Intraindividual variability of homocysteine and related thiols concentrations in follicular fluid

Michaela Kralikova · Jitka Melounova · Igor Crha ·
Milena Matejovicova · Jana Zakova · Jiri Jarkovsky ·
Jaroslava Tallova

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Abstract

Purpose To determine intraindividual variability in concentrations of homocysteine and related thiols in follicular fluids of particular follicles after ovarian stimulation and assess the differences between follicles with/without oocytes.

Methods HPLC-FD analysis of plasma and follicular fluid cysteine, homocysteine, cysteinylglycine and glutathione in women undergoing IVF.

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Capsule Study reveals a high intraindividual variability in concentrations of homocysteine and related thiols in follicular fluids. The same follicle should be observed for further analysis.

M. Kralikova (✉) · J. Melounova · M. Matejovicova · J. Tallova
Department of Biochemistry, Faculty of Medicine,
Masaryk University,
A16, Kamenice 5,
625 00 Brno, Czech Republic
e-mail: mkralik@med.muni.cz

I. Crha · J. Zakova
Department of Gynecology and Obstetrics, Faculty of Medicine,
Masaryk University and Faculty Hospital,
Obilni trh 11,
602 00 Brno, Czech Republic

J. Jarkovsky
Institute of Biostatistics and Analyses, Masaryk University,
Kamenice 5,
625 00 Brno, Czech Republic

Results In blood plasma, the homocysteine, cysteine, and cysteinylglycine concentrations decreased significantly during stimulation with rec FSH ($p < 0.001$). We found significant differences in concentrations of cysteine and glutathione between follicles with or without retrieved oocytes. High intraindividual variability in concentrations of thiols was determined.

Conclusions The concentration variability of thiols between single follicles is very high and we recommend mean at least from 3 follicles with/without oocytes for characterization of each woman. It is the best to examine individual follicles for further research and analysis of fertility outcomes.

Keywords Homocysteine · Thiols · Follicular fluid · Oocytes

Introduction

Hyperhomocysteinemia is associated with many pathologies, such as cardiovascular diseases, neurodegenerative disorders (e.g. Alzheimer's disease), hypothyroidism, birth defects and late pregnancy complications, such as pre-eclampsia, intrauterine growth retardation, preterm birth and intrauterine fetal death and abruption of placenta [1–6].

Both male and female factors are concerned in the causes of human infertility and changes of homocysteine (Hcy) levels in body fluids were observed in fertility disorders in both sexes [7–10].

Exogenous estrogens have been shown to affect several endocrine and metabolic pathways including homocysteine metabolism. Plasma estrogen and progesterone levels are higher in premenopausal women compared with postmen-

opausal women. Estrogen treatment of post-menopausal women significantly decreases the total Hcy levels [11]. Previous studies showed that the levels are significantly lower in the luteal phase than in the follicular phase of the menstrual cycle of premenopausal women [12]. And moreover, blood plasma total Hcy decreases during pregnancy from preconception onwards [13, 14].

A significant negative correlation between follicular fluid Hcy levels and oocyte and embryo quality was demonstrated in women undergoing assisted reproduction [8]. Ovarian stimulation deranges blood and follicular fluid biomarkers of the Hcy pathway and affects the Hcy concentration in follicular fluid [15]. High ovarian follicular fluid Hcy and folate levels may have detrimental effect on follicular development [17], Hcy inhibits the methylations and has a negative effect on IVF outcome [18]. Elevated Hcy may also suppress the synthesis of estradiol and consequently influence the oocyte maturation and fertilization in women with polycystic ovary syndrome [17, 18]. Because the cystathionine-beta-synthase is only poorly expressed or absent in the human oocyte [19], the remethylation pathway is the only alternative to remove Hcy.

Metabolism of Hcy is closely related to the metabolism of other thiols—cysteine (Cys), cysteinylglycine (CG) and glutathione (GSH). Cysteine generates in the transsulfuration pathway of Hcy metabolism and meets CG and GSH in gamma-glutamyl cycle. GSH is a very potent antioxidant protecting from oxidative stress and through direct conjugation, it can detoxify many organic and inorganic xenobiotics and carcinogens.

