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Effects of Hyperbaric Oxygen and α-Tocopherol on Skin Antioxidant Enzymes Defence in Rats

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ABSTRACT *: In order to test the effects of hyperbaric oxygen (HBO) and α-tocopherol on full-thickness skin grafts in rats, we performed full-thickness skin grafts bilaterally on rats. After surgery, we analyzed the tissue-concentrations of superoxide dismutase (SOD), catalase, and glutathione peroxidase(GPx)/reductase(GPr) on days 0, 2, 4, 7, 10, 14, 21 and 28. The four groups had similar patterns of change in SOD, catalase, GPx and GPr values. SOD increased initially, and was significantly increased at day 7, returning to the preoperative activity level on day 14 (control, HBO, and α-tocopherol treated alone) and 28 (HBO plus α-tocopherol). Catalase had a similar pattern of change as the SOD enzyme activity, except for the surgical control on day 2. Glutathione peroxidase/reductase activity in the four groups had a similar pattern of enzyme activity, with a significant increase from preoperative level on day 4, peaking during days 7 to 10, and returning to preoperative level on day 21(surgical control, HBO, and α-tocopherol-treated alone) and 28 (HBO plus α-tocopherol treated group). Hence, the clinical use of HBO and α-tocopherol mixture can be recommended as an adjunctive treatment for free skin grafts in rats. But, the antioxidant used, its dose, and the timing of its administration, as well as, the exposure time and the pressure of HBO, should be the subject of further research.*

Key Words *: Full-thickness skin grafts, Hyperbaric oxygen, Superoxide dismutase, Catalase, Glutathione*

I. INTRODUCTION

The ubiquity of molecular oxygen in our environment and the dependence of most organisms upon oxygen to generate the fuel for metabolic processes may obscure the fact that oxygen and its metabolites are often toxic(Del Maestro, 1979). Oxygen derived free radicals have been implicated as mediators of tissue damage in postischemic tissue injury in a variety of model systems (McCoid, 1985). The skin provides the outermost barrier against the invasion of infectious agents, reactive electrophiles, and free radicals. Free radical reactions are ubiquitous in living organisms. Oxidative damage is produced by such highly reactive oxygen species (ROS) as a consequence of the phagocytic process, production being initiated continuously *in vivo* by both enzymatic and non-enzymatic reactions. Although ROS provide a defence mechanism, there are situations, such as acute inflammation, where they may damage host tissue. The skin is particularly vulnerable to ROS due to its constant exposure to high oxygen tension, frequent exposure to ultraviolet (UV) light (Black, 1987) and the presence of considerable amounts of polyunsaturated fatty acids (PUFAs) (Camp, 1988). Several studies have provided evidence that supports the role of superoxide radicals in damaging the integrity of the microvascular architecture during or immediately after ischemia (Korthuis *et al.*, 1985). In particular, it has been demonstrated in experimental island skin flap models that ischemia, followed by reperfusion, results in tissue necrosis (Im *et al.*, 1984; Im *et al.*, 1985). The time course of

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ischemia and reperfusion of the rat skin flap has been previously documented (Saski and Pang, 1980).

Vitamins A, C, and E act as antioxidants and free radical scavengers in biological systems (Richard *et al.*, 1987). Vitamin E reacts with hydroxyl and organic free radicals much faster than with superoxide, unlike vitamin A(Fukazawa, 1983). The ubiquitous nature of vitamin E reactions has lead the many to consider vitamin E as the most important natural antioxidant and free radical scavenger (Burtom, 1981; Burton, 1983).

Administration of hyperbaric oxygen (HBO) is used extensively as an adjunctive treatment for human undergoing reconstructive procedures (Pelliteri *et al.*, 1992), and has been used to treat various problems in dogs and cats (Hosgood *et al.*, 1992). Intuitively, treatment with HBO should improve the acceptance of free skin grafts. However, HBO treatment may increase the accumulation of products of lipid peroxidation in the free skin graft. It was proposed that the antioxidant defense mechanisms of the grafts are overwhelmed by the increased production of toxic metabolites. This may be a factor in free skin graft failure, contradicting the indication for HBO therapy in the treatment of free skin grafts. If this is true, free skin grafts survival may be enhanced by using supportive antioxidant systems. For these reason, the study reported here was undertaken to determine the effect of HBO plus α-tocopheol on the survival of free skin grafts in rats. It also was designed to allow comparison of the results with those of another study, which evaluated the effect of HBO and α-tocopherol administration alone.

