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ORIGINAL PAPER

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# Pretreatment of diabetic rats with lipoic acid improves healing of subsequently-induced abrasion wounds

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**Abstract** The etiology of delayed or impaired wound-healing in diabetic individuals is multifactoral, but peripheral vascular dysfunction is an underlying factor in the majority of cases. Recent studies have shown that lipoic acid improves vascular function in diabetic skin and reduces the symptoms associated with the diabetic peripheral neuropathy. In this study, rats were made diabetic with streptozotocin (STZ) and treated systemically on alternative days with lipoic acid (100 mg/kg given via intraperitoneal injection) for 8 weeks. Untreated STZ-diabetic rats and non-diabetic rats served as control. At the end of the 8-week period, rats from all the three groups were subjected to abrasion wound formation. Skin wounds healed more rapidly in untreated non-diabetic rats than in the untreated diabetic rats. Wounds in lipoic acid-treated diabetic rats healed more rapidly than wounds in untreated diabetic rats. Subsequent in vitro studies demonstrated that lipoic acid protected endothelial cells from oxidant injury. At the same time, lipoic acid had no apparent effect on endothelial cell proliferation and had no measurable effect on fibroblast function (proliferation, collagen synthesis and matrix metalloproteinase expression). These findings suggest that prophylactic use of lipoic acid might be useful in preventing the development of non-healing skin ulcers from minor traumas in at-risk skin.

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## Introduction

R-α-lipoic acid (lipoic acid) is a thiol-containing pentanoic acid derivative found in both the prokaryotic and eukaryotic cells. Lipoic acid is a scavenger of several oxygen radical species including hydroxyl radical, superoxide, singlet oxygen, peroxyl radicals, hypochlorous acid and nitric oxide [1]. In vitro studies have demonstrated the ability of lipoic acid to prevent injury of vascular endothelial cells directly or to inhibit alterations in endothelial cell function that promote sensitivity to inflammatory injury [2–7]. Lipoic acid has been used for a number of years to treat the pain and numbness associated with peripheral neuropathy in diabetic patients [8–10]. While the exact mechanism(s) by which improvement is brought about remain(s) unclear, current thinking is that the ability of lipoic acid to retard peripheral microvascular damage and the attendant ischemia associated with endothelial cell dysfunction underlies the beneficial effects on the peripheral neuropathy.

It is well recognized that vascular damage (both macrovascular and microvascular) is a major underlying factor in the formation of chronic ulcers in diabetic patients, as well as in other individuals in which wound-healing capacity is limited. Inadequate arterial perfusion is a contributing factor in approximately 60% of diabetic subjects with non-healing foot ulcers and 46% of those who have a major amputation [11]. In addition to macrovascular disease, microvessel disease also contributes to the chronicity of diabetic foot ulcers [12]. A specific diabetic small vessel (capillary) disease has been implicated in the pathogenesis of diabetic foot lesions by some researchers [13–15]. Chronic ischemia in the poorly-perfused tissue leads to secondary changes that are the proximal cause of wound-healing failure. Since lipoic acid treatment is beneficial in treatment of other complications of diabetes that are presumed to reflect decreased vascular function, the present study was undertaken to determine if wound-healing might also be improved by this agent.

## Materials and methods

### Streptozotocin (STZ)-induced diabetes

Male nude rats with a start weight of 200–250 g were purchased from Charles River (Wilmington, MA, USA) and acclimated for 1 week upon arrival in the laboratory. Animals were randomized to control or treatment groups. Diabetes was induced by intraperitoneal injection of streptozotozin (STZ) (Sigma Chemical Co., St. Louis, MO, USA) (50 mg/kg in 0.2 ml of 10 mM citrate buffer, pH 5.5) as described previously [16]. After 48 h, blood glucose levels were assessed and rats included in the study if glucose concentrations were $>250$ mg/dl in heparinized tail vein blood (measured by glucometer). The University Committee on Use and Care of Animals approved all of the procedures used in this study.

### Lipoic acid treatment

Diabetic rats were divided into treatment and control groups. Starting 3 days after STZ administration, one group received 100 mg/kg lipoic acid (Sigma Chemical Co., St. Louis, MO, USA). Treatment was done on every other day for 8 weeks. A second group of diabetic rats (untreated) served as control. A third group of rats received no treatment at all (non-diabetic). All treated and control rats had ad libitum access to water and suitable rat diet. STZ-treated rats were given daily injections of a small dose of protamine zinc insulin (0.5–3.0 units per day) as required to maintain blood glucose levels in the 350 mg/dl range. At the end of the 8-week treatment, the rats were subjected to abrasion wound formation and included in wound-healing studies.

