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EFFECTS OF A SINGLE DOSE OF ULTRAVIOLET B IRRADIATION ON OXIDANT/ANTIOXIDANT BALANCE IN THE EYE OF WISTAR RATS

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ABSTRACT. Solar ultraviolet radiation (UV) is a major cause of ocular injury contributing to photokeratitis, cataract and pterygium development. The aim of the study was to investigate the oxidant/antioxidant status of Wistar rat eyes after exposure to various doses of UVB in correlation with morphological and structural changes. Five groups of 8 animals each, randomly divided, were treated as follows: group 1: control, no UVB irradiation; group 2: a single UVB exposure to a dose of 45 mJ/cm^2; group 3: a single UVB exposure to 90 mJ/cm^2; group 4: a single UVB exposure to 180 mJ/cm^2; group 5: a single UVB exposure to 360 mJ/cm^2. At 24 hrs after UVB irradiation the animals were anaesthetized and sacrificed by cervical dislocation. The rat eyes from 5 animals were extracted and used for biochemical determinations and from 3 animals were harvested and used for histopathological investigation. Our results demonstrated that a single UVB exposure at different doses disturbs the oxidant-antioxidant balance in the eye tissues by lipid peroxides generation, activation of CAT and SOD and adaptative increasing of GSH levels, particularly at high doses. Twenty hours following UVB irradiation the cornea showed significant lesions: inflammation, hemorrhage, superficial/deep ulcerous keratitis and epithelial exfoliation. Severity of injuries was dose-dependent. These data suggest that oxidative stress may be responsible for the corneal lesions induced by UVB irradiation.

Keywords: *ultraviolet radiation, oxidative stress, eye, Wistar rats*

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INTRODUCTION

The ocular surface is constantly exposed to noxious agents from environment such as atmospheric oxygen (Holly et al., 1977), pollutants, chemical compounds and ultraviolet radiations (Tsubota et al., 1993). A large epidemiological data showed an association between ultraviolet radiation (UV) exposure and anterior pole ocular pathology (Mc Carty and Taylor, 2002). Thus, UV, especially UVB, penetrates the cornea and after absorption into the lens induces its damage by several mechanisms: formation of protein cross-linking, alteration of membrane transport system, swelling (Ringvold et al., 1997; Torriglia and Zigman, 1988), subcapsular vacuoles, deregulation of normal matrix dynamics (Ardan and Cejkova, 2012) and changes in cellular DNA (Wolf et al., 2008). These alterations have a major impact on metabolic pathways in the lens and explain the mechanisms involved in photokeratitis and subsequently in cataract and pterygium development (Johnson et al., 2004) (Berwick, 2000).

Generation of oxygen reactive species such as anion superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radical is a well documented route for UVB-induced ocular damage (Andley, 1987; Goosey, 1980; Spector and Garner, 1981). Generally, cornea stops 92% of UVB and 60 % of UVA, especially its superficial layers (Ringvold et al., 1997). Moreover, it is known that anterior cornea is nourished by the tear film and posterior cornea by the aqueous humor, and both liquid media have protective, antioxidant, antibacterian and lubricant role. Thus, Crouch et al. reported that tears contain only superoxide dismutase (SOD) as antioxidant enzyme (Crouch et al., 1991) not catalase (CAT) or glutathione peroxidase (GPx). Other authors (Behndig et al., 1998) identified in human tears small quantities of SOD and large amounts of various non-enzymatic antioxidant including acid ascorbic, cysteine, lactoferrin (Kuizenga et al., 1987), tyrosine, glutathione and uric acid (Gogia et al., 1998). Aqueous humor has high quantities of ascorbate, glutathione and uric acid which remove reactive oxygen species generated by cellular metabolism and light exposure (Spector and Garner, 1991). The ascorbate protects the lens against lipooxygenase activity and counteracts the reactive oxygen species released by inflammatory cells during ocular inflammation (Williams and Paterson, 1986). The human cornea is rich in SOD, heme oxygenase-1 (HO-1) and NADPH cytocrome P450 reductase and the lens contain antioxidant enzyme such as SOD, CAT, GPx (Abraham et al., 1987; Behndig et al., 1998). The activities of all antioxidant enzymes dramatically decrease with age and after UV exposure and predispose cornea and lens to injury and diseases.

