Experience of measuring glutathione peroxidase activity in surgically induced endometrial-like lesions in rats

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BACKGROUND: Endometriosis is known to be linked with altered activities of antioxidant enzymes and with their gene polymorphisms. Progestins are known to induce glutathione peroxidase activity in the endometrium and promote reduction of endometrial lesions. It could be useful to estimate the correlation between the activity of glutathione peroxidase within endometrial lesions and their degree of reduction.

AIM: The present study was aimed at estimating glutathione peroxidase activity in surgically induced endometrial-like lesions of different degree of reduction in rat model of endometriosis.

MATERIALS AND METHODS: The method for determining glutathione peroxidase activity using hydrogen peroxide as a substrate and 5,5'-dithiobis(2-nitrobenzoic acid) for estimation of residual reduced glutathione was applied for quantitative analysis of the enzyme activity in endometriotic foci, surgically induced in female Wistar rats. An assay of glutathione peroxidase activity in tissue homogenates was performed at 37°C in a reaction medium containing Tris-HCl buffer supplemented with tetrasodium ethylenediaminetetraacetate and sodium azide (pH 8.5) in the presence of 0.55 mM reduced glutathione and 0.192 mM hydrogen peroxide. Before adding trichloroacetic acid, 40-second incubation was used. The correlation between the specific activity of the enzyme and protein amount in endometriotic foci was estimated.

RESULTS: In a rat model of endometriosis, there was a high, well-determined glutathione peroxidase activity in endometriotic foci. For the same endometriotic tissue sample, the enzymatic activity was proportional to the amount of protein in the reaction mixture. The range of specific glutathione peroxidase activity was 2.43–6.45 micromoles of consumed glutathione per minute per milligram of protein ($n = 7$). In most reduced endometriotic foci (with the minimum amount of endometriotic tissue), the highest specific activity of glutathione peroxidase was found (the Spearman's rho of -0.93 with $p = 0.0067$).

CONCLUSIONS: The method for determining glutathione peroxidase activity using hydrogen peroxide and 5,5'-dithiobis-(2-nitrobenzoic acid) is convenient for working with the endometriotic tissue in a rat model of endometriosis. We can accept, with $p < 0.01$, that weight of endometriotic foci is negatively linked with specific glutathione peroxidase activity within their tissue. The results are analogous to the previously obtained data on catalase activity and suggest the involvement of both antioxidant enzymes in reduction of endometrial lesions.

Keywords: endometriosis; correlation; glutathione peroxidase; enzyme; hydrogen peroxide; method.

Активность глутатионпероксидазы в эндометриоидных очагах при хирургическом моделировании эндометриоза

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Обоснование. Известна связь между полиморфизмами генов, кодирующих антиоксидантные ферменты, изменениями активностей каталазы и глутатионпероксидазы в ткани эндометрия и развитием эндометриоза. Некоторые формы глутатионпероксидазы активируются гестагенами. В то же время гестагены эффективны в обеспечении регресса эндометриоидных очагов. Представляет интерес оценка связи глутатионпероксидазной активности в эндометриоидных очагах со степенью их регресса.

Цель — проанализировать активность глутатионпероксидазы в смоделированных эндометриодных очагах, имеющих разную степень регресса.

Материалы и методы. Метод определения глутатионпероксидазной активности с использованием пероксида водорода в качестве субстрата и 5,5'-дитиобис(2-нитробензойной кислоты) для оценки убыли восстановленной формы глутатиона применен для количественной оценки активности фермента в эндометриодных очагах, смоделированных у лабораторных крыс линии Вистар. Определение проводили в гомогенатах ткани при 37 °С в реакционной среде на основе трис-HCl-буфера с этилендиаминтетраацетатом и азидом натрия (pH 8,5) в присутствии 0,55 мМ глутатиона и 0,192 мМ пероксида водорода (время инкубации — 40 с). Оценивали корреляцию между глутатионпероксидазной активностью и массой очагов.

Результаты. Для эндометриоидных очагов, смоделированных на лабораторных крысах, в целом характерен высокий, хорошо определяемый уровень глутатионпероксидазной активности. Величина глутатионпероксидазной активности для одних и тех же гетеротопий пропорциональна содержанию их белка в реакционной смеси. Диапазон значений удельной активности глутатионпероксидазы в разных очагах составил 2,43–6,45 мкмоль израсходованного глутатиона / мин на 1 мг белка ($n = 7$). В очагах, обладающих меньшей массой (сильнее подвергшихся регрессу), регистрировали более высокую глутатионпероксидазную активность — коэффициент ранговой корреляции Спирмена составил −0,93 ($p = 0,0067$).

