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ORIGINAL ARTICLE *Reproductive endocrinology*

Homocysteine concentrations in follicular fluid are associated with poor oocyte and embryo qualities in polycystic ovary syndrome patients undergoing assisted reproduction

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BACKGROUND: A poor quality of oocytes and embryos and a low fertilization rate have been found in polycystic ovary syndrome (PCOS) patients. An inverse association between follicular fluid homocysteine (Hcy) levels and oocyte and embryo quality has also been demonstrated. We examined the relationship between follicular fluid Hcy concentrations and oocyte and embryo quality in PCOS patients undergoing assisted reproduction.

METHODS: Fifty-two PCOS patients were included in the study, and underwent GnRH agonist/recombinant FSH treatment. The Hcy, folate, vitamin B_{12}, malonyldialdehyde (MDA) and estradiol (E_2) levels were measured in follicular fluid from single oocytes at time of retrieval. One follicle per ovary was sampled and 94 were analysed. Plasma hormones were also measured. Oocytes and embryos were graded (1–3) using standard approaches.

RESULTS: The concentrations of Hcy, E_2, vitamin B_{12}, folate and MDA in plasma were higher than in follicular fluid (all $P < 0.001$). Significant differences were observed in follicular Hcy levels between Grade 3 and Grade 2 oocytes ($P < 0.001$). Hcy levels were lower in Grade 1–2 embryos than that in Grade 3 embryos; follicular fluid vitamin B_{12} levels were lower in patients showing high concentrations of follicular fluid Hcy ($P < 0.01$). The follicular fluid Hcy levels were negatively correlated with follicular fluid vitamin B_{12} ($r = -0.44$), folate ($r = -0.68$) and fertilization rate ($r = -0.85$), and positively correlated with follicular fluid MDA ($r = 0.51$).

CONCLUSIONS: Concentrations of Hcy in follicular fluid on the dOPU may be a useful marker for fertilization rate, and oocyte and embryo quality in PCOS patients undergoing assisted reproduction.

Key words: homocysteine / polycystic ovary syndrome / malonyldialdehyde / assisted reproduction

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrinopathy involving ovulatory disturbances, hyperandrogenism, infertility and an increased miscarriage rate. Many studies detected elevated plasma homocysteine (Hcy) levels in women with PCOS (Yarali *et al*., 2001; Loverro *et al*., 2002; Schacter *et al*., 2003; Kaya *et al*., 2008). The presence of Hcy, folate and vitamin B_{12} in follicular fluid has been previously demonstrated (Steegers-Theunissen *et al*., 1993). A significant inverse association between follicular fluid Hcy levels and oocyte and embryo quality was demonstrated in women undergoing assisted reproduction (Steegers-Theunissen *et al*., 1993; Ebisch *et al*., 2006).

The clinical and preclinical data suggest poor oocyte and embryo quality, and a lower fertilization rate in PCOS patients undergoing assisted reproduction (Balen *et al*., 1993; Homburg *et al*., 1993; Kodama *et al*., 1995; Ludwig *et al*., 1999; Plachot *et al*., 2003; Heijnen *et al*., 2006). The composition of follicular fluid, to some extent, seems to reflect systemic Hcy metabolism, high or low Hcy levels in the follicular fluid may well occur (Steegers-Theunissen *et al*., 1992). In the gonadotrophin-dependent stages of follicle maturation, the primary oocyte undergoes both cytoplasmic and nuclear maturation, which render it competent to resume meiosis (Hardy *et al*., 2000). During this phase of development, both the systemic and the local ovarian milieu can have a profound influence on follicle

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growth and oocyte maturation. In anovulatory women with PCOS, granulosa cell function is abnormal (Franks 2002) and many studies have shown that gonadotrophin stimulation of women with PCOS is associated with a poor oocyte quality and lower fertilization rate than women with normal ovarian function (Kodama *et al*., 1995; Barnes *et al*., 1996; Cano *et al*., 1997; Doldi *et al*.,1999; Ludwig *et al*.,1999; Kovacs and Wood 2001). Although women with PCOS had embryos with less fragmentation which cleaved faster, cavitated earlier and had more cells at the blastocyst stage than embryos from a patient with tubal disease (Hardy *et al*., 1995, 2000), cumulative pregnancy rates in women with PCOS are not superior to those in control women (Sengoku *et al*., 1997; Weghofer *et al*., 2007). Therefore, various metabolic abnormalities in PCOS may also influence oocyte and embryo quality. It is possible that increased plasma Hcy levels could affect follicular fluid Hcy levels which, in turn, might affect oocyte and embryo quality in PCOS patients undergoing assisted reproduction. Oxidative stress occurs when the production of reactive oxygen species exceeds the antioxidant scavenging capacity of a tissue (Das *et al*., 2006). Malonyldialdehyde (MDA) is frequently anaylsed as one of the end products of the lipid peroxidation process, which is a marker of oxidative stress (Plachta *et al*., 1992; Betteridge, 2000). Oxidative stress has an impact on the production of granulosa cell steroid hormones, which is an important predictor of ovarian response (Appasamy *et al*., 2008). The level of MDA was significantly increased in the follicular fluid of patients with PCOS undergoing assisted reproduction as compared with patients with normal ovulation on IVF treatment (Yıldırım *et al*., 2007).

To date, the relationship between follicular Hcy levels and oocyte and embryo quality has not been specifically determined in PCOS women undergoing assisted reproduction. The principal goal of this study was to assess Hcy levels in follicular fluid of women with PCOS undergoing assisted reproduction and to clarify the relationship between oocyte and embryo quality and fertilization rate. In addition, follicular fluid vitamin B_{12}, folate, estradiol (E_2) and MDA were measured to examine the relationship between the follicular fluid Hcy and these parameters.

