OXIDATIVE STRESS AND ANTIOXIDATIVE DEFENCE IN THE SKIN OF RATS WITH THERMAL INJURY

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Summary: Changes in the activity and level of some antioxidative defence system components were determined in the rat skin during hypo- (ebb) and hypermetabolic (flow) phase of thermal trauma. At the same time, the effects of enzymatic (superoxide dismutase) and non-enzymatic (vitamin E and glutathione) antioxidants, as well as of L-arginine applied on the scalded skin area in different combinations in the form of a lyposomal ointment on endogenous antioxidative defence components were studied both in the injured and uninjured skin. In scalded skin during hypometabolic phase, a decrease in activity of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, as well as in the level of vitamin E was observed in comparison with the control. This decrease was accompanied by a complete loss of glutathione and the activity of glutathione-S-transferase and thioredoxin reductase. The same trend of changes was recorded in hypermetabolic phase. In the uninjured skin of scalded animals, the activities of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase were at the control level both in hypo- and hypermetabolic phase. Also, no changes in vitamin E content were found, while the activities of thioredoxin reductase and glutathione-S-transferase were increased. Glutathione level in this group of animals was decreased, the decrease being more prominent in hyper- then in hypometabolic phase. The ointments applied to the injured parts of the skin expressed protective effects observed as an increase in vitamin E level and an attenuation of glutathione reductase activity inhibition.

Kew words: oxidative stress, skin, thermal injury, antioxidant treatment, L-arginine

Introduction

Contemporary definition of early pathophysiological events induced by trauma i.e. traumatic shock includes both local reaction and a reaction of uninjured part of an organism to trauma, termed the general reaction (1). Posttraumatic fluid loss and a decrease of blood volume and afferent nerve impulses are in the basis of pathogenesis of a general reaction of an organism to trauma (2, 3). Acute local reaction is basically a local vasoconstriction followed by an inflammation accompanied by an increased vascular permeability as the most prominent feature of this reaction (4). However, an increased vascular perme-

ability in different tissues, different parts of microcirculation in the same tissue and different time periods in fluid loss would be difficult to explain by the effects of a single mediator (5). The mediators of an increased permeability are mainly generated by the platelets and leukocytes, endothelial cells of capillaries and venulae in injured tissue representing their primary target (6).

Although the mechanism(s) of such tissue injury remains unclear, increasing evidences implicated reactive oxygen species (ROS) as a causative agent of local and systemic damage. ROS are first generated from the burned skin (7). In addition, ischemia causes leukocyte adherence, activation and further injury. This injury appears to be mediated by both ROS and activated neutrophils (8). It has been suggested that infiltrated neutrophils lead to formation of toxic oxygen products thereby contribute to organ injury distant from the original burn wound. Several studies have demonstrated that burn injury is associated with lipid peroxidation, wich is an autocatalytic mechanism leading to oxidative destruction of cellular membranes, and their destruction can lead to the production of toxic, reactive metabolites and cell death (9, 10).

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Antioxidative defence (AD) of an organism plays a crucial role in reducing increased level of free radicals generated not only by local scalding, but also by inflammatory reaction, to physiological limits (11 13). AD involves some enzymatic components such as: superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GSH-Px, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2) and glutathione-S-transferase (GST, EC 2.5.1.18), but also some low molecular weight non-enzymatic components, e.g. reduced form of glutathione (GSH) and vitamins E and C. Although the picture on the composition and the regulation of AD is still incomplete, it is well documented that it maintains free radical equilibrium within the homeostasis limits and prevents propagation of the free radical chain reactions which could lead to tissue destruction. AD was shown to be species-, organ- and tissue-specific (14 18). Specifities of antioxidative defence in the skin can be seen not only at the level of its organization, but also at the level of its regulation. This is primarily related to the role of thioredoxin reductase (TR, EC 1.6.4.5), in defence mechanism especially in extracellular protection from ROS (superoxide anion radicals and hydrogen peroxide) (19). Mechanisms and types of protection under many pathological conditions in the skin are still insufficiently studied, primarily due to the lack of knowledge concerning mechanisms of antioxidative defence regulation in the skin.

