Correlation of superoxide dismutase activity distribution in serum and tissues of small experimental animals

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Abstract. A necessary link in the life of any cell is lipid peroxidation. This process underlies the renewal and restructuring of biological membranes, regulation of their composition, permeability and activity of membrane-bound enzymes. The increase in the level of POL (peroxidation of lipids) leads to oxidative stress, which provokes the formation of various pathological conditions in the body. One of the main components of the antioxidant protection of the body is a group of metal enzymes - superoxide dismutase (SOD), catalyzing the dismutation reaction of superoxide anion radicals and maintaining the concentration of these radicals in the cell at a low level. The mechanism of SOD functioning includes sequential renewal and oxidation of metal ions of variable valence in the active center of the enzyme. SOD is an endogenous acceptor of free oxygen radicals, the excessive accumulation of which in the cell is important in the development of a number of oxygen-dependent pathological processes (hypoxia, inflammation, intoxication, etc.). SOD removes superoxide radicals and prevents the formation of other, more dangerous for the body free radicals: hydroxyl radical and singlet oxygen. The activity of superoxide dismutase in blood and tissues is a marker of metabolic disorders. During the study of the relationship of the distribution of catalase activity in serum and tissues of rats, the following tasks were solved: the activity of SOD in serum and tissues of the liver, brain, heart, as well as in skeletal muscle tissues of rats was indicated; the relationship of the distribution of SOD activity in serum and tissues of rats was revealed. The paper presents the results of nonparametric correlation analysis to assess the relationship of the distribution of SOD activity in serum and tissues of small experimental animals.

1. Introduction
One of the indicators of homeostatic balance in the body is the action of nonspecific mechanism of tissue protection - the activity of the antioxidant system, neutralizing the effects of oxidative stress. Oxidative stress is the process of formation of pathological changes in the body, which is accompanied by increased formation of free radicals. Oxidative stress provokes a set of various factors that accompany the processes of life, from negative environmental conditions to depressive emotional states. Currently, oxidative stress is recognized as one of the reasons for the formation of a number of pathological conditions – malignant neoplasms, atherosclerosis, diabetes mellitus, neurodegenerative brain lesions, pathologies associated with adverse environmental conditions [1].
In the redox reactions of the body involved all components of its metabolism. First of all, reactive oxygen species (ROS) enter into chemical reactions with unsaturated fatty acids, triggering lipid peroxidation processes (POL). Activation of POL causes alterations of proteins, nucleic acids, lipid structures of the cell. In addition to fatty acids, ROS oxidize low-density lipoproteins of low-density (LPLD) of serum and other substrates. Stimulation of free radical processes in the tissues of internal organs causes increased generation of endogenous aldehydes inside and, as a consequence, the occurrence of carbonyl stress. The breakdown of lipoperoxides from oxidized LPLD generates unsaturated aldehydes and malonic dialdehyde (MDA) – one of the most common aldehydes formed as a result of peroxidation of arachidonic, eicosapentaenoic and docosahexaenoic acids. In turn, they are able to form complexes with amino groups of lysine residues of apoB-100 (apolipoprotein B) molecules, which causes a change in the structure of LPLD particles. In addition, MDA induces the process of carbonylation – the formation of oxidized modified proteins. Among the products of peroxidation a significant role in damage to cell structures and violations of its functioning belongs also to peroxides of nitrogen – nitrite, nitroprusside and peroxynitrite, which has strong toxic effects on cells and tissues of the body, including reducing vasodilation of the ability of the vascular endothelium. Damage of the vascular endothelium, cardiomyocytes and other cells with new substances triggers a vicious circle. Since it is impossible to determine the content of free peroxynitrite in blood plasma due to its high reactivity, its concentration is estimated by the content of 3-nitrotyrosine in blood serum [1,2,3].

Additional protein oxidation products (AOPP – advanced oxidation protein products) are considered as markers of oxidative stress severity. Increasing its serum levels is associated with the development of age-associated diseases. It should also emphasize the pathogenetic role of carbonyl stress in the formation of cellular damage to internal organs. Modified proteins are products of the toxic action of free radicals and active oxygen/nitrogen species, which are one of the powerful elements of cellular signaling. Due to the wide natural prevalence of proteins and the stability of its oxidation products, the evaluation of oxidative modification of proteins is considered a reliable marker of oxidative damage [2,3].

Excess production of free radicals can be reduced by converting it into metabolically inactive compounds or by rapid neutralization.

