THE ROLE OF IONIZED WATER AND ITS IMPACT ON THE ACTIVITY OF GLUTATHIONE PEROXIDASE ON RATS BLOOD SERUM DURING HYPERTHERMIC STRESS

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Abstract: Experimental animals respond to hyperthermic stress (HS) both systemically and cellularly. Overproduction of ROS brought on by HS decreases the activity of antioxidant defense mechanisms, increasing oxidative damage. Ionized water (IO) or electrolyzed reduced water (ERW) has already been the subject of various studies concerning its antioxidant properties. ERW has a very negative oxidation-reduction potential. By scavenging ROS, ERW with a high pH and a sizable negative redox potential (RP) imitates the actions of other antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT). Glutathione peroxidase (GPx) activity is a major antioxidant defense system that is essential and vital to the overall defensive mechanisms and tactics in biological systems. Our work used non-enzymatic antioxidants, glutathione, and vitamin C to examine the effects of ERW on glutathione peroxidase (GPx) activity in blood serum following hyperthermic stress. As an experimental model, white laboratory rats of the female Wistar breed, with a body weight of 180 to 220 grams, were used, divided into three groups (15 animals each, n = 45) for applying the appropriate treatment. The animals were housed at a temperature of 20°C ± 2 ˚C throughout the trial, with a light regime of 12:12 hours. Every animal used in the study received unlimited access to normal laboratory food and water. Three different groups of treated rats were created. The first or control group consisted of rats (CPM) drinking commercial mineral water, the second group of rats (TAM) drank electrochemically reduced water with pH = 9.4 (measured immediately after water activation), while the third group of rats (TAD) drank electrochemically reduced water (pH = 9.4) with additional glutathione and Vitamin C. The investigation was conducted for 21 days. The animals in the respective groups were subjected to a hyperthermic environment for five hours following their respective treatments on the twenty-first day, and this exposure continued until the animals reached secondary hyperthermia (a body temperature of 43 °C). Acute hyperthermic exposure caused a significant increase ($p < 0.001$) in GPx activity in the third group. In the period 7-14 days, when the rats were not exposed to high ambient temperature, CPM 7 and CPM 14 show a statistically significant difference in GPx activity ($p < 0.001$); also TAM 7 and TAM 14 show a statistically significant difference in GPx activity ($p < 0.01$). Acute hyperthermic exposure caused a significant increase ($p < 0.001$) in GPx activity in the TAD group on day 21. Regarding enzymes belonging to the glutathione redox cycle, IO treatment with the addition of glutathione and vitamin C during hyperthermic stress led to higher GPx activity in blood serum.
Keywords: glutathione peroxidase, ionized water, hyperthermic stress, glutathione, vitamin C

1. INTRODUCTION

Heat stress is one of the primary variables that might enhance the production of reactive oxygen species (ROS). (Li, L. et al., 2017). In mammalian cells, hyperthermia results in a variety of alterations to cellular structure, biochemistry, and function (Tabuchi, Y. et al., 2016). The activity of antioxidant defense mechanisms is decreased by excessive ROS generation brought on by heat stress, which increases oxidative damage (Ibtisham, F. et al., 2018). One of the crucial enzymes in the cellular antioxidative defense system is glutathione peroxidase (GPx) (Ugar, M et al., 2018). GPx is an enzyme that eliminates hydroperoxides produced in cells. It is assumed to be a selenoenzyme that defends cells against numerous harms because its subunits include a Selenium (Se) atom. Glutathione peroxidase activity is a major antioxidant defense system that is crucial and important among the numerous defensive mechanisms and techniques employed by biological systems. (Sarikaya, E. and Dogan, S., 2022). Mammalian glutathione peroxidases (GPx) are non-heme thiol peroxidases that contain selenocysteine and catalyze the conversion of organic hydroperoxides or H_2O_2 to water or the equivalent alcohols. They do this by utilizing reduced glutathione (GSH) or thioredoxin (TRX). In order to maintain the integrity of the membrane, GPx is an essential part of the systems that process ROS (Riyazuddin, R. et al., 2022). The glutathione peroxidase family of enzymes consists of two subgroups: the selenium-dependent glutathione peroxidase (Se-GPx), which can reduce both organic and inorganic peroxides, and the non-Se-GPx, which can only reduce organic peroxide. Up to 8 different GPx isoforms have been found in vertebrates. Of them, only GPx6 is a selenoprotein found in humans, while GPx1 till GPx4 are selenoproteins found in mammals. Cysteine is used in place of the selenocysteine residue (Sec) in the remaining isoforms. The GPx family contains the original and most prevalent form of GPx, known as GPx1 (Do, T. D. et al., 2019). With widespread distribution in the cell's cytoplasm and cellular membranes,