Заключение. Метод определения глутатионпероксидазной активности с использованием пероксида водорода и 5,5'-дитиобис(2-нитробензойной кислоты) пригоден для работы с тканью смоделированных эндометриоидных очагов. Чем более высокая активность глутатионпероксидазы в очагах, тем, как правило, меньше их масса. Результаты аналогичны полученным ранее данным по активности каталазы и позволяют предположить вовлеченность обоих антиоксидантных ферментов в механизм регресса эндометриоидных очагов.

Ключевые слова: эндометриоз; корреляция; глутатионпероксидаза; фермент; пероксид водорода; метод.

BACKGROUND

According to several studies, the development of endometriosis is associated with changes in the activity of antioxidant enzymes in the endometrial tissue as well as with polymorphisms of their genes. Endometriosis is characterized by changes in catalase activity [1, 2]. In addition, there exist data indicating the possibility of a connection between the polymorphism of the promoter of the catalase-encoding gene and the risk of endometriosis [3]. In our recent study of catalase activity in rat models of endometrioid heterotopias with varying degrees of regression, an inverse correlation was noted between the enzyme activity and the mass of heterotopic tissues ($p < 0.025$) [4]. In addition, endometriosis involves changes in the expression of the classic intracellular form of glutathione peroxidase (GPx1), owing to impairment in cyclic changes in endometrial GPx1 expression [5]. Catalase and GPx are functionally similar enzymes and they play a role in the detoxification of hydrogen peroxide and thus in the reduction of free radical oxidation level [6]. Previous data indicate assessing the activity of antioxidant enzymes in heterotopic tissues with different degrees of their regression, and this is important both for understanding the mechanisms of development and regression of endometrioid foci as well as for assessing the possibility of predicting disease course by the activity level of these enzymes. The present study, which determined GPx activity, is similar to the study conducted earlier that determined catalase activity in endometrioid foci [4].

The present study aimed to quantify GPx activity in endometrioid foci with varying degrees of regression, simulated in laboratory rats. Specifically, it assessed the possibility of using the previously developed method for determining GPx activity in endometrioid implant tissue and (in case of positive response) evaluated the relationship between GPx activity and their mass.

MATERIALS AND METHODS

For the experiment, sexually mature female Wistar rats weighing 230–320 g were enrolled. All laboratory animals were obtained from the Rappolovo Nursery of laboratory animals (Leningrad region) and were housed under the regulated conditions of the vivarium of the D.O. Ott Research Institute of Obstetrics, Gynecology, and Reproduction in compliance with all the rules for housing laboratory animals (time and order of quarantine, labelling of all animals, constant sanitary control, standard diet, free access to water and food, and automatic lighting mode of day and night [12:12 h]).

The animals were prepared for surgery, and endometriosis was stimulated according to previously reported methods [4, 7]. All surgeries were performed at the estrus stage, standardizing the conditions for transplantation of uterine fragments from animals at the same stage of the estrous cycle. Auto-transplantation of uterine tissue fragments of 3×3 mm in size was performed into areas of the animal abdominal cavity. During the surgery, all animals additionally underwent bilateral oophorectomy, followed by replacement therapy with ethinyl estradiol. After 2 weeks, a diagnostic laparotomy was performed to confirm implant viability. After another 3 weeks, animals were sacrificed using deep inhalation anesthesia. The excised endometrial foci were frozen and stored at −85°C until preparation of homogenates. One implant was used from each animal; the remaining implants were used for other studies.

Each implant was washed thoroughly in isotonic sodium chloride solution and was homogenized in a glass homogenizer in chilled 0.05-M K-Na-phosphate buffer (pH 7.8) for 60 s. The total volume of the buffer used for preparing the homogenate was 50–80 µL, depending on the sample size. The exact volume of the buffer required for homogenization was recorded for subsequent calculation of the mass of soluble protein in the tissue. The homogenates were centrifuged for 6 min at 4°C (1000 ×g). The supernatant was then used as a biomaterial to determine GPx activity.

