THE ALTERATIONS OF ANTIOXIDANT ENZYME LEVELS IN THE BLOOD SERUM BY ADDING ALKALINE WATER SUPPLEMENTED WITH SODIUM ASCORBATE DURING ACUTE HYPERTHERMIC EXPOSURE

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Abstract: Free radicals and other oxidants are toxic compounds in all metabolic processes. Reactive oxygen species (ROS) react with the main cellular components, causing damage of tissues and oxidative stress. The state of oxidative stress is initiated by metabolic activation and elevated oxygen consumption, caused by increased temperature of the environment. So, the stress response functions to enhance the survival of the species. Antioxidants are necessary for the maintenance of redox homeostasis in organisms. In order to have a proper physiological function, it is necessary a balance between ROS and antioxidants. Because of its antioxidant effects and its ability of alkalizing the organism, alkaline water (AW) is in the central focus of scientific interest. Adding AW and co-treatment of AW with sodium ascorbate (SA) is expected the organism to act preventively to hyperthermic stress. The aim of our research was to evaluate the effect of AW and SA on antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) during acute hyperthermic stress at white laboratory rats. The experimental animals used were thirty female Wistar rats, divided into three groups of ten rats each, wheighing 210-300gr. Oxidative stress was caused by acute hyperthermic exposure at 41˚C, for 21 days. The first group is the control group, the second group is treated with AW, and the third group with AW and SA. SOD levels were measured using the enzymol method and CAT levels using the spectrophotometric method. All variables were given with its mean ± standard deviation. One way ANOVA was used to analyze the data and the Tukey test was used for comparison. The critical *p* value was at 0.05, i.e. the difference is significant if p<0,05. Our results show that acute hyperthermic stress on the 21st day in the second group treated with AW significantly (p <0.001) decreased SOD activity as compared to the control group, while co-treatment of animals with AW and SA in the third group significantly increased the levels of the SOD enzyme on the 7th day (p<0.01), but not on the 21st day, although there values obtained show a tendency of increase. Treatment of animals with both AW and SA significantly increased the levels of SOD on the 21st day (p<0.001) as compared with the group of animals treated with AW. On the other hand, the levels of CAT were decreased in second group of rats, on the 14th day (p<0.05) as compared to control group, also on the 21st day the *p* value is close to significance. Co-treatment with AW and SA resulted in an significantly increase in the levels of CAT on the 21st day (p<0.0001), as compared to control group. Also, treatment of animals with both AW and SA significantly increased the levels of CAT on day 14th and 21st (p<0.001) as compared to group of animals treated with AW, showing a synergistic effect of these treatments. So, this present study showed that individual action of AW as well as synergism with SA caused a high protective effect on oxidative damage and treatment was effective.
Keywords: alkaline water, sodium ascorbate, hyperthermic stress, antioxidant enzymes, rats.

1. INTRODUCTION

Because of their ability to cause damage effects, free radicals and oxidants are in the central focus of scientific interest. The free radical is extremely reactive, unstable, and has a short half-life (Stohs, 1995). The accumulation of free radicals in the body results in generation of oxidative stress (Hacisefki, 2009). The imbalance between free radicals, reactive oxygen species (ROS) and the endogenous defense mechanisms is defined as oxidative stress (McCord, 2000). ROS are highly reactive chemical molecules and include free oxygen radicals, such as superoxide anion, hydrogen peroxide, and hydroxyl radicals (Cross *et al.*, 1987). Most ROS are generated as by-products during mitochondrial electron transport. The increased production of ROS during hyperthermic stress leads to peroxidation of lipids, oxidation of proteins, enzyme inhibition, damage to DNA and death of the cells (Schieber & Chandel, 2014). The antioxidant defense system can remove or scavenge the increased levels of ROS. This system comprises of the enzymatic or endogenous and non-enzymatic or exogenous antioxidants. There are several antioxidant enzymes that neutralize ROS, the most important are: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase. Superoxide radical is converted into hydrogen peroxide and molecular oxygen by SOD, while hydrogen