correspondingly, GPx1 and GPx4 are the two most common isoforms. For both the cytosolic and mitochondrial enzymes, the nuclear GPx1 gene provides the genetic code. While GPx4 is in charge of eliminating phospholipid peroxides from membranes, GPx1 breaks down hydrogen peroxides (Behnisch-Cornwell, S. et al., 2020). GPx inhibits lipid peroxides damage to the cell surface and other organelles by eliminating hydrogen peroxide and lipid peroxides by managing glutathione (GSH) levels (Shao, X. et al., 2020). Glutathione peroxidases (GPx), together with catalase (CAT) and superoxide dismutase (SOD), are the first line of defense for guarding against oxidative stress in cells and controlling cellular redox homeostasis. The primary endogenous antioxidant produced by cells is GSH, which plays a direct role in the destruction of free radicals and ROS as well as preserving the reduced (active) forms of exogenous antioxidants such as vitamins C and E (Habeeb, A. A., 2018). The degree of their synergistic effect in the direction of and to maintain cellular redox equilibrium determines the total antioxidant capacity in addition to the concentration/activity of individual antioxidants (Ighodaro, O. M. and Akinloye, O. A.,2019). Ionized water or electrolyzed reduced water (ERW) denotes a material with an alkaline pH, a high concentration of hydrogen molecules, and a negative oxidation-reduction potential (ORP) and ROS salvage capacity. By scavenging ROS, ERW imitates the actions of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Ridwan R. D. et al., 2017).

2. OBJECTIVES

The goal of our research resulted from our assumptions that the alkaline reserve in the body will be enriched through the consumption of ionized, or electrochemically reduced water (ERW). We emphasize this property of ionized water (ERW) in conditions of exposure of the organism to high external temperature as a stress factor. Our hypotheses were based on the assumption that IO (ERW), which serves as an antioxidant, will raise the body's tolerance to glutathione peroxidase (GPx) activity in blood serum.

3. MATERIAL AND METHODS

Experimental model and Ethical issues

The Macedonian Center for Bioethics authorized the Manual for the Care and Use of Laboratory Animals, and all experimental procedures were carried out in compliance with its guidelines. The procedures were authorized in accordance with the guidelines for animal-based biomedical research provided by the Council of International Organizations for Medical Sciences by the Animal Ethics Committee of the University of St. Cyril and Methodius in Skopje. Anesthesia was administered in accordance with the advice provided in the Welfare Directive 86/609/EEC of the European Commission.

As an experimental model, white laboratory rats of the female Wistar breed, with a body weight of 180 to 220 grams, were used, divided into three groups (15 animals each, n = 45) for applying the appropriate treatment. The animals were housed at a temperature of 20°C ± 2 ˚C throughout the trial, with a light regime of 12:12 hours. Every animal used in the study received unlimited access to normal laboratory food and water.

Three different groups of treated rats were created

The first or control group consisted of rats (CPM) drinking commercial mineral water,

The second group of rats (TAM) drank electrochemically reduced water with pH = 9.4 (measured immediately after water activation)

The third group of rats (TAD) drank electrochemically reduced water (pH = 9.4) with additional glutathione and Vitamin C.