GPx activity was determined using a modified method involving hydrogen peroxide as a substrate [8]. For reaction mixture I, we used the reagent A: 0.64-mM solution of the reduced form of glutathione (GSH) in a mixture of 0.1-M Tris-HCl buffer (pH 8.5) containing 0.34-mM EDTA and an aqueous solution of NaN_3 (26 mg/ml). A mixture of buffer and NaN_3 solution was prepared at a volume ratio of 17:1. Subsequently, reaction was initiated by adding reagent B (5-mM aqueous H_2O_2 solution). Reagent B was prepared by 4-fold dilution of an aqueous solution of H_2O_2 that had an absorbance of 0.872 at 240 nm (i.e., 4-fold dilution of a 20-mM hydrogen peroxide solution). The reaction was terminated by adding 30% (w/v) trichloroacetic acid. The concentration of trichloroacetic acid should not exceed this value to avoid complicating subsequent staining of samples in the reaction with Ellman's reagent [9].

Sample filling and determination progress

The reaction was performed at 37°C in a water bath by placing the reaction mixture in Eppendorf tubes. Aliquots of 90 µl of reagent A were added carefully to the bottom of the tubes, avoiding the formation of drops that separate and settle on tube walls. The tubes were then pre-incubated for a few minutes in a water bath. Subsequently, the reagents were added according to the four reaction schemes.

SUM: 4 µl of reagent B and 10 µl of biomaterial were simultaneously added and then incubated for 40 s. Then, 20 µl of trichloroacetic acid was added.

NEO: 4 µl of reagent B and 10 µl of biomaterial solvent (phosphate buffer) were simultaneously added and then

incubated for 40 s. Subsequently, 20 µl of trichloroacetic acid was added.

X: 10 µl of biomaterial was added, incubated for 40 s, and treated simultaneously with 4 µl of reagent B and 20 µl of trichloroacetic acid.

ST: 10 µl of the biomaterial solvent was added and incubated for 40 s. Then, 4 µl of reagent B and 20 µl of trichloroacetic acid were simultaneously added.

With the simultaneous adding of reagents, they are not pre-mixed. For this purpose, 4 µl of reagent B was preliminarily applied to the tube wall above the level of reagent A, and then the biomaterial (according to the SUM and NEO schemes) or trichloroacetic acid (according to schemes X and ST) was added, carefully pipetting, mixing, and rinsing the walls with a mixture of reagents.

If possible with the biomaterial amount used, proportionally increased volumes of reagents can be used (Fig. 1): 180 µl of reagent A, 20 µl of biomaterial, 8 µl of reagent B, and 40 µl of trichloroacetic acid solution.

After terminating the reaction, the samples were centrifuged at room temperature for 10 min at 1000 ×g. Reaction mixture II for each sample was collected as follows: 1160 µl of 0.1-M Tris-HCl buffer with 0.34-mM EDTA (pH 8.5) were sequentially added to 108 µl of each supernatant containing residual GSH, and then at the same intervals, 9 µl of a solution of 5,5'-dithiobis(2-nitrobenzoic acid) or Ellman's reagent (4 mg/ml of absolute methanol) was added to each sample (in the same sequence of samples). The mixture was allowed to react until a yellow color was registered. If the reagent is added to each subsequent sample with an interval of half a minute, 15 samples can undergo reaction within 7.5 minutes.
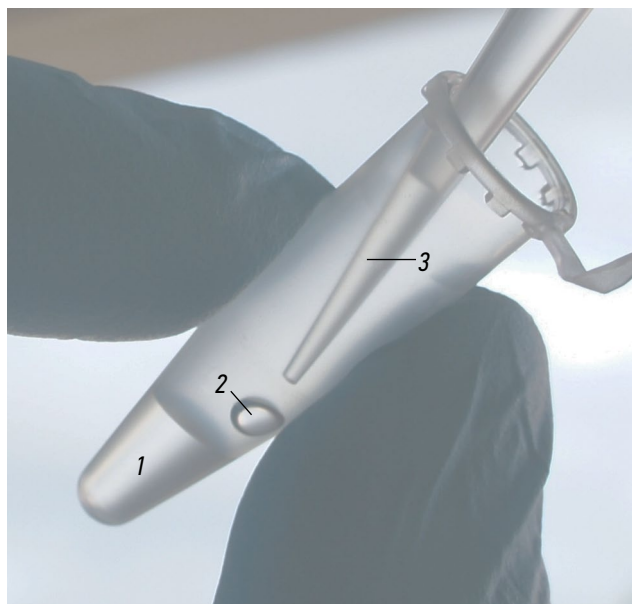

Fig. 1. Simultaneous adding of hydrogen peroxide and biomaterial into the reaction mixture: *1* — reagent A (180 µl); *2* — 5-mM H_2O_2 (8 µl); *3* — biomaterial (20 µl). Photo by A.D. Yushina

Subsequently, we recorded the absorbance at 412 nm after the next 7–8 min, scanning each sample photometrically at the same time interval as the Ellman's reagent was added. Sample staining and optical density measurement were performed at room temperature. For an accurate assessment of GSH concentration, the absorbance was measured against a sample in which, instead of 108 µl of the supernatant from reaction mixture I, 108 µl of the mixture are added, collected previously in the same way, but without GSH (90 µl of Tris-HCl buffer with NaN_3 and EDTA + 10 µl of phosphate buffer + 4 µl H_2O_2 solution + 20 µl trichloroacetic acid solution). However, as the difference between the absorption values for different samples is important for further calculations of the enzymatic activity, the measurement can be performed against distilled water.