peroxide is converted into water and oxygen by CAT and peroxidases (Fridovich, 1995). So, superoxide and hydrogen peroxide, two potentially harmful species, are converted into water. Moreover, there are also contributions from exogenous antioxidant molecules, such as: vitamin C, E, A, glutathione, carotenoids, polyphenols, etc. Vitamin C is a powerful antioxidant, that participates in a variety of metabolic functions. It is an electron donor, a potent reducing agent and a scavenger of free radicals (Carr & Maggini, 2017). Vitamin C can accumulate in phagocytic cells, like neutrophils. It is necessary for growth and development, it can strenghten chemotaxis, phagocytosis, generation of reactive oxygen species, and the microbial killing (Webb & Villamor, 2007). One form of vitamin C that has sodium components is sodium ascorbate, that can help to lower its acidity levels. In order for the vitamin C to be easily absorbed and stay longer in the body, the sodium content helps. If the oral intake of vitamin C is increased, then it would be potentially beneficial in order the oxidative damage to tissues to be reduced, by chemical reduction of oxidant species (Khassaf *et al.*, 2003). AW has a pH level greater than 7 and is generated by electrolysis, or by a chemical reaction with alkaline earth metals (Pérez-Hernández & Pedraza-Chaverri, 2011, Shirahata *et al.*, 2018). A number of studies have demonstrated that AW is ROS scavenger, especially it can scavenge hydrogen peroxide, which is thought to be related with protective effects against oxidative stress, such as: DNA and mitochondrial damage (Franceschelli *et al.*, 2016, Tsai *et al.*, 2009), alloxan-induced type 1 diabetes (Li *et al.*, 2011), hemodialysis-induced oxidative stress (Huang, Yang, Lee & Chien, 2003), atherosclerosis and neurodegenerative diseases (Hayashi & Kawamura, 2002). Shirahata *et al.*, (1997) have previously demonstrated that the defence mechanism of AW is brought about by active atomic hydrogen, which is a potent reducing agent. It is reported that the platinum nanoparticles (Ptn) present in AW are scavengers of ROS, and they strength the anti-oxidising action of dissolved hydrogen. So, Henry and Chambron (2013) have concluded that Ptn have a similar effect to SOD and CAT.

2. MATERIAL AND METHODS

2.1. Experimental design

The Guiding Principles for Care and Use of Laboratory Animals were applied and respected in all experimental procedures which were conducted, and this is approved by the Macedonian Center for Bioethics. In accordance with the International Guiding Principles for Biomedical Research Involving Animals all protocols were approved by the Animal Ethics Committee of the University "SS. Cyril and Methodius", Skopje, R. Macedonia, as it is issued by the Council for the International Organizations of Medical Sciences. Anesthetics were applied in coordination to the standards given by the guide of the EC Directive 86/609/EEC. An intraperitoneal injection of thiopental sodium (Rhone-Poulenc Rorer Limited, Nenagh, Co Tipperary, Ireland), was used to anesthetize the animals, 50 mg kg-1 b. wt. For all protocols were used Wistar female rats (n=30) on the age of 6 months, weighing 210-300gr. They were maintained on a 12:12 light: dark cycle and fed with standard rat chow and water ad libitum, at a thermo-neutral temperature of 22ºC. The experiment lasted 21 days.

2.2. Experimental protocol

The first group of animals referred to as the control group, who during the entire experimental period were under the conventional laboratory conditions and received only natural water. A second group of animals, who were treated intragastically with alkaline water. Third group of animals, who were treated intragastically with alkaline water and sodium ascorbate. Every morning, at the same time, treatment was applied intragastrically in portions of 2ml. On the 7^{th}, 14^{th} and 21^{st} day of the experiment,1,5 ml of blood was taken from the tail of the rats, with its incision, in order to determine the level of antioxidant enzymes. Blood serum for analysis was obtained after 5 minutes of centrifugation at 1500 rpm and it was frozen at -80 ℃ for the required analysis. Five hours after receiving appropriate treatment on day 21, animals in the respective groups were exposed to a hyperthermic environment until they reached a stage of secondary hyperthermia (body temperature of 43 ˚C). The exposure was performed individually in air chambers at 40 ± 1 ˚C for a duration of 80 minutes. Then, rats were sacrificed by subcutaneous administration of sodium thiopental. The tissues were perfused in 0.9% NaCl and instantly frozen in liquid nitrogen. All isolated materials were stored at -80˚C until further analysis.

2.3. Determination of serum superoxide dismutase

In order to determine the activity of SOD, was used the method that was described by Marklund and Marklund (1974). This method uses the property of SOD to inhibit the auto-oxidation of the pyrogallol. The reaction mixture was consisted of 50 mM Tris-HCl, pH 8.2, 1 mM diethylenetriamine pentaacetic acid and sample. At the beginning concentration of 0.2 mM pyrogallol was added to initiate the reaction. The absorbance was measured kinetically at 420 nm, 25 °C, for 3 min. The amount of sample needed to inhibit the pyrogallol oxidation by 50 % was defined as one unit of activity. The final results were given as U/g hemoglobin.

