SMJ Saratov Medical Journal

ISSN 2712-8253, Saratov Medical Journal

2023. Volume 4. Issue 1 (March). Article CID e0103
DOI: 10.15275/sarmj.2023.0103

Original article

Reprint

Changes in the parameters of pro-oxidant–antioxidant balance in the brain of rats with stepwise incomplete cerebral ischemia

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Received 11 May 2022, Accepted 10 March 2023

Abstract:
Objective: to evaluate the activity of oxidative stress in rats with stepwise incomplete cerebral ischemia (SICI).
Material and methods. Experiments were performed on 24 male outbred rats weighing 260±20 g, allocated into 3 subgroups (6 animals in each) based on time of ligation of both common carotid arteries (CCA). The control group comprised of sham-operated rats of the same sex and similar weights (n=6). To identify the pro-oxidant–antioxidant state of the brain based on its homogenates, the activity of lipid peroxidation processes, the content of thiobarbituric acid reactive substances (TBARS), the concentration of reduced glutathione (GSH), total thiol groups (TSH), and the activity of glutathione peroxidase were determined.
Results. SICI with ligation interval of both CCAs of 1 day and 3 days led to a significant decrease in the total SH groups of proteins and glutathione by 30% (p=0.038) and 46% (p=0.044), respectively, TBARS concentration by 29% (p=0.038) and 31% (p=0.043), respectively. SICI with the maximum interval between CCA ligations was manifested by less pronounced changes in the pro-oxidant–antioxidant state of the brain.
Conclusion. In SICI with ligation of both CCAs 7 days apart, at which histological changes were the least pronounced, changes in the pro-oxidant–antioxidant balance were insignificant. The most pronounced disorders of the pro-oxidant–antioxidant balance in the brain were observed in the subgroup with the minimum interval between CCA ligations, which implied the highest activity of oxidative stress.

Keywords: ischemia, pro-oxidant–antioxidant balance, brain homogenates

Cite as: Bon LI, Maksimovich NYe, Dremza IK, Kokhan NV, Burak IN. Changes in the parameters of pro-oxidant–antioxidant balance in the brain of rats with stepwise incomplete cerebral ischemia. *Saratov Medical Journal* 2023; 4 (1): e0103. https://doi.org/10.15275/sarmj.2023.0103

Introduction

Oxidative reactions and the substances formed as a result of their occurrence are of great importance for the vital activity of the cells in entire body and brain in particular. It is known that oxygen radicals perform the functions of a messenger, being responsible for the activity of neurons, and also regulate blood flow in the brain, apoptosis, and other processes necessary for the functioning of the brain [1].

However, excessive production of radicals can lead to membrane damage, accumulation of oxidation products of lipids, proteins, and nucleic acids (aldehydes, ketones), shortage of reduced pyridine nucleotides and phospholipids of mitochondrial membranes, and later on to electrolyte imbalance, mitochondrial swelling, uncoupling of oxidation and phosphorylation processes, and death of neurons during ischemia. Damage by radicals to the mitochondrial DNA unprotected by histones leads to inhibition of the synthesis of electron carrier proteins [2-4]. That is why the study of oxidative stress and the activity of the antioxidant system is of great importance [5, 6].

The objective of our study was to evaluate the activity of oxidative stress in rats with stepwise cerebral ischemia.

Material and methods

The experiments were performed on 24 male outbred rats weighing 260±20 g in compliance with the Directive of the European Parliament and of the Council No.2010/63/EU of September 22, 2010 on the protection of animals used for scientific purposes.

The choice of experimental animals was due to the similarity of the brain angioarchitectonics in rats and humans. Modeling of cerebral ischemia was carried out under conditions of intravenous thiopental anesthesia (40-50 mg/kg).

Stepwise incomplete cerebral ischemia (SICI) was performed by sequential ligation of both common carotid arteries (CCA) with an interval of 7 days (subgroup 1), 3 days (subgroup 2), or 1 day (subgroup 3). The sampling of brain material was carried out 1 hour after ligation of the second CCA in each subgroup. The control group consisted of sham-operated rats of the same sex and similar weights.

Method for examining the pro-oxidant–antioxidant state of the brain. To determine the pro-oxidant–antioxidant state of the brain in its homogenates (20% dilution in phosphate-buffered saline, PBS, with pH=7.2), we examined the activity of lipid peroxidation (LPO) processes, the content of

thiobarbituric acid reactive substances (TBARS), the concentration of reduced glutathione (GSH), total thiol groups (TSH), and glutathione peroxidase activity.

TBARS are found in the body as a result of breakdown of polyunsaturated fats by reactive oxygen species. Thiobarbituric acid (TBA) is a marker of LPO activity and oxidative stress.