The total volume of the photometrically scanned solution according to this technique was 1277 µl; therefore, a DU-65 spectrophotometer (Beckman Coulter, USA) was used to measure the absorbance using a conventional spectrophotometric cuvette with an optical path length of 1 cm and the same distance between its side walls. When working with a Lambda 25 spectrophotometer (PerkinElmer, USA), it is recommended to use a "microcuvette" with an optical path length of 1 cm and concave side walls (with a distance of 0.45 cm between them), or in the case of a conventional cuvette, to proportionally increase the volumes of all reagents (e.g., by 2 times).

The loss of GSH in reaction mixture I was calculated from the difference in the absorption values at 412 nm in reaction mixture II. For this purpose, the coefficient of millimolar absorption of the reaction product of GSH with Ellman's reagent at 412 nm was used ($14.15 \ mM^{-1} \ cm^{-1}$) [10] along with the dilution of the reaction mixture. Reagent A with 0.64-mM GSH (90 µl) was diluted to a fully collected reaction mixture (104 µl) by 1.15 (5) times to a GSH concentration of 0.55385 mM (i.e., working concentration of GSH). Then, trichloroacetic acid was added (up to the total volume of the mixture of 124 µl) by 1.1923 times. The supernatant was then diluted 11.824-fold with reaction mixture II (1277 µl/108 µl). The total dilution of the reaction mixture I was as follows: (124/104) (1277/108) = 14.09793: this was approximately equal to the millimolar absorption coefficient of the reaction product of GSH with Ellman's reagent. Thus, 1 mmol of colored product per 1 mmol of GSH was formed. Accordingly, 1 absorption unit (1.000) of the reaction mixture II at 412 nm corresponded approximately to 1-mM GSH (exactly 0.9963201-mM GSH) in reaction mixture I. The working concentration of GSH used in this study in reaction mixture I corresponded to the optical density of reaction mixture II (0.556). Thus, arithmetic operations with the optical densities at 412 nm for different samples would reflect well the changes in GSH concentrations in the reaction mixture I.

The enzymatic activity was calculated using an equation based on the determination of the loss of GSH concentration in various processes during the analytical procedure:

$$\Delta A_{GPx} = [(A_{ST} - A_{SUM}) - (A_{ST} - A_{NEO}) - (A_{ST} - A_X)] \times 1.5,$$

where ΔA_{GPx} is the difference in absorbance at 412 nm and the numerically equal decrease in GSH concentration in enzymatic oxidation per 1 min, expressed in mM (μmol/ml).

Thus, the decrease in the non-enzymatic reaction $(A_{ST} - A_{NEO})$ and the decrease in GSH oxidation by the biomaterial without the participation of GPx $(A_{ST} - A_X)$ were subtracted from the total GSH loss $(A_{ST} - A_{SUM})$. The difference in the last part of the equation $(A_{ST} - A_X)$ can be a negative value if the biomaterial does not oxidize GSH without the involvement of GPx but contains an additional amount of free sulfhydryl groups. The coefficient 1.5 was used to express activity per 1 min (60 s/40 s).

In this study, the protein concentration in the homogenate supernatant was determined using a simplified turbidimetric procedure [11] using several dilutions of the same biomaterial. A human serum albumin solution (Cormay, Poland) was used as a standard. The protein concentration in the reaction mixture was calculated considering dilution of the biomaterial with the reaction mixture (104 μl/10 μl). To calculate the specific activity of GPx (in micromoles of GSH per minute per 1 mg of protein), the decrease in GSH concentration in enzymatic oxidation over 1 min (μmol/min/ml) was divided by the protein concentration in the reaction mixture (mg/ml).

The proportionality of the reaction rate to biomaterial content in the reaction mixture was assessed using the coefficient of determination (R^2); to assess the correlation between the specific activity of GPx and the mass of the biomaterial, the Spearman's rank correlation coefficient (*rho*) was applied. The calculations were performed in the R software environment (version 3.4.0) [12].

