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Depletion of Reduced Glutathione, Ascorbic Acid, Vitamin E and Antioxidant Defence Enzymes in a Healing Cutaneous Wound

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In the present investigation the involvement of free radicals in a self-healing cutaneous wound has been demonstrated. The levels of different enzymatic and non-enzymatic antioxidants have been studied in 2, 4, 7 and 14 days old wounds and compared with normal skin. Except for glutathione reductase (GR), all other enzymatic and non-enzymatic antioxidants were found to decrease following wounding. The decrease was 60–70% in superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) at 2, 4 and 7 days, while in the case of catalase (CAT) the decrease was 40–60% during this period. Although a complete recovery in the activity of CAT was observed, SOD and GPx did not recover completely and GST was found to be slightly elevated on 14th day post wounding. Non-enzymatic antioxidants viz. ascorbic acid, vitamin E and glutathione were also found to decrease to about 60–70% and except glutathione none of them was found to recover completely at 14th day postwounding. Interestingly thiobarbituric acid reactive substance (TBARS) expressed as malondialdehyde (MDA) equivalent, a marker of lipid peroxidation, decreased following wounding which could be because of meagre availability of lipid substrate and/or of ascorbic acid. The results indicate that wounding results in loss of different free radical scavengers both enzymatic and non-enzymatic which either partially or completely recover following healing.

Keywords: Wound, wound healing, antioxidants, free radicals

INTRODUCTION

Wound healing proceeds through a series of coordinated and cytokine-mediated events that require the concerted action of many cell types.[1,2] The first phase of healing is governed primarily by various inflammatory cells that accumulate within the wound. Platelets are among the earliest inflammatory mediators to arrive at the site of injury, whereas neutrophils and macrophages abound during the first two days after wounding and typically precede the influx of fibroblasts, lymphocytes and endothelial cells.[3] The well orchestrated series of events in which these cell types interact include the release of cytokines, growth factors and other bioactive molecules like free radicals.[4] These substances hold regulatory roles in the local process of repair.

The skin is potentially the target of significant oxidative injury because of its constant exposure

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to high oxygen tension and its frequent exposure to uv light, which is known to promote the generation of oxygen radicals and lipid peroxides. Markedly elevated lipid peroxide levels have been demonstrated in certain inflammatory skin lesions such as traumatic wounds and radiation dermatitis.[5] These observations indicate that free radicals may play an important role in healing of a wound. Very little is known regarding the role of free radicals and their scavengers in a self healing cutaneous wound. The present study investigates the profile of different free radical scavengers (enzymatic and non-enzymatic) and lipid peroxidation at different time points of a healing wound. It may provide a lead for future therapeutic interventions in wound healing by using free radical scavengers.

MATERIALS AND METHODS

Male Sprague-Dawley rats (180–200 g) obtained from the CDRI animal breeding colony were used for experiments, Four circular skin deep (full thickness, completely transdermal) wounds were made on preshaved, sterilized (with 70% alcohol) dorsal surface of rats with the help of Acupunch (Acupunch, Acuderm Inc. Loudero, USA), 8 mm in diameter. All surgical procedures were carried out under thiopentone anaesthesia (25 mg/kg i.p.). Animals were allowed to recover and housed individually in cages kept under standard animal house conditions. The animals had a free access to a pellet diet and tap water. All wounding procedures were performed aseptically and animals were maintained on autoclaved paper cuttings to avoid infections. The rats used in the study had completely uninfected wounds.

Wound Tissue Excision

Wound tissue excision was done on second, fourth, seventh and fourteenth day of wounding using the same 8 mm Acupunch, which excises only the newly formed tissue in the wounded area thus avoiding contamination with non wounded tissue. At the same time zero day wound tissues (normal skin) were also collected from fresh animals for a comparison using the same procedure.