Increased levels of oxidants disturbs the balance between generation of free radicals and their inactivation by antioxidant defense systems and leads to alterations of epithelial membrane proteins, lipid oxidation, changes in ion

concentration and nuclear fragmentation (Soderberg, 1988). In addition, loss of thiol groups, methionine oxidation (Kovacic and Somanathan, 2008) and decreased proteasome activity disturbed redox balance and increase apoptosis (Wilhelm et al., 2007). Moreover, ROS are involved in activation of intracellular signaling pathways including nuclear factor kappa-B (NF-kB) and mitogen activated protein kinase (MAPK) with important consequences in early defensive reactions, in apoptosis and cell proliferation. In parallel, CAT, SOD and GPx activities decrease in the lens (Ringvold et al., 1997) and proinflammatory citokines are released.

Experimental *in vivo* studies certified that the rat lens has a maximum sensivity to UV around 300 nm (Merriam et al., 2000). Setting a maximum tolerable dose of UVB which does not induce damage and consequently cataract is a new goal in preventing ultraviolet radiation-induced changes. The purpose of the study was to investigate the oxidant/antioxidant status of rat eyes after exposure to various doses of UVB in correlation with morphological and structural changes.

RESULTS AND DISCUSSION

MDA is a useful marker for photooxidative damage. Exposures to 45 and 90 mJ/cm^2 of UVB did not reveal any changes in MDA levels in eye homogenates (0.12±0.02 respectively 0.16±0.02 nmoles/mg protein) compared to untreated eyes (0.25±0.16 nmoles/mg protein; $p>0.05$). The irradiation with high doses of UVB (180 and 360 mJ/cm^2) induced a 2.91 respectively 2.75 fold increase in lipid peroxidation as compared to eye exposed to 45 mJ/cm^2 UVB ($p<0.01$) (Figure 1). The increasing of lipid peroxidation was not dose dependent, the differences between MDA levels in groups irradiated with 180 and 360 mJ/cm^2 being insignificant ($p>0.05$).

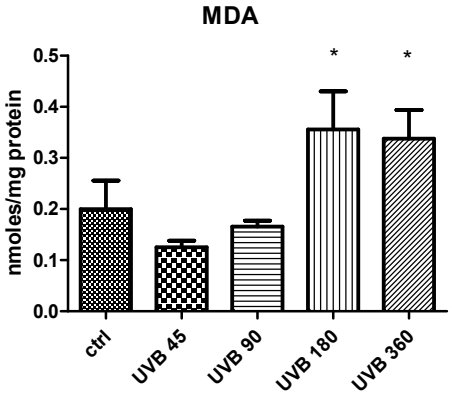

Figure 1. MDA level in eye homogenates exposed to single UVB irradiation

GSH levels increased significantly (6.0 fold) in the irradiated group with 45 mJ/cm^2 compared to the non UVB exposed group (5.16±0.84 vs. 0.86±0.66 nmoles/mg protein; $p<0.001$) (Figure 2). At 90 mJ/cm^2 of UVB the GSH generation also increased significantly compared to control group (3.53 fold; 3.04±0.70 vs. 0.86±0.66 moles/mg protein; $p<0.01$). The two high doses of UVB (180 respectively 360 mJ/cm^2) maintained low levels of GSH in eye tissues, insignificant compared with controls (0.83 ± 0.53 respectively 1.35 ± 0.94 nmoles/mg protein; $p>0.05$).

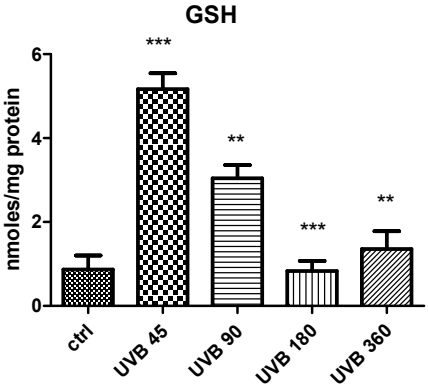

Figure 2. Glutathione reduced level in eye homogenates exposed to single UVB irradiation

GPx activity, under our experimental conditions, decreased insignificantly ($p>0.05$) at all doses administered (Figure 3).