### Superficial wound formation protocol

Rats were anesthetized with an intraperitoneal injection of ketamine/xylazine (100 mg/kg ketamine and 10 mg/kg xylazine) and the paravertebral skin was cleansed with 70% alcohol. Abrasion-type superficial wounds were made in the paravertebral dorsal skin by application of 100% acetone to the skin followed by rubbing with a coarse emery board. A circular wound area with approximate dimensions of 35 $cm^2$ was made. The depth of the wound was made just to the point where oozing of fluid into the abraded tissue could be seen. The superficial wounds were designed to mimic the type of wound typically seen in human skin following minor scrapes.

Wounds were photographed using a digital camera on day 0 and on days 2, 4 and 6. Wound area was calculated as a multiple of *x*- and *y*- axes of the wound. At the time of wound closure, animals were killed by cervical dislocation. Biopsies of skin from three different sites—i.e., wound center, wound edge and beyond the original wound margin—were taken from each animal. The tissue was immediately fixed in 10% buffered formalin and used for histology.

### Endothelial cells and fibroblasts

An immortalized line of rat endothelial cells [17] was used in these studies. Dulbecco's Minimal Essential Medium supplemented with non-essential amino acids and 10% fetal bovine serum (DMEM-FBS) was used as culture medium. Fibroblasts were isolated from the dermis of young Sprague-Dawley rats. These cells were also grown in DMEM-FBS. Both cell types were maintained at 37°C in an atmosphere of 5% $CO_2$ and 95% air, and subcultured as required.

### Endothelial cell injury protocol

Sensitivity of endothelial cells to injury by hydrogen peroxide was assessed as described previously [18]. Briefly, cells were plated in 24-well dishes at $4\times10^4$ cells per well and incubated for 2 days (37°C and 95% air/5% $CO_2$) in DMEM-FBS containing either 5 mM or 25 mM glucose as culture medium. Although 10% serum was used for most of the experiments, in some cases, 5% or 1% FBS was used. In some experiments, lipoic acid was examined for ability to inhibit cytotoxicity. When used, it was included in the incubation medium at the desired concentration. At the end of the incubation period, cells were washed two times and then incubated in Hanks' balanced salt solution with varying amounts of hydrogen peroxide (10–100 μM). After exposure to the hydrogen peroxide for 2 h, the cells were harvested with trypsin/EDTA and replated in DMEM-FBS. Eighteen hours later, the cells that had reattached were counted. The percentage of the initially plated cells that had successfully reattached was determined, and this value was used as a measure of viability.

### Proliferation assay

Endothelial cells were plated at $4\times10^4$ cells per well in DMEM-FBS supplemented with either 5 mM or 30 mM glucose or treated with lipoic acid as above. After incubation for 3 days, cells were harvested and counted. Fibroblast proliferation was also assessed in the presence or absence of lipoic acid. Fibroblast growth was assessed as with endothelial cells except that serum-free DMEM supplemented with 200 μg/ml BSA (DMEM-BSA) was used in addition to DMEM-FBS.

### Substrate-embedded enzymography

Substrate embedded enzymography (zymography) [19] was used to assess MMP production by fibroblasts. Briefly, SDS-PAGE gels were prepared from 30:1 acrylamide/bis with the incorporation of gelatin (1 mg/ml) before casting. The gels were routinely 7.5% acrylamide. Samples of fibroblast culture fluid and molecular weight standards were electrophoresed at constant voltage of

150 V under non-reducing conditions. After electrophoresis, gels were removed and washed twice for 15 min in 50 mM Tris buffer containing 1 mM $Ca^{2+}$ and 0.5 mM $Zn^{2+}$ with 2.5% Triton X-100. The gels were then incubated overnight in Tris buffer with 1% Triton X-100 and stained with Brilliant Blue R Concentrate the following day. After destaining, zones of gelatin hydrolysis were detected as clear areas against a dark background. Zymographic images were digitized. Negative images were created and quantified by scanning densitometry. Using the digitized images, levels of MMP-2 (72-kD gelatinase A) and MMP-9 were obtained.

### Soluble collagen assay

The Sircol collagen Assay (Biocolor Ltd., Newton Abbey, Northern Ireland) [20] was used as a way to assess soluble collagen levels in the same organ culture fluids. This is a quantitative dye-binding method designed for the analysis of acid soluble collagen released into culture medium by mammalian cells during in vitro culture. Briefly, a collagen standard at four concentrations (5, 10, 25 and 50 μg of the supplied reference collagen solution) and 100 μl of test samples in separate 1.5 ml conical microcentrifuge tubes were mixed with 1.0 ml of the Sircol dye reagent at room temperature for 30 min on a mechanical mixer. The tubes were then centrifuged for 10 min at 10,000 *g* and the supernatants discarded. The remaining pellets were mixed with 1.0 ml of alkali reagent and resuspended using a vortex mixer. The absorbance of samples, standards and assay blanks was measured using a spectrophotometer at the wavelength of 540 nm after 10 min. The values obtained with the culture fluids were compared directly with the values obtained from the control wells and the standard curve.