Procedure of wound formation and wound tissue excision followed was from a well accepted method of Werner *et al.*[6]

Sample Preparation

Healthy and newly formed tissues (consisting of all kinds of cells invoved in repair mechanism) were collected in a petri dish kept over ice and were washed with normal saline to remove adhering blood. Skin punches were weighed and cut into small pieces. A 10% homogenate was made in chilled 0.15 M KCl containing 5 mM EDTA using Ultra-Turrax. Each sample was homogenized by giving it four strokes of 15 second each. The presnce of EDTA and low temperature help to protect antioxidants from destruction during processing. Samples were sonicated (10 bursts of 5 second each with 5 second rest between two bursts) and aliquots were withdrawn for reduced glutathione, vitamin E and ascorbic acid estimation. To the rest of the homogenate Triton X-100 was added to a final concentration of 0.1% (v/v). Samples were left at 4°C for two and half hours with occasional shaking. The homogenate was centrifuged at 3000 rpm (1000 g) for 10 minutes and supernatant was used for the estimation of different enzymatic antioxidants and lipid peroxides.

Recovery Experiments

Skin pieces were homogenized in 0.15 M KCl with 5 mM EDTA and known amounts of standard. The homogenate was sonicated and treated according to the method given for determination of different parameters. The recovery of the standards was calculated by comparing the values in the skin sample without added standards. The recovery of the added standards was found to be in the range of 95

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± 3%, that showed no appreciable losses of nonenzymatic antioxidants during processing of the tissue. The methods used for various biochemical analyses were found to be reproducible both within batch and day to day experiments.

Analysis of Non-Enzymatic Antioxidants

Reduced Glutathione (GSH). Sonicated homogenate was centrifuged at 3000 rpm for 10 minutes. From the supernatant an aliquot of 0.7 ml was withdrawn and added to 0.7 ml of triple distilled water. To this diluted sample 0.35 ml of 25% metaphosphoric acid was added and kept at 4°C for one hour. The samples were centrifuged at 4000 rpm for 10 minutes and 0.1 ml of supernatant was used to estimate glutathione by the fluorometric method.[7]

Vitamin C. Ascorbic acid estimation was done by using the method of Rae[8] in which the coloured complex formed by the reaction of ascorbic acid with dinitrophenylhydrazine (DNPH) was monitored at 540 nm.

Vitamin E. Vitamin E estimation was done fluorometrically after saponification with KOH and extraction with purified hexane.[9]

Assays of Enzymatic Antioxidants

Superoxide Dismutase (SOD). Total SOD activity was assayed by monitoring the inhibition of coloured osazone formation due to the transfer of electrons from nitroblue tetrazolium (NBT) to phenazenemethosulfate (PMS) in the presence of NADH. The reduction in the colour intensity was followed at 560 nm. One unit of the SOD activity was defined as the amount of enzyme which inhibited the colour formation by 50%.[10]

Catalase (CAT). The activity of CAT was measured as disappearance of hydrogen peroxide (H_2O_2) at 240 nm.[11]

Glutathione Reductase (GR). GR activity was estimated according to the procedure of Beutler,[12] using oxidized glutathione as a substrate. The oxidation of NADPH was monitored at 340 nm at 37°C.

Glutathione-S-Transferase (GST). Procedure of Habig[13] was used to assay the activity of this enzyme in presence of chlorodinitrobenzene (CDNB) as a substrate. Glutathione conjugate formation was monitored at 340 nm at 37°C.

Glutathione Peroxidase (GPx). Se-dependent and total Gpx were assayed in presence of cumene hydroperoxide (CHP) and, H_2O_2 respectively as substrates by the coupled enzyme assay method of Flohe and Gunzler.[14]

Thiobarbituric Acid Reactive Substance. This is a marker of lipid peroxide and expressed as malondialdehyde equivalent. It was measured as thiobarbituric acid (TBA) reactive material by the procedure of Ohkawa et al.[16] The pink colored adduct formed at 85°C was read at 532 nm.

Protein. Protein estimation was done using Folin-phenol reagent, following the method of Lowry et al.[15] Bovine serum albumin was used as standard.

Special care was taken to avoid animal suffering and metal contamination. Appropriate and documented ethical criteria have been met. All procedures were carried out at 0 to 4°C and unnecessary exposure to oxygen was also minimized by following each and every step quickly.