II. MATERIALS AND METHODS

1. Animals

Thirty-five SPF Sprague-Dawley male rats were purchased from Genetic Engineering Institute (range of body weight; 200~300 g) and were randomly allotted to 5 groups (non-surgical control, surgical control, HBO-treated, α-tocopherol treated, and HBO plus α-tocopherol treated). General anesthesia was induced by injecting a combination of ketamine (100 mg/*ml*) and xylazine (8 mg/*ml*) intraperitoneally at a dosage of 1 *ml*/kg of body weight. The hair over the dorsal of each rat was clipped, and the skin disinfected with povidone iodine and alcohol surgical scrub.

2. Surgical technique of free skin grafts

Using a marking pen, a 30×30 mm area was outlined bilaterally on the dorsal of each rat. The skin was incised along the perimeter of the outline and was completely elevated from the panniculus carnosus muscle. The free skin graft was then replaced on the panniculus carnosus, and sutured using a simple continuous suture with 4-0 nylon at the perimeter of the graft. All rats were given antibiotics (ointment) after surgery.

3. Treatments

HBO-treated rats received hyperbaric oxygen at 2 ATA (atmospheres absolute) for 90 minutes (with an additional 15-minute compression and decompression) twice daily for 28 days, surgical control rats were not treated with HBO. α-tocopherol treated rats received the agent (1,000 IU/kg *in corn oil*) via oral gastric tube daily for 3 days preoperatively and a fourth dose 1 to 2 hours postoperatively. HBO plus α-tocopherol treated rats received HBO and α-tocopherol as mentioned above.

4. Skin graft biopsy

The grafts of each rat were visually divided into quadrants (4 regions/rat). Biopsy specimens (3.5 mm in diameter) from skin grafts were taken from each quadrant at specific times after grafting. Biopsy specimens also were taken from nongrafted skin on the day of grafting to serve as presurgical controls. Biopsy specimens of the grafts were taken immediately after grafting (day 0) and on days 2, 4, 7, 10, 14, 21 and 28. Biopsy specimens were immersed in a liquid nitrogen tank immediately after harvesting, and stored at -70°C until needed.

5. Antioxidant enzyme activity assay

Buffer A [sodium chloride 130 mM, glucose 5 mM, disodium ethylenediaminetetraacetic acid (EDTA) 1 mM, and sodium phosphate 10 mM; pH 7.0], 0.75~1.5 *ml*,

was used for homogenization. Each sample was homogenized with a Teflon homogenizer rotated by an electric drill at maximum speed for 2 min and then centrifuged with a bench-top Eppendorf centrifuge (10,000×g for 10 min). The supernatant was kept on ice and used for enzyme assay. We verified that this technique produced a supernatant that contained all the enzyme activities by treatment with Triton-X100. The activities of SOD, catalase, glutathione peroxidase, and glutathione reductase were assayed spectrophotometrically on an Ultrospec 3000 (Pharmacia Biotech) according to the procedures described in the cited references (Aebi, 1974; Floche and Gunzler, 1984; Floche and Otting, 1984). One enzyme unit is equivalent to 1 μmol of product or 1 μmol of substance disappearance/minute under the defined conditions, except for SOD. In the case of SOD, the amount of SOD inhibiting the cytochrome c reduction rate by 50% under the given assay conditions was defined as 1 unit.

1) Activity of superoxide dismutase was determined, using a modification of the technique described by Floche and Otting (1984). Using this techniques, the following solutions were prepared.

solution A : 0.76 mg (5 μmol) xanthine in 10 *ml* of 0.001 N sodium hydroxide and 24.8 mg (2 μmol) cytochrom C were admixed with 100 ml of 50 mM phosphate buffer pH 7.8 and 0.1 mM EDTA(the resulting solution is stable for 3 days at 4°C)

solution B : xanthine oxidase 0.2 U/*ml* in 0.1 mM EDTA (freshly prepared) kept in ice.