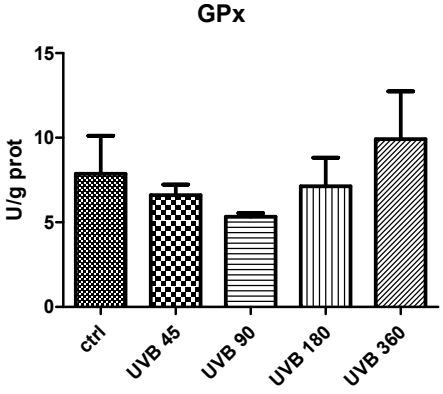

Figure 3. Glutathione peroxidase activity in eye homogenates exposed to single UVB irradiation

CAT activity increased significantly after exposure to 45 mJ/cm^2 (2.55 fold; 0.23±0.04 U/mg protein), 90 mJ/cm^2 (2.0 fold; 0.18±0.04 U/mg protein) and 180 mJ/cm^2 (2.88 fold; 0.26±0.10 U/mg protein) of UVB compared to no exposed animals (0.09±0.06 U/mg protein; $p<0.05$) (Figure 4). A dose of 360 mJ/cm^2 UVB decreased significantly CAT activity (0.08±0.05 U/mg protein; $p>0.05$), the values being similar to control group (Figure 4).

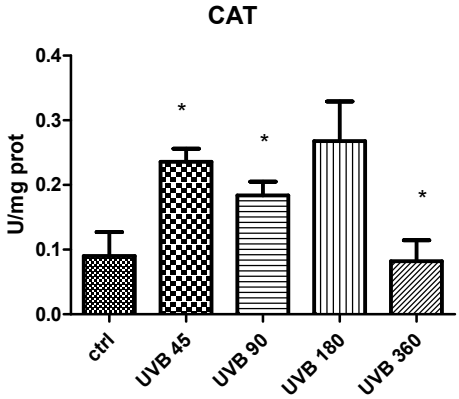

Figure 4. Catalase activity in eye homogenates exposed to single UVB irradiation

MnSOD activity at 24 hrs after the UVB irradiation increased significantly compared to control group only at dose of 360 mJ/cm^2 (2.23 fold; 1984±788.4 vs. 887.7±286.5 U/mg protein; $p<0.05$). Comparison remained significant between group treated with high dose and groups which received low doses of UVB. UVB exposures at doses of 45 mJ/cm^2 and 90 mJ/cm^2 (852.9±44.45 respectively 624.2±68.38 U/mg protein) maintain the reduced MnSOD activity in eye homogenates near to control group (Figure 5).

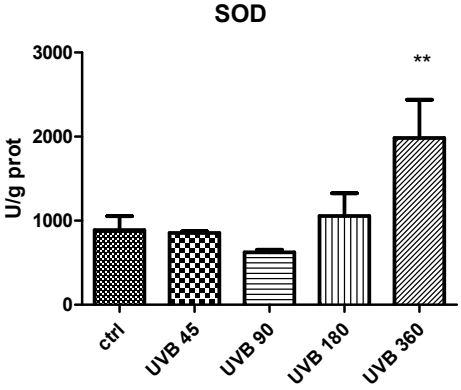

Figure 5. Superoxid dismutase activity in eye homogenates exposed to single UVB irradiation

The exposure of rats at different UV radiation doses was followed by the emergence of different intensity lesions in the cornea, related to the utilized radiation dose. Obvious lesions appear from 90 mJ/cm^2 dose and consist of corneal hypertrophy (1.5 fold), descuamation and zonal epithelial necrosis (superficial ulcerative keratitis), marked stromal edema, massive infiltration with polymorphonuclear cells in the vicinity of the ulcerated area, moderate and diffuse in the rest of the stroma (Figures 6 and 7).

