteine was assessed by fluorescence polarization immunoassay (FPIA) adapted to the IMx analyzer (Abbott Laboratories), with the high-performance liquid chromatography (HPLC) method with fluorescent detection. Measuring range: 4.5-15.0 $\mu\text{mol/L}$. The intra and inter assay coefficient of variation were 2.7% and 3.4%, respectively.

Electrochemiluminescence immunoassay Elecsys Estradiol III Assay (ECLIA) was used for the in vitro quantitative determination of estradiol in human serum and plasma. Measuring range (25-3,000 pg/mL); Intermediate imprecision was 4.5%.

Determination of MTHFR polymorphisms

Peripheral blood was collected for genomic DNA analysis to determine the substitution of cysteine by thymine (C677T). Genomic DNA was isolated from whole blood using the QIAamp DNA Blood Mini Kit (Berlin, Qiagen, Germany). DNA quality and quantity were assessed by a UV spectrophotometer at 260 and 280 nm. The following primers were used (5'-TGA AGG AGA AGG TGT CTG CGG GA-3') and MTHFR-2 (5'-AGG ACG GTG CGG TGA GAG TG-3'). The assays were performed using TaqMan Genotyping Master Mix with 50 ng DNA. The PCR conditions were 35 cycles of denaturation at 94°C for 5 min and annealing/extension at 62°C for 1 min, as recommended by the manufacturer. The amplified products were digested with *Hinfl* since the C677T mutation creates a restriction site for this enzyme (converting the 198 bp product in two fragments of 175 and 23 bp). Homozygous individuals were detected when a single product of 198 bp (C/C) or 175 (T/T) was detected. Heterozygous displayed the two fragments (C/T).

Calculations and statistical analysis

Data are expressed as mean \pm standard deviation of the mean (SD). Data were normally distributed. Statistical comparisons were performed by paired Student t-test or Pearson correlation coefficient. A power study was conducted to calculate the number of samples for plasma folate, intracellular folate and homocysteine levels. ($\alpha=0.05$ and the desired power = 0.80). Calculations were performed with Libre Office 4.3.2.2 spreadsheet and statistical analysis with GraphPad Prism version 4.00 for Windows, GraphPad Software (San Diego, CA, USA). A probability (p) value $p<0.05$ was considered statistically significant.

Results

General features of the individuals included in this study

A total of 49 patients (groups A1 and A2) and 14 egg donor women (Group B; control group) were included in this study. The general features of these groups are listed in Table 1. These include the age, the body mass index, the smoking habits, the number of days with hormonal stimulation, the total dose of FSH, the number of follicles of 14 mm or more, the number of retrieved oocytes and embryos produced, the fertilization rate, the number of embryos transferred and the pregnancy rate. As expected, individuals in group B displayed higher number of follicles (> 14 mm) and number of oocytes, which is strictly related to the fact that these women are younger and do not present fertility problems.

Table 1

General features of the studied population. Group A1. patients who became pregnant after the treatment. Group A2: patients that did not achieved pregnancy. Group B: control group (patients of our egg donor program).

	Group A1 n = 11 mean (range)	Group A2 n = 38 mean (range)	Group B n = 14 mean (range)
Age	32.0 (26–38)	31.6 (23–39)	23.6 (20–30)
Body mass index	25.1 (21.1–27.2)	27 (20.5–35.6)	22.5 19.6–28.8
Smokers (%)	0	2	4
Days of stimulation	9.9 (9–12)	10.1 (7–12)	9.4 (6–11)
Dose of FSH dose (IU)	2681.8 (1575–3375)	2893.5 (1125–6000)	2287.5 (1575–3300)
Follicles > 14 mm	7.4 (2–17)	7.9 (2–24)	11.6 (4–17)
Number of oocytes	10.6 (2–30)	7.7 (2–17)	12.5 (6–22)
Number of embryos	8.4 (2–24)	5.6 (0–15)	7.1 (2–14)
Fertilization (%)	79.5 (42–100)	75.2 (0-100)	59 (11.7–100)
Number of embryos transfered	2.8 (2–4)	2.6 (0–4)	2.9 (2–3)

In group A, 11 out of 49 patients become pregnant (positive beta hCG; group A1). Of those, two interrupted the pregnancy (one presented an ectopic pregnancy and the second one, a miscarriage). The embryos produced from oocytes of group B produced 5 biochemical pregnancy's and all of them resulted in childbirths.

Concentration of folic acid and homocysteine did not significantly change during hormonal stimulation

The blood levels of folic acid, homocysteine and estradiol were measured in groups A1, A2 and B at two different times: before and after initiating the hormonal stimulation. The second one coincided with the day of oocyte retrieval.