Materials and Methods

Subjects

Women with PCOS ($n = 52$) undergoing assisted reproduction comprised the study group. All patients have failed to conceive in three ovulatory cycles after ovulation induction with gonadotrophins. They had all been investigated by hysterosalpingograph and some of them also by laparoscopy and hysteroscopy. All of the male partners had normal semen quality according to World Health Organization (WHO, 1999) criteria. PCOS patients with accompanying male factor infertility, endometriosis or tubal factor were excluded. Patients who had a history of smoking were excluded as well. The diagnosis of PCOS was made according to menstrual history, hirsutism, hormonal concentrations and ultrasound examination and was based on the revised Rotterdam ESHRE/ASRM criteria (2004). All women had oligomenorrhoea or amenorrhoea (eight or fewer menstrual cycles per year), had clinical (hirsutism) and/or biochemical (raised free androgen index) (Vermeulen *et al*.,1999) evidence of hyperandrogenism, and polycystic ovaries on ultrasound scans. Patients with congenital adrenal hyperplasia, Cushing's syndrome, androgen-secreting tumours and thyroid disease were excluded and all patients had normal prolactin levels. All of the patients met these inclusion criteria. In agreement with the inclusion criteria, no patient suffering from any other aetiology of infertility has been enrolled. All subjects were asked to give a written consent and the institutional review boards of hospitals approved the study.

Cycle monitoring and protocol for controlled ovarian stimulation

All patients received a standard GnRH agonist (leuprolide acetate) regimen starting on Day 21 of a spontaneous menstrual cycle. Leuprolide acetate (0.5 mg Lucrin, daily injection, Abbott, Istanbul) was administered for 10–14 days until complete pituitary desensitization was documented. Recombinant FSH stimulation (150 IU, recFSH) was initiated on the third day of subsequent withdrawal bleeding and at that time the dose of leuprolide acetate was decreased to 0.25 mg/day. Further recFSH doses were determined according to the standard criterion for follicular maturation, assessed by ultrasound and serum E_2 measurements. HCG (10 000 IU, Pregnyl, Organon, the Netherlands) was administered when at least three follicles had reached a diameter of >18 mm. Oocyte retrieval was performed 36 h later under transvaginal ultrasound guidance and i.v. sedation. All patients received luteal phase support of 600 mg/daily of vaginally administered micronized progesterone (Progestan, Koçak, Istanbul) starting from the day after oocyte retrieval. The oocytes were graded (Veeck, 1999) and subsequently inseminated by ICSI. Pronucleolus score was noted 16–18 h after insemination. Embryo quality was assessed before embryo transfer, and a maximum of three embryos were transferred to each patient approximately 48 h (4-cell stage) after insemination (Martikainen *et al*., 2001). Grade 1–2 embryos were identified on Day 3 as those having no multinucleated blastomeres, four or five blastomeres and $<20\%$ anucleated fragments. Grade 3 embryos were identified on Day 3 as those having 20–50% anucleated fragments or number of multinucleated blastomers (Van Royen *et al*., 1999). Embryos were transferred on the third day after oocyte retrieval. Depending on the woman's age and the embryo quality, one to three embryos were transferred using an Edwards Wallace catheter (Simcare Ltd, UK) under transabdominal sonographic guidance. Biochemical pregnancy was established when serum β-HCG level was >20 IU/l on the 12th day after embryo transfer, and clinical pregnancy was defined as the presence of a gestational sac on ultrasound performed at 6 weeks after embryo transfer.

Follicular fluid collection

To obtain the exact Hcy level within a single follicle and avoid contamination from blood, flush medium or mixed follicular fluid during oocyte retrieval, only the follicular fluid from the first retrieved follicle from bilateral ovaries was collected. The presence or absence of blood contamination was graded by visual inspection, and samples that looked cloudy or blood stained were discarded: meticulous care was taken to include only uncontaminated samples. Follicular fluid was not collected from follicles of <17 mm in diameter size. One hundred and four follicular fluid samples were collected and 94 samples were suitable for analysis. The collected follicular fluids were processed by centrifugation at 3000*g* for 15 min at 4°C to eliminate cellular elements and subsequently frozen at −80°C until biochemical and hormonal analysis. For the analysis, 104 follicles bigger than 17 mm in diameter were collected. Ninety-four samples were suitable for analysis. About 74 of the 94 collected follicles were graded as Grade 3 oocyte (metaphase II) and 20 of them were graded as Grade 2 oocyte (metaphase I). Then, on day 2, samples of follicular fluid were classified again based on subsequent embryo formation as Grade 1–2 embryo and Grade 3 embryo. All embryos were then cultured for 72 h and subsequently transferred back to the uterus. All assays of follicular fluid for each patient were performed in duplicate. Time elapsed

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Table I Patient characteristics in the low (<25th), average (25th–75th) and high (>75th) follicular Hcy groups, for 52 women with PCOS

Variable[b]	dOPU follicular Hcy (μmol/L)[a]		
	<7.1	7.1–9.8	>9.8
Age (years)	29.4 ± 3.1	29.1 ± 3.7	30.1 ± 3.3
BMI (kg/m^2)	23.2 ± 3.9	23.8 ± 3.8	24.3 ± 3.5
FSH (IU/l)	6.2 ± 1.8	6.4 ± 1.7	6.5 ± 1.8
LH (IU/l)	9.2 ± 1.9	9.6 ± 2.4	9.0 ± 2.7
>14 mm, follicle (*n*)	11.2 ± 2.6	11.7 ± 2.1	11.8 ± 2.1
Totally oocyte (*n*)	16.0 ± 4.9	15.4 ± 5.7	16.6 ± 5.8
MII oocyte (*n*)	10.1 ± 2.6	10.7 ± 3.0	10.5 ± 3.3
Total gonadotropin dose (IU)	2250 ± 378	2170 ± 329	2215 ± 375
rFSH/day	12.2 ± 1.7	12.6 ± 1.3	11.7 ± 1.5
>17 mm follicle (*n*)	9.9 ± 3.4	9.4 ± 3.3	9.0 ± 3.6
E_2 (pg/ml)/on day of hCG	2445 ± 448	2286 ± 475	2420 ± 430