The goals of this study were to measure the levels of total Cys, Hcy, CG and GSH in blood plasma and follicular fluids of women undergoing assisted reproduction techniques on the day of oocyte retrieval, assess differences in thiol concentrations between the individual follicles, determine the differences between follicles with and without oocytes, and define recommendations for further analyses.

Materials and methods

Patients and samples

Forty-nine women (22–44 years old) undergoing ovarian stimulation (recFSH, GnRH agonists in long protocol) were enrolled in this study. All of them signed an informed consent for their participation in the experiment. Venous blood samples were drawn from each woman just before FSH application and before oocyte retrieval in vacutainer tubes containing anticoagulant. Blood samples were centrifuged at 2 000 g for 10 min. and plasma samples were stored at -80°C until assayed.

Particular follicular fluid concentrations were examined after the FSH stimulation. After the oocyte retrieval for the IVF or ICSI procedure, samples of follicular fluids from 2–8 follicles were centrifuged separately at 4 000 g for 10 min, then frozen without preservatives and stored at -80°C until analysis. Only follicular fluids without blood contamination were used. The presence of blood was tested by HemoPhan Test stripes (Lachema, Czech Republic). In total, 279 (169 with and 110 without oocyte) follicular fluid samples were collected and further assayed.

Biochemical assays

A method of high-performance liquid chromatography with fluorescence detection (HPLC-FD) and preceding derivatization was used for determination of thiols in blood plasma and follicular fluid.

We used 25 μl of 0.5 mmol/l N-Acetyl-L-cysteine (Fluka, Switzerland) as an internal standard, which was added in the first step to 100 μl of each sample. Next, 25 μl of 10% solution of Tributylphosphine (TBF; Sigma Aldrich, Canada) in N,N-dimethylformamide (DMF; Sigma Aldrich, Germany) was added. After 30 min reduction at 4°C , samples were deproteinated by 100 μl of 70% (v/v) perchloric acid (PCA; Fluka, Switzerland), 0.6 mol/l PCA containing 1 mmol/l EDTA- $\text{Na}_2 \cdot 2\text{H}_2\text{O}$ (Fluka, Switzerland), for 10 min and centrifuged at 21 910 g for 15 min. Subsequently, 25 μl of the deproteinated sample was derivatized with 7-fluorobenzofurazane-4-sulfonic acid (SBD-F; Fluka, Switzerland) for 1 h at 60°C by adding 50 μl of borate buffer (125 mmol/l $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$ (Fluka, Germany) and 4 mmol/l EDTA- $\text{Na}_2 \cdot 2\text{H}_2\text{O}$ (Fluka, Switzerland), pH 10.1) and 25 μl of SBD-F solution (1 mg/ml of borate buffer; 125 mmol/l $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$ and 4 mmol/l EDTA- $\text{Na}_2 \cdot 2\text{H}_2\text{O}$, pH 9.5). The derivatization was stopped by an ice-bath and the samples were stored at 4°C until the next day HPLC analysis.

20 μl of each derivatized sample was injected on the column. We used a gradient elution at flow rate of 1 ml/min: 0.1 M phosphate buffer, pH 3.0 : acetonitrile (ACN; Scharlau, Spain) 100:3 (v/v) for 1.9 min, 0.1 M phosphate buffer, pH 3.0 : ACN 100:7 (v/v) for another 6.1 min, and 0.1 M phosphate buffer, pH 3.0 : ACN 100:3 (v/v) for last 2 min of analysis.

We applied a solvent delivery system (UFLC prominence liquid chromatograph LC-20 AD, Shimadzu, USA) and fluorescence detector (RF 10AXL, Shimadzu, USA) operating at an excitation wavelength of 385 nm and an emission wavelength of 515 nm. We used the separating column Supelcosil LC-18, $150 \times 4.6 \text{ mm} \times 5 \mu\text{m}$ particle size (Supelco, USA) and the guard column C18, $4 \times 3 \text{ mm}$ (Phenomenex, USA). Obtained data were analysed with the Clarity chromatography data system (DataApex, Czech

