

## The effect of finger millet feeding on the early responses during the process of wound healing in diabetic rats

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### Abstract

In the present study, the role of finger millet feeding on skin antioxidant status, nerve growth factor (NGF) production and wound healing parameters in healing impaired early diabetic rats is reported. Hyperglycemic rats received food containing 50 g/100 g finger millet (FM). Non-diabetic controls and diabetic controls received balanced nutritive diet. Full-thickness excision skin wounds were made after 2 weeks prior feeding of finger millet diet. The rate of wound contraction, and the levels of collagen, hexosamine and uronic acid in the granulation tissue were determined. The skin antioxidant status and lipid peroxide concentration were also monitored during the study. In hyperglycemic rats fed with finger millet diet, the healing process was hastened with an increased rate of wound contraction. Skin levels of glutathione (GSH), ascorbic acid and  $\alpha$ -tocopherol in alloxan-induced diabetic rat were lower as compared to non-diabetics. Altered activities of superoxide dismutase (SOD) and catalase (CAT) were also recorded in diabetics. Interestingly, thiobarbituric acid reactive substances (TBARS) were elevated in the wound tissues of all the groups, when compared to normal (unwounded) skin tissues. However, in diabetic rats the TBARS levels of both normal and wounded skin tissues were significantly elevated ( $P < 0.001$ ) when compared with control (non-diabetic) and diabetics fed with FM. Impaired production of NGF, determined by ELISA, in diabetic rats was improved upon FM feeding and further confirmed by immunocytochemical observations reflects the increased expression of NGF in hyperglycemic rats supplemented with FM-enriched diet. Histological and electron microscopical evaluations revealed the epithelialization, increased synthesis of collagen, activation of fibroblasts and mast cells in FM-fed animals. Thus, increased levels of oxidative stress markers accompanied by decreased levels of antioxidants play a vital role in delaying wound healing in diabetic rats. However, FM feeding to the diabetic animals, for 4 weeks, controlled the glucose levels and improved the antioxidant status, which hastened the dermal wound healing process.

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**Keywords:** Finger millet; Wound healing; Diabetic

### 1. Introduction

Cutaneous wound healing is a self-motivated physiological process of cell regeneration which occurs without any external stimuli. In general, dermal injury is accomplished by fibroplasia, angiogenesis and migration of fibroblasts, endothelial cells and epithelial cells lead to wound contraction [1]. The healing process starts with the formation of granulation tissue and ends with scar formation. The degree

of wound healing is determined by inflammation [2], a vital and protective response offered by the injured cells at wound site that actually starts the process of tissue repair [3].

As a result of inflammation, reactive oxygen species (ROS), such as superoxide anion radical ( $O_2^-$ ) and non-radical hydrogen peroxide ( $H_2O_2$ ), are generated continuously.  $H_2O_2$  produced by the injured endothelial cells and smooth muscle cells exerts a positive role in regulating microvascular blood flow, which facilitates proper nutrient supply for the repairing activities and proliferation of new cells [4].

Normally, the free radicals and other cytotoxic chemical species are effectively detoxified by the scavenging substances called 'antioxidants', and thus perfect healing of

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injured tissues occur. Such a controlled process can be interrupted in disease conditions like diabetes and age-associated biochemical phenomenon [5]. Critical modulation of inflammatory responses by various factors is also evident from the previous studies [2].

Recently ESR spectroscopic studies suggest that patients with diabetes mellitus (DM) are susceptible to increased levels of oxidative stress [6]. ROS are increased in diabetes due to the autoxidation of glucose, advanced glycation and abnormal mitochondrial function [7,8]. It is well documented that healing is impaired and delayed in the diabetic condition [9].

An imbalance between the oxidant and antioxidant defense mechanisms leads to oxidative stress status, which results in lipid peroxidation (LPO), DNA damage, and enzyme inactivation, including free radical scavenger enzymes [10]. Further, oxygen free radicals or oxidants contribute to tissue damage in the events following skin injury and are known to impair healing process [11,12]. Antioxidants, on the other hand, significantly prevent tissue damage and stimulate the wound healing process. However, the molecular mechanisms that delay the healing process are poorly understood.

At present, a number of antioxidants are being developed for clinical use [13]. Though several studies demonstrated the negative role of free radicals on wound healing, antioxidant therapeutic trials against such oxidative stress-mediated interventions have not been adequately documented. Many plant extracts and medicinal herbs have shown potent antioxidant and antidiabetic activities. Flavonoids, phenols and tannins, the major components of plant extracts, act as powerful free radical scavengers [14]. In a previous investigation, we demonstrated the antioxidant effects of methanolic extracts from finger millet *Eleusine coracana* in vitro [15]. Millets were found to have high content of phenolics and tannins [15].