Student's 't' test was applied to calculate the significance of the difference between two groups. P values less than 0.05 were taken as significant.

RESULTS

Non-Enzymatic Antioxidants

All the three non-enzymatic antioxidants studied, were found to decrease following wounding. Decreases were found to be significant at all the time points and percent decrease was almost

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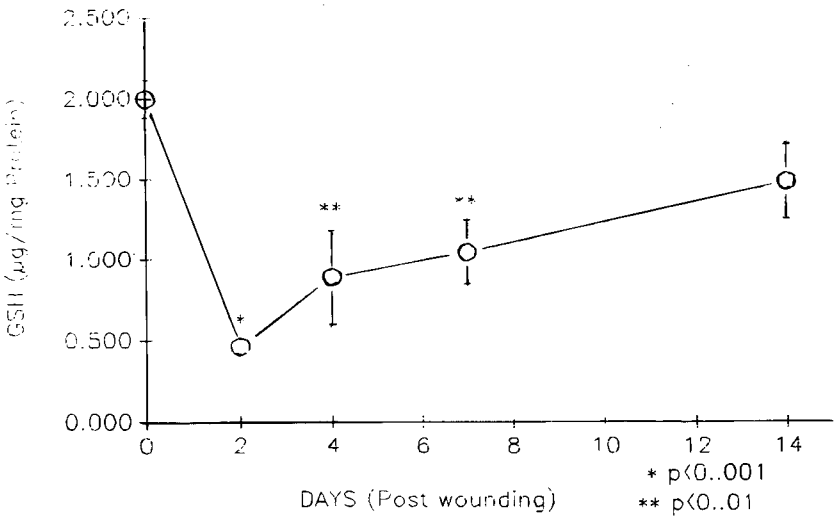

FIGURE 1 Reduced glutathione (GSH) levels of a healing cutaneous wound in rat. Values represent mean ± SE of six experiments. *p < 0.001 and **p < 0.01 as compared to zero day wound (normal skin).

similar at 2, 4 and 7th day post wounding. At 14th day although glutathione levels were slightly higher than control levels (Fig. 1), vitamin C and vitamin E did not recover completely (Fig. 2).

Enzymatic Antioxidants

Out of various enzymes studied, except for glutathione reductase, a marked decrease was observed in all the enzyme activities at 2, 4 and

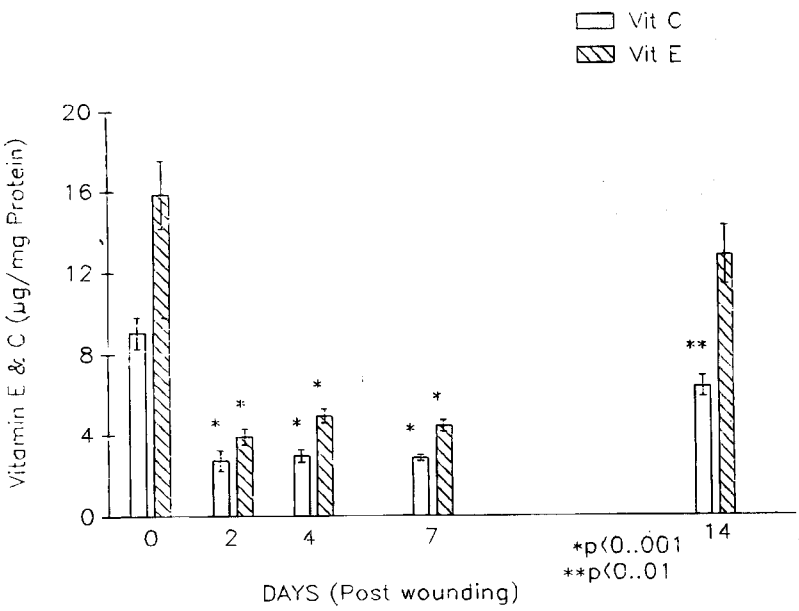

FIGURE 2 Vitamin E and ascorbic acid levels of a healing cutaneous wound of rat. Values represent mean ± SE of six experiments. *p < 0.001 and **p < 0.01 as compared to zero day wound (normal skin).