Experimental protocol

The three groups of rats in an experimental period lasting 21 days, were treated daily in the morning with appropriately modified natural water. The control group received only natural water during the specified period. The other two groups received IO (ERW) and IO with added glutathione and vitamin C, respectively. This type of functional water was prepared in portions every three days at the Institute of Chemistry at the Faculty of Science and Mathematics in Skopje. Water was applied intragastrically in volumes of 2 ml each. Samples for analysis of selected parameters were taken on the 7th, 14th, and 21st days of treatment. Blood required for analysis on day 7 and day 14 was drawn from the tail of the rats and collected into appropriately marked Eppendorf tubes. Blood serum for analysis was obtained after 5 minutes of centrifugation at 1500 rpm and it was frozen at -80℃ for the necessary analyses. Five hours after receiving the respective treatment on the 21st day, the animals of the respective groups were exposed to a hyperthermic environment until reaching the stage of secondary hyperthermia (body temperature of 43 ˚C). The exposure was performed individually in air-conditioned chambers at 40 ± 1 ˚C for a duration of 80 minutes. Rectal temperature was also monitored during the hyperthermic exposure. Body temperature was measured every 20 minutes, and 10 minutes after the last measurement the animals were sacrificed by subcutaneous

application of 3 ml of thiopental. Blood was taken from the abdominal aorta. The resulting blood serum was further aliquoted and frozen at -80 ˚C until further analyses.

Determination of glutathione peroxidase (GPx) activity

Glutathione peroxidases are a group of enzymes found in mammalian cells that help prevent lipid peroxidation of membranes by breaking down free peroxides in the cell. The enzyme acts on lipid hydroperoxides, and cholesterol hydroperoxide, and can also hydrolyze hydrogen peroxide at low concentrations. It uses glutathione as a cofactor and functions in a system with glutathione reductase.

Principle of the method

GPx activity was determined according to the method of Lawrence and Burk (1976), with some modifications. It involves monitoring the oxidation of NADPH at a wavelength of 340 nm for 3 min (25 ˚C) in the presence of both GR and GSH. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, 1 mM sodium azide, 2 mM GSH, 0.2 mM NADPH, 1 U/ml GR, 1.5 mM cumene hydroperoxide, and a sample. Absorption was monitored for 5 minutes at 25 ˚C.

Test procedure

The reaction was started by adding cumene hydroperoxide. One unit of enzyme activity is defined as the amount of enzyme required to catalyze the oxidation of one µmol of NADPH for 1 minute under the above conditions. The final results are expressed as U/mg-proteins.

$$\text{GPx activity (U/mg)} = \frac{\text{The direction of the curve}}{0.5433*6220*(\text{mg/m protein})*10^{6}}$$