Each biopsy specimen was homogenized with 1% triton X-100 (1:9), and 2.9 *ml* of solution A and 50 μ*l* of sample were added into a 3 *ml* cuvette. The enzymatic reaction was initiated by the addition of 50 μ*l* of solution B. After mixing, the change of absorbance at 550 nm was recorded after initiation of the reaction. Enzyme activity was calculated as follows :

$1/\Delta E\ min^{-1}$

2) The activity of catalase was determined, using a modification of the technique described by Aebi (1974). The following reagents were prepared: Phosphate buffer(a) consisting of 6.81 g of potassium dihydrogen phosphate in distilled water (1000 *ml*) pH 7.0, and phosphate buffer(b) consisting of 8.90 g of dibasic sodium phosphate in distilled water (1000 ml), pH 7.0. Solution (a) and (b) were mixed in the proportion 1:1.5 (v/v). Hydrogen peroxide 30 mM was diluted with 0.34 *ml* of 30% hydrogen peroxide and phosphate buffer to 100 *ml*. Each biopsy sample was homogenized with 1% Triton X-100 (1:9). The homogenate was centrifuged at 1,000×g for 19 minute, at 4°C. and the supernatant diluted with phosphate buffer pH 7.0 (1:100). The enzymatic reaction was initiated by the addition of 1 *ml* of 30 mM hydrogen peroxide and the change of absorbance at 240 nm was recorded for about 30 sec after reaction initiation.

3) The activity of glutathione peroxidase/reductase was determined, using a modification of the technique described by Pagalia and Valentiene (1976). This procedure measures the rate of GSH oxidation by H_2O_2 catalyzed by the glutathion peroxidase (GPx) present in the homogenate. The rate of oxidized glutathione (GSSG) formation is measured by following the decrease in the absorbance of the reaction mixture at 340 nm, as NADPH is converted to NADP.

Each biopsy specimen was homogenized in 1 *ml* of 0.25 M sucrose in 0.02 M Na EDTA, and centrifuged at 10,000×g for 20 minute at 4°C. 0.1 *ml* of supernatant was then added to 0.05 M potasium phosphate buffer in 0.005 M EDTA (1.74 *ml*), 0.0084 M NADPH (0.065 *ml*), 100 enzyme units/*ml* of GSSG reductase (0.010 ml), 1.125 M NaN_3 (0.007 *ml*), and 0.15 M GSH (0.05 *ml*). These mixture were allowed to equilibrate at 20°C for 10 minutes. The enzymatic reaction was initiated by the addition of 0.002 M H_2O_2 (0.1 *ml*) to this mixture. The conversion of NADPH to NADP was monitored by continuously recording the change in absorbance (ΔA) of the system at 340 nm between 2 and 4 minutes of initiating the reaction. Enzyme activity was calculated using the following : $EU/ml = (\Delta A \times 2.052)/(6.22 \times 0.1)$.

6. Statistical evaluation

Statistical analysis of the results was performed using the by least significant difference (LSD) test, with the statistical analysis software (SAS program). Statistical significance was determined at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$.

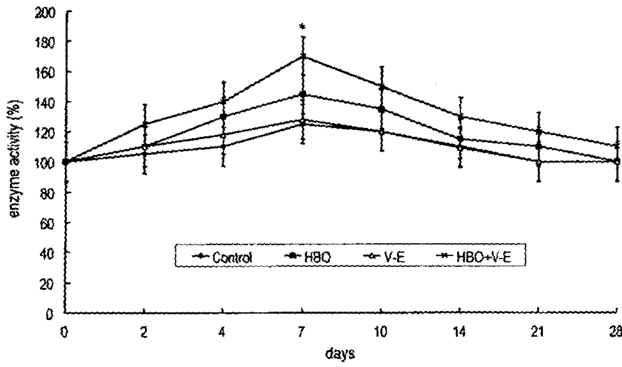

Fig. 1. Changes of SOD activity of free skin grafts in rats. n=7. *$p<0.05$, enzyme activity significantly different from control group. Control : surgical control, HBO : Hyperbaric oxygen, V-E : α-tocopherol.

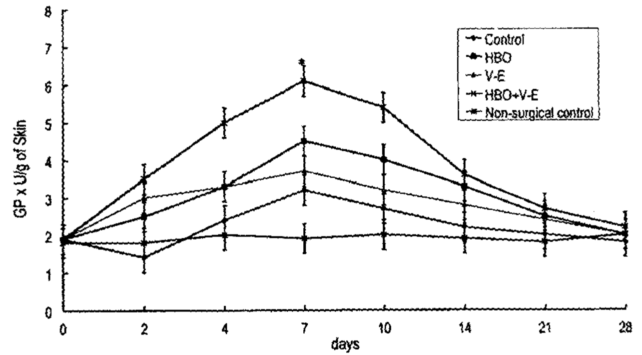

Fig. 3. Change of glutathione peroxidase(GPx) activity of free skin grafts in rats; n=7. *$p<0.05$, enzyme activity significantly different from control group. Control : surgical control, HBO : hyperbaric oxygen, V-E : α-tocopherol.