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7th day, which either partially or totally recovered on 14th day.

SOD activity decreased significantly and remained decreased at 2, 4 and 7th day post wounding. At 14th day partial recovery in activity was observed (Fig. 3). CAT decreased significantly and peak decrease was observed on 4 and 7th day post wounding although on 2nd day also decrease in activity was noticed. A complete recovery was observed at 14th day. Fold decrease in activity was less as compared to SOD (Fig. 3). Conservation in activity of only GR enzyme was observed following wounding. No significant difference was observed in activity of GR as compared to control at any time point (Fig. 4). GST also showed a decrease in its activity at 2, 4 and 7th day following wounding. Peak decrease was observed at 2nd and 4th day. However, at 14th day activity of GST was found to be slightly more than control (Fig. 4). Both Se-dependent and Se-independent Gpx were found to decrease following wounding. Although the Se form recovered completely at 14th day only partial recovery in activity was observed with non-Se form (Fig. 5).

MDA. The levels of malondialdehyde a marker of lipid peroxidation was measured as thiobarbituric acid reactive material were found to decrease following 2, 4 and 7 day post wounding and slightly increased on 14th day when estimated as MDA formed per mg of protein as compared to control (normal skin) (Fig. 6).

DISCUSSION

Although there is some direct as well as indirect evidence[17–19] in existing literature to show the involvement of free radicals in wound healing, the status of free radical scavengers during wound healing is not yet clear. The present investigation was undertaken to investigate the status of different enzymatic and non-enzymatic free radical scavengers and lipid peroxidation at different time points of a normally healing cuta-

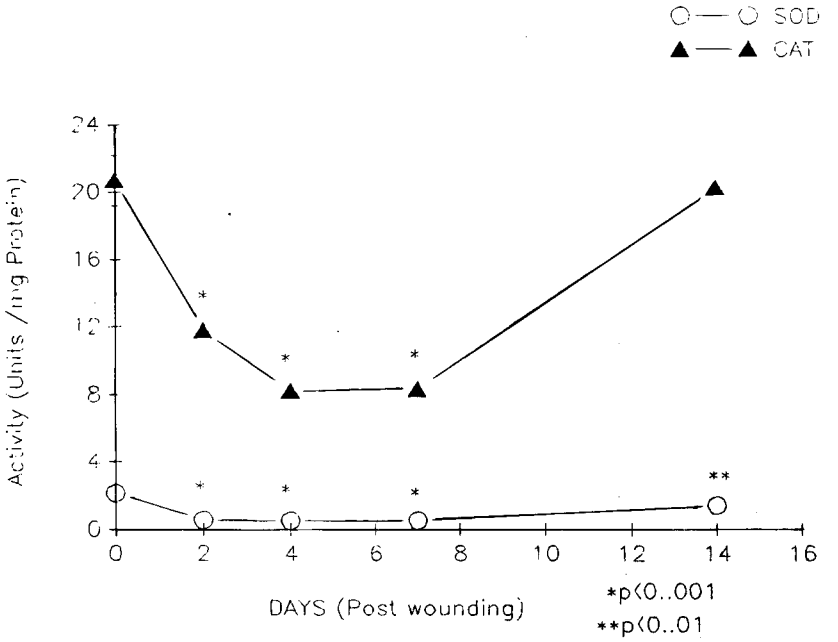

FIGURE 3 Superoxide dismutase (SOD) and catalase (CAT) activity in a healing cutaneous wound of rat. Values represent mean ± SE of six experiments. *$p < 0.001$ and **$p < 0.01$ as compared to zero day wound (normal skin).