The levels of folic acid were measured in two different ways: in plasma, which represent the dietary folate intake of the last 24 hours and in red blood cells, which represents the dietary folate intake of the last 3 month. In Table 2, it is shown the levels of these compounds in the groups studied. Overall, despite that positive correlations were noted in all groups, there were no significant differences in the levels of folic acid before and after the hormonal stimulation.

Table 2

Levels of folic acid, homocysteine and estradiol in the groups studied. Group A1: patients who became pregnant after the treatment. Group A2: patients that did not achieved pregnancy. Group B: control group (patients of our egg donor program).

Metabolite (normal range)	Group	First sample Mean \pm SD (range)	Second sample Mean \pm SD (range)	Correlation r	T-test T
Plasma folate (3–17 ng/ml)	Group A1 (n = 11)	19.1 \pm 6.3 (4–24)	16.3 \pm 5.6 (5.5–24.4)	0.77	NS
	Group A2 (n = 38)	13.9 \pm 6.1 (4–24)	13.2 \pm 5.9 (4.1–26.3)	0.58	NS
	Group B (n = 14)	12.1 \pm 4.5 (4.2–22.4)	12.6 \pm 5.3 (4.2–22.5)	0.40	NS
Intracellular folate (175–700 ng/ml)	Group A1 (n = 11)	562.8 \pm 230.5 (150–1022)	537.2 \pm 185.2 (157.5–804.5)	0.70	NS
	Group A2 (n = 38)	566.3 \pm 241.6 (170–1273)	596.9 \pm 243.1 (180–1260)	0.84	NS
	Group B (n = 14)	482.7 \pm 207.1 (140–855.4)	529.9 \pm 193.2 (210–834.5)	0.76	NS
Homocysteine (4.5–15.0 μmol/L)	Group A1 (n = 11)	6.7 \pm 1.0 (4.9–8.1)	5.7 \pm 1.3 (3.1–7.6)	0.80	NS
	Group A2 (n = 38)	6.3 \pm 1.4 (4.2–9.1)	5.8 \pm 1.7 (3.3–10.3)	0.76	NS
	Group B (n = 14)	7.9 \pm 1.1 (5.3–9.4)	6.4 \pm 1.1 (4.1–7.1)	0.64	p < 0.05
Estradiol (> 25 pg/ml)	Group A1 (n = 11)	37.1 \pm 42.8 (5–167)	1725 \pm 1007.1 (484–4300)	-0.17	p < 0.001
	Group A2 (n = 38)	27.7 \pm 35.5 (4–171)	1448.8 \pm 1078.0 (317–4044)	0.08	p < 0.001
	Group B (n = 14)	55.0 \pm 87.3 (9.4–306)	1659.1 \pm 1108.9 (611–4300)	0.41	p < 0.001

The levels of plasmatic homocysteine in all groups before and after hormonal stimulation are also presented in Table 2. It is observed that the values measured after stimulation were slightly lower compared to those obtained in before hormonal treatments. All groups presented positive correlations but only in group B the difference was statistically significant. As a control, the levels of estradiol were also measured in these groups and are shown in Table 2. As expected, the levels of this hormone increased after the stimulation in all groups studied.

In order to evaluate the association between the levels of homocysteine with the levels of folic acid and estradiol in the groups studied, a Pearson correlation coefficient was used. The results are presented in Table 3. Overall, it is observed that there are not significant correlations between these parameters.

Table 3

Correlation between the levels of estradiol, folic acid and homocysteine. Group A1: patients who became pregnant after the treatment. Group A2: patients that did not achieved pregnancy. Group B: control group (patients of our egg donor program).

		Group A1 (n = 11)	Group A2 (n = 38)	Group B (n = 14)
		r	r	r
Homocysteine 1st sample	Plasma Folate 1st sample	0.06	0.07	0.53
Homocysteine 1st sample	Intracellular Folate 1st sample	0.47	0.29	0.53
Homocysteine 1st sample	Estradiol 1st sample	0.28	0.15	0.08
Homocysteine 2nd sample	Estradiol 2nd sample	0.09	0.15	0.29

Patients who achieved pregnancy possessed higher frequency of 677T MTHFR allele.