### Detection of intracellular reactive oxygen species (ROS)

2′,7′-Dichlorodihydrofluorescin diacetate (DCFH-DA, Molecular Probes, Eugene, OR, USA) was prepared as a 10-mM stock solution in DMSO prior to each use. Cells growing in 48-well plates were loaded (30 min, 37°C) with DCFH-DA (100 μM) added directly to culture media. The cells were then washed, placed in fresh DMEM-FBS and treated with 5 mM or 30 mM glucose (5 mM glucose + 10 μM hydrogen peroxide served as a positive control). After 1, 2 and 3 h, the fluorescence of the oxidized product 2′,7′-dichlorofluorescin (DCF) was monitored by flow cytometry using a FACSCalibur (BD Bioscience, San Diego, CA, USA). For each sample, 10,000 events were recorded and the data analyzed to determine the percentage of the total cells in which fluorescence above background levels could be detected.

### Statistical analysis

Differences between groups in experiments with multiple groups were analyzed for statistical significance by ANOVA followed by paired-group comparisons. Where there were only two groups, the Student's *t* test was used. Data were analyzed as paired or unpaired as appropriate.

## Results

### Plasma glucose values and body weights of non-diabetic and diabetic rats

A total of 30 hairless rats were included in the study. Twenty of the rats were made diabetic by giving STZ as described in the Materials and methods section. The diabetic rats were divided into two groups. One group was treated with 100 mg/kg lipoic acid by intraperitoneal injection for 8 weeks as described in the Materials and methods section. The second group of diabetic rats and the group of non-diabetic rats were kept as controls. Mean body weights and blood glucose values for each of the three groups of rats were obtained on day 1 of treatment with lipoic acid, on day 56 of treatment (immediately following the final treatment and before wounding) and immediately before sacrifice (6–10 days later). The values are presented in Table 1.

### Wound formation and healing

At the end of the treatment period, circular abrasion wounds with a surface area of approximately 35 $cm^2$ were created in the paravertebral skin of rats from all three groups. Wound healing was followed and time to

**Table 1** Body weight and blood glucose levels of rats used in wounding experiments

| Treatment group | Body weight | | | Blood glucose | | |
|---|---|---|---|---|---|---|
| | Day 1 | Day 56 | Days 62–64 | Day 1 | Day 56 | Days 62–64 |
| Non diabetic | | | | | | |
| Vehicle | 350 ± 30 | 512 ± 43 | 503 ± 41 | 78 ± 11 | 75 ± 15 | 89 ± 27 |
| Diabetic | | | | | | |
| Vehicle | 347 ± 25 | 393 ± 29 | 384 ± 27 | 365 ± 98 | 406 ± 94 | 408 ± 62 |
| LA | 348 ± 24 | 425 ± 41 | 413 ± 54 | 373 ± 73 | 407 ± 73 | 430 ± 92 |

Values are means and SD based on five to eight rats per group. Blood glucose in mg/dl. Body weight in grams

wound closure was measured as described above. As seen in the upper part of the Fig. 1, the mean wound surface area on day 0 of wounding was similar in all three groups. Wound closure occurred over time in all the three groups. As early as day 2, a difference was observed between the untreated non-diabetic and lipoic acid-treated diabetic rats on the one hand, and the non-treated diabetic rats on the other hand. The difference in closure rates was much greater at days 4 and 6 where the residual wound area was significantly larger in the non-treated diabetic rats than in either of the other two groups. Although only data through day 6 are presented in Fig. 1, wounds in all of the non-diabetic rats and lipoic acid-treated diabetic rats were completely healed by day 8, while wounds in the untreated diabetic rat were still unhealed at this time. In some diabetic rats, wounds were not completely healed by day 10 (not shown). The lower part of the Fig. 1 demonstrates gross features of wounds (day 6) in a single rat from each of the three groups of animals. The still scabbed-over wound on the untreated diabetic rat can be seen, while the wounds of the lipoic acid-treated rat and untreated non-diabetic rat have healed completely.

At the time of wound closure in each rat, skin biopsies were obtained from three sites—from the wound center (most recently healed skin), from the edge of the initially wounded area and from the site beyond the margin of the original wound. Biopsies were stained with hematoxylin and eosin and examined by light microscopy. The control site tissue from all three groups was similar (Fig. 2). The only difference was that the epidermis of the untreated diabetic skin was (on average) thinner than the skin of the untreated non-diabetic rats. Skin from diabetic rats treated with lipoic acid was similar to the skin of untreated diabetic rats—i.e., the epidermis appeared to be thinner than the epidermis of non-diabetic rats. However, overall differences among the three groups of animals were small, and the variability within tissue from the individual rats and variability within each group of rats was such that the differences were not statistically significant.