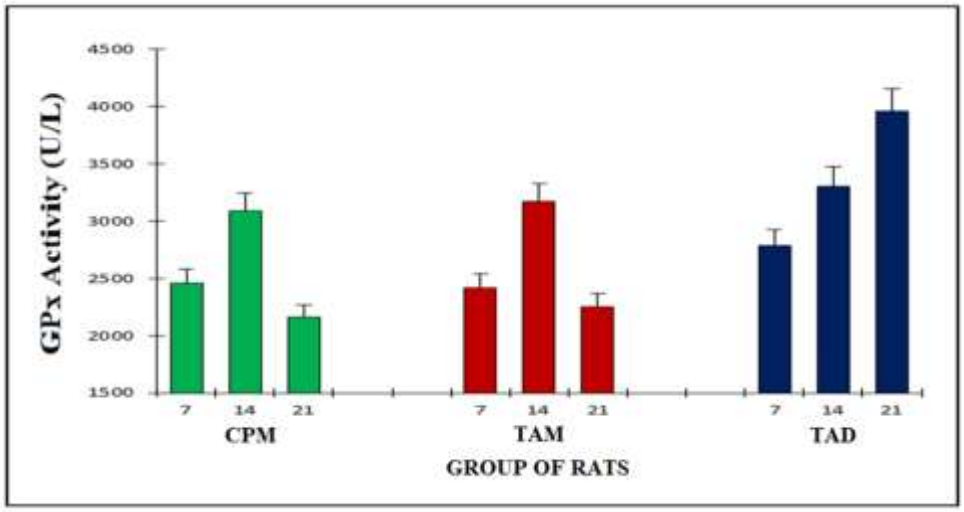
Analytical statistics

The statistical processing of the results obtained during the experiment was performed with the statistical program "InStat". Results are presented as mean values with standard error (SEM). The effect of individual treatment with ionized water, as well as adding vitamin C and GSH to it, combined with hyperthermic exposure of the experimental model, was determined by applying a One-way analysis of variance (ANOVA).

4. RESULTS

Graph 1 displays the findings of our study on the effects of therapy with ionized water on GPx activity, both with and without the addition of suitable antioxidants. Acute hyperthermic exposure was also added on the 21st day of treatment.

Graph 1. Glutathione peroxidase activity in blood serum



Legend: CPM – control group treated with natural water; TAM – group treated with ionized water; TAD – group treated with ionized water with added glutathione and vitamin C

Table 1. Results of the statistical analysis of data on the activity of GPx in serum

Statistical analysis – GPx activity				
Compared groups			Results	
CPM 7	vs	CPM 14	p < 0,001	***
CPM 7	vs	CPM 21	p < 0,01	**
CPM 14	vs	CPM 21	p < 0,001	***
TAM 7	vs	CPM 14	p < 0,01	**
TAM 7	vs	TAM 21	p < 0,001	***
TAM 14	vs	TAM 21	p > 0,05	Ns
TAD 7	vs	TAD 14	p < 0,01	**
TAD 7	vs	TAD 21	p < 0,001	***
TAD 14	vs	TAD 21	p < 0,01	**
CPM 7	vs	TAM 7	p > 0,05	Ns
CPM 7	vs	TAD 7	p > 0,05	Ns
TAD 7	vs	TAD 7	p > 0,05	Ns
CPM 14	vs	TAM 14	p > 0,05	Ns
CPM 14	vs	TAD 14	p > 0,05	Ns
TAM 14	vs	TAD 14	p > 0,05	Ns
CPM 21	vs	TAM 21	p > 0,05	Ns
CPM 21	vs	TAD 21	p < 0,001	***
TAM 21	vs	TAD 21	p < 0,001	***

All three groups in relation to the respective treatment that each of them individually received and the time of its application show a statistically significant difference in the activity of GPx, in the period 7-14 days, when the rats were not exposed to high ambient temperature. In the control group treated with natural water on the 14th day, an increased activity of GPx is observed, which decreases during hyperthermic exposure in the same group. GPx activity during hyperthermic exposure also decreases in the second group treated with ionized water. In the third group treated with ionized water with added glutathione and vitamin C, a higher activity of GPx was registered. Acute hyperthermic exposure caused a significant increase ($p < 0.001$) in GPx activity in the third group. In the period 7-14 days, when the rats were not exposed to high ambient temperature, CPM 7 and CKPM 14 show a statistically significant difference in GPx activity ($p < 0.001$); also TAM 7 and TAM 14 show a statistically significant difference in GPx activity ($p < 0.01$). Acute hyperthermic exposure caused a significant increase ($p < 0.001$) in GPx activity in the TAD group on day 21.