III. RESULTS

1. Change of antioxidative enzyme activity

1) Superoxide dismutase activity : Changes of SOD enzyme activity in the four groups showed a similar pattern. Increased activity was apparent between day 2 and 7, and was significant only in the combined group at day 7, returning to the preoperative level at day 14 (control, HBO, and α-tocopherol treated alone) and day 28 (HBO plus α-tocopherol treated; Fig. 1).

2) Catalase activity : Catalase enzyme activity change in the four groups had a similar pattern as the SOD enzyme activity, except for the surgical control group on day 2. Activity was started to increase between days 2 and 7, with a significant increase on day 7, and returned base-line level on day 10 (control, HBO, and α-tocopherol treated alone) and day 28 (HBO plus α-tocopherol treated). The enzyme activity of combined group differed significantly from the surgical control (Fig. 2).

3) Glutathione peroxidase/reductase activity : Glutathione peroxidase(GPx)/reductase(GPr) activity change in four groups had a similar pattern of enzyme activity, with a significant increase from pre-operative activity by day 4, peaking during days 7 to 10, and returning to the pre-operative activity level at day 21 (surgical control, HBO, and α-tocopherol-treated alone) and day 28 (HBO plus α-tocopherol treated; Fig. 3 and Fig. 4).

4) Relative percentages of the enzyme antioxidants : From the results of enzymic antioxidants activity measurement, specific changes occurred on day 7, and so, we determined the relative percentage contri-

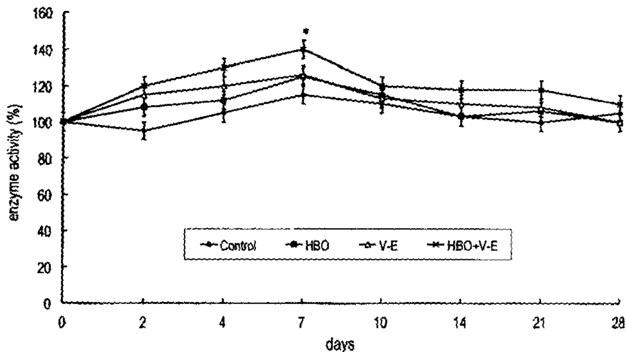

Fig. 2. Chang of catalase activity of free skin grafts in rats; n=7. *$p<0.05$, enzyme activity significantly different from the surgical control group. Control : surgical control, HBO : hyperbaric oxygen, V-E : α-tocopherol.

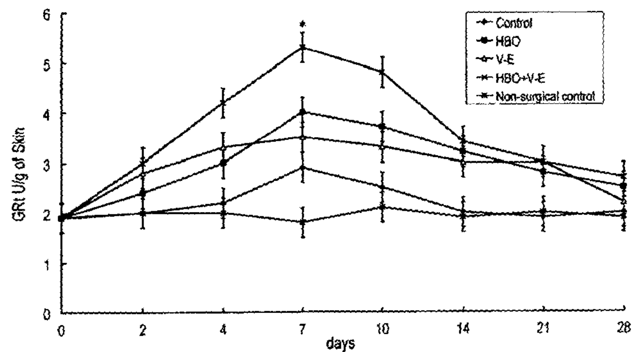

Fig. 4. Change of glutathione reductase(GPr) activity of free skin grafts in rats; n=7. *$p<0.05$, enzyme activity significantly different from control group. Control : surgical control, HBO : hyperbaric oxygen, V-E : α-tocopherol.

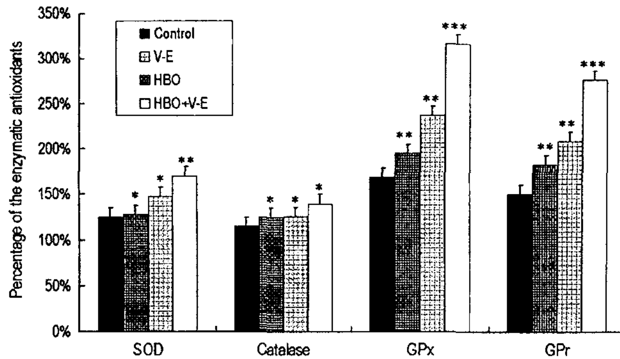

Fig. 5. Relative percentages of the enzymic antioxidants in free skin grafts of rat on day 7 after surgery; n=7; results are mean±SE; compared with surgical control; *p<0.05, **p<0.01, ***p<0.001. Control : surgical control, HBO : Hyperbaric oxygen, V-E : α-tocopherol.

bution of each enzymic component on day 7 for each of the groups. The relative percentage of SOD activity increased significantly in the four groups, compared with the surgical control (HBO, and α-tocopherol treatment alone : $p<0.05$; HBO plus α-tocopherol-treated : $p<0.01$), catalase activity increased compared with the surgical control at the $p<0.05$ significance level and glutathione peroxidase/glutathione reductase activity also increased significantly compared with the surgical control at the $p<0.01$ and $p<0.001$ level, respectively (Fig. 5).