In comparison to control site tissue, skin from the wound center in all the three groups demonstrated the significant differences. As expected, there was a hyperplasia in the epidermis and a provisional matrix separated the recently-healed skin from the dermis below. Consistent with what we have reported previously [21], the provisional matrix in the skin from untreated diabetic rats appeared to be less well-organized. There were fewer spindle-shaped cells in the provisional matrix from the untreated diabetic rats than in the provisional matrix from the other two groups and a greater number of inflammatory cells. These findings are demonstrated in Fig. 2. Skin from the wound edge was intermediary between the other two skin sites (not shown).

## Lipoic acid protection of endothelial cells against oxidant injury

A series of experiments were conducted to assess the effects on lipoic acid on endothelial cell function in monolayer culture. In the first experiments, rat endothelial cells were cultured for 2 days in DMEM-FBS

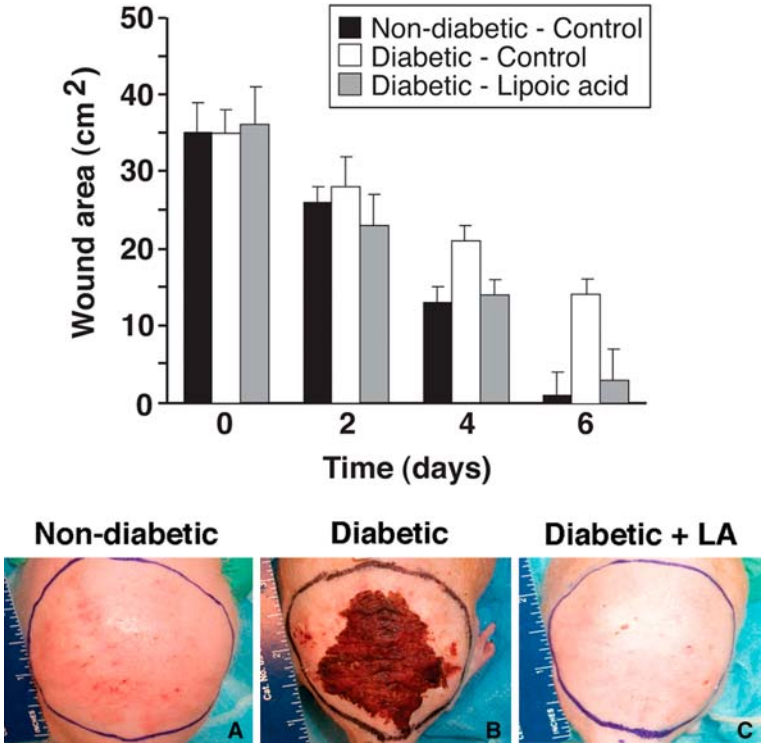


**Fig. 1** *Upper panel* Wound-closure times in untreated non-diabetic rats, untreated diabetic rats and diabetic rats following 8-weeks treatment with lipoic aicd prior to wounding. Values shown are means and SDs based on 5–8 rats per group. Values at each time point were compared by ANOVA followed by paired-group comparisons. Residual wound area in the untreated diabetic rats on days 4 and 6 was significantly greater than residual wound area in the other two groups at the $P < 0.05$ level. *Lower panel* Gross appearance of wounded skin from an untreated non-diabetic rat, an untreated diabetic rat and a diabetic rat treated with lipoic acid. It can be seen that much of the original wound in the untreated diabetic rat still has a scab (day 6 after wounding), while the wounds have completely healed in the skin from the untreated non-diabetic rat and the lipoic acid-treated diabetic rat