Differences among groups are not statistically significant.
MII, metaphase II oocyte; E_2, estradiol; dOPU, day of oocyte retrieval; recFSH, recombinant FSH; PCOS, polycystic ovary syndrome.
[a,b]Data are mean ± SD.

between follicular aspiration and follicular fluid cryopreservation did not exceed 30 min.

Biomarker measurements in serum, plasma and follicular fluid

Blood samples obtained at time of oocyte retrieval were assayed for Hcy, vitamin B_{12}, folate, E_2 and MDA. Plasma and follicular fluid Hcy levels were determined as total Hcy technique by high-performance liquid chromatography using Chromsystems kits with fluorescence detector (Mannheim, Germany). The intra- and inter-assay coefficients of variation (CV) were <2%. Serum and follicular fluid vitamin B_{12} and folate levels were measured with a specific electrochemiluminescence immunoassay (ELECYS 2010 HITACHI, Roche Diag. Germany). Mean and intra- and inter-assay CV for vitamin B_{12} and folate were 5.2, 3.4, 4.9% and 6.8, 7.9, 5.4%, respectively. Serum E_2 levels were determined by an automated chemiluminescence technique (ELECYS 2010 HITACHI, Roche Diag. Germany). The intra- and inter-assay CV were 2.7 and 3%, respectively, and the sensitivity of the assay was 10 pg/ml. Plasma and follicular MDA levels were determined by the 2-thiobarbituric acid reactive substances (TBARS) method. 1,1,3,3-Tetraethoxypropane (Sigma, UK) was used as a standard, and results were expressed as nanomolar per millilitre. In order to avoid possible bias as a result of follicular fluid volume variability, hormone concentrations in the follicular fluid were adjusted for protein content (Spitzer *et al.*, 1996), measured using the pyrogallol-red molybdate complex (colorimetric method) (Cobas Integra 700/800, Roche Diag. Germany).

Statistical analysis

Data analysis was performed by using the Statistical Package for the Social Sciences for Windows, version 11.5. Continuous variables were tested for normal distribution using Shapiro–Wilk test. Hcy concentrations were assigned to the 25th centile or below (low Hcy), between the 25th and 70th centiles (average Hcy) or above the 75th centile (high Hcy) of measurements. Data were shown as median (25th–75th) percentiles. Wilcoxon Sign Rank test was applied for intra-group comparisons. The differences among follicular fluid Hcy percentiles were evaluated by Kruskal–Wallis test. When the Kruskal–Wallis test was significant, a multiple comparison test was used to determine which groups differed from which others. Degree of association between continuous variables was calculated by Spearman's *rho* correlation coefficient. The effects of follicular fluid Hcy concentration on fertilization, oocyte and embryo quality were analysed with multiple linear and multinomial logistic regression analyses after adjusting for age, dose of FSH and the number of transferred embryos. In multiple linear and multinomial logistic regression analyses, the effects of follicular Hcy tertials on fertilization, oocyte and embryo quality were evaluated after adjusting for age, FSH dose and the number of embryos transferred. In the multiple linear and multinomial logistic regression analyses, fertilization rate, oocyte and embryo quality are the dependent variables and the mean age, total FSH dose and number of embryos transferred are the independent variables. A *P*-value of <0.05 was considered statistically significant.

Results

Table I shows patient characteristics in the groups of the low, average and high follicular Hcy. The concentrations of Hcy, E_2, vitamin B_{12}, folate and MDA in plasma and follicular fluid are presented in Table II. The concentrations of Hcy, E_2, vitamin B_{12}, folate and MDA in plasma were higher than in follicular fluid. Ninety-four follicular samples were suitable for Hcy analysis. The mean ± SD value of follicular fluid Hcy was 8.7 ± 2.2 μmol/l with a range from 4.0 to 19.9 μmol/l and a median of 8.0 μmol/l. The associations between follicular Hcy levels and vitamin B_{12}, folate, E_2 and MDA levels are presented in Table III. Follicular fluid vitamin B_{12} levels and E_2 were lower, whereas MDA levels were higher, in patients showing higher follicular fluid Hcy.

Significant differences were observed in terms of follicular fluid Hcy levels between the groups. Hcy levels were lower in Grade 3 oocyte group than Grade 2 oocyte group (Fig. 1A). Hcy levels were lower in Grade 1–2 embryo group than Grade 3 embryo group (Fig. 1B). We also compared follicular fluid Hcy levels between patients who became pregnant after IVF/ICSI and those did not (Fig. 1C). Decreased follicular fluid Hcy levels were noted in pregnant group. Based on percentile

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Table II Concentrations of Hcy, vitamin B_{12}, folate, E_2 and MDA in plasma or serum and in follicular fluid (follicular fluid) in 52 PCOS patients undergoing assisted reproduction

Variable	Plasma or serum	Follicular fluid (per mg protein)	P
Hcy (μmol/l)	11.7 ± 2.9	9.1 ± 2.7	<0.001
Vitamin B_{12} (pg/ml)	328.2 ± 56.9	271.0 ± 62.5	<0.001
Folate (ng/ml)	12.8 ± 3.9	9.9 ± 6.6	<0.001
E_2 (pg/ml)	2518.1 ± 708	1991.0 ± 677	<0.001
MDA (nmol/l)	7.5 ± 3.3	5.2 ± 3.3	<0.001

