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SOME PROBLEMS OF MEASUREMENT OF THIOLS AND DISULFIDES N CLINICAL MATERIAL REVIEW

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Measuring of the level of oxidized and reduced glutathione and other thiols and disulfides in tissues and blood is a sensitive marker of disturbances of the redox balance. Nevertheless, methodical approaches to their estimation require careful observation of the storage and sample preparation conditions for biological material for analysis, the choice of an optimal, specific, sensitive analytical method giving well-reproducible results[1, 2].

Despite the fact that the glutathione system is one of the most important systems in maintaining the redox balance in tissues, an accurate measurement of the GSH / GSSG ratio represents a certain methodological problem until now. The levels of both GSH and GSSG in tissues fall within a range easily measured by most currently used methods (1-5 mM and 0.01-0.05 mM, respectively). However, the measurement of GSH in blood plasma may present certain problems due to the sensitivity required for quantification (1-10 μM) and the easy transition of GSH to GSSG during blood sampling and sample preparation[3, 4, 5].

Glutathione is normally found in the form of GSH, and the GSSG content does not exceed 5% of the level of its reduced form, but given the rapid transition ability of the reduced form of glutathione to oxidized during manipulation of tissue sampling, careful attention should be paid to sample preparation for analysis. Potential errors in such measurements are often not fully recognized or evaluated.

To exclude postmortem oxidation of GSH in blood samples, the following conditions must be met. Blood is recommended to be taken to vacutainers (vacuum tubes for blood sampling) with EDTA to stabilize thiols or EDTA + NEM – in the measuring of oxidized glutathione[6, 7, 8]. It is allowed to store samples at -80 ° C for several months. To prevent the formation of disulfides in the storage of samples, an acidic extract is prepared on a cooled sulfosalicylic[7] or metaphosphoric acid [9], or on perchloric acid with the addition of EDTA [10]. Acidic extract should be prepared immediately after blood sampling or after centrifugation of blood in case of working with plasma.

One of the most popular methods for measuring GSH in the literature is Ellman's method, proposed in 1959 to estimate common tissue sulfhydryl groups[11]. Unfortunately, the extinction coefficient of the resulting product for dilute solutions was underestimated in the original publication of 1959 (13,600 M-1 · cm-1), and, as noted in[12], this error has been preserved in the literature. The determination of GSH with Ellman's reagent usually gives excessive amounts of this compound, since in the reaction with Ellman's reagent the total content of sulfhydryl groups of not only GSH but also other minor non-protein thiols (Cys, γ-GCys, etc.) is determined. Based on the fact that the cysteine content, as a rule, is more than two orders of magnitude lower than that of glutathione, they are usually neglected in the routine determinations of glutathione. At the same time, the definition of GSH by the Ellman method is incorrectly used to evaluate its content in blood and tissues when thiol-containing drugs are introduced (N-acetylcysteine, lipoic acid, unitiol, etc.), since these compounds themselves can react with Ellman's reagent.

For the specific measuring of GSH, enzymatic analysis methods exist that allow a high level of accuracy in assessing the actual level of GSH provided that the correct procedure for sample preparation of biological material for analysis is performed. The enzymatic method, the most common and popular now, is the recycling enzymatic method with glutathione reductase (GR). This method allows to estimate the content of both total glutathione (GSH + 2GSSG) and oxidized glutathione (GSSG). The content of the latter is measured in the presence of a masking reagent to eliminate free sulfhydryl GSH groups[4, 6, 7, 9, 10]. The GSH content is calculated from the difference between the total glutathione content (GSH + 2GSSG) and the GSSG

To measure the GSSG content, a masking alkylating reagent – N-ethylmaleimide (NEM) or 2-vinylpyridine (VP) is added to the sample to bind the free sulfhydryl GSH groups. In the case of NEM, there is a need to remove its excess, since NEM is an inhibitor of GR[6, 10]. For this purpose, the extraction of excess NEM with dichloromethane[6] or diethyl ether or purification on a Sephadex-10 chromatographic column, QAE-25 Sephadex[10] is used, which complicates the analysis procedure, especially with its routine using. If VP is used as a masking agent for sulfhydryl

groups , the excess of the VP should not be removed, since it is active only at an acidic pH value[9]. Due to the limited solubility of VP in water, it is recommended to use ethanol as its solvent.

An analysis of the content of redox-forming compounds (thiols and disulphides) in blood and tissues provides important information about the disturbances of redox processes. Thiol-disulfide redox status can be partially estimated or by measuring the total content of thiols and disulfides in the cell (total content), or by their individual fractions: GSH / GSSG (key fraction of the non-protein thiol disulfide system), PSH / PSSP (fraction protein thiols and disulfides). An increase in the level of PSSP occurs in cells in pathological processes associated with oxidative stress. Protein thiols / disulfides can be extracellular, forming part of membranes and subcellular structures, involved in the regulation of redox homeostasis, or they can participate in allosteric enzymatic and receptor-mediated reactions. It is assumed that catalytically important SH groups in PSH can be protected from oxidative stress by reversible reaction with glutathione to form S-glutathionylated proteins (PSSG). There are known approaches to the determination of total thiolation of proteins, which are used in the clinical analysis of the blood plasma of patients[13].

In addition to glutathione, animal tissues also contain non-protein (NPSH) thiols and disulfides (cysteine, cystine, homocysteine, γ-glutamylcysteine, coenzyme A, mixed non-protein disulfides) and protein thiols (PSH, cysteine and cystine containing proteins) and protein disulfides PSSP, disulfides mixed with proteins)[4, 5].

The measuring of PSH in most cases is carried out in a colorimetric manner using the Ellman's reagent. To estimate the total protein in the tissues of rats, a precipitated protein from the homogenate obtained by precipitation with a deproteinizing reagent (most commonly for this purpose using TCA) is used. After solubilizing the protein in 6 M guanidine chloride or 8 M urea, a reaction with Ellman's reagent is carried out. It should be noted that in calculating the sulfhydryl groups of proteins, the molar extinction coefficient of 13600 $M^{-1} \cdot cm^{-1}$ should be used in this case. The PSH is determined by the difference between the total content of free sulfhydryl groups in the sample and the content of NPSH[4, 5].

To estimate the disulfide groups of PSSP proteins, the solubilized protein is treated with a reducing reagent, which is a solution of sodium borohydride or dithiothreitol[4, 14, 15]. After reduction of the disulfide groups, the protein is precipitated with TCA, washed off from excess reagent and solubilized in 6M guanidine chloride or 8M urea and reacted with Ellman's reagent. As a result of this analysis, the total content of sulfhydryl and disulfide groups of proteins is obtained, therefore, to calculate the latter, the calculation is made by the difference[16, 17, 18].

Thus, the measuring of the level of oxidized and reduced glutathione and other thiols and disulfides in tissues and blood plasma is a sensitive marker of violation of the redox balance in the body. Nevertheless, methodological approaches to their estimation require careful fulfilment of storage conditions and sample preparation of biological material for analysis, first of all, careful observance of all the nuances of sample preparation of the test material, in order to exclude the possibility of postmortem oxidation of thiols in time storage of samples, which may then give incorrect values of GSH / GSSG ratio, protein and nonprotein thiols and disulfides.

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