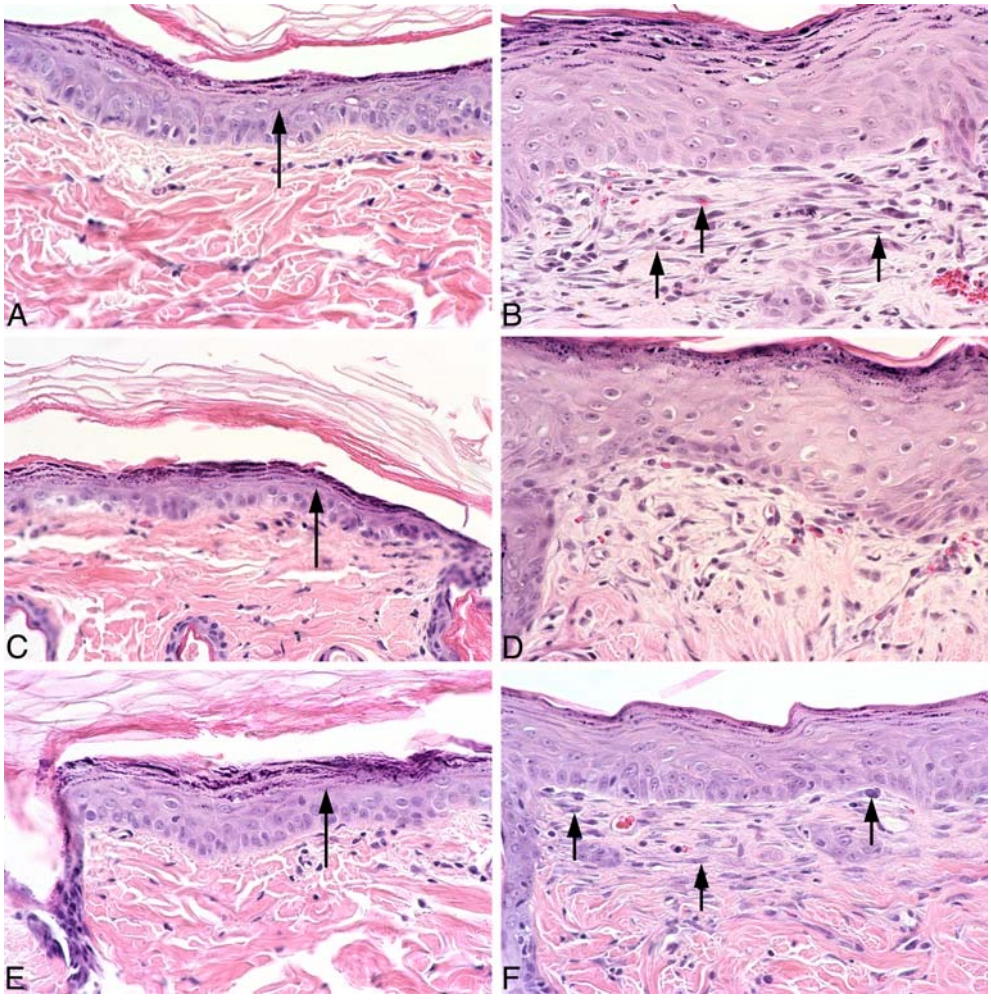


**Fig. 2** Histological appearance of control site (non-wounded) skin (*left panels*) and post-wounded skin (*right panels*) **a,b** Untreated non-diabetic rat; **c,d** untreated diabetic rat; **e,f** diabetic rat treated with lipoic acid; Superficial wounds were created in the paravertebral dorsal skin of each rat as described in the Materials and methods section. At the time of wound closure, the animals were sacrificed and pieces of skin from just beyond the edge of the original wound and from the wound center (most recently healed) were obtained. There was little difference in the appearance of control site skin among the three groups, although the epidermis of the untreated diabetic skin (**c**) was slightly thinner than the epidermis of skin from the other two groups (*long arrows*). In the recently healed skin, the epidermis was thickened in all the three groups while the provisional matrix of untreated diabetic rats was thinner and more loosely organized. There appeared to be more spindle-shaped cells in post-wounded untreated non-diabetic rat skin and post-wounded diabetic rat skin with lopoic acid (indicated by *short arrows* in *b* and **f**) (Hematoxylin and eosin, 330)

containing either 5 mM glucose (low glucose) or 30 mM glucose (high glucose). At the end of the incubation period, cell numbers were assessed and intracellular oxidant levels were determined using DCF fluorescence as a measure of intracellular oxidant load. As shown in the upper panel of Fig. 3, net growth was lower in high glucose-containing medium than in the medium with low-glucose supplementation. Similar results were obtained regardless of whether there was 10% FBS in the culture medium (standard) or whether the FBS level was reduced to 5%. In additional experiments (not shown), cells were grown in DMEM containing 1% FBS as well as in DMEM-BSA. Similar results to those shown here were obtained. That is, in all the cases there were fewer cells in the medium supplemented with high-glucose than in the medium supplemented with low-glucose.

The differences in the growth did not appear to reflect differences in the intracellular oxidant levels. Cells were grown for 2 days in the medium supplemented with 5 mM or 30 mM glucose and then examined for DCF fluorescence after incorporation of DCFH-DA. There was no significant differences in DCF fluorescence between the cells grown under the two conditions (Fig. 3, middle panel).

The lower panel of Fig. 3 shows the effects of lipoic acid on endothelial cell proliferation in the high-glucose (30 mM) medium. At no lipoic acid concentration examined was there growth simulation, but at high concentrations of the agent (greater than 32 mg/ml), slight inhibition of growth was observed.