Statistical significance was defined as $P < 0.05$. Follicular fluid Hcy, vitamin B_{12}, folate, E_2 and MDA concentrations in the follicular fluid were adjusted for protein content. Hcy and MDA were measured in plasma while E_2, vitamin B_{12} and folate were measured in serum.
Values are mean ± SD.

of follicular fluid Hcy, the association between follicular Hcy levels and oocyte and embryo quality and fertilization rate are presented in Table IV. Grade 3 oocyte, Grade 1–2 embryo and fertilization rate were significantly lower in patients showing higher follicular fluid Hcy.

The follicular fluid Hcy levels were significantly and negatively correlated with follicular fluid vitamin B_{12}, folate and fertilization rate (Fig. 2A, B and D). The follicular fluid Hcy levels were significantly and positively correlated with follicular fluid MDA levels (Fig. 2C).The follicular fluid Hcy levels were negatively correlated with follicular fluid E_2 but this did not reached a statistical significance ($r = -0.19$, $P = 0.09$, data not shown). Serum concentrations of Hcy, folate, vitamin B_{12} and MDA appeared to significantly correlate with corresponding concentrations in follicular fluid (data not shown). However, no significant associations were observed between follicular fluid Hcy levels and follicular fluid folate levels. No correlation was found for the other mentioned parameters.

The effects of follicular fluid Hcy tertiles on fertilization, oocyte and embryo quality were analysed with multiple linear and multinomial logistic regression analysis. In the multiple linear regression analyses (Table V), follicular fluid Hcy is an independent determinant of oocyte quality and embryo quality [standardized β coefficient = −0.43, $P < 0.001$, 95% CI (−0.25 to 0.09); standardized β coefficient = −0.836, $P < 0.001$, 95% CI (−0.49 to 0.311), respectively]. In multinomial logistic regression analyses according to follicular fluid Hcy levels of <7.1, when follicular fluid Hcy levels are between 7.1 and 9.8, Grade 1–2 embryo rate decreases 4.17 times. According to follicular fluid Hcy levels <7.1, when follicular fluid Hcy levels are >9.8, Grade 1–2 embryo rate decreases 14.67 times [odds ratio = 4.1 (95% CI: 1.2–13.6, $P < 0.01$), odds ratio = 14.6 (95% CI: 1.5–13.5), respectively] (Table V).

Discussion

This is the first study about the role of Hcy at the ovarian level and reproductive outcome in PCOS patients undergoing assisted reproduction. In the present study, there was a negative association between follicular fluid Hcy levels and fertilization rate and of oocyte and embryo quality in PCOS patients undergoing assisted reproduction.

The presence of folate and Hcy in ovarian follicular fluid has been previously demonstrated (Steegers-Theunissen *et al*., 1993). More recently, it was demonstrated that preconception folic acid supplementation significantly alters folate and total Hcy levels in follicular fluid (Boxmeer *et al*., 2008b). Thus, the composition of follicular fluid to some extent seems to reflect systemic Hcy metabolism, and high Hcy levels or low methionine levels in the follicular fluid may well occur. However, several studies have previously found that the serum Hcy levels were significantly higher in PCOS women (Yarali *et al*., 2001; Loverro *et al*., 2002; Schacter *et al*., 2003; Kaya *et al*., 2008). In this study, the concentrations of Hcy, E_2, vitamin B_{12}, folate and MDA in plasma were higher than in follicular fluid (Table II). Serum concentrations of Hcy, folate, vitamin B_{12} and MDA appeared to be significantly correlated with corresponding concentrations in follicular fluid. Therefore, it may be assumed that increased plasma Hcy levels can affect follicular fluid Hcy levels.

In the present study, there was a significant association between follicular fluid Hcy levels and the grade of oocytes. Significant differences were observed in terms of follicular Hcy levels between Grade 3 and 2

Table III Concentrations of Hcy, vitamin B_{12}, folate, E_2 and MDA in the low (<25th), average (25th—75th) and high (>75th) follicular Hcy groups in 52 PCOS patients with undergoing assisted reproduction

Variables	In follicular fluid Hcy (μmol/L)[a]			Overall P-value[b]
	Low <7.1	Median 7.1–9.8	High >9.8	
Vitamin B_{12} (pg/ml)/mg protein	312.5 ± 61.1	217.1 ± 69.2[d]	167 ± 62.2[c]	<0.01
Folate (ng/ml)/mg protein	11.9 ± 4.4	10.2 ± 4.7	10.9 ± 4.1	0.78
E_2 (pg/ml)/mg protein	1878 ± 474	1586 ± 522[e]	1082 ± 451[e]	<0.01
MDA (nmol/l)/mg protein	4.1 ± 1.1	5.9 ± 1.6[d]	8.2 ± 2.1[c]	<0.01

[a]Data are shown as low (<25th), median (25th—75th) and high (>75th) percentiles. Follicular fluid Hcy, vitamin B_{12}, folate and E_2 and MDA concentrations in the follicular fluid were adjusted for protein content.
[b]Overall *P*-values was determined by Kruskal–Wallis test.
[c]Kruskal–Wallis multiple comparison test were used to know which group differ from which others <0.001.
[d]Kruskal–Wallis multiple comparison test were used to know which group differ from which others ≤0.002.
[e]Kruskal–Wallis multiple comparison test were used to know which group differ from which others ≤0.01.