5. DISCUSSION

Electrochemically activated water (reduced, alkaline water) is counted among the natural agents that can strengthen the body's antioxidant defense. The most researched type of useful water is electrolyzed water. Electrochemically reduced water (ERW) is produced near the cathode and electrochemically oxidized water (EOW) is produced near the anode (Shirahata et al., 2012). Active hydrogen in ERW can be considered an ideal ROS "cleaner" because after reduction it does not produce oxidized molecules, as is the case with organic antioxidants (vitamins C and E and polyphenols) (Li, L. et al., 2002). Shirahata and colleagues (Shirahata, S. et al. 2018), on the other hand, suggest that the function of ERW similar to SOD and CAT is not due to dissolved molecular hydrogen, but to active atomic hydrogen, which has a higher reducing ability. A crucial part of the host's defense against oxidative stress in the cytosol is played by the enzyme glutathione peroxidase (GPx). A selenium-containing enzyme called glutathione peroxidase uses glutathione as a reducing agent to detoxify hydrogen peroxide and other hydroperoxides (Palathingal, P. et al., 2022). An antioxidant enzyme that can also release free radicals is glutathione peroxidase. In this way, it helps to prevent lipid peroxidation and maintain intracellular homeostasis, as well as redox balance (Mulgund, A. et al., 2015). A crucial ROS scavenger, glutathione peroxidase (GPx), joins forces with catalase (CAT), superoxide dismutase (SOD), and other enzymes to build an antioxidant defense system. By controlling glutathione (GSH) levels, GPx prevents peroxidative damage to the cell membrane and other organelles by removing hydrogen peroxide and lipid peroxides (Shao, X. et al., 2020).

As a result of exposing the rats to a high ambient temperature, a thermal gradient is created, which results in the ability of the animals to absorb heat from the environment until their body temperature equalizes with the ambient temperature. Animals begin to raise their body temperature entering a state of hyperthermia. In addition to this, a higher body temperature leads to higher enzyme activity and intensification of metabolism, resulting in metabolic heat being produced, which contributes to an additional rise in body temperature. Heat stress is the initial state of hyperthermia, and in the case of thermoregulatory collapse, conditions are created for progression to heat shock, a state in which the body temperature reaches over 40 ℃ and ends with multiorgan dysfunction syndrome. Exposure to acute temperature stress leads to the initiation of thermoregulatory mechanisms in rats in order to maintain

temperature homeostasis. These adaptive responses to exposure to high ambient temperature include cellular and systemic responses. Changes in the cardiovascular, endocrine, and nervous systems are systemic responses that occur in all mammals early in hyperthermic exposure.

Acute temperature stress speeds up metabolic processes by increasing oxygen intake, which is followed by an increase in oxygen flow via the mitochondrial respiratory chain, which again results in the production of ROS. Heat stress causes oxygen radicals to form at the level of the entire cell as a result of an increase in oxygen flow via the mitochondrial electron transport chain. We are confident that the above-mentioned mechanisms for the temperature stress-oxidative stress pathway were active in the rats during our study, and we can state without a shadow of a doubt that the rats experienced acute temperature stress on day 21 of the treatment and strong oxidative stress as a result.

The antioxidant capacity has a particularly significant role in circumstances of exposure of the body to stressful conditions. Stressful factors, of an emotional and/or physical nature, end up generating free radicals in the body. That is why it is essential to work on strengthening the antioxidant defense of the body. In our research, we achieved this goal by supplementing the experimental model with ionized water, glutathione, and vitamin C. Maintaining the variability of homeostasis at a low level means the normal functioning of the entire organism, with the exception of conditions favorable for the appearance and development of pathological conditions.

According to the results we obtained from our research, in the control group CPM, treated with natural water, during the 14th day, we observed an increased activity of GPx, which decreases during hyperthermic exposure in the same group. GPx activity during hyperthermic exposure also decreases in the second TAM group treated with IO. In the third TAD group, treated with IO and with added glutathione and vitamin C, higher GPx activity was registered. Acute exposed heat caused a significant increase in GPx activity in blood serum in the third group.

6. CONCLUSION

Regarding enzymes belonging to the glutathione redox cycle, IO treatment with the addition of glutathione and vitamin C during hyperthermic stress led to higher GPx activity in blood serum.

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