Figure 4 presents results of experiments in which endothelial cells were exposed to hydrogen peroxide under conditions of low- or high-glucose. For these studies, cells were grown for 2 days in DMEM-FBS in the medium containing either 5 mM or 30 mM glucose. At the end of the incubation period, cells were exposed to various concentrations of hydrogen peroxide for a 2-h period, and the percentage of cells killed was determined. As shown in the upper panel, the percentage of the cells killed by hydrogen peroxide was higher when the cells were grown in medium with 30 mM glucose than in medium with 5 mM glucose.

Next, cells were incubated in medium containing 30 mM glucose and 32 μg/ml lipoic acid. The concentration of lipoic acid was chosen based on the preliminary studies showing that 32 μg/ml was the highest concentration that would not inhibit endothelial cell growth in the same medium. Cells were incubated for

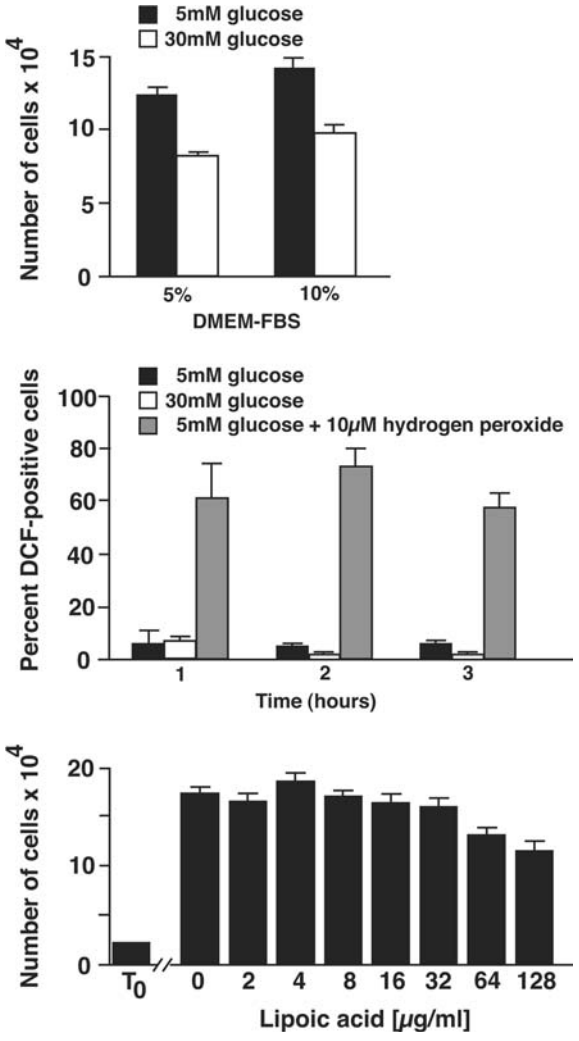


**Fig. 3** Effects of glucose concentration and lipoic acid on endothelial cell function. *Upper panel* Proliferation: growth studies were carried out in 5% and 10% serum-containing DMEM supplemented with 5 mM or 30 mM glucose. Values shown represent mean number of cells per well after 72-h in culture ± SE based on eight separate experiments with duplicate or triplicate samples per data point. Values for high- and low-glucose at each serum concentration were compared using the Student's *t* test. Differences between high- and low-glucose were significant at the $P < 0.05$ level. *Middle panel* Intracellular oxidant levels: DCF fluorescence levels were assessed at the indicated time-points as described in the Materials and methods section. Values shown represent the mean number of cells ± standard deviations with fluorescence levels over baseline values in a single experiment. The experiment was conducted two times with virtually identical results. *Lower panel* Proliferation: growth studies were carried out in 10% serum-containing DMEM supplemented with 30 mM glucose. Cells were concomitantly treated with the indicated concentrations of lipoic acid. Values shown represent mean number of cells per well at time 0 or after 72 h in culture ± SE based on five separate experiments with duplicate or triplicate samples per data point. Values were compared by ANOVA followed by paired-group comparisons. None of the differences between lipoic acid-treated endothelial cells (up to 32 μg/ml) and untreated cells were significant at the $P < 0.05$ level

2 days and then washed several times to remove non-cell-associated lipoic acid. Following this, the cells were exposed to hydrogen peroxide for 2 h. At a high concentration of hydrogen peroxide (100 μM), there was essentially no protection by lipoic acid against lethal injury. However, at lower hydrogen peroxide concentrations (10, 25 and 50 μM), lipoic acid pretreatment offered significant protection against lethal injury. In subsequent experiments (not shown), protection with lipoic acid was shown to be dose-dependent (protection seen at 16 μg/ml but not at lower concentrations) and time-dependent (pretreatment for 1 h was not effective while protection was observed after 24 and 48 h).

