Effect of intradermal human recombinant copper-zinc superoxide dismutase on random pattern flaps in rats

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ABSTRACT: *Background.* Studies have focused on enhancing flap viability using superoxide dismutase (SOD), but only a few used SOD from human origin, and most gave the compound systemically. We evaluated the ability of SOD to improve random skin flap survival using human recombinant copper-zinc superoxide dismutase (Hr-CuZnSOD) in variable doses, injected intradermally into the flap.

Methods. Seventy male Sprague Dawley rats were randomly assigned into 4 groups. Cephalic random pattern flaps were elevated on their backs and intradermal injections of different dosages of Hr-CuZnSOD were given 15 minutes before surgery. Flap survival was evaluated by fluorescein fluorescence. Analysis of variance (ANOVA) and *t* test statistical analyses were performed.

Results. Flap survival in all treated groups was significantly better than in the controls.

Conclusions. The beneficial effect of HR-CuZnSOD on flap survival is attained when it is given intradermally into the flap tissue. Theoretically, Hr-CuZnSOD delivered with local anesthetics used in flap elevation may be a valuable clinical tool. © 2012 Wiley Periodicals, Inc. *Head Neck* **35:** 1265–1268, 2013

KEY WORDS: SOD, flaps, survival, rats, oxygen free radicals

INTRODUCTION

Skin flaps are a versatile tool for dealing with complex skin defects. Flaps are limited by the risk of ischemia and necrosis, especially in the part distal to the vascular inflow. Improving flap survival continues to be a consummate goal in reconstructive surgery. Numerous studies have investigated methods for improving skin flap survival. Many have focused on enhancing flap viability through pharmacologic manipulation. During the first few hours, which are the most critical for flap survival, there is vasoconstriction, which is caused by various factors. This vasoconstriction changes to vasodilatation, which causes reperfusion of the relatively ischemic tissue and carries with it a new danger, reperfusion injury. Reperfusion injury research has focused on the administration of free radical scavengers, such as deferoxamine, mannitol, superoxide dismutase (SOD), and free radical formation blockers such as allopurinol.[1–6] Other modalities have included the use of hyperbaric oxygen[7] and methods to reduce neutrophils through leukocyte filters, antibodies, radiation, and chemotherapeutic agents such as cyclophosphamide.[8] Many studies have been reported on the use of SOD, but only a few have used SOD from human origin, and most have given the compound systemically. In this work, we evaluated the ability of SOD to improve random skin flap survival in a rat model using human recombinant copper-zinc superoxide dismutase (Hr-CuZnSOD; provided free of charge by Biotechnology General, Rehovot, Israel) in variable doses injected intradermally in the area of the flap.

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MATERIALS AND METHODS

This work was approved by the Assaf Harofeh Medical Center Institutional Review Board for animal experiments and institutional guidelines regarding animal experimentation were followed.

Seventy male Sprague Dawley rats (average weight 250 grams) constituted the study group. The rats were randomly assigned into 4 groups: 3 different dosages of Hr-CuZnSOD (50 mg/Kg, 15 mg/Kg, and 5 mg/Kg; provided free of charge from Biotechnology General, Rehovot, Israel) and a control group (Table 1). All animals were housed and treated according to the rules of the Animal Experiments Review Board at our institution.

Hr-CuZnSOD was used in a rat model rather than rat recombinant CuZnSOD, because we know that there are great similarities between these 2 enzymes, and the effect and safety of Hr-CuZnSOD should first be examined in a well-established rat model before use in humans.

Before flap elevation, all animals were anesthetized with intraperitoneal injections of Ketamine (50 mg/Kg) and Xylazine Hydrochloride (5 mg/kg; Rompun; Bayer Animal Health). The animal's back was shaved and disinfected with 70% alcohol. Using a flap template measuring 80 × 20 mm, flap borders were outlined on the back of the animal. The flap was elevated with its base at the

TABLE 1. Results of the effect of human recombinant copper-zinc superoxide dismutase on flap survival.

Group no.	Hr-CuZnSOD dosage	No. of animals	Flap length survival	Statistical significance, *p* value, *t* test
1	5 mg/kg	12	47.91	<.01
2	15 mg/kg	11	44.36	<.05
3	50 mg/kg	24	49.18	<.001
4 (control)	Saline	23	34.84	–

Abbreviation: Hr-CuZnSOD, human recombinant copper-zinc superoxide dismutase.
Human recombinant superoxide dismutase was provided (free of charge) by Biotechnology General, Rehovot, Israel.

cephalic end (cephalic random pattern flap), including the skin and the intimately attached panniculus carnosus (Figure 1). The flap was completely separated from the underlying fascia up to its base and then immediately sutured back to the donor bed using 3-0 silk on a swedged-on cutting needle. Each rat was caged separately to prevent cannibalism. The rats were given water and food ad libidum.