Hence, the current study was taken up to investigate the in vivo antioxidant effects of finger millet supplementation on dermal wound healing process in diabetic-induced oxidative stress-mediated modulation of inflammation. Further, special emphasis was given on the production of nerve growth factors (NGF) in healing-impaired diabetic rats.

## 2. Materials and methods

### 2.1. Chemicals

Thiobarbituric acid (TBA), catalase (CAT), glutathione reductase, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), NGF, NGF-monoclonal antibodies, glutathione (GSH), bathophenanthroline, bovine serum albumin (BSA) and other fine chemicals were purchased from Sigma (St. Louis, MO, USA). Secondary antibodies and other chemicals were of good quality and analytical grade, obtained from standard chemical companies.

### 2.2. Animals

Wistar strain, male albino rats weighing  $160 \pm 10$  g were used for the study. They were maintained in individual metabolic cages, in hygienic air-conditioned ( $25\text{--}27$  °C) room with a 12-h dark and 12-h light cycle; they were fed with corn starch-based balanced nutrient diet and water ad libitum. This study was approved by the Ethics Committee of the Government of India.

### 2.3. Dietary regimen

An isoproteic and isocaloric diet comprised of 10% fat, 20% casein and 65% carbohydrate in the form of cornstarch was formulated as per ISI and NIN guidelines. Adequate minerals and vitamins were also added to the formulated diet. Finger millet (FM) diet was formulated by adding 50 g (for every 100 g feed) of whole grain milled flour instead of corn starch.

### 2.4. Experimental design

The animals were divided into three groups with six or more animals each.

Group I (Control)	Non-diabetic, fed with corn-based, isocaloric and isoproteic diet.
Group II (Experimental)	Diabetic-induced and fed with the above corn-based balanced diet.
Group III (Experimental)	Diabetic-induced and fed with finger millet (FM) diet (isocaloric and isoproteic). FM diet feeding was started 2 weeks before the induction of diabetes.

### 2.5. Induction of DM

Alloxan was used to induce type 1 DM. The animals were fasted for 12 h. Freshly prepared alloxan, 80 mg/kg of body weight, in 0.89% autoclaved saline, was intraperitoneally injected for 2 alternative days. Blood sugar levels were estimated at [16] various time periods, to confirm the development of DM. These animals developed type 1 mild DM in approximately 10–12 days after the first injection of alloxan. Only those animals showing substantial increase in blood sugar due to mild hyperglycemia (fasting blood sugar  $\geq 200$  mg/dl) were considered to have type 1 mild DM. Control animals received only 0.89% saline.

### 2.6. Wound creation and evaluation

The fur on the backside of the rats was shaved under mild ether anesthesia. Subsequently, open excision wounds of standard size ( $1.5 \times 1.5$  cm) were made on the 15th day after the induction of diabetes. The progress of wound healing was evaluated periodically by monitoring the biochemical and histopathological parameters from granulation and skin

tissues obtained at 0, 5, 10 and 15 days after wound creation.

### 2.7. Wound contraction assessment by planimetry

The contour of the individual wounds of both control and experimental animals was periodically measured using transparent graph sheet and the rate of healing was calculated and expressed as percentage contraction [17]. The following formula was used to calculate the percentage of wound contraction.

Wound contraction %

$$= \frac{\text{Wound area day 0} - \text{wound area day}(n)}{\text{Wound area day}(n)} \times 100$$

### 2.8. Biochemical analyses

Collagen in granulation tissue was estimated as per Wossner [18] and hexosamine as per Elson and Morgan [19]. Uronic acid was extracted from the granulation tissue as per Schiller et al. [20] and estimated by the method of Bitter and Muir [21].

### 2.9. Determination of NGF levels from skin samples by ELISA

#### 2.9.1. Extraction of NGF from skin tissues

Skin tissue samples (0, 1, 4, 7, 10 and 15 days) were homogenised with ice-cold PBS buffer containing 0.4M NaCl and subjected to ultrasonic disintegration for 10–20 s. at 4 °C. Then they were centrifuged at 12,500 × g for 60 min, and the supernatants were used for the quantitation of NGF. NGF levels in skin tissue samples were determined with human monoclonal anti-NGF antibody using ELISA procedure as mentioned [22].

### 2.10. Estimation of skin antioxidants/enzymes

Total reduced GSH was determined by the method of Moron et al. [23]. Aliquots of 10% skin homogenates were taken and precipitated by adding 5% TCA and then centrifuged. To 0.5 ml of phosphate buffer (0.2 M), pH

8.0, 0.5-ml supernatant was added, followed by 2.0 ml of DTNB reagent (0.6 mM). The color developed was read at 412 nm against a blank containing 5% TCA instead of sample.