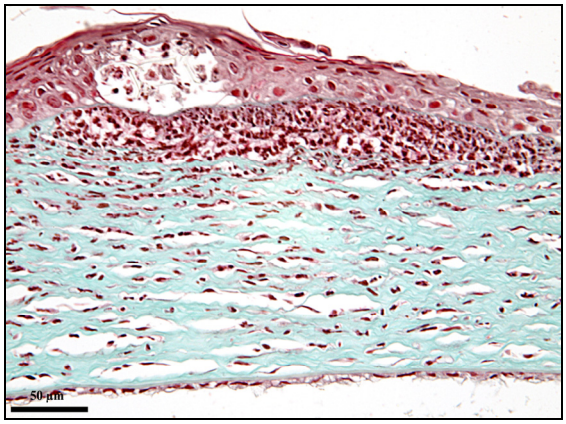

Figure 6. Group III – Cornea: superficial ulcerative keratitis, massive zonal infiltration with polymorphonuclear cells (Goldner's Trichrome)

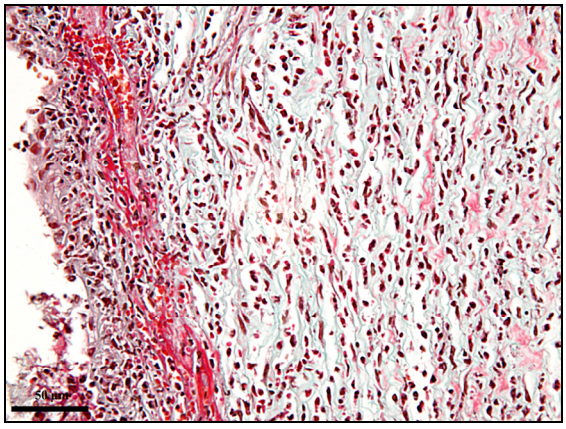

Figure 7. Group V – Cornea: generalized infiltration with polymorphonuclear cells, zonal hemorrhages (Goldner's Trichrome)

In order to estimate *in vivo* the threshold of UVB toxicity on the rat eye we investigated the parameters of oxidative stress in corelation with morphological changes under irradiation with different doses of UVB. It is known that direct

exposure to ambient ultraviolet light affect the cornea and the first changes are those caused by oxidative stress. Moreover, hydrogen peroxide is chronically present in the aqueous environment surrounding the anterior lens and may additional contribute to oxidative injury UVB-induced (Ringvold, 1980). To defend against damaging free radical-mediated reactions, cells possess antioxidant defense mechanisms. Thus, ocular tissues and fluids contain both nonenzymatic antioxidants (ascorbic acid, glutathione and α-tocopherol) and enzymatic antioxidants (catalase, superoxide dismutase, glutathione peroxidase and reductase). Superoxide dismutase protects the ocular tissue from the superoxide radicals and was detected in corneal epitelium and endothelium, lens epitelium, inner segments of the photoreceptor cell layer of the retina and in retinal pigment epitelium (Rao et al., 1985). The dismutation of superoxide by SOD leads to the formation of hydrogen peroxide, which is subsequently converted to water and oxygen through a reaction that is catalyzed by catalase (CAT) or glutathione peroxidase (GPx).

Our results demonstrated that acute UVB exposure at different doses disturbs the oxidant-antioxidant balance in eye tissues by lipid peroxides generation, activation of CAT and SOD and adaptative increasing of GSH levels, especially at high doses. Thus, MDA levels in eye homogenates increased at high doses of UVB in parallel with the decrease of GSH levels.

The UVB doses used in our experiment were chosen to reflect the human exposure of cornea to summer sunlight. It was known that during exposure of human cornea to sunlight 10 hours this received a dose of 0.105 J/cm^2 of UVB (Zigman, 1995). This dose evoked a significant increase in corneal hydration and light absorption.

