The Dynamics of the Main Oxidative Stress Chemical Markers in the Serum of Rats Stressed by Various Behavioural Tasks

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Oxidative damage is a biochemical event currently incriminated in the occurrence of many disorders. The range of affections varies, from psychiatric illnesses, like depression, schizophrenia, Alzheimer's disease, for instance, to somatic disorders such as carcinogenesis or diabetes. Moreover, ophthalmological pathology is also affected in their development by the oxidative stress factor. In the present study, we focused on following the changes which occur in the serum of rats exposed to environmental stress conditions (swimming and treadmill exercises) by determinations of superoxide dismutase (SOD), glutathione peroxidase (GPX) specific activity and malondialdehyde (MDA) concentration. There are important biomarkers of oxidative stress, suspected to play a relevant role in the pathology of dry eye syndrome. Our preliminary oxidative stress analysis of the serum, sampled from animals exposed to physical stress in attempt to induce dry eye syndrome pathology, showed that the levels of SOD and GPX enzymes were higher as compared to controls. Also, MDA concentration was decreased with a significant value attributed to the swimmer rats compared to controls.

Keywords: superoxide dismutase, glutathione peroxidase, malondialdehyde, catalase, rat animal models, oxidative stress, environmental stress

Oxidative damage is a biochemical event currently incriminated in the occurrence of many disorders ranging from psychiatric (Alzheimer disease [1], depression [2], schizophrenia [3]) to somatic disorders (diabetes [4], carcinogenesis [5]). Even ophthalmologic disorders have an oxidative stress factor held accountable for their development [6]. Our research group is already studying oxidative modifications that manifest in ophthalmology related pathologies [7]. By evaluating antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPX) in the context of keratoconus, an important decrease was indicated in the activity of these two antioxidant enzymes, determined from the patients' serum compared with controls [7]. Also, following the same ophthalmological pathology, it was observed that an augmentation of malondialdehyde (MDA) levels was present in the serum of patients diagnosed with keratoconus as opposed to those without the disease, indicating an increased lipid peroxidation activity [8]. The results of the serum biochemical determinations indicate that alterations of oxidative balance with a possible developing of an inflammatory mechanism for keratoconus.

Physical exercise is another factor that is currently debated because of its implications in the oxidative stress processes and its various effects on oxidative levels, by either increasing or decreasing markers of oxidative damage [9, 10]. Current research is concentrating on the different outcomes that physical activities (such as running on a treadmill, or swimming) have in regard to oxidative process. Similarly, a part of our group has formerly assessed the biochemical differences induced by treadmill exercises expanding on a short period of time in running rats, and it was demonstrated that increased levels of oxidative damage were traced in the rat's serum [11].

Considering the recent interest in understanding the connection between oxidative alterations and ophthalmological pathology (e.g. glaucoma [12], cataracts [13]) along with our previous experience in studying ocular disorders in connection with oxidative imbalance [7, 8], our group has taken interest in studying the dry eye syndrome, another pathological condition of the eye that involves oxidative stress modifications [6]. This disease is characterized by an alteration of tears and ocular surface with multifactorial background leading to symptoms of visual disturbance and discomfort, coupled with vulnerability, high osmolarity of tear film and inflammatory processes [14]. The relevance of this connection is highlighted by the relationship between inflammation, oxidative stress and dry eye pathology. To study this link in a thorough manner we applied several stressors to an animal model in the attempt to create an experimental model of the dry eye syndrome and obtained promising results marked by an important decrease in the tear catalase (CAT) levels, a marker enzyme of the antioxidant process known to catalyse the reaction of turning hydrogen peroxide into water and oxygen [15].

Therefore, in the present study we focused on following the changes which occur in the serum of rats exposed to environmental stress conditions and subjected to swimming and treadmill exercises by following preliminary determinations of SOD, GPX specific activity (relevant antioxidants enzymes) and MDA concentration, important biomarkers of oxidative stress, suspected to play a relevant role in the pathology of dry eye syndrome.