The aim of the present study was to examine the changes in AD system in injured and non-injured skin of scalded rats and to investigate the effects of exogenously applied compounds known to influence free radicals equilibrium and lead to the restoration of optimal AD functioning.

Material and Methods

Animal model

Male Wistar rats, (220 ± 20 g, 3-month-old), were used. They were housed in individual plastic cages with free access to food and water. The food was removed 17 h prior to scalding and all animals were deprived of food and water during the first 24 h after traumatization. The skin of the back and stomach was clipped one day before the scalding. A special mould was constructed allowing the scalding of about 20% of the total body surface as a single thermal injury performed under light ether anaesthesia. Anaesthetized animal was put into the mould and the clipped skin immersed in hot water (83 °C) for 30 s (20). Upon removal from the water bath, each rat was quickly dried by rolling in a towel and returned to the cage. This type of injury was nonlethal during 30 days observation. Total body surface was calculated by the formula of Lee (21). Such a single scalding produces a moderate and transient fall of body temperature (22). This early hypometabolic (ebb) phase is the most

expressed 6 h after injury, while in the hypermetabolic (flow) phase, 48 h after injury, there is an increase in heat production.

Biochemical procedures

The skin was dissected out within 3 min after the sacrifice. Minced tissue was thoroughly rinsed to wash out traces of blood and the hypodermis was removed using a scalpel. Dermis with epidermis was homogenized (a Janke and Kunkel Ka-Werke Ultra-Turrax homogenizer, 0 4 °C) in a solution containing 0.25 mol/L sucrose, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA) and 0.05 mmol/L Tris-HCl buffer, pH 7.4 and the homogenates were sonicated as suggested by Takada et al. (23). Total SOD was determined by a modified method of Misra and Fridovich (24) and the activity was expressed in U mg $^{-1}$ protein. One unit was defined as the amount of protein causing 50 % inhibition of epinephrine autooxidation into adrenochrome in 3.2 mL reaction mixture containing 3×10^{-4} mol/L epinephrine, 10^{-4} mol/L EDTA and 0.05 mol/L Na_2CO_3, pH 10.2, at 26 °C. CAT was assayed as suggested by the supplier (SIGMA Chemicals, St. Louis, MO, U.S.A.) and activity was expressed as μmol H_2O_2 min $^{-1}$ mg $^{-1}$ protein. GSH-Px was determined using t-butylhydroperoxide as a substrate (25) and the activity was expressed as nmol of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidized min $^{-1}$ mg $^{-1}$ protein. Activity of GR was assayed by the method of Glatzle et al. (26) and expressed as nmol NADPH min $^{-1}$ mg $^{-1}$ protein. GST was measured after the procedure of Habig et al. (27) and the activity was expressed as μmol GSH min $^{-1}$ mg $^{-1}$ protein. TR activity was examined by the method of Luthman and Holmgren (28) and activity was expressed as nmol NADPH min $^{-1}$ mg $^{-1}$ protein. Total glutathione (GSH + GSSG) was measured by an enzyme recycling assay (29) and vitamin E according to Desai (30), expressed as nmol g $^{-1}$ tissue and μg g $^{-1}$ tissue, respectively. Protein content was estimated by the method of Lowry et al. (31).

Experimental design

Animals were divided into five groups: sham-scalded rats served as the control; I scalded animals; II, III and IV, consisted of scalded rats with topically applied ointments A, B and C, respectively, onto the injured skin area.

Ointment A represents a mixture of liposomes (multilamellar vesicles of dipalmitoyl phosphatidylcholine stearylamine cholesterol with an average diameter of 350 400 nm) and glycerol (1:1).

Ointment B: 50 mL of ointment A supplemented with 500 mg of vitamin E, 500 mg of GSH and 30 mg of SOD which were encapsulated by sonication.