RESULTS AND DISCUSSION

In the case of surgically induced endometriosis in rats, a high, well-defined level of GPx activity is detected in heterotopic tissues. The specific activity of GPx ranged from 2.43 to 6.45 μmol of GSH consumed in the enzymatic reaction, calculated per 1 minute and per 1 mg of protein (*n* = 7).

The rate of enzymatic reaction (after subtracting the rate of non-enzymatic oxidation of GSH by peroxide and the rate of GSH oxidation by biomaterial without the involvement of GPx and peroxide) for the same heterotopic tissues was proportional to the level of their protein in the reaction mixture. The linearity index of this relationship (R^2) for five separate foci varied from 0.9943 to 1, which corresponded

to a high and very high degree of linearity, respectively. In the case of the same sample, this implied that the specific activities determined at different protein concentrations were identical. This indicated that the comparison of the enzyme specific activity obtained at different protein concentrations for different tissue samples was correct.

The contribution of non-enzymatic oxidation of GSH with peroxide $(A_{ST} - A_{NEO})$ to the overall reaction at protein concentrations in the reaction mixture of 57–125 μg/ml was only 12–16%. The $(A_{ST} - A_X)$ contribution to the overall reaction was close to zero. Thus, in the above-described conditions of the reaction mixture, GSH was primarily consumed due to the enzymatic activity of GPx.

The weight of soluble protein in the simulated endometrioid heterotopic tissue was 47–415 μg. This means that the formed foci underwent significantly different degrees of regression in different animals. A statistically significant and negative correlation was noted between the specific activity of GPx in heterotopic tissues and their mass, estimated indirectly by the amount of soluble protein present in them: *rho* = −0.93, *p* = 0.006746 (Fig. 2).

Based on the data presented in Fig. 2, it can be assumed that endometrioid implants are initially characterized by a certain non-zero level of GPx specific activity, regardless of their size (which is quite logical as initially it is normal endometrial tissue), which, however, further increases in the event of their regression.

The method used to present data shows that, as in the case of catalase activity [4], GPx activity was assumed to be a probable component of the mechanism underlying endometrioid focus regression. Accordingly, we placed the enzyme activity on the horizontal axis of the graph and
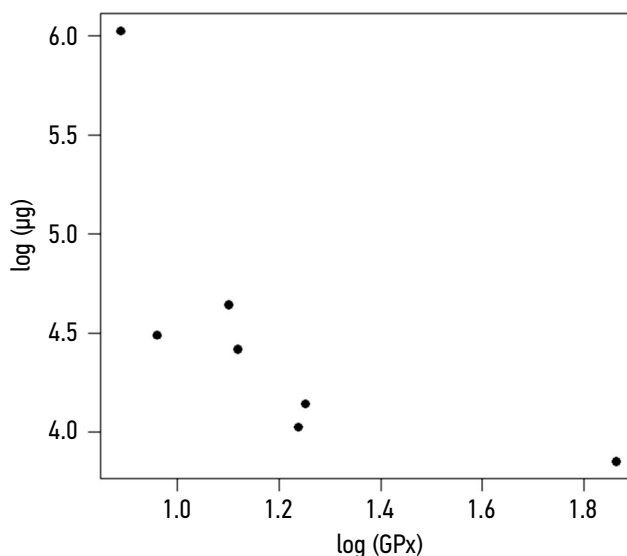

Fig. 2. Inverse correlation between the specific activity of GPx in simulated endometriotic lesions and their mass. log (GPx) — natural logarithm of GPx activity (μmol/min per 1 mg of protein); log (μg) — natural logarithm of the mass of the entire soluble protein in the focus (micrograms), extracted during homogenization

considered the mass of heterotopic tissues as the dependent variable [4]. The result of the correlation assessment obtained suggested that the foci underwent regression owing to the additional induction of GPx, and the growth of the endometrioid foci might be attributed to the decrease in GPx activity. At the same time, we understand that the presence of a correlation does not prove a causal relationship, and the increased GPx activity in reduced foci can only reflect the response of the antioxidant system to the work of other, basic mechanisms that cause regression of endometrioid formations.

In general, the data obtained are consistent with the conclusions made earlier by other authors [1, 2] regarding the involvement of oxidative stress in endometriosis progression. It is advisable to test the contribution of GPx in suppressing oxidative stress in the endometrioid tissue and in the regression of endometrioid foci in experiments using selenium compounds, which is required for GPx biosynthesis, and whose consumption increases the detectable activity of this enzyme in tissues.

ADDITIONAL INFORMATION

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