Methylene tetrahydrofolate reductase (MTHFR) is the rate-limiting enzyme in the methyl cycle, and it is encoded by the *mthfr* gene. There are DNA sequence variants (genetic polymorphisms) associated with this gene. One of the most investigated is C677T. The MTHFR nucleotide at position 677 in the gene has two possibilities: cytosine (C) or thymine (T). C at position 677 (resulting in an alanine at amino acid 222) is the normal allele. The 677T allele (resulting in a valine substitution at amino acid 222) encodes an enzyme with reduced activity. The most common genotype is the one with two copies of 677C (C/C). In contrast, those with 677TT (T/T) have lower MTHFR activity than CC or CT (heterozygous). There is ethnic variability in the frequency of the T allele. Hispanics tend to have higher frequency than Caucasians or Africans and individuals with 677TT tend to display a mild hyperhomocysteinemia due to less active MTHFR available to produce 5-methyltetrahydrofolate.

To evaluate the frequency of these polymorphisms in our studied group, we have determined the genotype of the individuals enrolled in this study. The results are shown in Table 4. The frequency of the T allele (T/T and C/T) in group A1, A2 and B was similar (62%, 63.7 and 69.5, respectively). However, none of the studied patients presented either severe or mild hyperhomocysteinemia. Interestingly, when

we analyzed the frequency for homozygous T/T, group A1 displayed a much higher frequency (27.4%) compared to the other groups.

Table 4
Incidence of MTHFR polymorphisms in groups A1, A2 and B.

MTHFR polymorphism	C/C	C/T	T/T
Group A1 (n = 11)	4 (36.3%)	4 (36.3%)	3 (27.4%)
Group A2 (n = 36)	11 (30.5%)	21 (58.3%)	4 (11.2%)
Group B (n = 13)	5 (38.0%)	7 (54.0%)	1 (8.0%)

Discussion

Previous evidence demonstrated a high correlation between the levels of homocysteine and folates in follicular fluid and serum indicating that the growing oocyte and the embryos are exposed to changes in these metabolites [23]. The goal of this paper was to study the level of these metabolites and the prevalence of different polymorphism of MTHFR in patients undergoing IVF cycles. In particular, because women undergoing assisted reproduction treatments are subjected to different protocols for hormonal stimulation that may cause changes in the concentration of these metabolites.

Previous studies have shown that homocysteine levels higher than 18 $\mu\text{moles/L}$ were associated with increased frequency of recurrent miscarriage [24]. Supporting these observations, in our study none of our patients possessed homocysteine levels higher than 10.3 $\mu\text{moles/L}$ and did not present recurrent miscarriages. In addition, our results also found no differences between patients that achieved pregnancy compare to those who did not. The overall levels of homocysteine reported by Jerzak and coworkers [24] were significantly higher than those that we observed in the Mexican population. This may be caused by the ethnic differences and the diet in the population studied.

The levels of homocysteine displayed a decrease after hormonal stimulation where the levels of estradiol are significantly higher. This is in agreement with findings reported by Wouters and coworkers [25], who observed a significant decrease in the levels of homocysteine in a group of women undergoing menopause that used hormone replacement therapy. Other reports did not find significant relations between the levels of homocysteine and estradiol [26]. However, these observations may be altered by the small number of patients involved in those studies.

In this study, we did not find significant differences in the folate concentration either before and after hormonal stimulation or, between pregnant and non-pregnant women. Nelens and coworkers [10] reported that low levels of folates may be associated with early pregnancy loss. Folic acid is normally used as a supplement by women during pregnancy to reduce the risk of neural tube defects in the baby but there is limited information about changes in this metabolite during IVF cycles. In our study, none of the patients

displayed low levels of folates, which is in agreement with the fact that Mexican women are usually not exposed to deficiency of this vitamin in their diets.

Regarding the genetic polymorphisms associated with the *mthfr* gene, there is ethnic variability in the frequency of the T allele. Hispanics tend to have higher frequency than Caucasians or Africans. In the Mexican population, we observed a very high incidence of the T allele (range 62% – 69.5%) with a particular high prevalence of the T/T homozygous (27.2%).

Surprisingly, we found a high incidence of the T/T homozygous in patients that achieve pregnancy. Although this study was not aimed to investigate the relationship between the presence of this allele and the occurrence of pregnancy or miscarriage, previous reports showed that in a group of women that underwent unexplained miscarriages, there was a high incidence of the T allele [27]. In addition, low folate intake affects individuals with the 677TT genotype to a greater extent than those with the 677CC/CT genotypes. However, it is important to mention that because the levels of folates and homocysteine in all patients studied were normal, the presence of the T allele is not of critical risk for the pregnancy outcome.

Conclusions

In summary, we found that the levels of folic acid and homocysteine in women undergoing hormonal stimulation did not significantly change in the presence of high levels of estrogens. These results are of great importance when considering therapeutic options in the Mexican population, that displays a high incidence in this polymorphism.

Declarations

Declarations

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