Experimental part

Materials and methods

In this experiment, we used 21 adult Wistar rats acquired from the Victor Babes National Institute of Research and Development, Bucharest, Romania, with an average weight of 275+/-5g. The rats were housed in cages with constant temperature and humidity and in circadian cycle

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light conditions (12h light/12h darkness), with no food or water privations.

The experiment respected the guidelines of animal bioethics stated in the Act on Animal Experimentation and Animal Health and Welfare Act from Romania and was authorized by The Ethics Committee of Grigore T. Popa University of Medicine and Pharmacy. The procedures applied respected the European Communities Council Directive of 24 November 1986 (86/609/EEC).

The rats were equally divided (n=7) into separate groups as follows: group 1: control group, group 2: runners, group 3: swimmers. The rats included in the groups 2 and 3 were trained for five days. The rat from group 2 ran with 4 km/h for 15 min at the end of the fifth week of the experiment. The swimmers from the group 3 swam in a custom-made rat pool at a constant temperature, increasing progressively the time spent swimming to 45 minutes after five weeks. The detailed animal model was described in our previous study about decreased catalase specific activity in the tears as a result of environmental stress [15].

At the end of the fifth week, the rats were anesthetized (ketamine 100mg/kg, xylazine 10mg/kg) and blood was collected intracardiac. Blood was centrifuged at 3000 rpm for 15 min. Following centrifugation, the supernatant was separated and pipetted into tubes.

Superoxide dismutase determination

Superoxide dismutase (SOD) activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 substrate (a water-soluble tetrazolium dye) and xanthine oxidase using a SOD Assay Kit (Fluka, product number: 19160) in accordance with the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the coloured product of WST-1 reaction with superoxide) after 20 min of reaction time at 37°C. The percent inhibition was normalized by mg protein and presented as SOD activity units [16].

Glutathione peroxidase determination

Glutathione peroxidase (GPX) activity was measured using the GPX cellular activity assay kit CGP-1 (Sigma Chemicals). This kit uses an indirect method, based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalysed by GPX, which is then coupled with recycling GSSG back to GSH utilizing glutathione reductase (GR) and NADPH. The decrease in NADPH at 340 nm during oxidation of NADPH to NADP is indicative of GPX activity [17].

Malondialdehyde determination

Malondialdehyde (MDA) concentrations were determined by thiobarbituric acid reactive substances (TBARs) assay. 200µL of supernatant was added and briefly mixed with 1 mL of trichloroacetic acid at 50%, 0.9 mL of TRIS-HCl (*p*H 7.4) and 1 mL of thiobarbituric acid 0.73%. After vortex mixing, samples were maintained at 100°C for 20 min. Afterwards, samples were centrifuged at 3000 rpm for 10 min and supernatant read at 532 nm. The signal was read against an MDA standard curve and the results were expressed as nmol/mg protein [18, 19].

Results were statistically analysed using one-way analysis of variance (ANOVA) by using SPSS. All of the results are described as mean ± SEM and statistical significance outcome was considered to be at p values smaller than 0.05 ($p<0.05$).

Results and discussions

Measurement procedures of SOD enzymatic activity determined from the serum of rats subjected to physical training stressors (e.g. swimming and running), showed a statistically significant increased activity of SOD enzyme levels in rats exposed to both running and swimming ($p<0.05$) with higher significance in the case of runner rats compared to controls (fig. 1).