Table 1 Concentrations of thiols in blood plasma

Analyte	Before FSH stimulation ($\mu\text{mol/l}$) ^a	After FSH stimulation ($\mu\text{mol/l}$) ^a	Differences between concentrations ($\mu\text{mol/l}$) ^a	<i>p</i> -value
Cys	242.6 (184.0; 424.4)	221.0 (148.9; 332.3)	−27.4 (−145.6; 42.7)	< 0.001
Hcy	10.4 (6.6; 17.0)	8.1 (5.2; 12.7)	−2.1 (−6.4; 0.8)	< 0.001
CG	51.8 (31.1; 104.6)	40.7 (28.9; 54.1)	−10.1 (−58.4; 7.3)	< 0.001
GSH	8.2 (3.6; 16.2)	7.4 (3.6; 12.1)	−1.1 (−11.6; 6.5)	0.251

^a Data are median (5th; 95th percentile)

Republic). Concentrations of homocysteine were calculated using a calibration curve, which was obtained from homocysteine standard solutions (Sigma Aldrich, England) of concentrations 3.125 $\mu\text{mol/l}$, 6.25 $\mu\text{mol/l}$, 12.5 $\mu\text{mol/l}$, 25 $\mu\text{mol/l}$, and 50 $\mu\text{mol/l}$ for follicular fluid and 6.25 $\mu\text{mol/l}$, 12.5 $\mu\text{mol/l}$, 25 $\mu\text{mol/l}$, 50 $\mu\text{mol/l}$ and 100 $\mu\text{mol/l}$ for blood plasma. In the same way, the concentrations of other thiols were calculated; the concentrations of standard solutions were 12.5 $\mu\text{mol/l}$, 25 $\mu\text{mol/l}$, 50 $\mu\text{mol/l}$, 100 $\mu\text{mol/l}$ and 200 $\mu\text{mol/l}$ (follicular fluid) and 25 $\mu\text{mol/l}$, 50 $\mu\text{mol/l}$, 100 $\mu\text{mol/l}$, 200 $\mu\text{mol/l}$ and 400 $\mu\text{mol/l}$ (blood plasma) for Cys, 6.25 $\mu\text{mol/l}$, 12.5 $\mu\text{mol/l}$, 25 $\mu\text{mol/l}$, 50 $\mu\text{mol/l}$ and 100 $\mu\text{mol/l}$ (follicular fluid) and 12.5 $\mu\text{mol/l}$, 25 $\mu\text{mol/l}$, 50 $\mu\text{mol/l}$, 100 $\mu\text{mol/l}$ and 200 $\mu\text{mol/l}$ (blood plasma) for CG and 1.5625 $\mu\text{mol/l}$, 3.125 $\mu\text{mol/l}$, 6.25 $\mu\text{mol/l}$, 12.5 $\mu\text{mol/l}$ and 25 $\mu\text{mol/l}$ (follicular fluid) and 3.125 $\mu\text{mol/l}$, 6.25 $\mu\text{mol/l}$, 12.5 $\mu\text{mol/l}$, 25 $\mu\text{mol/l}$, and 50 $\mu\text{mol/l}$ (blood plasma) for GSH.

All samples were analysed in duplicate in the same run. The limit of detection (signal/noise 5 : 1) was 0.2 $\mu\text{mol/l}$.

Statistical analysis

Statistical significance of changes from the start of FSH stimulation to the date of follicle puncture was analyzed by means of Wilcoxon paired test. Non-parametric Spearman correlation was used for the analysis of dependance on age and intraindividual variability was measured using the ratio of difference between average value of follicles with and without eggs against range of individual follicles values. The values of mean, median, standard deviation and range of values of follicles were computed from the patient's follicles and the median supplemented by 5th and 95th percentile of these patient's characteristics was then computed. Statistical significance of thiols between follicles with and without eggs was analyzed by means of Wilcoxon paired test.

Results

The concentrations of cysteine, homocysteine, cysteinylglycine and glutathione in blood are presented in Table 1.

The differences between thiol levels in follicles with or without oocytes are shown in Table 2. Intraindividual variability of thiols in follicular fluid with or without oocytes is presented in Table 3 and individual values of follicular thiols concentrations are shown in Fig. 1.