Ointment C: 50 mL of ointment B enriched with 500 mg of L-arginine, encapsulated by sonication.

The ointments (1.0 mL) were carefully smeared on the injured skin area. All animals sacrificed 6 h after the scalding (ebb phase) were treated with ointment only once immediately after the injury. The rats sacrificed 48 h following the scalding (flow phase) were smeared with the ointments immediately after the injury and three more times, the treatment being spaced 12 h apart.

Statistical analysis

Differences between two means were analyzed by Student's t test (32) and p values under 0.05 were considered as statistically significant.

Results

The data obtained in experiments on injured skin are listed in *Table I*. Following the scalding in the ebb phase, the activities of all measured enzymes and the content of vitamin E were significantly lower in comparison with the control (p<0.005). Activities of GST and TR, as well as the level of glutathione were under the values detectable by the methods applied. The same trend of changes was detected in the flow phase. However, activities of both SOD and CAT were decreased to a lesser extent than that of GSH-Px. Application of the ointment A, containing only liposomes and glycerol prevented this activity decline of SOD and GSH-Px and decrease of vitamin E content in the ebb phase, but did not affect these values in the flow phase. This ointment did not influence the other examined parameters. Ointment B enriched with antioxidants led to a statistically significant increase of vitamin E level in the injured skin in both post-trauma phases (p<0.005). Under the same conditions, GSH-Px activity remained at the control level in the ebb phase. Ointment C, supplemented with antioxidants and L-arginine, also led to statistically significant increase of vitamin E content in both phases (p<0.005) and prevented SOD inhibition in the flow phase.

In the uninjured skin of scalded animals, activities of SOD, CAT, GSH-Px and GR, as well as vitamin E content were not significantly changed (data not shown). Changes in GST activity in the uninjured skin of scalded animals are presented in *Figure 1*. Although the activity of this enzyme was increased in all groups of animals in both phases as compared to the controls, this increase was statistically significant only in scalded animals treated with ointment B in the flow phase (p<0.02). Activity of TR in the non-scalded skin and the effect of the ointments applied are shown in
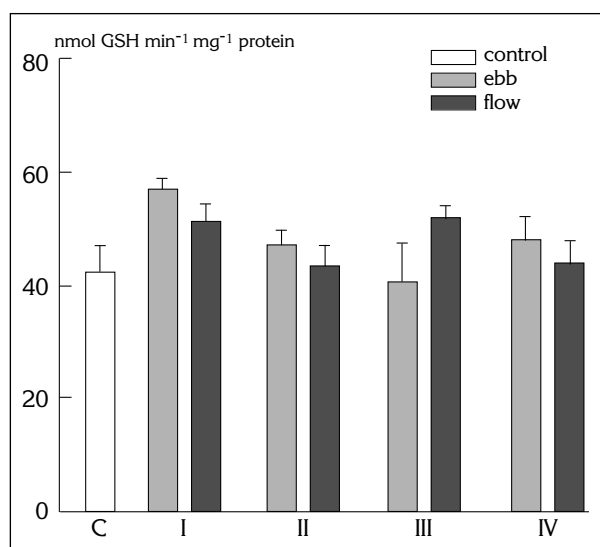

Figure 1.   Changes in activity of glutathione-S-transferase in uninjured skin of scalded animals upon local application of antioxidant-containing ointments.

C   control; I   scalded group; II, III and IV   groups of scalded animals topically treated with ointments A, B and C, respectively. Bars represent SEM. Statistical significance of the differences is given under RESULTS.

Table I   The effect of scalding and local application of antioxidant-containing ointments on antioxidative components in injured skin areas of the ebb and flow phase of the trauma.