2.4. Determination of serum catalase

The method of Beers and Sizer (1951) was used to determine the activity of CAT. This method, spectrophotometrically, gives the wanted results by measuring the exponential disappearance of H_2O_2 (10 mmol/L) at 240 nm in the presence of cellular homogenates. The enzyme activity was expressed as µmol H_2O_2 mg protein^{-1} min^{-1}. By diluting 0.15 ml of superoxol (Merck) with 25 ml of 0.05 M phosphate buffer, pH 7.0 was prepared an approximately 5 X 10^{-3} M solution of hydrogen peroxide. In each of four cuvettes 2 ml of buffered catalase solution was pipetted, except in the control one, in which 1 ml of buffer was added. The cuvette housing unit was covered quickly. Then every 10 seconds optical density readings were taken. The average standard velocity constant that was calculated for 1 M catalase, was 2.O ± 0.17 X 10^7 liters mole^{-1} sec^{-1}.

3. STATISTICAL ANALYSIS

All variables are given with its mean ± standard deviation. One way ANOVA was used to analyze the data and the Tukey test was used for comparison. The critical *p* value was at 0.05, i.e. the difference is significant if $p<0,05$. For all analyses we used the statistical software package Addinsoft (2021) XLSTAT statistical and data analysis solution, New York, USA.

4. RESULTS

All analysis that are made, show that there is a significant difference mostly after the 21st day. However the correlation matrices show that the correlation is absent or positive low and we can see some correlation between the results of the control group and the results after the 21st day. Serum antioxidant enzymes activities are shown in *Figure 1* and *Figure 2*.

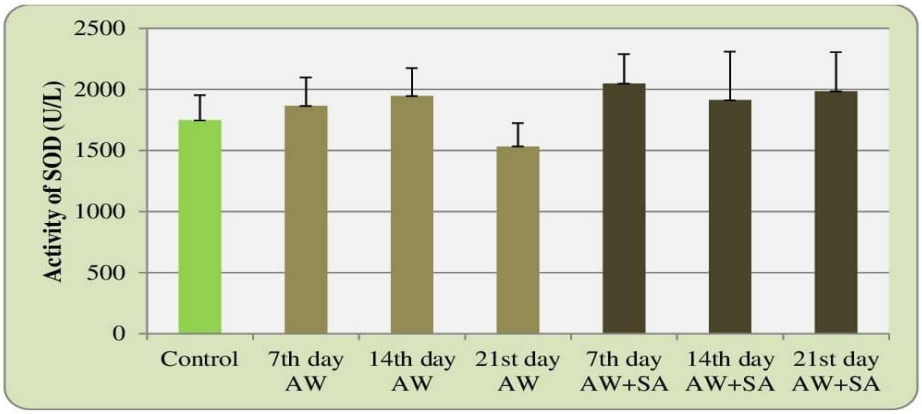
Superoxide dismutase

The results show that levels of SOD have been decreased in the group of animals treated with AW on the 21st day after treatment ($p<0.001$), but not on the 7th and 14th day as compared to control group. Co-treatment of animals with AW and SA significantly increased the levels of the enzyme SOD on the 7th day ($p<0.01$), but not on the 21st day, although there values obtained show a tendency of increase in SOD levels in the treated group (*Table 1*).

Table 1. The comparison of SOD serum levels among groups.

SOD±SD	Control	*P*	After 7 days	*P*	After 14 days	*P*	After 21 days
AW	1746.7±207	>0,05	1868±230	>0,05	1947.3±228.5	<0,05	1533.5±189.7
AW+SA	1746.7±207	<0,05	2050.1±237.3	>0,05	1912.3±399	<0,05	1985.5±318.6

Figure 1. The level of SOD (U/L) in blood serum in all three groups.



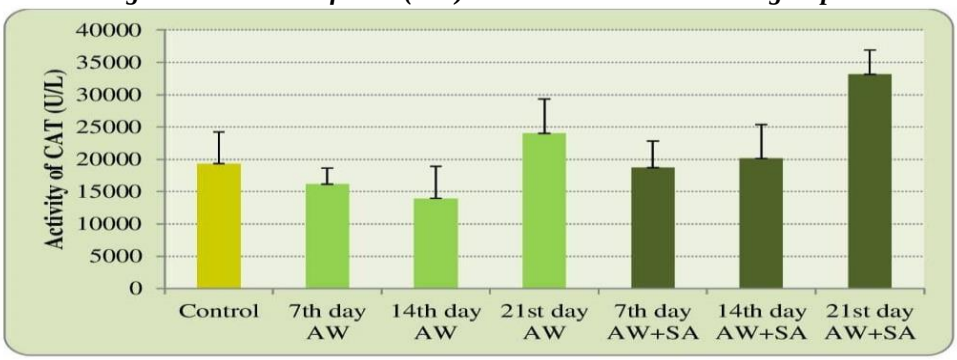
Catalase

Treatment with AW significantly decreased the levels of catalase on the 14th day ($p<0.05$) as compared to control group, but there was not a significant change on the 7th and 21st day, although on the 21st day the *p* value is close to significance. Treatment with AW and SA significantly increase the levels of CAT on the 21st day ($p<0.0001$), but not on the 7th or 14th day (*Table 2*). These results are shown also in *Figure 2*.