The Hcy, Cys and CG concentrations in blood plasma decreased significantly after the stimulation with rec FSH (2300 ± 600 IU; achieved estradiol level 15.7 ± 11.3 nmol/l) ($p < 0.001$; Table 1). The decrease of GSH concentration was also observed but this did not reach a statistical significance ($p = 0.251$).

We found significant differences in Cys ($p = 0.022$) and GSH ($p = 0.027$) levels between follicles with or without oocytes (Table 2). The mean $\pm$ SD value of Cys in follicles without oocyte was 157.9 ± 26.8 $\mu\text{mol/l}$ with a median of 155.0 $\mu\text{mol/l}$ while the mean $\pm$ SD value in follicles with oocyte was 147.6 ± 22.2 $\mu\text{mol/l}$ with a median of 149.4 $\mu\text{mol/l}$. The mean $\pm$ SD value of GSH in follicles without oocyte was 17.0 ± 5.3 $\mu\text{mol/l}$ with a median of 17.6 $\mu\text{mol/l}$ while the mean $\pm$ SD value in follicles with oocyte was 18.0 ± 4.2 $\mu\text{mol/l}$ with a median of 18.0 $\mu\text{mol/l}$. Hcy and CG concentration was higher in follicles without oocyte (mean $\pm$ SD = 6.0 ± 0.9 $\mu\text{mol/l}$ and 25.4 ± 4.6 $\mu\text{mol/l}$) than in follicles containing the oocyte (mean $\pm$ SD = 5.2 ± 0.8 $\mu\text{mol/l}$ and 24.1 ± 3.4 $\mu\text{mol/l}$), but the difference was not statistically significant ($p = 0.262$ and $p = 0.190$, respectively).

High concentration variability of all thiols was found in different follicles of each woman, regardless of whether they contained the oocyte or not. Concentrations of thiols in individual follicles of each patient are shown in Fig. 1.

Table 2 Differences between concentrations of thiols in follicles with and without oocytes

Analyte	Follicles without oocytes ($\mu\text{mol/l}$) ^b	Follicles with oocytes ($\mu\text{mol/l}$) ^b	<i>p</i> -value
Cys	155.0	149.4	0.022
Hcy	6.1	5.2	0.262
CG	25.0	24.1	0.190
GSH	17.6	18.0	0.027

^b Data are median

Table 3 Concentrations of thiols in follicular fluid and intraindividual variability

Follicles	Mean ($\mu\text{mol/l}$) ^c	Median ($\mu\text{mol/l}$) ^c	SD ($\mu\text{mol/l}$) ^c	Range ($\mu\text{mol/l}$) ^c
Without oocytes				
Cys	157.9 (100.0; 214.1)	155.0 (95.6; 231.7)	26.8 (12.7; 79.6)	56.8 (20.1; 167.6)
Hcy	6.0 (3.2; 9.1)	6.1 (2.8; 9.1)	0.9 (0.2; 2.8)	1.9 (0.5; 5.1)
CG	25.4 (15.9; 43.3)	25.0 (15.4; 43.3)	4.6 (1.5; 12.4)	9.6 (4.0; 27.0)
GSH	17.0 (4.8; 31.5)	17.6 (4.7; 30.5)	5.3 (0.9; 11.5)	12.0 (1.5; 21.9)
With oocytes				
Cys	147.6 (93.0; 211.9)	149.4 (91.2; 209.4)	22.2 (1.3; 63.1)	46.2 (1.9; 138.1)
Hcy	5.2 (2.6; 9.1)	5.2 (2.5; 9.4)	0.8 (0.1; 2.8)	1.8 (0.2; 4.7)
CG	24.1 (13.2; 38.7)	24.1 (13.2; 39.2)	3.4 (0.6; 9.7)	6.4 (0.9; 18.9)
GSH	18.0 (6.1; 33.9)	18.0 (6.1; 33.9)	4.2 (0.5; 10.6)	7.9 (0.8; 20.5)

^c Data are median (5th; 95th percentile)

Discussion

Ovarian stimulation deranges blood and follicular fluid biomarkers of the Hcy pathway and affects the Hcy concentration in follicular fluid [15]. It was demonstrated that ovarian stimulation in women undergoing IVF treatment is associated with a decrease of total Hcy in blood. It may be due to estradiol mediated induction of general enzyme activity and in particular activity of cystathionin- β -synthase through which the catabolism of Hcy might be increased [8, 16, 17]. This study also confirmed the decrease of homocysteine in the blood plasma after ovarian stimulation.