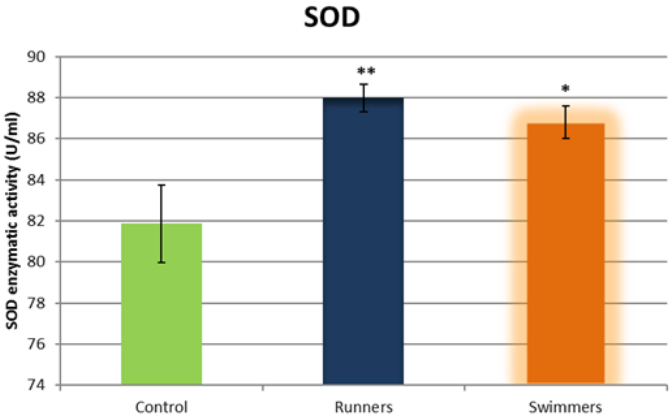

Fig. 1. The influence of physical exercise vs. control on SOD levels of enzymatic activity (U/mL) from rat serum (n=21). The values are explained as mean ± SEM (n=7 animals per group). **p<0.001 vs. control *p< 0.05 vs. control group

These results suggest an increased antioxidant activity, because of the known role that SOD has in the dismutation of superoxide, an event that reduces the damaging action of the superoxide anion.

Following the activity of GPX enzyme there were also noted significant increases in the levels of GPX enzymatic activity measured in the serum of physical active rats as compared to control ones ($p<0.05$), with a higher enzymatic value attributed to the runner rats (fig. 2). These results also point to an increased antioxidant activity, given that GPX is part of the antioxidant system.

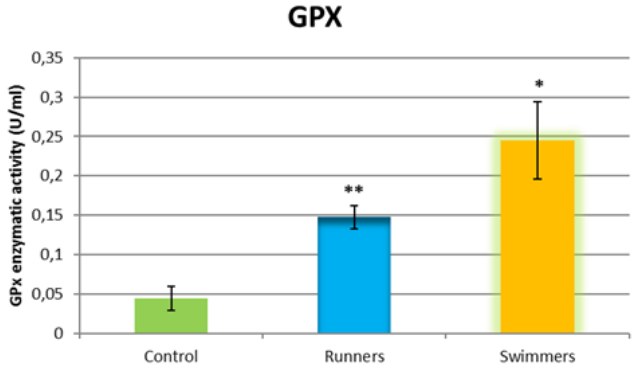

Fig. 2. The influence of physical exercise vs. control on GPX levels of enzymatic activity (U/mL) from rat serum (n=21). The values are explained as mean ± SEM (n=7 animals per group). **p<0.001 vs. control *p< 0.05 vs. control group

We determined the values of MDA concentration in the serum of rats subjected to environmental physical stress (e.g. swimming, running) and controls. The results indicated to a significantly decreased MDA concentration in the case of swimmer rats as compared to controls ($p<0.05$).

Considering our preliminary results, we observed the presence of an increased antioxidant activity through the high levels of SOD and GPX and also a decreased rate of lipid peroxidation indicated by low levels of MDA concentrations.

Although the results indicate a decreased presence of oxidative stress remarked through high rates of antioxidant enzymes and low levels of the MDA, it must be emphasized that serum obtained from the peripheral blood is a

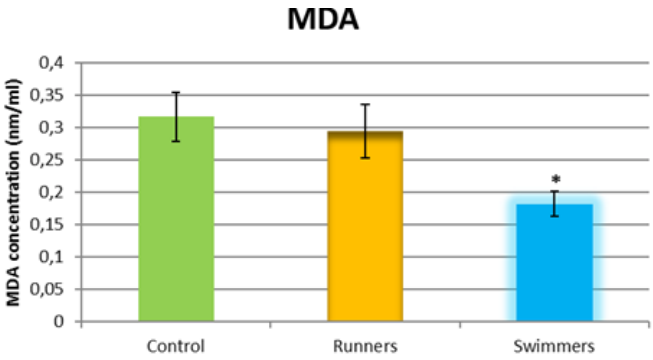

Fig. 3. The influence of physical exercise vs. control on MDA levels of enzymatic activity (U/mL) from rat serum (n=21). The values are explained as mean ± SEM (n=7 animals per group). *p<0.05 vs. control group

component subjected to the influence of many factors as opposed to the brain and tears which are enclosed systems, therefore this might be one of the reasons why there are encountered variations in regard to oxidative markers.