		SOD	CAT	GSH-Px	GR	VIT E
C		11.4 ± 1.3	22.4 ± 2.3	29.7 ± 2.9	15.7 ± 0.7	6.5 ± 0.8
I	ebb	6.7 ± 0.7	1.6 ± 0.3	22.9 ± 3.2	5.0 ± 0.4	3.8 ± 0.5
	flow	8.2 ± 0.9	3.1 ± 0.4	8.9 ± 2.0	5.1 ± 1.1	3.0 ± 0.7
II	ebb	11.9 ± 0.7	2.7 ± 0.5	31.1 ± 4.2	6.1 ± 0.3	5.3 ± 1.0
	flow	7.4 ± 1.1	3.7 ± 0.6	10.9 ± 2.7	3.8 ± 0.8	4.2 ± 0.8
III	ebb	7.9 ± 0.8	2.2 ± 0.2	35.7 ± 3.2	6.9 ± 0.8	9.4 ± 1.3
	flow	8.3 ± 0.6	2.8 ± 0.5	7.2 ± 0.9	5.6 ± 0.4	15.6 ± 1.7
IV	ebb	9.4 ± 1.2	2.1 ± 0.2	30.8 ± 2.2	7.9 ± 0.4	10.9 ± 0.7
	flow	10.4 ± 1.1	2.6 ± 0.5	6.9 ± 2.4	6.6 ± 0.9	19.5 ± 1.8

C   control; I   scalded group; II, III and IV   groups of scalded animals topically treated with ointments A, B and C, respectively immediately after the injury (ebb phase) and three more times (flow phase). GST and TR activities, as well as GSH content in the controls were: 41.6 ± 5.7, 49.3 ± 7.1 and 63.5 ± 11.3, respectively. In all other groups of animals they were under detectable limits.
Bars represent SEM. Statistical significance of the differences is given under RESULTS.
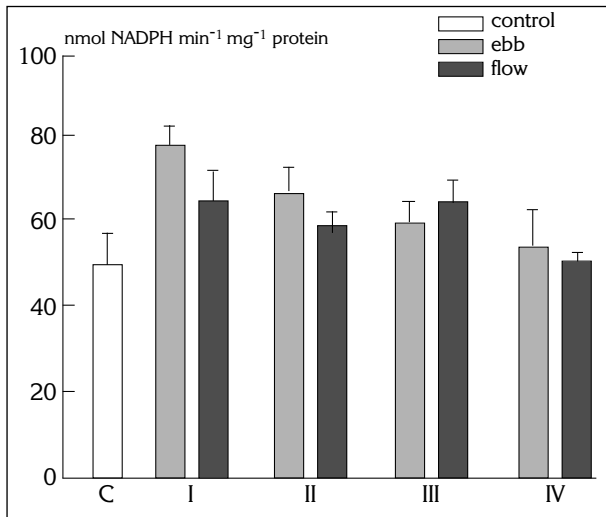
Figure 2. Influence of antioxidant-containing ointments
on thioredoxin reductase activity in uninjured skin
of the scalded rats.

C — control; I — scalded group; II, III and IV — groups
of scalded animals topically treated with ointments A, B and C,
respectively. Bars represent SEM. Statistical
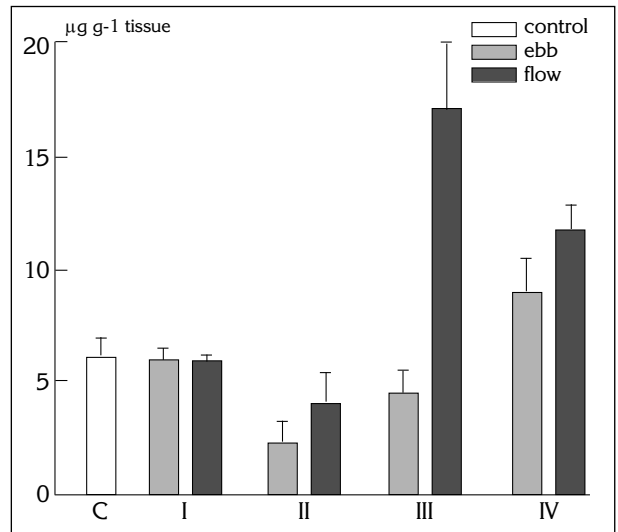significance of the differences is given under RESULTS.