We found similar decrease in blood plasma Cys concentration. The mechanism is probably analogous to

the Hcy decline and observed decrease of blood plasma CG concentration in the present study appears to be based on the same mechanism. The observed changes in GSH blood levels are not statistically significant. In human organism, GSH is synthesized in a gamma-glutamyl cycle. Except its role as a potent antioxidant, preventing damage of important cellular components caused by reactive oxygen species (ROS), GSH is a substrate in conjugation reactions. To conclude, estradiol mediated induction of enzyme activities can affect both the enzymes of GSH synthesis—gamma-glutamylcysteine synthetase and glutathione synthetase—and enzymes related to GSH function—glutathione S-transferase, glutathione peroxidases and glutathione reductase.

Fig. 1 Individual values of follicular thiol's concentrations. Note: ● follicles with an oocyte, ○ follicles without an oocyte

The presence of Hcy in follicular fluid was demonstrated already in 1993 [20]. More recently, new studies assessed the concentration of Hcy in follicular fluid [17, 18] and our levels are a little lower than the published with mean $\pm$ SD value of 6.0 ± 0.9 $\mu\text{mol/l}$ and median of 6.1 $\mu\text{mol/l}$ in follicles without oocyte and 5.2 ± 0.8 $\mu\text{mol/l}$ and median of 5.2 $\mu\text{mol/l}$ in follicles containing the oocyte. It was also reported that low follicular fluid Hcy concentration is associated with a higher degree of the oocyte maturation [20] and that there is a significant association between follicular fluid Hcy levels and the grade of oocytes. High follicular fluid Hcy levels are negatively correlated with oocyte fertilization and the quality of embryo, which indicates that follicular fluid Hcy may play an important role in the development of oocytes and fertilization [18]. Hcy is a well-known inhibitor of methylation reactions and this could explain why high Hcy levels negatively affect methylations and consequently the oocyte and embryo development. This effect on methylation reaction should be considered mainly because of the trend to culture early human embryos in medium lacking essential amino acids including methionine. So, methionine is not available during the first days of in-vitro culture when methylation is of major importance [21].

On the other hand, lower concentrations of Hcy in more mature follicles can be due to physiological transudation process, which is more expressed in the latter stages of maturity and can cause dilution of some metabolites.

The presence of other thiols in follicular fluid of oocyte-containing follicles was also already demonstrated [8]. Unfortunately, we cannot compare this data to our results because of different units of concentration. Also, we measured thiols concentrations in follicles without oocytes. The assessed values are 157.9 ± 26.8 $\mu\text{mol/l}$ in follicles without oocyte and 147.6 ± 22.2 $\mu\text{mol/l}$ in follicles with oocyte for Cys, 25.4 ± 4.6 $\mu\text{mol/l}$ in follicles without oocyte and 24.1 ± 3.4 $\mu\text{mol/l}$ in follicles with oocyte for CG and 17.0 ± 5.3 $\mu\text{mol/l}$ in follicles without oocyte and 18.0 ± 4.2 $\mu\text{mol/l}$ in follicles with oocyte for GSH.

This is the first study focused on the intraindividual variability of thiols concentrations in follicular fluid between single follicles and reveals that this variability, especially in some of patients, is very high. We consider these findings to be the most important. Our recommendation is to observe one and the same oocyte-containing follicle and potential pregnancy originated from this oocyte for analysis of fertility outcomes. Only when this is not possible, use the mean from at least three oocyte-containing follicles for characterization of each woman. This variability should also be taken into account when evaluating the fertility outcomes of the woman.

Conclusion

Because of the very high intraindividual variability of concentrations of thiols (Hcy, Cys, CG and GSH) in follicular fluids from particular follicles we recommend to examine the individual oocyte-containing follicle for further research and analysis of fertility outcomes. It is practical to use the mean from at least three follicles only if this is not possible.

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