Nonetheless, in our attempt to create an animal model of dry eye pathology, as we previously illustrated, oxidative stress is present in the tears of rats which were inflicted with the same kind of physical stress, determined through the low level of catalase, an important antioxidant factor [15]. Although, the determination was made on another fluid the parameters followed are still indicators of the oxidative stress presence.

As formerly presented, physical stress alone or together with psychological stress, represent elements that contribute to the formation of oxidative damage, which in turn contributes to the occurrences of different diseases either psychiatric or somatic [5, 20, 21]. The oxidative mechanism through which these disorders develop are based on the imbalance between production and reduction of reactive oxidative species (ROS) [22]. Altogether, we must not forget that oxidative stress is not only harmful, but it contributes to the overall protection against pathogens in physiological circumstances [23].

The problem with oxidative stress starts when the balance between pro-oxidants and antioxidants is destroyed and among other factors psychological stress is incriminated [24]. Considering this feature, we applied it to our animal model trying to mimic dry eye syndrome, knowing that according to Wang's study, where the whiskers of a mouse were cut off inducing an enormous psychological stress to the animal, high registered rates of oxidative stress were recorded [25].

Another element that we used in inducing the dry eye animal model was the physical stress, acknowledged to produce oxidative imbalances in rats [9, 26, 27], being considered a stressor feature for the animals: swimming [28] as well as running [29, 30] induced elevated plasma catecholamines and glucocorticoid, known indicators of stress. Noteworthy are the varied responses that were encountered towards physical stress, some reports describing an elevated rate of oxidative stress marked through the low plasma levels of vitamin A (antioxidant determinant) in running rats [26], while others demonstrate a diminished oxidative stress rate, sustained by high CAT levels [27].

Continuing the oxidative stress polemic, the SOD marker appears to be either intensified in physical training environment [31, 32] or without change in the case of acute versus chronic exercise in rats [27]. In the same fashion, GPX behaves in a comparable manner, with either high levels encountered in the case of rats constrained to an intensive treadmill training [33] or without variation in the serum GPX rates of human individual subjected to physical activity of mild intensity [34].

No matter how remarkable these fluctuations might be, the potential reason of the different oxidative stress outcomes in regard to any physical training, is the history of previous physical practice attributed to the examined subject. Therefore, in individuals that are without any previous physical experience, the levels of lipid peroxidation rise, whereas individuals with former training, have no alterations in the levels of lipid peroxidation when exposed to physical exercise [27].

Likewise, a different research, highlighted that individuals practicing bicycle exercise without former training, but who were administered vitamin C before the effort, showed decreased oxidative stress as compared to those who did not take the antioxidant [35]. Similarly, a part of our group, already demonstrated that increased oxidative stress rates are recorded if untrained rats are bound to run on a treadmill a limited small amount of time [11].

Furthermore, other studies conducted in collaboration with our group, demonstrated the involvement of oxidative stress in the ophthalmologic pathology by assaying MDA levels from the serum of patients diagnosed with keratoconus in comparison with healthy subjects obtaining increased MDA concentrations, an important marker of the lipid peroxidation [8] and significantly decreased rates of SOD and GPX enzymatic activity, valuable antioxidant markers [7].

In this context, we must remark that, to our best of knowledge, this is the first time when SOD, GPX and MDA along with dry eye syndrome are conjoined, although there is still need of immunohistochemical analysis for solid proof in this matter.

Conclusions

This preliminary analysis of the serum for oxidative markers sampled from the animal model exposed to physical stress in attempt to induce dry eye syndrome pathology showed that the levels of SOD and GPX enzymes were higher compared to controls. Also, MDA concentration was decreased with a significant value attributed to the swimmer rats compared to controls.

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