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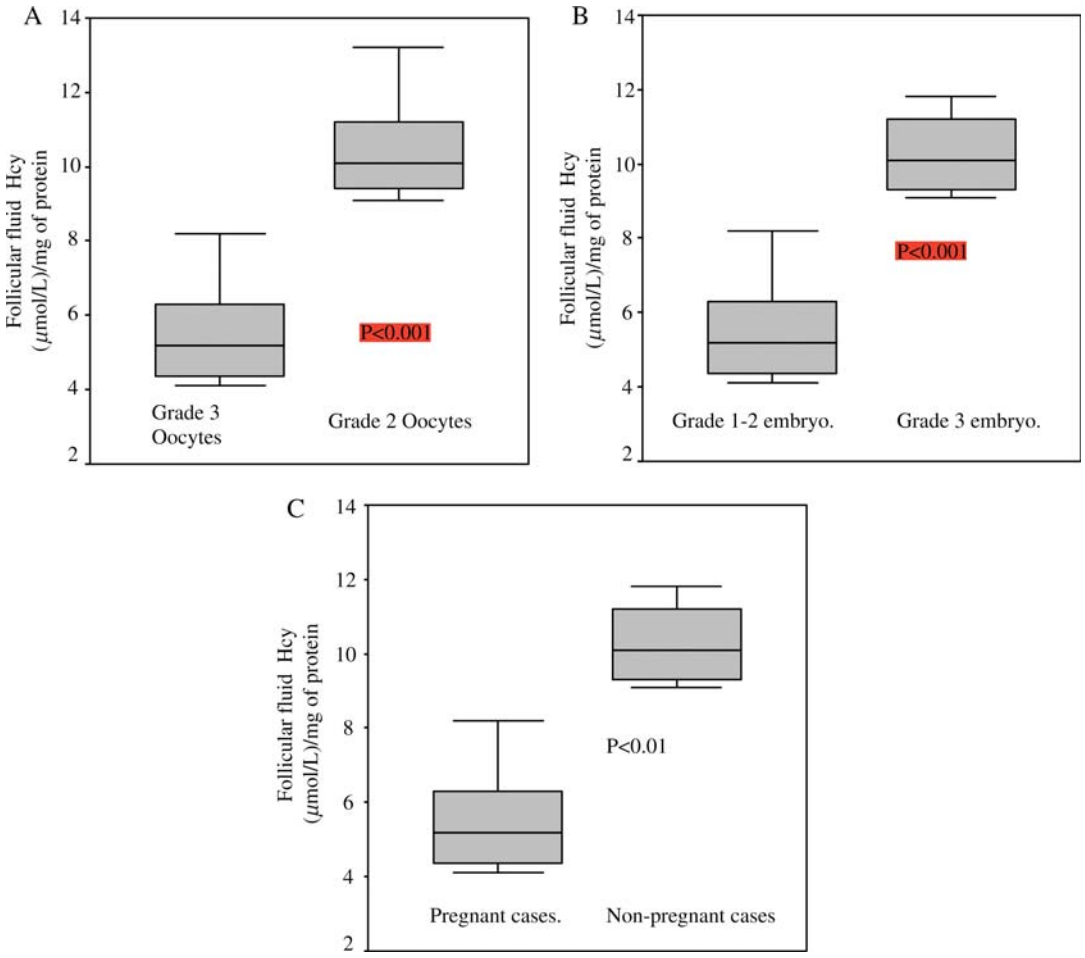

Figure 1 Box and whisker plots depicting the levels of Hcy in follicular fluid ($n = 94$ samples).

Solid lines inside boxes depict the median follicular Hcy level, whereas the upper and lower limits of the boxes and whiskers indicate 75th, 25th and 95th and 5th percentiles. Follicular fluid Hcy concentrations were adjusted for protein content. (**A**) follicular Hcy levels in Grade 1 oocytes and Grade 2 oocytes. (**B**) Follicular Hcy levels in Grade 1–2 and Grade 3 embryos. (**C**) Follicular Hcy levels in women who did or did not become pregnant.

oocyte groups. When PCOS patients showing higher follicular Hcy concentration are compared with those with the lower follicular Hcy concentration, there were fewer Grade 3 oocytes and more Grade 2 oocytes in the higher Hcy level group (Table IV). The follicular fluid Hcy levels were negatively associated with follicular fluid E_2. So far, follicular fluid Hcy levels and the associations with follicular fluid E_2 have not been reported in PCOS patients undergoing assisted reproduction. In addition to this, high follicular fluid Hcy levels are negatively correlated with oocyte fertilization, which indicates that follicular fluid Hcy may play an important role in the development of oocytes and fertilization. The E_2-enriched follicular fluid in women undergoing assisted reproduction positively correlated with oocyte fertilization, cleavage and implantation (Wramsby *et al.*, 1981; Carson *et al.*, 1982; Botero-Ruiz *et al.*, 1984; Foong *et al.*, 2005), whereas the absence of E_2 production in patients with 17α-hydroxylase deficiency is associated with *in vitro* embryonic developmental arrest and an inability to conceive (Rabinovici *et al.*, 1989; Pellicer *et al.*, 1991; Tesarik and Mendoza 1995). In addition, Teissier *et al.* (2000) showed that E_2 follicle concentration, following recFSH induction, is correlated with oocyte quality regardless of the endocrine profiles of PCOS women. Taking all these results together, we may speculate that higher follicular fluid Hcy levels may suppress E_2 production and interfere with the development of dominant follicles, oocyte maturation and fertilization in women with PCOS undergoing assisted reproduction. Previously, a low concentration of follicular fluid Hcy has been reported to be associated with a higher degree of maturation of the oocyte (Steegers-Theunissen *et al.*, 1993). In our study also, high follicular fluid Hcy levels were negatively correlated with oocyte fertilization, which indicates that follicular fluid Hcy may play an important role in the development of oocytes and fertilization. In the multiple linear regression analysis, follicular fluid Hcy is independently associated with oocyte and embryo qualities. These findings suggest that follicular fluid Hcy levels do reflect the fertilization potential of oocytes in PCOS patients undergoing assisted reproduction. Therefore, we may speculate that higher follicular fluid Hcy may lead to a number of development defects of the oocyte that can impede fertilization and/or the reproductive outcome in PCOS patients undergoing assisted reproduction. However, low fertilization