To determine the content of TBARS, 2.4 mL of 0.07 N sulfuric acid solution and 0.3 mL of 10% phosphotungstic acid solution were sequentially added to the test sample of 10% brain homogenate (0.3 mL). To the precipitate, washed twice and dissolved in 3.0 mL of double distilled water, 1 mL of 0.85% TBA aqueous solution was added, dissolved in 25 mL of acetic acid with the addition of H2O (5 mL). The color reaction takes place in hermetically sealed tubes at 96 ˚C for 60 minutes. After cooling them in water for 5 minutes, the optical density of the centrifuged supernatant was determined on a PV 1251C spectrophotometer (Solar, Belarus) at wavelengths of 532 nm and 580 nm [7].

The concentration of TBARS was calculated using the formula: TBARS = (E532–E580)/0.156×K, where E is the molar extinction coefficient at the corresponding wavelengths, V1 is the volume of the TBA solution; V2 is the volume of the test sample; K is the dilution factor of the brain sample (147.7).

Calculation of TBARS concentration was carried out using the absorption coefficient for the resulting product, ε532=1.56×105 M-1×cm-1. It was expressed in nanomoles per gram of protein (gram of tissue).

When measuring the concentration of GSH, 0.2 mL of 25% trichloroacetic acid was added to 1 mL of 15% brain homogenate. The mixture was shaken and centrifuged at 5,000 rpm for 5 minutes. To the resulting supernatant (0.2 mL), we added 1.2 mL of 0.5 M phosphate buffer (pH=7.8) and 50 μL of Ellman's reagent. The GSH concentration was calculated using the molar extinction coefficient (ε412=13,600 M-1 cm-1) via determining the optical density of the samples under study at λ=412 nm on a PV 1251C spectrophotometer.

Determination of TSH concentration was carried out as follows. We added 30 μL of 3% sodium dodecyl sulfate solution to 60 μL of brain homogenate; 25 μL of the obtained mixture was combined with 1.2 mL of 0.5 M phosphate buffer (pH=7.8) and 50 μL of Ellman's reagent. After 10 min of incubation at room temperature, the optical density was measured on a PV 1251C spectrophotometer at λ=412 nm, taking into account the molar extinction coefficient. The value of the molar extinction coefficient in this particular case was 13,600 M-1 cm-1.

To measure the activity of glutathione peroxidase, we added 0.1 mL of brain homogenate and 20 mM of tert-butyl hydroperoxide to 0.8 mL of Tris-HCl buffer (pH=7.25) containing 0.012 M solution of sodium azide, 0.001 M solution of ethylenediaminetetraacetic acid and 4.8 mM of GSH; then, incubated for 10 minutes at 37 °C. The reaction was stopped by adding 0.02 mL of 25% trichloroacetic acid solution. To identify a zero point, a similar procedure was carried out immediately after the introduction of tert-butyl hydroperoxide. The samples were centrifuged (5,000 rpm, 5 min), and 30 μL of the resulting supernatant and 30 μL of Ellman's reagent were added to 1 mL of phosphate buffer (pH=7.8). Next, the optical density was measured at λ=412 nm and λ=700 nm [8].

To exclude systematic measurement errors, the brain samples of the control and experimental groups were examined in identical conditions.

As a result, we obtained quantitative continuous data. Since the experiment used small samples with a non-normal distribution, the analysis was carried out by nonparametric statistics using the licensed computer program Statistica 10.0 for Windows (StatSoft, Inc., USA). The data were presented as Me (LQ; UQ), where Me is the median, LQ is the value of the lower quartile; UQ is the value of the upper quartile. Differences between groups were considered significant at $p<0.05$ (Kruskal – Wallis test with Bonferroni correction) [9].

Results

The results of examining the activity of oxidative stress and the antioxidant system of the brain in rats with stepwise cerebral ischemia are presented in *Table*. Against the background of the control group, the subjects in SICI subgroup 1 (with an interval between ligations of both CCAs of 7 days) exhibited a decline in the content of total SH-groups of proteins and glutathione by 8% (p=0.047) and GSH concentration by 20% (p=0.049), along with an increase in the activity of glutathione peroxidase by 5% (p=0.049.) The content of the LPO of TBARS increased by 23% (p=0.046).

In SICI subgroup 2 with both CCAs ligations 3 days apart, the subjects were compared with the control group. We observed a reduction in the content of total SH groups of proteins and glutathione by 30% (p=0.038), GSH concentration by 31% (p=0.046), along with an increase in the activity of glutathione peroxidase by 8% (p=0.049). The content of TBARS increased by 29% (p=0.038).