Figure 4. Indirect effects of local application of ointments
A, B, and C to the scalded skin areas on the content
of vitamin E in uninjured skin of the same animal.

C — control; I — scalded group; II, III and IV — groups
of scalded animals topically treated with ointments A, B and C,
respectively. Bars represent SEM. Statistical
significance of the differences is given under RESULTS.

Figure 3. Changes in glutathione content
in uninjured skin of scalded rats.

C — control; I — scalded group; II, III and IV — groups
of scalded animals topically treated with ointments A, B and C,
respectively. Bars represent SEM. Statistical
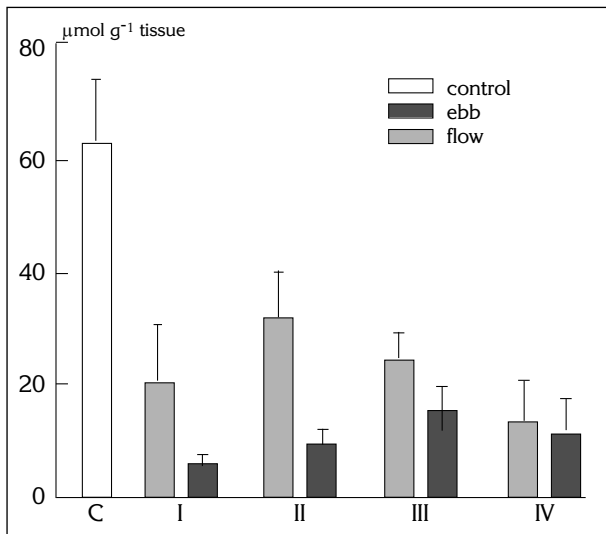significance of the differences is given under RESULTS.

Figure 2. It can be seen that the scalding of the animals leads to a significant increase of TR activity both in the ebb ($p < 0.01$) and the flow phase ($p < 0.05$). The application of ointment C maintained activity of this enzyme at the control level, while an increase of TR activity was recorded only in the flow phase when ointment B was used ($p < 0.05$). Changes in glutathione

content in uninjured skin are depicted in *Figure 3*. It can be seen that glutathione content was significantly decreased in all groups of animals in comparison with the controls. The ointment A, consisting of liposomes and glycerol led to a decrease in vitamin E level in uninjured skin in the ebb phase, as compared to the controls ($p < 0.01$). At the same time, the ointments supplemented with antioxidants and L-arginine, not only restituted the amount of vitamin E in the ebb phase, but led to a statistically significant increase of this vitamin level in uninjured skin in flow phase as seen from *Figure 4* ($p < 0.005$). The ointment C containing antioxidants and L-arginine significantly reduced CAT activity in uninjured skin of scalded animals in the flow phase (CAT activity in scalded animals without application of the ointment was 20.6 ± 1.6 and in the scalded animals treated with ointment C 12.5 ± 2.6, $p < 0.005$).

Discussion

A concept of sequential development of general physiological reaction of an organism to trauma is based on the energy metabolism changes (1). According to this concept, a hypometabolic phase appears immediately after trauma and is induced by changes in the central mechanisms of thermoregulation (33). The first line of defence is based on selective and regional vasoconstriction and redistribution of the blood volume, providing an adequate blood pressure for perfusion to vital organs (brain, heart, lungs), despite a decreased blood volume (34). Lipid peroxi-

des formed at the site of scalded skin could represent toxic products related to free radicals in the ebb phase of trauma (35). Our results observed in the ebb phase support this concept, since only a slight increase of GSH-Px and TR activities and no changes in SOD, CAT and GR activities were recorded in the uninjured skin of scalded animals. Also, a decrease in GSH level occurred (both in ebb and flow phase) suggested the occurrence of unbalanced oxidation-reduction status in the skin. The inhibition of antioxidative enzymes in the skin injured by scalding is primarily due to thermal inactivation.