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Table IV The oocyte, fertilization rate and quality of embryo for PCOS patients in relation to follicular fluid Hcy levels

Variables	In follicular fluid Hcy (μmol/l)[a]			Overall *P*-value[b]
	Low <7.1	Median 7.1–9.8	High >9.8	
Grade 3 oocytes (%)	77.8 ± 5.1	49.7 ± 3.7[e]	26.0 ± 4.3[e]	<0.001
Grade 2 oocytes (%)	24.2 ± 4.7	35.3 ± 4.1[c]	48.0 ± 5.8	<0.001
Fertilization rate (%)	75.7 ± 3.4	64.2 ± 10.4[e]	50.7 ± 8.1[e]	<0.001
Grade 1-2 embryo (%)	70.8 ± 6.4	39.1 ± 4.4[d]	19.2 ± 5.8[d]	<0.001
Grade 3 embryo (%)	29.2 ± 5.6	60.9 ± 4.8[d]	80.8 ± 6.4[e]	<0.001

[a]Data were shown as low (<25th), median (25th—75th) and high (>75th) percentiles.
[b]Overall *P*-values was determined by Kruskal–Wallis test.
[c]Kruskal–Wallis multiple comparison test <0.001.
[d]Kruskal–Wallis multiple comparison test ≤0.002.
[e]Kruskal–Wallis multiple comparison test ≤0.01.

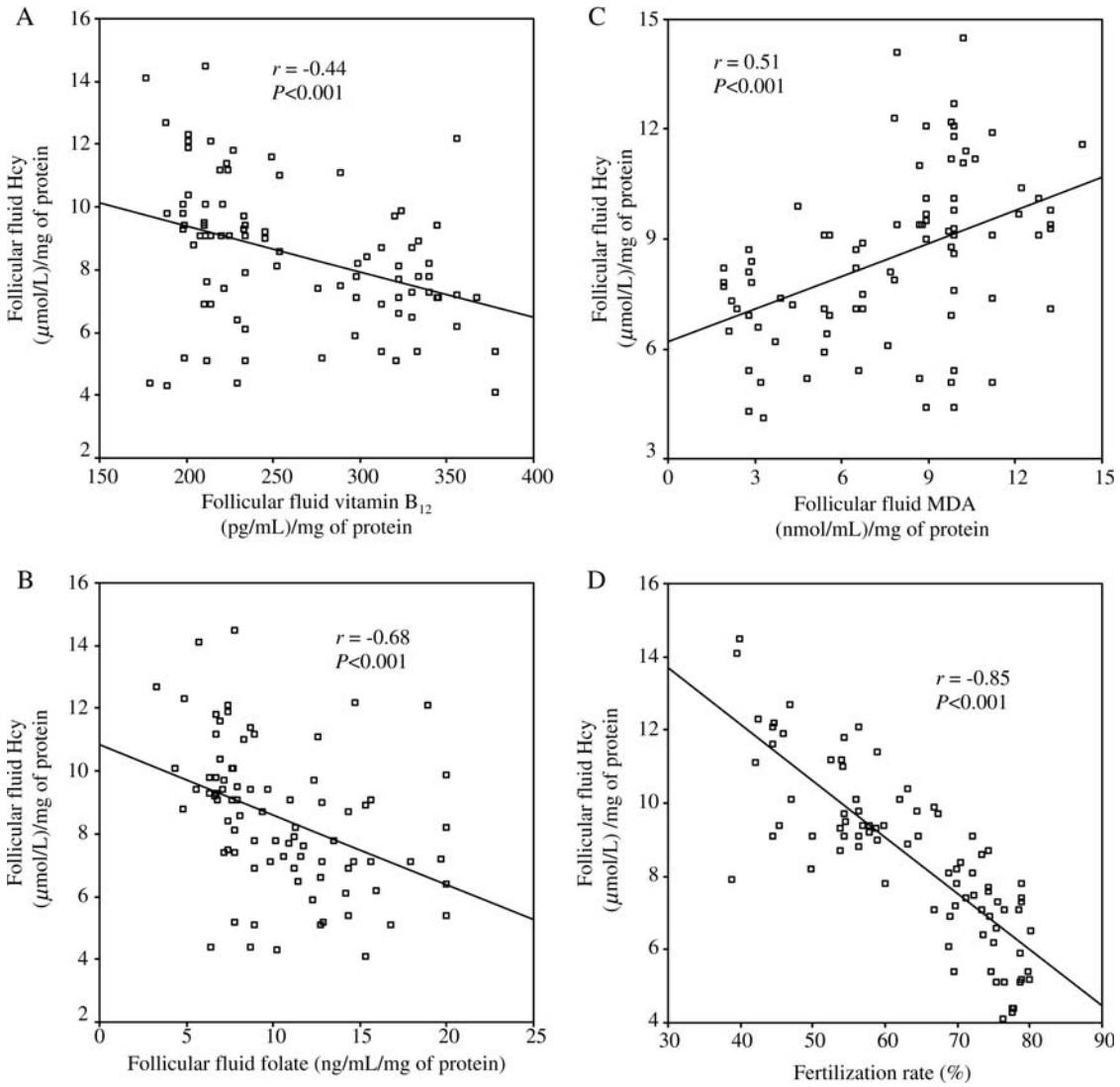

Figure 2 (**A**–**D**) Correlation between follicular Hcy levels ($n = 94$ samples) and vitamin B_{12}, folate, MDA and fertilization rate.
Follicular fluid Hcy concentrations were adjusted for protein content.