Antioxidant system (AOS) regulates free radical processes, represented by a set of protective mechanisms of the body. In parallel of the antioxidant function, compounds that reduce the intensity of free radical processes, participating in various types of metabolism, prevent the accumulation of toxins and create structural elements of the cell [1].

One of the main components of the antioxidant protection of the body is a group of metal enzymes - superoxide dismutase (SOD), catalyzing the dismutation reaction of superoxide anion radicals and maintaining the concentration of these radicals in the cell at a low level [4,5,6].

Also, SOD reduces in general the probability of singlet oxygen formation, whose activity is 3-4 orders of magnitude higher than that of superoxide anion radicals [7]. First superoxide dismutase has been described by scientists MacCord and Fridovich in 1969. Depending on the type of metal ion, this enzyme is divided into several isoforms: copper and zinc-containing superoxide dismutase (Cu, Zn-SOD), Mn-SOD (found in the matrix of mammalian mitochondria) and Fe-SOD (found in prokaryotes). Copper and zinc-containing superoxide dismutase present in the cytosol of aerobic cells and liver lysosomes of warm-blooded animals are more common and most active [4,7].

Zinc and copper ions in the enzyme molecule, interacting with each other, are so close that the resulting changes in the environment of one ion affect the environment of another.

Copper ions catalyze enzyme activity, but there is no direct correlation between copper absorption and SOD activity. Copper ions perform a structural role, conforming the protein to the active center of the enzyme operation.

Monovalent anions (chlorine, hydroxide) are competitive enzyme inhibitors, binding copper ions of the active center. The enzyme is inhibited by cyanide. Inactivation of SOD by hydrogen peroxide is
accompanied by luminescence and cytochrome C reduction. However, at low concentrations hydrogen peroxide acts as an enzyme reducing agent. The reduced enzyme is quite resistant to oxygen [4,7,8].

There is also an extracellular form of superoxide dismutase, which was first isolated in 1982 and is a glycoprotein consisting of two dimers connected by a disulfide bridge. Each of the 4 subunits has a molecular weight of about 30 kDa, contains copper and zinc ions [9]. All enzyme subunits have the same amino acid sequence. However, the subunits of extracellular and cytoplasmic Cu-Zn-SOD differ in amino acid composition, antigenic properties [10], gene locus encoding the amino acid sequence of apoenzyme and catalytic activity.

If the cytoplasmic Cu-Zn-SOD is a homodimer, each subunit of which has four cysteine residues, the extracellular superoxide dismutase is a tetramer, whose subunits contain six cysteine residues [11].

Extracellular SOD is present mainly in extracellular spaces. It is found in fibroblasts and glial cells [12] and is the main isoenzyme of plasma, lymph, synovial fluid [13]. High levels of extracellular superoxide dismutase were found in the lungs, heart [9], kidneys, placenta [10]. It is inhibited by cyanide, inactivated by hydrogen peroxide [5], and iron ions generally block enzyme secretion [14,15].

The mechanism of SOD functioning includes sequential reduction and oxidation of metal ions of variable valence in the active center of the enzyme. SOD is an endogenous acceptor of free oxygen radicals, the excessive accumulation of which in the cell is important in the development of a number of oxygen-dependent pathological processes (hypoxia, inflammation, intoxication, etc.). SOD removes superoxide radicals and prevents the formation of other, more dangerous to the body free radicals: hydroxyl radical and singlet oxygen. In addition, SOD prevents the accumulation of neutrophils in the focus of inflammation, which secrete significant amounts of lysosomal enzymes that destroy nearby tissues [15,16,17].

Extracellular SOD plays the role of the main regulator of bioactivity of nitrogen monoxide (NO), which reacts with oxygen anion radicals to form a highly toxic peroxynitrite. Nitrogen monoxide is synthesized by endothelial cells, macrophages and neutrophils, is an endothelial relaxing factor, maintains the necessary level of vasodilation and has antiaggregatory activity, preventing thrombosis [16,17,18].

Superoxide dismutases catalyze the dismutation reaction of superoxide anion radicals and maintain the concentration of these radicals in the cell at a low level, as well as reduce the probability of formation of singlet oxygen, whose activity is 3-4 orders of magnitude higher than that of superoxide anion radicals. The rate constant of enzymatic dismutation reaction at pH 7.0 and temperature 20-25°C is \((1.8-2.3) \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}\) and depends little on the pH value in the range 4.8-9.7. The rate constant of spontaneous dismutation decreases with increasing pH: at pH 4.8 its value is \(8.5 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}\), at pH 7.4 - \(1.2 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}\). Among isoforms, Cu-, Zn-containing superoxide dismutase has the greatest activity. The mechanism of SOD functioning includes sequential reduction and oxidation of metal ions of variable valence in the active center of the enzyme. The enzyme belongs to the group of antioxidants-catalysts of direct action [7,8, 11,18].