Hr-CuZnSOD was injected intradermally into the flap area 15 minutes before the operation. The purpose of the intradermal injection was to prepare the flap for the ischemic change before elevation, and to imitate the future use of this substance with local anesthesia. We also speculated that by using an intradermal injection of the substance, its protective effect would last for longer than with any other method. The injected volume was 1.5 cc in all groups, spread evenly across the flap's surface, and a grid was drawn every 5 mm within the flap area, creating 15 equal rectangles. Each rectangle was evenly injected with 0.1 cc of Hr-CuZnSOD. Control animals received appropriate sham injections (an equal volume injection of normal saline in the same manner). Seven days after flap elevation, all animals were re-anesthetized and flap survival was evaluated by fluorescein fluorescence. Fluorescein was given by an intravascular injection (0.3 mL of 5% sol) and after 15 minutes, fluorescence was assessed under the Wood light. The perfused and nonperfused portions of these flaps were then marked on clear plastic sheets (Figure 2). We found that at 7 days postoperatively the fluorescein perfusion was all or none, which correlated with the clinical findings. At the end of the experiment, all animals were killed humanely.

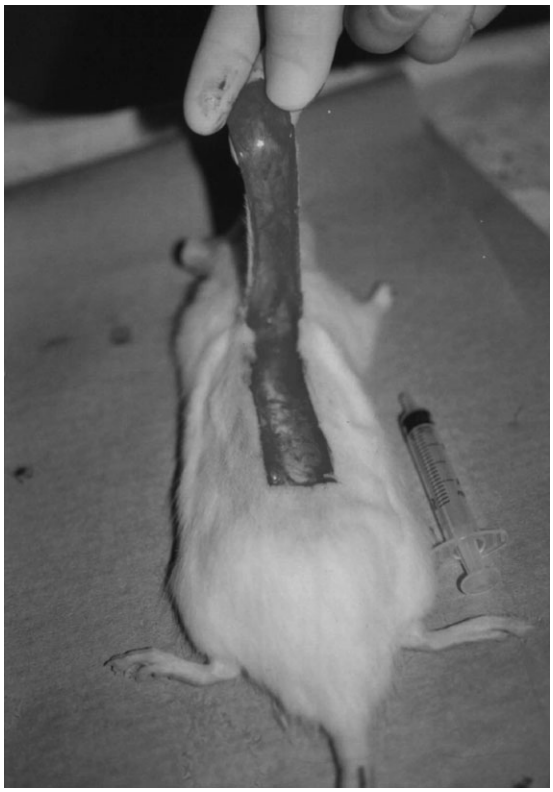

FIGURE 1. With use of a flap template measuring 80 × 20 mm, flaps were elevated with their base at the cephalic end (cephalic random pattern flap), including the skin and the intimately attached panniculus carnosus. The flaps were completely separated from the underlying fascia up to their bases and then immediately sutured back to the donor bed.

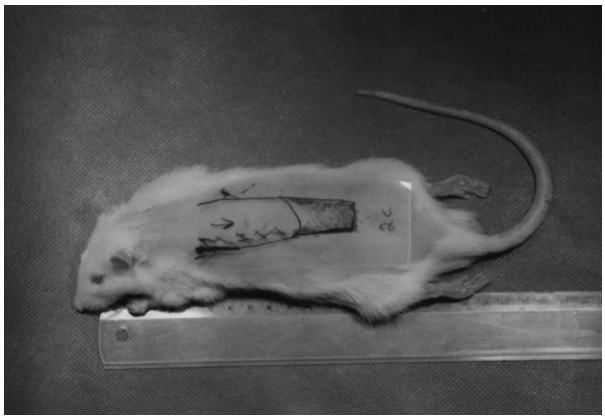

FIGURE 2. Perfused and nonperfused portions of the flaps were marked on clear plastic sheets. Flap survival was determined by averaging the viable flap length at both lateral edges and at the center of the flap.

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One-way analysis of variance (ANOVA) and a *t* test statistical analysis of survival relationships were performed using the Origin statistical program.

RESULTS

All rats survived surgery and behaved normally until humanely killed.

In the control group, there were 18 rats with an average survival length of 34.84 mm (SE, 2.5). In the 5 mg/Kg Hr-CuZnSOD group, there were 17 rats with an average survival length of 47.91 mm (SE, 3.4), which proved to be significantly better than the control group ($p < .01$) when using the *t* test. In the 15 mg/Kg Hr-CuZnSOD group, there were 17 rats with an average survival length of 44.36 mm (SE, 4.3), which proved to be significantly better than the control group ($p < .05$) when using the *t* test. In the 50 mg/Kg Hr-CuZnSOD group, there were 18 rats with an average survival length of 49.18 mm (SE,

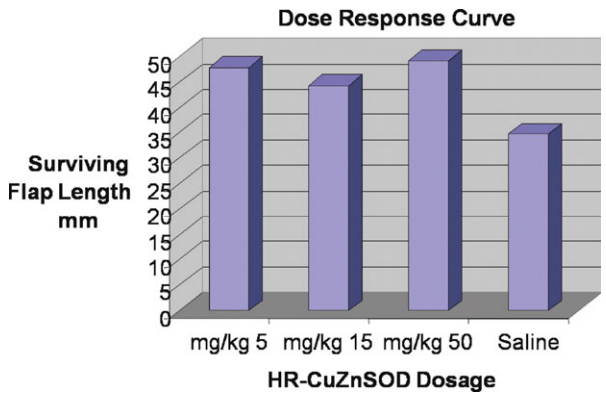

FIGURE 3. Human recombinant copper-zinc superoxide dismutase (Hr-CuZnSOD) dose response curve displays the effects of different dosages of Hr-CuZnSOD on flap survival. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

3.4), which was also found to be significantly better than the control group ($p < .001$) when using the t test (Figure 3). Using the 1-way ANOVA test, a statistical difference was found between all the study groups ($p < .01$).