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Table V Multiple linear regression analysis of relationship between follicular fluid Hcy concentrations and selected reproductive variables in 52 PCOS patients after adjusting for maternal age, total FSH dose and the number of transferred embryos

Dependent variables	Independent variables	Coefficient of regression (β)	Standardized β	*P*	95% CI for β	
					Lower	Upper
Fertilization rate	Hcy *F* [7.1–9.8]	−0.178	−0.437	<0.001	−0.257	−0.099
	Hcy *F* >9.8	−0.404	−0.836	<0.001	−0.498	−0.311
	Age (years)	−0.007	−0.137	0.091	−0.016	0.001
	Total FSH dose	0.00005	0.042	0.607	−0.0001	0.0002
	Embryo transfer (*n*)	−0.011	−0.024	0.762	−0.084	0.062
Oocyte quality	Hcy *F* [7.1–9.8]	−0.004	−0.010	<0.001	−0.115	0.107
	Hcy *F* >9.8	−0.016	−0.032	<0.001	−0.147	0.116
	Age	0.014	0.263	0.019	0.002	0.026
	Total FSH dose	0.0001	0.045	0.686	−0.0002	0.0003
	Embryo transfer (*n*)	−0.022	−0.046	0.674	−0.124	0.081
Embryo quality	Hcy *F* [7.1–9.8]	−0.220	0.509	<0.001	0.116	0.324
	Hcy *F* >9.8	−0.272	0.530	<0.001	0.149	0.395
	Age	0.004	−0.064	0.526	−0.015	0.008
	Total FSH dose	0.0001	−0.057	0.567	−0.0003	0.0002
	Embryo transfer (*n*)	0.011	−0.022	0.827	−0.107	0.085

CI: confidence interval.

rate may be a result of suboptimal response to ovarian stimulation but all the patients underwent the same stimulation protocol in this study. Therefore, here-presented data indicates that low fertilization rate was independent of ovarian stimulation protocol in our PCOS series.

In this study, a significant negative association between follicular fluid Hcy levels and the quality of embryo has been observed. Significant differences were observed in terms of follicular Hcy levels between Grade 1–2 and Grade 3 embryos. The follicular fluid Hcy levels were higher in Grade 3 embryo group than Grade 1–2 embryo group. Therefore, the follicular fluid Hcy levels were associated with poor embryo quality. There were fewer Grade 1–2 embryos in patients showing higher follicular Hcy. In the multiple linear regression analysis, follicular fluid Hcy is an independent determinant of the quality of embryo and in the multinomial logistic regression analysis, as the levels of follicular fluid Hcy increased, Grade 1–2 embryos decreased. These findings reveal that there is a direct relationship between follicular fluid Hcy levels and the quality of embryo in PCOS patients undergoing assisted reproduction. Our findings are in good agreement with the reports of Ebisch *et al.* (2006), where inverse correlation between the follicular fluid Hcy concentrations and embryo quality were reported. Boxmeer *et al.* (2008b) determined a significant effect of folic acid supplementation on the follicular fluid Hcy concentrations. We also found that the follicular fluid Hcy levels were significantly and negatively correlated with follicular fluid folate levels. This situation suggests folate and vitamin B_{12} might play an important role in the follicular Hcy levels in PCOS patients. Boxmeer *et al.* (2008a, b) showed that high ovarian follicular fluid total Hcy and folate levels may have detrimental effects on follicular development. The results of our study also seem to suggest that high levels of Hcy have detrimental effect on the quality of oocyte and embryo. Based on the results of the present study, the effect of follicular fluid Hcy levels on quality of embryo may be mediated through follicular fluid vitamin B_{12} or methionine or lipid peroxidation. Vitamin B_{12} levels were lower whereas MDA levels were higher in patients showing higher follicular Hcy. As expected, the levels of follicular fluid Hcy were significantly and negatively associated with follicular fluid vitamin B_{12} levels. Therefore, follicular fluid vitamin B_{12} levels are closely associated with follicular fluid Hcy levels. The importance of vitamin B_{12} in the remethylation of Hcy to methionine is well recognized, and hyperhomocysteinaemia is also a feature of vitamin B_{12} deficiency (Selhub and Miller 1992; Fonseca *et al.*, 1999; McCarty 2000). The follicular fluid low vitamin B_{12} may prevent the generation of methionine from Hcy (Selhub and Miller 1992; Fonseca *et al.*, 1999). Methionine is converted to *S*-adenosylmethionine, which is an essential methyl donor in the body for DNA. Therefore, methionine is important for DNA synthesis as well as the regulation of DNA expression by methylation. Thus, follicular fluid methionine may be an important nutrient contributing to the growth of oocytes. In this study, as the follicular fluid Hcy increased, follicular fluid vitamin B_{12} decreased. Thus, low follicular fluid vitamin B_{12} may be lead to a decrease of methionine in follicular fluid. The follicular fluid low methionine levels may lead to poor quality embryo formation. However, this hypothesis requires further biological investigation. Therefore, these findings are to be confirmed by prospective cohort studies before low follicular methionine becomes a possible prospective identification marker in PCOS women prone to develop poor oocyte and poor embryo.