## Rat dermal fibroblast function following treatment with lipoic acid

In a final set of experiments, rat dermal fibroblasts were plated in wells of a 24-well dish ($6 \times 10^4$ cells per well) and treated for 2 days with lipoic acid (32 μg/ml) or with buffer alone. At the end of the treatment period, cells were harvested and counted. As seen in Fig. 4 (upper

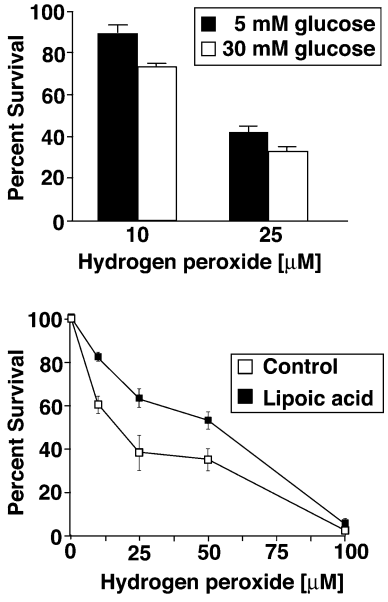


**Fig. 4** Endothelial cell susceptibility to lethal injury by hydrogen peroxide. *Upper panel* Effect of glucose concentration on injury. Cells were incubated in DMEM-FBS supplemented with 5 or 30 mM glucose. After 2 days, cells were exposed for 2 h to 10 or 25 μM hydrogen peroxide. Values shown represent mean number of viable cells ± SEs based on six separate experiments with duplicate or triplicate samples per data point. Values for high- and low-glucose at each serum concentration were compared using the Student's *t* test. Differences between high- and low-glucose were significant at the $P < 0.05$ level. *Lower panel* Cells were incubated in DMEM-FBS with 30 mM glucose and treated with lipoic acid (32 μg/ml) for 48-h. Injury studies were then carried out as described in the Materials and methods section. Values shown represent mean number of viable cells ± SE based on five separate experiments with duplicate or triplicate samples per data point. Values for each concentration of hydrogen peroxide were compared using the Student's *t* test. Differences between lipoic acid-treated endothelial cells and the corresponding group of untreated cells at 10, 25 and 50 μM hydrogen peroxide were statistically different at the $P < 0.05$ level

panel), there was no significant difference in the number of cells recovered from control versus lipoic acid-treated cultures (i.e., no growth response to lipoic acid). In addition to harvesting and counting cells, the culture medium (DMEM-FBS or DMEM-BSA) was collected and assayed for soluble collagen and for MMPs as described in the Materials and methods section. As seen in the middle and lower panels of Fig. 5, lipoic acid treatment had no measurable effect on collagen production or MMP expression.

## Discussion

In healthy skin, abrasion wounds typically heal without consequence. However, in individuals with diabetes (as well as in individuals with other conditions where the peripheral vasculature has been compromised), minor wounds often go on to form slow-healing ulcers with high morbidity [22–25]. In diabetic patients, chronic ulcers of the lower limb and foot often result in amputation. Various approaches have been tried to prevent chronic wound formation in at-risk skin in diabetic patients. Among these are design of optimized footwear, development of strategies for "off-loading" weight from the affected limb, development of new antibiotics, use of growth factors for epithelial, dermal or vascular components of the skin, and the use of proteinase inhibitors, anti-inflammatory agents, and growth-promoting cytokines. The current study suggests that prophylactic use of lipoic acid might also be beneficial. A number of past studies have demonstrated the beneficial effects of lipoic acid in the treatment of diabetic peripheral neuropathy [8–10], but this is the first indication that the same treatment might enhance wound-healing in the skin. To the extent that failure of at-risk skin to heal following minor traumas leads to chronic ulceration, treatment with lipoic acid might counteract the tendency to form the non-healing ulcers that affect the lower limbs of diabetic patients.

The mechanism by which lipoic acid pre-treatment promotes healing of wounds is not fully understood. A number of studies have shown that oxidants contribute significantly to the "chronic wound" environment. It is generally felt that vascular insufficiency results in ischemic conditions and this, in turn, promotes accumulation and activation of oxidant-generating neutrophils and other inflammatory cells [26–28]. As noted above, lipoic acid is a potent scavenger of several oxidant species [1]. Based on this, and on the knowledge that vascular endothelial cells are particularly oxidant-sensitive [29, 30], experiments were conducted to determine if lipoic acid could protect endothelial cells from oxidant injury. The results of these studies were clearly positive. Pre-treatment of rat endothelial cells with lipoic acid (followed by washing) resulted in significant protection against subsequent injury induced by hydrogen peroxide. It is of interest to note that rat endothelial cells maintained in the medium with a high concentration of glucose (30 mM) demonstrated no increase in intracellular oxidant levels (assessed by DCF fluorescence) as compared to the cells maintained in the medium with