DISCUSSION

During the first 12 to 18 hours after flap elevation, flow diminishes dramatically, especially in the distal portion of the flap, due to a combination of decreased perfusion pressure as well as the release of sympathetic vasoconstrictors and the progressive leukocyte-mediated endothelial injury. As sympathetic neurotransmitters are depleted during the ensuing 12 to 24 hours, and as angiogenesis from the flap bed occurs in 2 to 3 days, flap perfusion is gradually restored. Because oxygen is available again, cells change to aerobic metabolism and start to degrade waste products that were formed in the anaerobic state. A major degradation product of adenosine triphosphate is hypoxanthine, which is then oxidized to form uric acid and reactive oxygen species (ie, free oxygen radicals).

If natural protection mechanisms such as the cellular and mitochondrial SODs are not available, the newly formed reactive oxygen species can cause a chain reaction and produce more free radicals in a process known as oxidative stress. Some well-studied consequences of oxidative stress are the alteration of membrane integrity, DNA instability, and a decline in enzymatic activities.[9]

Various pharmacologic agents have been investigated for their efficacy in preventing or reversing skin flap ischemia. Sympatholytics, vasodilators, topical vasoactive agents, calcium channel blockers, hemorheological agents, prostaglandin inhibitors, anticoagulants, glucocorticoids, and free radical scavengers are among the drugs thought to be beneficial for flap survival.[10] The major drawback associated with these substances is the need for systemic application, at relatively high doses, to achieve significant improvements in flap survival, which increase the possibility of potential systemic side effects. Theoretically, these unwanted side effects could be reduced if a pharmacological agent could be applied locally.

SODs are metalloenzymes widely distributed in prokaryotic and eukaryotic cells.[11] They are defined between kingdoms by virtue of their bound metal ion, namely the CuZn-SOD class, Mn-SOD class, and Fe-SOD class. The latter exists in prokaryotic cells and some green plants. They constitute an enzyme family that catalyzes the conversion of superoxide anion to hydrogen peroxide (H_2O_2) (Figure 3). The effect of SOD in preventing distal necrosis of a random pattern flap has already been reported in many studies, but most dealt with systemic application.[12–15] Local application has been reported by only a few. Im et al[16] succeeded in salvaging distal necrosis in a random pattern region of an island flap by infusion of SOD directly into the flap. Suzuki et al[17] demonstrated that simple application of Hr-CuZnSOD ointment to the upper surface of a random pattern flap on a rat's back had no effect, whereas its application to the under surface of the flap or using it in an occlusive dressing was effective for salvaging distal flap ischemia.

A great advantage of Hr-CuZnSOD is its human origin, which decreases the possibility of an immunologic reaction, making it more effective for clinical application in treating human ischemic tissues. We did not find any signs of local or systemic adverse effects in any of the groups of rats.

The study was designed to include 3 different doses of Hr-CuZnSOD in order to evaluate whether a dose response curve could be found. We found that the 3 different doses had almost the same effect. This could be explained by the fact that the maximal effect found in this specific model could be achieved at very low doses. Moreover, the fact that the higher doses did not have any deleterious effect is also encouraging, because the possibility of an inverse effect that might accrue in which higher doses of Hr-CuZnSOD would produce more oxygen-free radicals that the other pathways would be unable to handle, thus creating more damage. Therefore, this study proves the safe use of this substance even at very high doses.

Our present experiment indicates that the beneficial effect of Hr-CuZnSOD on flap survival may be attained by changing the local tissue milieu when given intradermally into the flap tissue. The amount needed to attain this local effect is much less than that needed for systemic delivery of the drug. At the moment, Hr-CuZnSOD is certified by the Food and Drug Administration for clinical use in bronchopulmonary dysplasia of neonates. Nevertheless, it is still very expensive and may not be cost-effective for routine clinical use. Used locally, however, lower dosages are needed and thus clinical application may become more reasonable. Hr-CuZnSOD delivered along with local anesthetics used in flap elevation may be valuable. We find our results very encouraging and we intend to further investigate the clinical and experimental options of SOD use.

In conclusion, flap survival in all treated groups was significantly better than in the control group. Our experiment indicates that the beneficial effect of Hr-CuZnSOD on flap survival is attained when it is given intradermally into the flap tissue. Theoretically, Hr-CuZnSOD delivered along with the local anesthetics used in local flap elevation may be a valuable clinical tool.

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