GSH is a major non-protein thiol in living organisms, which plays a central role in coordinating the body's antioxidant defense process. GSH directly scavenge free radicals and acts as cofactor for GPx during the metabolism of hydrogen peroxide or lipid peroxides. Normally, in the cornea, GSH is found in the millimolar range (4-7 mM), the highest levels being found in the epithelium (Dalton et al., 2004). Generally, UVB exposure activates the transcriptional regulator NF-E2-related factor 2 (Nrf2) and consequently increases the activity of ROS-detoxifying enzymes and stimulates the production of antioxidants, including the glutathione (Schafer et al., 2010). In our study, the GSH enhancement after UVB exposure was important at low doses and was part of adaptive mechanisms triggered to remove reactive oxygen species. At high doses of UVB, GSH decreased dose-dependent due to counteract of reactive oxygen species generated in excess or leakage out of GSH from the lens due to injured of membrane (Hightower and McCready, 1992).

Several previous studies confirmed that UV exposure induced GSH depletion in lens of rabbits (Hightower and McCready, 1992) and rats irradiated (Risa et al., 2004; 2005; Tessem et al., 2006; Wang et al., 2010). Thus,

Hightower exposed cultured rabbit lenses to high doses of UV 315 nm and found a loss of 40 % GSH after irradiation. Wang noticed an average loss of 14% GSH concentration at 7 days after exposure to 8 kJ/m^2 UV 30 nm. Risa used for irradiation of rat lenses doses of 2-15 kJ/m^2 UV 300 nm.

Data in literature concerning these parameters following UV irradiation are rather contradictory probably due to differences in experimental design, UVB light sources used and variable interval between irradiation and evaluation. Thus, Ayala and co-workers did not find a depletion of GSH after UVB exposure of rats (Ayala and Soderberg, 2004). Risa et al. showed that *in vivo* exposure to UV light did not induce a detectable change of total lens GSSG levels (Risa et al., 2004; 2005). In our study, the GPx activity doesn't evolve in parallel to the level of GSH in the eye homogenates.

It is known that glutathione peroxidase (GPx) is a selenoprotein that catalyses the conversion of UV-induced hydrogen peroxide into water and molecular oxygen using GSH as unique hydrogen donor. It is possible to decrease the substrate due to activation of catalase, an enzyme that removes also hydrogen peroxide and protects superoxide dismutase from hydrogen peroxide-induced inactivation.

The results obtained support this hypothesis, the low and moderate doses of UVB increasing the activity of this enzyme. Other authors found that irradiation with 254 nm UV rays for two minutes lead to a decrease in catalase activity in the corneal epithelium, corneal endothelium and lens epithelium (Cejkova and Lojda, 1994). The same results were obtained when corneal epithelium of rabbits was exposed to four doses (1.1 – 1.6 J/cm^2) of UVB (Cejkova et al., 2001). The dose-dependent decline in catalase activity after UV exposure is explained by the direct photodestruction (Afaq and Mukhtar, 2001; Hellemans et al., 2003). In our experiment, MnSOD activity increased after high dose of UVB in order to dismute anion superoxide in hydrogen peroxide. Moreover, it has shown that UVB irradiation induced a release of soluble factors (IL-1α, Il-1β, TNF-α) that amplified MnSOD activity by a paracrine mechanism (Hachtroudi et al., 2002).

Experimental studies showed that ROS generated by UVB cause morphologic alterations in the cornea. A single UVB exposure of cornea blocked the proliferation of epithelial cells, determined the loss by autolysis of superficial corneal epithelial cells. The presence of the inflammatory polymorphonuclear cells infiltrate, directly proportional with the severity of the lesions, represents a morphologic evidence of the oxidative stress involvement in the lesions of the UVB exposed cornea (polymorphonuclear cells are a source of ROS). The presence of leukocytes in the irradiated corneal stroma was reported along with a gradual increase of the xanthine oxidase (Cejkova et Lojda, 1996) and D-amino acid oxidase activities in the corneal epithelium and endothelium (Čejkova et al., 2001). The experimental

generation of superoxide anion in the anterior chamber of the eye, in rabbit, determined the development of an early leukocytic infiltrate in the first 4 hrs from the irradiation (Cejkova et al., 2001).