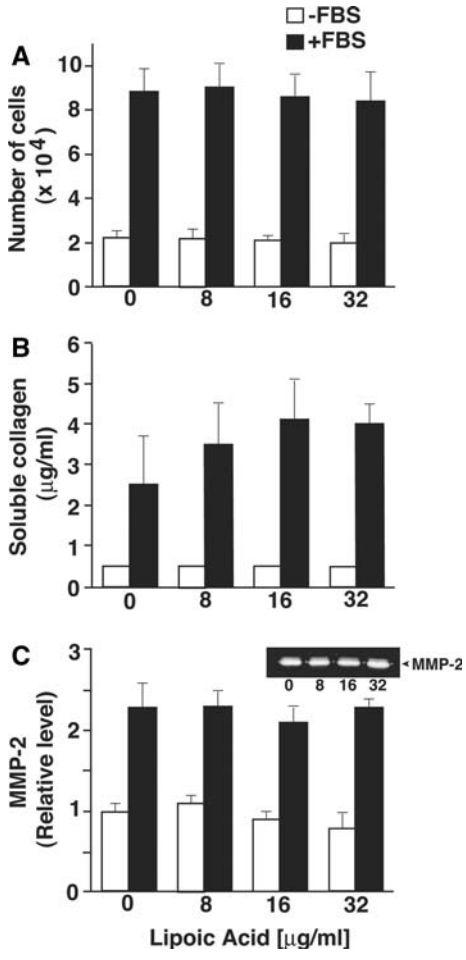


**Fig. 5** Effects of lipoic acid on dermal fibroblast function. Five different isolates of rat dermal fibroblasts were used. Studies were carried out in serum-containing and serum-free DMEM as described in the Materials and methods section. *Upper panel* proliferation. Values shown represent mean number of cells per well at time-zero or after 72-h in culture ± SEs based on five separate experiments with duplicate samples per data point. Seventy-two hour values were compared by ANOVA followed by paired-group comparisons. None of the differences between lipoic acid-treated dermal fibroblasts and untreated cells were significant at the $P < 0.05$ level. *Middle panel* Collagen levels. Soluble collagen was assessed using the Sircol assay as described in the Materials and methods section. Values shown represent microgram of collagen per milliliter after 72 h ± SE based on five separate experiments with duplicate samples per data point. Values were compared by ANOVA followed by paired-group comparisons. None of the differences between lipoic acid-treated dermal fibroblasts and untreated cells were significant at the $P < 0.05$ level. *Lower panel* MMP levels. Gelatin zymography was used to assess MMP-2 levels. Gels were scanned and negative images prepared. Images were quantified by densitometry. Values shown represent number of pixels in the appropriate bands ± SEs based on four separate experiments with duplicate samples per data point. Values were compared by ANOVA followed by paired-group comparisons. None of the differences between lipoic acid-treated dermal fibroblasts and untreated cells were significant at the $P < 0.05$ level

5 mM glucose, but did show increased sensitivity to the cytotoxic effects of exogenous hydrogen peroxide. Taken together, these data are consistent with the hypothesis that endothelial cells maintained in a high-glucose environment undergo physiological changes that do not, in and of themselves, cause oxidant damage, but increase the cells' sensitivity to exogenous oxidants (generated, presumably, in the context of the inflammatory response [17, 18]). Lipoic acid, by virtue of its anti-oxidant activity, counteracts this sensitivity. While consistent with what is currently known, this hypothesis does not rule out other possible beneficial effects of lipoic acid on endothelial cell function or other mechanisms of wound healing. It does not appear, however, that lipoic acid acts to directly stimulate endothelial cell proliferation. No growth-promoting activity was observed when endothelial cells (in high-glucose medium) were examined over a wide range of lipoic acid concentrations.

Likewise, lipoic acid does not appear to modulate fibroblast function. Rat dermal fibroblasts were exposed to lipoic acid at various concentrations and then examined for proliferation, collagen production and MMP levels. No significant effect of lipoic acid was observed on any of these functions. Recently, we showed in the same model of STZ-induced diabetes that topical treatment of the rats with all-trans retinoic acid (RA) improved the healing of similar abrasion wounds (wounded after 8 weeks pretreatment with RA) [21]. Unlike lipoic acid, retinoic acid treatment induced fibroblast proliferation and this was associated with the increased collagen synthesis/decreased MMP elaboration in both rat and human skin [20, 21, 31]. Since topical retinoid treatment is known to promote the same changes in dermal fibroblast physiology in the context of aged and photoaged skin [32–36], it is not unreasonable to suggest that similar changes might occur in diabetic skin. Taken in conjunction with these past studies, the current works suggest that mechanistically, lipoic acid's beneficial effects on wound healing in diabetic skin are different from those of the biologically-active retinoids. Since the two agents have different target cells, apparently different mechanisms of action and different routes of delivery, optimal beneficial effects might be achieved with the two agents in combination. Ongoing studies in our laboratory are addressing this issue.

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