IV. DISCUSSION

SOD is a major enzyme that maintain $O_2^{\cdot}$ levels in skin and catalyses $O_2^{\cdot}$ dismutation to the potentially damaging H_2O_2 and O_2. Human skin contains both CuZn-SOD and Mn-SOD, but their activities are considerably lower than that of heart, liver or kidney, suggesting that thioredoxin reductase and GPx are more important in the skin (Charles *et al.*, 1992). The changes of SOD enzyme activity in the four groups had similar patterns. Activity increased from day 2 to 7, with a significant increase from the preoperative activity level on day 7, and returned to its preoperative activity level on day 14 (surgical control, HBO, and α-tocopherol treated alone) and day 28 (HBO plus α-tocopherol treated). This results is not in agreement with those (decrease of 36% and 48% in epidermis and dermis compared to the control, respectively) of Yasuko *et al.* (1993) rats (Simonsen strain), this may be due to the different animal strain and drug used. In addition, our results differ those of Kim and Lee (1987) for human skin, which may represent a difference between human skin and rat skin.

When comparing free skin grafts and skin flaps in dogs, free skin grafts were found to have a greater depletion of ATP in the first 4 days after grafting than ischemic skin flaps (Kim and Lee, 1987). In addition, the concentration of superoxide dismutase in free skin grafts was significantly lower than that in ischemic skin flaps with venous drainage, at 1, 2, and 4 days after surgery (Angel *et al.*, 1992). In fact, during the 4th day after surgery, the superoxide dismutase concentration remained low in free skin grafts, whereas the concentration actually increased in the ischemic flaps (Angel *et al.*, 1992). Thus, we suggest that although the ischemic skin flaps developed cellular tolerance to the oxidative stress of reperfusion, the free skin grafts did not adapt during the first 4 days. This may reflect the substantial compromise of cellular function in the free skin graft and the low concentration of reperfusion. Intracellular H_2O_2 is removed by catalase, but levels of this enzyme are low especially in skin disorders, including vitiligo (Schallreuter *et al.*, 1991). Changes of catalase enzyme activity in the four groups showed a similar pattern as the SOD enzyme activity, except the surgical control on day 2.

Activity started to increase from day 2 to 7, with a significant increase from the preoperative activity level by day 7, and returned to the preoperative activity level by day 10 (control, HBO, and α-tocopherol treated alone) and day 28 (HBO plus α-tocopherol treated). Enzyme activity was significantly different between each group and the non-surgical control group. The results reported in this paper may not be directly comparable with others, because, to our knowledge, this paper is the first to examine HBO plus α-tocopherol effects in rats, however, our results show a similar patterns to others (Kaelin *et al.*, 1990; Yasuko *et al.*, 1993) Changes of glutathione peroxidase(GPx)/reductase(GPr) activity in four groups showed a similar pattern of enzyme activity, with a significant increase from the preoperative activity level on day 4, peaking during days 7 to 10, and returning to the preoperative activity level on day 21 (surgical control, HBO, and α-tocopherol-treated alone) and day 28 (HBO plus α-tocopherol treated). These results are in agreement with the results of Jacques *et al.* (1987), and Rose *et*

al. (1988).

The increase in GPx activity also suggests an increase in lipid peroxidation in the free skin grafts, resulting in enzyme induction as a defence mechanism. The increase in GPx activity occurred after (day 4) the increase in thiobarbituric acid-reactive substances (TBARS) absorbance (day 2). This was attributed to the time necessary for the enzyme to respond to the stimulus of lipid peroxidation. But, our results for GPx (6.1 U/g of skin) do not agree with those of Yasuki *et al.* (4.6 U/g of skin, 1993). We suggest that this discrepancy may be due to our use of HBO plus α-tocopherol, whereas the study of Yasuko examined the effect of UV radiation on the rat skin.

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