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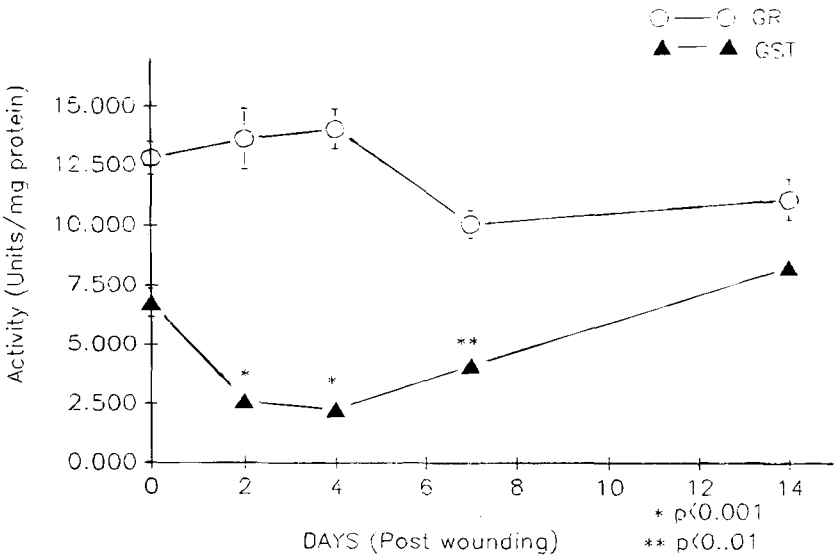

FIGURE 4 Glutathione-s-transferase (GST) and glutathione reductase (GR) activity in a healing cutaneous wound of rat. Values represent mean ± SE of six experiments. *p < 0.001 and **p < 0.01 as compared to zero day wound (normal skin).

neous wound. It appears from the results of the present investigation that all the three non-enzymatic antioxidants viz. ascorbic acid, vitamin E and reduced glutathione (GSH) were found to remain decreased after 2nd, 4th and 7th day of wounding and a partial recovery in their content was observed following healing (at 14th day). The depleted levels of GSH and ascorbate might be related to findings of Hemila *et al*[20] and Thomas *et al*[21] which demonstrate that activated neutrophils oxidize ascorbate and GSH. Depleted levels of vitamin E and ascorbic acid may contribute

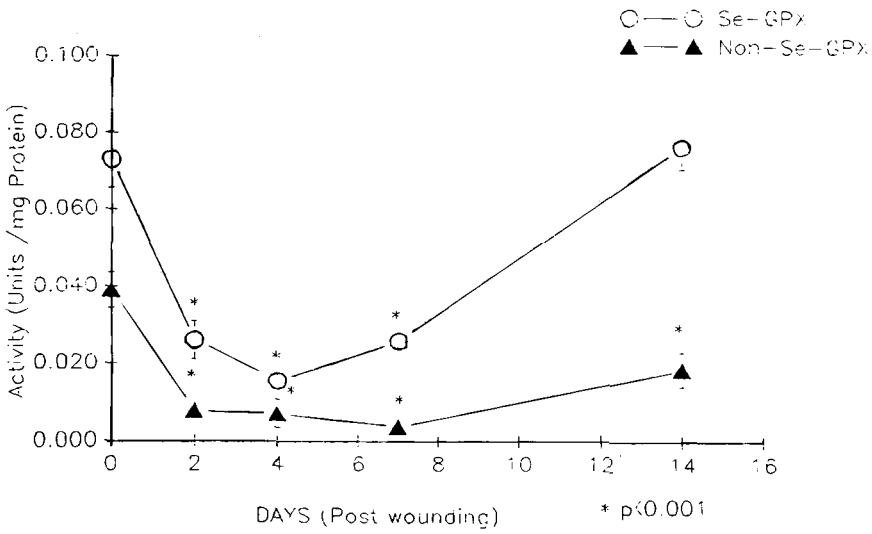

FIGURE 5 Glutathione peroxidase (Gpx) activity in a healing cutaneous wound of rat. Values represent mean ± SE of six experiments. *p < 0.001 as compared to zero day wound (normal skin).