On the other hand, follicular MDA levels were higher in patients showing higher follicular Hcy levels. These findings suggest that increased levels of the follicular fluid Hcy in PCOS leads to lipid

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peroxidation in follicular fluid. These results suggest a direct link between homocysteinemia and lipid peroxidation in follicular fluid. Close correlations were found between plasma levels of Hcy and oxidative LDL (Bautista *et al*., 2002; Homocystein Studies Colloboration, 2002). Therefore, hyperhomocysteinemia in follicular fluid of PCOS patients undergoing assisted reproduction may accelerate lipid peroxidation in follicular fluid and it may occur during oxidative stress in follicular fluid. Inconsistent results related to the detrimental and beneficial levels of reactive oxygen species and lipid peroxidation have been reported (Ho *et al*., 1998; Jozwick, 1999; Attaran *et al*., 2000; Oyawoye *et al*., 2003; Agarwal *et al*., 2003, 2005). Nevertheless, the relationship between oxidative stress parameters in follicular fluid and reproductive outcome has not yet been extensively investigated. However, Yıldırım *et al*. (2007) showed that lipid peroxidation was significantly increased in the follicular fluid of patients with PCOS undergoing assisted reproduction as compared with patients with normal ovulation on IVF treatment. In our study, follicular fluid Hcy and MDA affects the growth of oocytes and the quality of embryos through the same mechanism and their effects may be additive rather than interdependent in PCOS patients. Thus, the complex interaction between hyperhomocysteinemia and lipid peroxidation may result in the lower fertilization rate or fertilization failure, or poor embryo quality seen in PCOS patients. As a result, increase in follicular fluid Hcy and MDA and decrease in vitamin B_{12} may cause abnormal oocyte maturation, fertilization and possibly poor embryo formation in PCOS patients undergoing assisted reproduction. In the present study, we did not examine the relationship between quality of embryo and pregnancy rate. But our results showing lower follicular fluid Hcy concentrations in women who became pregnant compared with those who did not, infers that follicular fluid Hcy levels may affect pregnancy outcome in PCOS undergoing assisted reproduction. However, there is no difference in the levels of Hcy between clinical and biochemical pregnancy.

This study revealed that there was a negative association between the follicular fluid Hcy levels and fertilization rate and oocyte and embryo qualities in PCOS patients undergoing assisted reproduction. In the literature, there are conflicting data about the fertilization rate in women with PCOS. Several retrospective and prospective controlled studies reported that the fertilization rate was lower in PCOS patients (Regan *et al*., 1990; Salat-Baroux *et al*., 1988; Ashkenazi *et al*., 1989; Dor *et al*., 1992; Balen *et al*., 1993, 2003; Homburg *et al*., 1993; Homburg *et al*., 1988; Kodama *et al*., 1995; Heijnen *et al*.,2006), whereas other studies reported that the fertilization rate was normal (Engmann *et al*., 1999; Ludwig *et al*., 1999; Franks, 2002; Mulders, 2003; Jabara *et al*., 2003; Sahu *et al*., 2008). Not surprisingly, in view of this uncertainty, the fertilization rate in PCOS patients undergoing assisted reproduction remains controversial. Despite significantly higher oocyte yields, cumulative pregnancy rates in women with PCOS are not superior to those in control group (Homburg *et al*., 1993; Pellicer *et al*., 1996; Ludwig *et al*., 1999; Weghofer *et al*., 2007). A conceptual agreement, therefore, suggest that oocytes and embryos are of poor quality from patients with PCOS (Homburg *et al*., 1993; Pellicer *et al*., 1996; Cano *et al*., 1997; Ludwig *et al*., 1999; Franks, 2002; Plachot *et al*., 2003). Therefore, certain metabolic abnormalities in PCOS may influence oocyte or embryo quality (Franks, 2002; Mulders, 2003). It is possible that these findings in PCOS patients are related—in part—to high follicular fluid Hcy levels and that follicular fluid Hcy concentrations may play a causative role in the quality of oocytes, fertilization rate and the quality of embryos in PCOS patients undergoing assisted reproduction.

When the results of our study are compared with the study of Ebisch *et al*. (2006), which evaluated pre-ovulatory follicular fluid Hcy levels, the results in our study population are lower than fertile, idiopathic and endometriosis groups. The reasons for the differences between the findings are unclear because we do not know the factors affecting pre-ovulatory Hcy levels in fertile, idiopathic infertility and PCOS patients. In this study, there was no correlation between recFSH dose and follicular fluid Hcy. However, other unknown factors might play a role in follicular fluid Hcy regulation causing the differences between the groups. If the factors such as obesity, hyperinsulinemia and hiperandrogenemia associated with ovarian disfunction in PCOS are evaluated to clarify their role in follicular Hcy regulation, then the differences might be better explained. In the study of Ebisch *et al*. (2006), in assisted reproduction technology cycles a correlation was found between Hcy levels and embryo quality. Our results confirm this in women with PCOS.

We included PCOS couples with unsuccessful ovulation induction and PCOS women with both male factor, endometriosis or tubal factor were excluded. A strength of our study is the number of couples undergoing an ICSI procedure ($n = 52$) and thus we believe that the results obtained do not merely reflect chance in PCOS women who underwent ICSI following unsuccessful ovulation induction.

In conclusion, these results suggest that an increase in follicular fluid Hcy levels may affect the fertilization rate, the quality of oocytes and embryo through a decrease in follicular fluid vitamin B_{12}, E_2 and an increase in follicular fluid MDA in PCOS patients undergoing assisted reproduction. Concentrations of Hcy in the follicular fluid may constitute a useful marker for the quality of oocytes, fertilization rate and the quality of embryos in PCOS patients undergoing assisted reproduction.

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