The stromal inflammatory infiltrate can be induced by the release of interleukin 1β (IL-1β) from the damaged epithelial cells. IL-1β is a multipotent cytokine, involved in the acute neutrophils and macrophages inflammatory response (Wang et al., 2007). This experiment demonstrated the presence of lipid peroxidation in eye tissues in UVB exposures possibly due to release of ROS from polymorphonuclear cells which infiltrate the tissue injured.

Cornea of the rats exposed to 180 mJ/cm^2 presented more advanced lesions: over 2 fold thickening of the cornea, superficial ulcerative keratitis on a larger area in comparison to the anterior dose, very marked stromal edema and massive infiltration of polymorphonuclear cells. The lesions are more advanced at the 360 mJ/cm^2 dose. The cornea appears over 3 fold hypertrophyed, the ulceration from the central area reaches the Bowman membrane and extends into the depth. On the very marked edema background, the stromal liquefaction (keratomalacia) on large areas takes place. The polymorphonuclear infiltrate is very marked and generalized. In addition, diffuse hemorrhages are present and blood vessels appear in the corneal stroma.

CONCLUSIONS

Our results demonstrated that a single UVB exposure at different doses disturbs the oxidant-antioxidant balance in eye tissues by lipid peroxides generation, activation of CAT and SOD and adaptative increasing of GSH levels, particularly at high doses. Reactive oxygen species generated by UVB exposure may be responsible for morphologic alterations of the cornea.

EXPERIMENTAL SECTION

Materials

Trichloroacetic acid, o-phtalaldehyde, t-butyl hydroperoxide, glutathione reductase, glutathione reduced, Bradford reagent, cytochrome c, xanthine, xanthineoxidase, β – nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH) were purchased from Sigma-Aldrich Chemicals GmbH (Germany). 2-thiobarbituric acid and EDTA-Na_2 were obtained from Merck KGaA Darmstadt (Germany) and absolute ethanol, hydrogen peroxide and n-butanol from Chimopar (Bucuresti).

Experimental design

Forty female rats, 8 weeks old, weighing 120 ± 5 g, kept on normocaloric standard diet (VRF 1) and water *ad libitum,* were used. The animals were housed (5 animals/cage) at room temperature (24 ± 2^0C), with a 12/12 hrs light dark cycle. The rats were acclimatized to the laboratory for one week before the experiments. Five groups of 8 animals each, randomly divided, were treated as follows: group 1: control, received vehicle, no UVB irradiation; group 2: a single UVB exposure to a dose of 45 mJ/cm^2; group 3: a single UVB exposure to 90 mJ/cm^2; group 4: a single UVB exposure to 180 mJ/cm^2; group 5: a single UVB exposure to 360 mJ/cm^2. Before eye irradiation, the animals were anaesthetized with an i. p. injection of ketamine xylazine cocktail (90 mgkg^{-1} b.w. ketamine, 10 mgkg^{-1} b.w. xylazine). UVB irradiation was performed with a Waldmann UV 181 broadband UVB source, with 1.35 mW/cm^2 intensity, at 10 cm distance from the source. The UVB emission was monitored before each exposure with a Variocontrol radiometer (Waldmann GmbH, Germany). Irradiation doses were established using the formula: dose (mJ/cm^2) = exposure time (sec.) x intensity (mW/cm^2). All the experiments were performed according to the approved animal-care protocols of the Ethical Committee on Animal Welfare of the "Iuliu Hatieganu" University of Medicine, in accordance with the Romanian Ministry of Health and complied with the Guiding Principles in the Use of Animals in Toxicology. At 24 hrs after UVB irradiation the animals were anaesthetized and sacrificed by cervical dislocation. The rat eyes from 5 animals were extracted and used for biochemical determinations and from 3 animals were harvested and fixed in 10% buffered formalin for 48 hours (each eye ball was punctured laterally for a better fixation and crystalline extraction). The eye samples were then embedded in paraffin, cut at 5-µm thickness, and mounted on glass slides. Goldner's tricrome stain was performed for histological examinations. The eye sample harvesting and histological processing was realized in the Department of Histology, Faculty of Veterinary Medicine, Cluj-Napoca, Romania.