In the case of non-lethal injury, such as in our experiments, the hypometabolic phase is followed by the hypermetabolic phase of trauma. In the latter pha-se, free radicals production is increased in generally, resulting from an increased metabolic rate, but it is also increased at the very site of the injury due to its inflammation (36, 18). Optimal functioning of antiox-idative defence system under such circumstances may improve the recovery not only of the injured skin, but also of a whole organism.

Overproduction of free radical species led to fur-ther inhibition of GSH-Px in scalded skin in the flow phase which was impossible to prevent by any of the ointments used, while further decrease in vitamin E level was reversed upon the application of the vitamin E-containing ointment. Although the ointment was applied only at the injured skin area, its effects on the uninjured skin of the same animal were obvious. Restitution of SOD activity in flow phase under the influence of L-arginine-containing ointment applied at

injured skin (II versus III 48 h injured skin) seems to support predicted role of L-arginine as a metabolic NO generator via nitric oxide synthesizing enzymes in endothelial cells (37). The observed increase of TR and GST activities represents an event with beneficial effects on elimination of hydrogen peroxide and lipid hydroperoxides from uninjured skin in both ebb and flow phase of thermal injury.

The exhaustion of the glutathione pool in ther-mal injury has been observed to occur in other tissues, as well (38). Glutathione is the predominant low molecular weight thiol in mammalian cells and plays a major role in cellular defenses against oxidative stress. Glutathione exist in the reduced (GSH) and oxidized (GSSG) form. The ratio of GSH to GSSG under phys-iological conditions is 100 : 1. Sabeh et al. (38) report-ed significant decreases in GSH/GSSG ratio in liver and lung after thermal injury to the skin, and reflects disturbances of free radical equilibria. Application of L-arginine-supplemented ointment seems to act po-tentiating exhaustion of glutathione pool in the skin.

The results obtained throughout the present stu-dy showed that ointments supplemented with anti-oxidants and vitamin E expressed beneficial local and systemic effects. Addition of L-arginine to antioxidant-containing ointment stimulated restitution of GR activ-ity in the injured skin simultaneously leading to a more expressed reduction of glutathione level in the unin-jured skin of the scalded animals.

OKSIDATIVNI STRES I ANTIOKSIDATIVNA ODBRANA U KOŽI PACOVA SA TERMALNOM POVREDOM

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Kratak sadržaj: Promene u aktivnosti i količini komponenti antioksidativne odbrane u koži pacova praćene su u hipometaboličkoj (ebb) i hipermetaboličkoj (flow) fazi termalne traume. Efekti primene egzogenih enzimskih (superoksid dismutaza) i neenzimskih (vitamin E i glutation) antioksidanata i L-arginina, nanesenih na oštećenu kožu u obliku lipozomalne kreme, na endogene komponente antioksidativne odbrane ispitivani su i u oštećenoj i u neoštećenoj koži. U oštećenoj koži u hipometaboličkoj fazi dolazi do smanjenja aktivnosti superoksid dismutaze, katalaze, glutation peroksidaze, glutation reduktaze i količine vitamina E, i potpunog gubitka glutationa i aktivnos-ti glutation-S-transferaze i tioredoksin reduktaze. Promene su iste i u hipermetaboličkoj fazi u poredjenju sa kon-trolama. U koži, koja nije direktno oštećena, aktivnost superoksid dismutaze, katalaze, glutation peroksidaze i glu-tation reduktaze i količina vitamina E se ne menja u odnosu na kontrolu ni u hipo ni u hipermetaboličkoj fazi. Aktivnost tioredoksin reduktaze i glutation-S-transferaze povećava se u obe faze. Količina glutationa smanjuje se više u hipermetaboličkoj nego u hipometaboličkoj fazi. Efekti supstanci, nanesenih na oštećenu kožu, izraziti su u smislu povećanja količine vitamina E. Takođe njihov protektivni efekat prisutan je i kada je u pitanju nivo GR aktivnosti u oštećenoj koži.

Ključne reči: oksidativni stres, koža, termalna povreda, tretman antioksidantima, L-arginin

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