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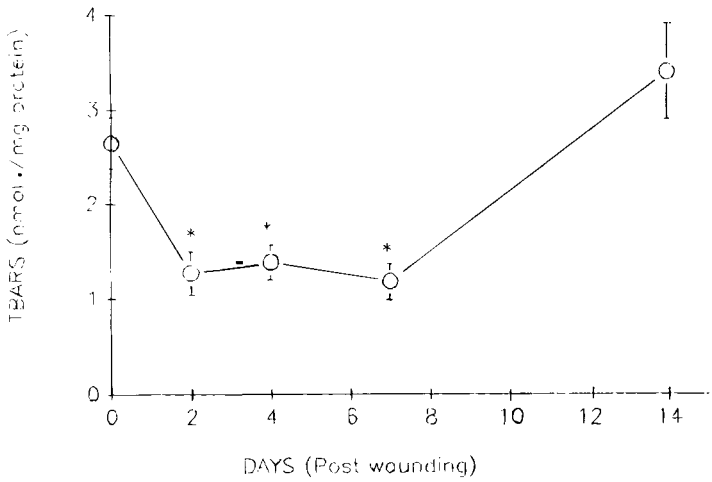

FIGURE 6 Thiobarbituric acid reactive substance (TBARS) levels of a healing cutaneous wound of rat. Values represent mean ± SE of six experiments. *p < 0.001 as compared to zero day wound (normal skin).

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to delayed healing, since it has been demonstrated by Pascoe[22] that vitamin E protects against cellular injury. Also, ascorbic acid has been shown to enhance collagen expression in fibroblast under *in vitro* conditions which can be correlated to enhanced wound healing.[23] The changes we observed are not due to influx of new inflammatory cells to the wound as the concentration of GSH and ascorbic acid in inflammatory cells are remarkably low compared to skin [GSH(ug/mg protein); neutrophils-2.0, platelets-0.7, skin-2.0: vitamin E (ug/mg protein); neutrophils-0.58, platelets-0.38, skin-16: vitamin C (ug/mg protein); neutrophil-3.2, platelets-2.8, skin-9] and are almost equal to their levels in wound tissue. Since wound tissue is not completely made up of inflammatory cells and a good percentage of total wound weight be derived from newly formed tissue itself, therefore almost equal concentration of GSH and ascorbic acid in wound tissue can result only when there is a decreased concentration of these antioxidants in newly formed skin tissues.

The activities of enzymatic antioxidants viz. SOD, CAT and Gpx and the activity of detoxifying enzyme GST were decreased significantly after 2, 4 and 7 day of wounding and the activities recovered either partially or completely at 14th day. An interesting observation of the study was that among several enzymes studied only GR showed conservation in its protein throughout healing period, and no change in the activity was noticed at any point of the healing wound. This could be either because of new cells migrating to wound carry with them an exceptionally good amount of this enzyme or new proteins are being synthesized in wounded area itself so that enzyme level is maintained continuously. Similar to our observation, a marked decline in SOD and heat shock protein 70 (HSP 70) levels has also been observed in burn injury of rats.[24] Topical application of epidermal growth factor along with a protease inhibitor increased the synthesis of SOD in rat skin which contributed to accelerated wound healing in burn wound.[24] In guinea pigs also SOD activities were found to decrease following burn.[25] However, not much is studied about status of other enzymes and their role in wound healing. Considering the decreased levels of these antioxidants it also appears that rat skin is not adaptogenic i.e. elevation in activity of different enzymes was not observed following injury. Although a few reports are available on SOD, the present investigation describes a novel

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report on the activities and contents of different enzymatic and non-enzymatic antioxidants following healing of an open cutaneous wound. Our findings also revealed that lipid peroxide levels in wounded skin of rats were significantly decreased possibly due to unavailability of substrate (polyunsaturated fatty acids) or of ascorbic acid which helps in keeping iron in its reduced form. On 14th day when a wound appears almost completely healed, lipid peroxide levels were found to be slightly higher than in the normal skin. Although there are reports showing increased lipid peroxidation following injury but none of them dealt with open excision type of wound. The protein content of wounded tissue was slightly higher than control but not so high so as to alter the interpretation of our results.

Based on the results of this study it can be concluded that injury resulting in open cutaneous wound leads to decreased peroxide levels and decreased enzymatic and non-enzymatic antioxidants. Most of the changes tend to recover either partially or completely with healing of wound. The study, suggests the fact that different free radical scavengers or preparations which can elevate the level of different antioxidants *in situ* can be utilized to facilitate the healing of a wound.

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