Measurement of oxidative stress parameters

Briefly, eye tissues were homogenized with a Polytron homogenizer (Brinkmann Kinematica, Switzerland) for 3 min on ice in phosphate buffered saline (pH7.4), added in a ratio of 1:4 (w/v). The suspension was centrifuged for 5 min at 3000 x g and 4 °C to prepare the cytosolic fractions. The proteins levels in homogenates were measured with Bradford method (Noble and Bailey, 2000). To evaluate the oxidative/antioxidative status we assessed the malondialdehyde, as marker of oxidative attack of reactive oxygen species on lipids, and the antioxidant enzymes activities (superoxide dismutase, catalase and glutathione peroxidase). In addition, we evaluated the level of glutathione reduced as antioxidant no enzymatic parameter.

Malondialdehyde (MDA) was determined using the fluorimetric method with 2-thiobarbituric acid described by Conti (Conti et al., 1991). The eye homogenate samples were heated in a boiling water bath for 1 h with a solution of 10 mM 2-thiobarbituric acid in 75 mM K_2HPO_4, pH 3 solution. After cooling the reaction product was extracted in n-butanol. The MDA was spectrofluorimetrically determined and the values are expressed as nmoles/mg protein.

Superoxide dismutase (SOD) activity was determined using cytochrome c reduction test with some adjustments (Beauchamp and Fridovich, 1971). Skin homogenates were introduced in a cytochrome c solution (2 μM in phosphate buffer 50 mM, pH 7.8) containing xanthine (5 μM). The reaction was started by adding xanthine oxidase (0.2 U/ml in 0.1 mM EDTA). The increasing absorbance at 550 nm, indicating cytochrome c reduction was recorded for 5 min. One unit of SOD inhibits the rate of increase in absorbance at 550 nm by 50% of those produced for a control sample without SOD under the conditions of the assay. Results were expressed in U/mg protein.

Catalase activity (CAT) was assayed according to Pippenger method (Pippenger et al., 1998) in a reaction mixture containing 10mM hydrogen peroxide in 50mM potassium phosphate buffer, pH7.4. The reduction in absorbance at 240 nm was recorded for 3 minutes. The enzyme quantity which produced an 0.43 reduction in absorbance per minute at 25^0 was defined as one unit of catalase activity and expressed as units/mg protein

Glutathione peroxidase activity (GPx) was determined with Flohe and Gunzler method, slightly modified (Flohe and Gunzler, 1984). The reaction mixture consisted in 1mM GSH, 0.24U/ml glutathione reductase and 0.15 mM NADPH (final concentrations) in 50 mM phosphate buffer (pH 7.0). The reaction mixture was incubated at 37º for 5 minutes with appropriate amounts of tissue homogenates. The assay was initiated with a (12mM) t-butyl hydroperoxide solution. The decrease in absorbance at 340 nm was recorded for 3 min. GPx activity was expressed as μmoles of NADP produced/min/mg protein and calculated using a molar absorbtivity for NADPH of $6.2x10^{-6}$, at 340 nm.

Reduced glutathione (GSH) was measured fluorimetrically using o-phtalaldehyde (Hu, 1994). Samples were treated with trichloroacetic acid (10%) and centrifuged. A solution of o-phtalaldehyde (1mg/ml in methanol) was added to supernatants diluted with sodium phosphate buffer 0.1M/EDTA 5mM, pH8.0. After 15 minutes, the fluorescence was recorded (350nm excitation and 420nm emission). GSH concentration was determined using a standard curve and expressed as nmoles/mg protein.

Statistical analysis

The data are expressed as the means± SD in five animals. Each measurement was done in triplicate. Comparisons were made by one-way ANOVA, with Tukey multiple comparisons test, using a GraphPad Prism software program, version 5.0 (GraphPad, San Diego, Ca, USA). $p < 0.05$ was considered as significant.

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