Table. Parameters of pro-oxidant–antioxidant balance in the brain of rats with stepwise incomplete cerebral ischemia, Me (LQ; UQ)

Groups		SH, mmol/L	GSH, mmol/L	GP, mmol GSH, min×L	TBARS, mmol/L
Control		5.5(5.4; 5.6)	4.6(4.4;4.8)	70(70;72)	19.9(13.8;22.7)
SICI	Sub 1 (7-day interval)	5.1(5.1; 5.1)*	3.6(3.6;3.8)*	74(74;75)*	25.5(24.8;26.0)*
	Sub 2 (3-day interval)	3.8(3.7; 4.0)*+	3.1(3.0;3.2)*+	76(76;77)*+	27.8(23.8;30.6)*
	Sub 3 (1-day interval)	2.9(2.8; 3.1)*+#	2.0(1.9;2.1)*+#	77(77;79)*+	29.5(28.9;30.5)*

* – differences are significant vs. the control group, + – differences are significant vs. the SICI subgroup 1; # – differences are significant vs. the SICI subgroup 2; SICI – stepwise incomplete cerebral ischemia, sub – subgroup, GP – glutathione peroxidase, TBARS – thiobarbituric acid reactive substances, GSH – reduced glutathione, SH – total SH-groups of proteins

The SICI subgroup 2 subjects compared against the background of subgroup 1 (7 days between consecutive ligations) exhibited a decrease in the content of total SH-groups of proteins and glutathione by 26% (p=0.042) and GSH content by 14% (p=0.048), as well as an increase in activity of glutathione peroxidase by 3% (p=0.049).

The SICI subgroup 3 with CCA ligations only 1 day apart against the background of the control group exhibited a decline in the content of total SH-groups of proteins and glutathione by 46% (p=0.044) and GSH concentration by 57% (p=0.038), as well as an increase in glutathione peroxidase activityby9% (p=0.05). The content of TBARS increased by 31% (p=0.043).

In SICI subgroup 3 with both CCAs ligations 3 days apart, the subjects compared with the subgroup 1 exhibited the reduction in the content of total SH-groups of proteins and glutathione by 42% (p=0.035) and GSH concentration by 46% (p=0.029), while the activity of glutathione peroxidase was higher by 4% (p=0.049). SICI subgroup 2 vs. SICI subgroup 3 exhibited a decrease in the content of total SH-groups of proteins and the concentration of GSH by 22% (p=0.037) and by 38% (p=0.034), respectively. The activity of glutathione peroxidase did not change.

Discussion

It is well known that oxidative stress is involved in the pathogenesis of numerous diseases, including cerebral ischemia [10, 11].

Excessive generation of reactive oxygen species in mitochondria can lead to damage of the Krebs cycle enzymes and a deficiency of reduced pyridine nucleotides and phospholipids of mitochondrial membranes, followed by the occurrence of electrolyte imbalance and swelling of mitochondria and uncoupling of oxidation and phosphorylation processes in them, thereby playing a decisive role in the pathogenesis of ischemic neuronal death. Damage by reactive oxygen species to mitochondrial DNA unprotected by histones is accompanied by inhibition of the synthesis of electron carrier proteins. However, the anaerobic pathway cannot compensate for the need of cells in macroergic compounds, which leads to an energy deficit [10].

Due to the accumulation of incompletely oxidized products of carbohydrate, lipid, and protein metabolisms, an excessive formation of H+ ions occurs, and metabolic acidosis takes place. Acidosis and deficiency of high-energy compounds inhibit metabolic processes and disrupt ion transport, which leads to a passive outflow of K+ ions from cells and an influx of Na+, Ca++, and hydrogen ions into neurons. Due to the accumulation of free Ca++ ions, the glutamate-calcium cascade is triggered, cell edema develops (glutamate excitotoxicity) with changes in the physicochemical properties of neuron membranes and vascular endothelium [10, 11].

As the ischemic period extends or the time interval between ligation of the CCAs decreases, the most pronounced stress on the mechanisms of antioxidant defense is noted, which reflects a decrease in the amount of SH groups, GSH concentration, and an increase in the activity of glutathione peroxidase. In turn, an increase in the content of products that react with TBA implies the activation of peroxide processes [6, 12].

Our results are consistent with published data, according to which the culmination of oxidative stress is due to an imbalance between prooxidants and antioxidants, and excessive production of reactive oxygen species involved in the pathogenesis of ischemic brain damage [6].

When modeling incomplete cerebral ischemia, there were no pronounced changes in the pro-oxidant–antioxidant balance, probably, due to compensation of blood circulation in the circle of Willis [13].

In stepwise cerebral ischemia with ligation of both CCAs with an interval of 7 days, in which histological changes were the least pronounced, changes in the pro-oxidant–antioxidant balance were insignificant. There was no suppression of the antioxidant defense in the brain as a manifestation of the activation of compensatory mechanisms: the efficiency of the processes of utilization of both oxygen and oxidation substrates and their delivery to the mitochondria of neurons increased, along with an increase in the synthesis of nucleic acids and proteins, transport of O2 and metabolic substrates, and predominance of anabolic processes over catabolic activity [11, 14-16], which reduced the severity of oxidative stress.

Conclusion

Hence, the most pronounced disorders in the activity of oxidative stress and the antioxidant system of the brain were observed in the SICI subgroup 3 where the minimum interval between CCA ligations was 1 day. These results implied the highest activity of the oxidative stress. The obtained results when extrapolated to the human body can be used to assess the severity of cerebral ischemia, and also serve as a marker for evaluating the development of compensatory responses and the use of corrective therapy.

Conflict of interest. The authors declare no conflicts of interest.

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