The content and activity of isoenzymes in organs and tissues are different. The total amount of Cu-, Zn-SOD in humans can reach 3900 mg. High levels of Cu-, Zn-SOD and Mn-SOD are found in the liver, in erythrocytes, which allows to use the blood as a source of enzyme secretion.

The content of these isoforms in organs and tissues varies less compared to extracellular Cu-, Zn-SOD, which is inferior to them in quantitative terms [13,14,18].

With age, there is a significant decrease in the specific activity of cytoplasmic superoxide dismutase. In this case, there is a drop in the catalytic activity of the enzyme.

Decrease in peroxidase activity in the postmitochondrial fraction of the brain in contrast to mitochondria in aging is associated with weakening of enzyme biosynthesis and with characteristic age-related posttranslational changes due to oxidative modification of the polypeptide chain of the protein. Activation of lipid peroxidation, an increase in hydrogen peroxide and a decrease in glutathione levels, accompanied by a decrease in the activity of SOD, glutathione peroxidase and glutathione reductase in testicular mitochondria of rats with age, according to some scientists, may
indicate a weakening of the antioxidant system and affect such physiological functions as steroidogenesis and spermatogenesis [18,19]. Also, the activity of the enzyme can be reduced with a deficiency of metal ions in the body. Oxidative stress can both reduce and increase the activity of SOD in comparison with the physiological norm.

The pathological process is a complex of successive reactions developing in the body in response to the action of the pathogenic factor and the role of free radicals in it is not always clearly indicated. Pathogenic factors cause the development of pathogenic processes in the body, as complex responses, in which the role of free radicals is not fully studied. The effect of different stages of free radical oxidation on the formation of chronic diseases is different, for example, in atherosclerosis reactions of lipid peroxidation are characteristic, in neurodegenerative diseases-oxidative damage of proteins [1,2,3,6], and the factor of its activation may be the insufficiency of antioxidant protection enzymes in the body.

According to studies, the decrease in peroxidase activity contributes to the increase of hypertension, the development of arthritis, diabetes, diabetic retinopathy, diabetic nephropathy and other pathological processes accompanied by the development of oxidative stress [20,21].

All of the abovementioned suggests that the assessment of the activity of superoxide dismutase in the blood and tissues is necessary to determine the causes and mechanisms of development of a pathological process in the body and offer ways to treat diseases. It was found that no similar studies to identify the correlation of the distribution of superoxide dismutase activity in blood and tissues outside the pathological processes in small experimental animals were carried out.

Thus, the aim of our study was to study the relationship of the distribution of superoxide dismutase activity in serum and tissues of white mongrel rats.

To achieve this goal, the following tasks had to be solved: to determine the activity of superoxide dismutase in serum and liver, brain, heart tissues, as well as in skeletal muscle tissues of rats; to identify the relationship of the distribution of SOD activity in serum and tissues of rats.

2. Materials and methods

The study was conducted using white mongrel sexually mature healthy male rats of one month of birth, weighing 180-200 g in the amount of 150 pieces, which were contained in the vivarium under standard conditions.

The activity of superoxide dismutase was determined by the method of V. S. Gurevich and co-authors [22].

In accordance with the procedure, the tissue homogenate was centrifuged with cooling at 6000 rpm for 10 minutes. The 3 ml of phosphate buffer (pH 7.4) was added to 1 ml of homogenate (serum), homogenized and centrifuged for 15 minutes at 5000 rpm. Then 1 ml of chloroform solution with methanol in a ratio of 2:1 was added to 0.5 ml of the upper phase to precipitate hemoglobin. The mixtures were cooled and thoroughly mixed for 10 minutes. The contents of the tubes were then centrifuged to remove hemoglobin and chloroform. The top layer was drained and diluted with phosphate buffer in 20 times. Then 0.2 ml of this solution were added to the incubation mixture containing 57 µm nitro blue tetrazolium (NBT), with 98.5 µm NAD·N, 16 µm fenasistrasalud (PMS). The reaction takes 10 minutes in a 0.5 M phosphate buffer with EDTA (pH 8.3) at a temperature of 250°C in aerobic conditions. The enzyme was not introduced into the control sample. Then the mixture was photometrically at a wavelength of 540 nm.

