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 liposomal ointment on endogenous antioxidative defence components were studied both in the injured and uninjured skin. In scalded skin during hypermetabolic 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.

Key words: oxidative stress, skin, thermal injury, antioxidative 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 permeability 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 venules 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, which 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).
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). Specificities 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 deprived of food and water during the first 24 h after scalding. A special mould was constructed allowing the scalding of about 20% of the total body surface as a single thermal injury per-registration. The skin was dissected out within 3 min after the sacrifice. Mincing 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 Na2CO3, 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 H2O2 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 nmol 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 dipalmityl 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.](image)

<table>
<thead>
<tr>
<th></th>
<th>SOD (nmol GSH min⁻¹ mg⁻¹ protein)</th>
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<tbody>
<tr>
<td></td>
<td>control</td>
</tr>
<tr>
<td>C</td>
<td>11.4 ± 1.3</td>
</tr>
<tr>
<td>I ebb</td>
<td>6.7 ± 0.7</td>
</tr>
<tr>
<td>flow</td>
<td>8.7 ± 0.9</td>
</tr>
<tr>
<td>II ebb</td>
<td>11.9 ± 0.7</td>
</tr>
<tr>
<td>flow</td>
<td>7.4 ± 1.1</td>
</tr>
<tr>
<td>III ebb</td>
<td>7.9 ± 0.8</td>
</tr>
<tr>
<td>flow</td>
<td>8.3 ± 0.6</td>
</tr>
<tr>
<td>IV ebb</td>
<td>9.4 ± 1.2</td>
</tr>
<tr>
<td>flow</td>
<td>10.4 ± 1.1</td>
</tr>
</tbody>
</table>

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.
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 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 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.

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 phase, 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 antioxidative 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 further 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 (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 thermal 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 physiological conditions is 100 : 1. Sabeh et al. (38) reported 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 potentiating exhaustion of glutathione pool in the skin.

The results obtained throughout the present study showed that ointments supplemented with antioxidants and vitamin E expressed beneficial local and systemic effects. Addition of L-arginine to antioxidant-containing ointment stimulated restitution of GR activity in the injured skin simultaneously leading to a more expressed reduction of glutathione level in the uninjured skin of the scalded animals.

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OXSIDATIVNI STRES I ANTI OXSIDATIVNA ODBRANA U KOŽI PACOWA SA TERMALNOM POVRĐOM

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Kratki sažazij: Promene u aktivnosti i količini komponenti antioksidativne odbranе u koži pacova prateone su u hipometaboličkoj (ebb) i hipermetaboličkoj (flow) fazi termalne traume. Efekti primene egzogenih enzimskih (superoksid dismutaza) i neenzimskih (vitamin E i glutat) antioksidanata i L-arginina, naneseneh na oštećenu kožu u obliku lipozomalne kreme, na endogene komponente antioksidativne odbране ispitivani su i u oštećenoj i u neoštećenoj koži. U oštećenoj koži u hipometaboličkoj fazi dolazi do smanjenja aktivnosti superoksid dismutaze, katalaze, glutat peroksidaze i kolijune vitamina E, i potpunog gubitka glutatina i aktivnosti glutat-S-transferaze i tioreoksidim reduktaze. Promene su iste i u hipermetaboličkoj fazi u poredjenju sa kontrolom. U koži, koja nije direktno oštećena, aktivnost superoksid dismutaze, katalaze, glutat peroksidaze i glutat reduktaze i kolijuna vitamina E se ne menja u odnosu na kontrolu ni u hip ni u hipermetaboličkoj fazi. Aktivnost tioreoksidim reduktaze i glutat-S-transferaze povećava se u obe faze. Količina glutationa smanjuje se više u hipermetaboličkoj nego u hipometaboličkoj fazi. Efekti supstanci, naneseneh na oštećenu kožu, izraziti su u smislu povećanja količine vitamina E. Takože njihov protetivni efekat prisutan je i kada je u pitanju nivo GR aktivnosti u oštećеноj koži.

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


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