Table 2. The comparison of CAT serum levels among groups.

CAT±SD	Control	*P*	After 7 days	*P*	After 14 days	*P*	After 21 days
AW	19316.5±4918.7	>0,05	16183.9±2440.7	>0,05	13958.6±4962.7	<0,05	24047.2±5296.3
AW+SA	19316.5±4918.7	>0,05	18720.7±4081.2	>0,05	20143.8±5247	<0,05	33157.8±3728.7

Figure 2. The level of CAT (U/L) in blood serum in all three groups.



5. DISCUSSION

The present study evaluated the potential protective effects of AW and co-treatment of AW with SA against oxidative stress caused by hyperthermia in white laboratory rats. It was demonstrated that AW is a potent antioxidant and can defend the organism from oxidative damage (Shirahata *et al.*,1997, Li *et al.*, 2011, Henry & Chambron, 2013). The scavenging ability of AW for ROS is thought to be complicated. AW contains hydrogen molecules and mineral nanoparticles. Active hydrogen and hydrogen molecules are considered to be new redox regulation factors, that have the ability of inducing the gene expression of antioxidative enzymes. To manifest more potent reducibility of hydrogen molecules, they may be converted to active hydrogen by catalyst action of metal nanoparticles (Shirahata, Hamasaki & Teruya, 2012). Also, Shirahata *et al.*, (1997) demonstrated that AW can destroy ROS, especially superoxide radicals (O^{2-}) and hydrogen peroxide (H_2O_2), in the same way as the action of SOD and CAT. These reactions can occur if is present dissolved hydrogen, but also it must be active in its atomic form. However, it was supposed that the activated hydrogen has been stabilized by Ptn, that are released during the process of degradation of the electrodes (Shirahata *et al.*, 2002). The results of our study are in accordance with the previous studies, which report that such properties of AW are shown in the second group, where acute hyperthermic exposure on the 21st day significantly ($p < 0.001$) decreased SOD activity, but not on the 7th and 14th day, as compared to control group. Co-treatment of animals with AW and SA significantly increased the levels of the enzyme SOD on the 7th day ($p<0.01$), also the values on the 21st day show a tendency of increase in SOD levels in the treated group. Moreover, treatment of animals with both AW and SA significantly increased the levels of SOD on the 21st day ($p<0.001$) as compared with the group of animals treated with AW, showing a synergistic effect of these treatments. SOD and CAT are the most important antioxidant enzymes, that form the front line of defense against oxidative damage (Landis & Tower, 2005, Younus, 2018). CAT use hydrogen peroxide as its substrate and maintains cellular redox homeostasis (Buettner, 2011). The results of our study show that the levels of CAT were decreased in second group of rats, on the 14th day ($p<0.05$) as compared to control group, but there was not a significant change on the 7th and 21st day, although on the 21st day the *p* value is close to significance. Interestingly, co-treatment of AW and SA in rats resulted in an significantly increase in the levels of CAT on the 21st day ($p<0.0001$), but not on the 7th or 14th day, as compared to control group. Also, treatment of animals with both AW and SA significantly increased the levels of CAT on the 14th and 21st day ($p<0.001$) as compared to group of animals treated with AW, showing a synergistic effect of these treatments. This increasement could be due to the expression of CAT, which can be induced by some kinds of stresses, especially from peroxide stress (Zaidi & Banu, 2004). Vitamin C is a potent non-enzymatic antioxidant, reacts with a wide range of biological oxidants, inhibits the formation of lipid and protein peroxidation and DNA damage (Fraga et al. 1991). For reducing the stress and especially its negative effects, a vitamin C or AW treatment are demonstrated to be effective (Paul, 2019, Shirahata *et al.*, 1997).

6. CONCLUSION

The exposure to high ambient temperature leads to increased production of oxidative stress indicators and to the reduction of the activity of antioxidant enzymes, leading to oxidative damage. SOD and CAT are a first line defense antioxidants and they have the most important role in the total defense mechanisms in living systems. This present study showed that individual action of AW as well as synergism with SA, were effective in increasing the SOD and CAT levels in Wistar rats during hyperthermic stress, causing a high protective effect on oxidative stress. But, the effect of oxidative damage is less presented in rats treated with AW and SA, of course because they have a stronger antioxidant defense that prevents oxidative modification of all biomolecules in the body.

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