The activity of superoxide was calculated by the formula 1:

\[ A = \frac{T\%}{100\% - T\%}, \]

where A is the enzyme activity in conventional units, calculated per mg of protein in grams; T% - percentage of inhibition of HBT reduction reaction or epinephrine oxidation in the sample for 1 minute (50 % of inhibition of the reaction corresponds to 1 conventional unit).

The activity of superoxide dismutase was studied in the tissues of liver, heart, brain and skeletal
muscle tissue of rats, as well as in serum. To do this, the rats were killed in accordance with ethical norms under ether anesthesia by decapitation, then the necessary tissues were extracted, which (except for blood serum) were washed with saline solution and immediately frozen. Homogenates were prepared by mechanical grinding of tissues weighing 1 g with 9 ml of Tris buffer (pH 7.4), at a speed of 5000 rpm in a vessel with double walls, constantly cooled by running water [22].

The digital material was statistically processed by nonparametric Spearman correlation analysis, as well as using gamma correlation coefficients and Kendall Tau.

3. Results of the study
As a result of the experiments, an array of numerical data of SOD activity in blood serum and tissues of rats was obtained. The results were subjected to statistical processing (table 1). At the first stage of the statistical analysis was tested for compliance with the normal distribution of SOD activity in the blood serum and tissues of rats. The Kolmogorov – Smirnov single-sample criterion was used for this purpose. As a result, it was found that the distribution of SOD activity in serum and tissues does not correspond to normal. In this regard, in the further statistical processing we have used nonparametric methods of analysis.

Table 1. Distribution of SOD activity values in serum and tissues of small experimental animals.

| Descriptive statistics of combined groups | N | M  | Me  | Min | Max  | 25 Perc | 75 Perc | 10 Perc | 90 Perc |
|------------------------------------------|---|----|-----|-----|------|---------|---------|---------|---------|
| Blood serum                             | 150 | 29.01 | 29.10 | 27.30 | 30.70 | 28.40 | 29.60 | 28.00 | 30.20 |
| Liver                                   | 150 | 200.43 | 200.45 | 198.30 | 202.60 | 199.70 | 201.10 | 199.30 | 201.60 |
| Brain                                   | 150 | 75.64 | 75.70 | 74.10 | 77.40 | 75.20 | 76.20 | 74.80 | 76.50 |
| Heart                                   | 150 | 96.96 | 96.80 | 94.80 | 98.90 | 96.30 | 97.70 | 95.60 | 98.45 |
| Skeletal muscle                         | 150 | 37.07 | 37.00 | 35.70 | 38.60 | 36.40 | 37.50 | 36.10 | 38.40 |

To assess the relationship between the distribution of SOD activity in serum and tissues of small experimental animals, a study of correlations within the observation group was carried out by the nonparametric Spearman correlation coefficient (table 2), and using gamma correlation coefficients (table 3) and Kendall Tau (table 4).

Table 2. Spearman correlation coefficient on SOD activity distribution in blood serum and tissues of rats and p value.

| Correlation by Spearman all the joint measurements | Valid N | Spearman R | p-level |
|---------------------------------------------------|---------|------------|---------|
| Blood serum & liver                               | 150     | 0.213389   | 0.008745|
| Blood serum & brain                               | 150     | 0.161438   | 0.048423|
| Blood serum & heart                               | 150     | -0.079408  | 0.334076|
| Blood serum & muscle                              | 150     | 0.011671   | 0.887281|

According to the data presented in table 2, the apparent presence of weak forces reliable direct correlation between the activity of SOD in serum and liver tissues (of 0.21 at p ≤ 0.008745) and serum and brain tissues (0.16 at p ≤ 0.048423).

Since no other relationships between the activity of superoxide dismutase in blood serum and tissues of rats were detected by the Spearman correlation coefficient, it was decided to conduct an analysis using gamma correlation criteria (table 3) and Kendall Tau (table 4).
Table 3. Gamma correlation coefficient for distribution of SOD activity in blood serum and tissues of rats.

| MD pairwise deleted | Marked correlations are significant at p <.05000 | Valid N | Gamma | Z     | p-level |
|---------------------|--------------------------------------------------|---------|-------|-------|---------|
| Blood serum & liver | 150                                              | 0.150943| 2.628770 | 0.008569 |
| Blood serum & brain | 150                                              | 0.119430| 2.061217 | 0.039282 |
| Blood serum & heart | 150                                              | -0.056349| -0.985468 | 0.324394 |
| Blood serum & muscle| 150                                              | 0.009665| 0.167139 | 0.867261 |

Table 4. Kendall Tau coefficient correlation on distribution of SOD activity in serum and tissues of rats.

| MD pairwise deleted | Marked correlations are significant at p <.05000 | Valid N | Kendall Tau | Z     | p-level |
|---------------------|--------------------------------------------------|---------|-------------|-------|---------|
| Blood serum & liver | 150                                              | 0.144763| 2.628770 | 0.008569 |
| Blood serum & brain | 150                                              | 0.113509| 2.061217 | 0.039282 |
| Blood serum & heart | 150                                              | -0.054268| -0.985468 | 0.324394 |
| Blood serum & muscle| 150                                              | 0.009204| 0.167139 | 0.867261 |

According to the data presented in tables 3 and 4, it can be seen that the study of the distribution of SOD activity in blood serum and tissues of rats revealed direct reliable weak force correlations between SOD activity in serum and liver tissues, as well as in serum and brain tissues.

4. Conclusions

Thus, all three methods of nonparametric correlation analysis used to assess the relationship between the distribution of SOD activity in serum and tissues of small experimental animals revealed that the activity of SOD in rats within the physiological norm is determined significantly weak direct correlation between the activity of SOD in serum and liver tissues, as well as in serum and brain tissues.

References

[1] Pavlova O, Simakova S 2011 Medical and physiological problems of human ecology: Materials of the IV all-Russian conference with international participation (Ulyanovsk: USU) pp 244-246
[2] Gorshunova N, Lazarenko V, Mal G et al. 2014 Exp. Clin. Cardiol. 20 7 pp 1614-1622
[3] Grivennikova V 2013 Advances in biological chemistry 53 pp 245-296
[4] Pushkina T, Tokaev E, Popova T, Borodina E 2016 Journal by name of N. V. Sklifosovsky “Emergency medical care” 4 pp 42-47
[5] Kupchinskaya S 2014 Togliatti medical Council 1-2 pp 56-59
[6] Romano A, Serviddio G, de Matthaeis A, Bellanti F, Vendemiale G 2010 J. Nephrol. 23 15 pp 29-36
[7] Sirota T, Zakharchenko M, Kondrashova M 2014 Biomedical chemistry 60 1 pp 63-71
[8] Griess B, Tom E, Domann F, Teoh-Fitzgerald M 2017 Free Radical Biology and Medicine 112 pp 464-479
[9] Ighodaro O, Akinloye O 2018 Alexandria Journal of Medicine 54 4 pp 287-293
[10] Horspool A, Chang H 2018 Redox Biology 17 pp 377-385
[11] Sun D, Sun X, Xu Y, Wu T, Tao L 2019 Experimental Gerontology 118 pp 72-77
[12] Volykhina V, Shafranovskaya E 2009 Bulletin of Vitebsk state medical University 8 4 pp 1-18
[13] Skrzycki M, Majewska M, Podsiad M, Czeczot H, Grytnyer-Zięcina В 2011 Experimental Parasitology 129 2 pp 158-163
[14] Seo J, Park J, Choi J et al. 2012 The Journal of Neuroscience 32 28 pp 9690–9699
[15] Iversen M, Gottfredsen R, Larsen U, Enghild J, Petersen S 2016 Free Radical Biology and Medicine 97 pp 478-488
[16] Morales K, Olesen M, Poulsen E, Larsen U, Petersen S 2015 *Free Radical Biology and Medicine* **81** pp 38-46
[17] Zhang J, Wang B, Wang H, He H, Yu C 2018 *Free Radical Biology and Medicine* **129** pp 268-278
[18] Forstermann U, Sessa W 2012 *European Heart Journal* **33** 7 pp 829-837
[19] Bonda D, Wang X, Perry G et al. 2010 *Neuropharmacology* **59** 4–5 pp 290–4
[20] Tokuda E, Nomura T, Ohara S, Watanabe S, Furukawa Y 2018 *Biochimica et Biophysica Acta (BBA) Molecular Basis of Disease* **1864** 6 A pp 2119-2130
[21] Fujita H 2009 *J. Am. Soc. Nephrol* **20** 6 pp 1303-1313
[22] Maksimovich D, Korik E 2017 *Internauka* **12-1** 16 pp 10-12