Ascorbic acid was estimated by the method of Omaye et al. [24]. To 1.0 ml of the supernatant solution, obtained after treating with 5% TCA, 0.2 ml of DTC (3.0-g dinitrophenylhydrazine, 0.4-g thiourea and 0.05-g copper sulfate) was added, and incubated for 3 h at 37 °C. Then, 1.5 ml of ice-cold 65% H<sub>2</sub>SO<sub>4</sub> was added, mixed well and allowed to stand at room temperature for an additional period of 30 min. Optical density was measured at 520 nm.

Alpha-tocopherol was estimated by a modified version of the method of Desai [25]. Dried hexane extracts of skin tissue were dissolved in 3.0 ml of absolute ethanol; 0.2 ml of 0.2% bathophenanthroline reagent was added and the contents were mixed thoroughly. The assay was carried out very rapidly from this point. Sufficient care was taken to avoid exposure to direct light. Ferric chloride reagent (0.2 ml; 0.001 M) was added and mixed thoroughly using a vortex mixer. After 1 min, 0.2 ml of orthophosphoric acid reagent was added and the tubes were mixed thoroughly once again. The absorbances of blank (purified ethanol), test and standards were read at 536 nm.

#### 2.10.1. Antioxidant enzymes

Superoxide dismutase (SOD) was assayed according to the method of Misra and Fridovich [26]. It is based on the inhibition of epinephrine-adrenochrome transition by the enzyme. Adrenochrome produced in the reaction mixture containing 0.2 ml of EDTA (0.6 mM), 0.4 ml of sodium carbonate (0.25 M) and 0.2ml of epinephrine (3.0 mM) in a final volume of 2.5ml was measured at 470nm. Transition of epinephrine to adrenochrome was inhibited by the addition of the required quantity of enzyme.

Beers and Seizer [27] method was used to determine the activity of the enzyme CAT. Three milliliters of reaction mixture containing 1.9 ml of phosphate buffer (0.05 M) pH 7.0, 1.0 ml of hydrogen peroxide (5.0 mM) and 0.1 ml of diluted enzyme (skin homogenate) was used in this assay. The activity was measured as the change in OD at 240 nm at 30-s interval for 3 min.

Table 1  
Percentage of wound contraction of control and experimental rats

Groups	Days				
	0	5	10	15	20
Control	(2.25)	15.50 ± 1.15 <sup>a</sup> (1.901)	61.18 ± 3.81 <sup>a</sup> (0.874)	98.91 ± 1.02 <sup>a</sup> (0.024)	–
Diabetic	-do-	8.21 ± 2.10 <sup>b</sup> (2.065)	32.84 ± 4.92 <sup>b</sup> (1.511)	60.13 ± 5.12 <sup>b</sup> (0.897)	83.92 (0.362)
Diabetic + FM	-do-	12.43 ± 2.18 <sup>a</sup> (1.971)	56.83 ± 1.41 <sup>a</sup> (0.971)	93.18 ± 6.12 <sup>a</sup> (0.154)	–

Values are expressed as  $\bar{X} \pm \text{S.D.}$  for six animals in each individual experiment. Within a column, values without a common letter are significantly different from the control group at  $P < 0.05$  as determined by ANOVA. Values given in brackets indicate unepithelialized area in cm<sup>2</sup>.

Table 2

Levels of collagen, hexosamine and uronic acid content (mg/100 mg dry wt) in the granulation tissue of control and experimental groups

Group	5th day	10th day	15th day
<b>Collagen</b>			
Control	3.12 ± 0.03 <sup>a</sup>	14.83 ± 0.71 <sup>a</sup>	9.41 ± 0.48 <sup>a</sup>
Diabetic	2.42 ± 0.04 <sup>b</sup>	8.41 ± 0.81 <sup>b</sup>	5.38 ± 0.44 <sup>b</sup>
Diabetic + FM	3.08 ± 0.02 <sup>a</sup>	12.69 ± 0.68 <sup>a</sup>	8.28 ± 0.31 <sup>a</sup>
<b>Hexosamine</b>			
Control	2.41 ± 0.06 <sup>a</sup>	1.98 ± 0.09 <sup>a</sup>	1.28 ± 0.05 <sup>a</sup>
Diabetic	1.92 ± 0.08 <sup>b</sup>	1.70 ± 0.06 <sup>b</sup>	1.63 ± 0.07 <sup>b</sup>
Diabetic + FM	2.18 ± 0.09 <sup>a</sup>	1.96 ± 0.10 <sup>a</sup>	1.34 ± 0.04 <sup>a</sup>
<b>Uronic acid</b>			
Control	0.86 ± 0.04 <sup>a</sup>	1.58 ± 0.08 <sup>a</sup>	0.81 ± 0.05 <sup>a</sup>
Diabetic	0.51 ± 0.03 <sup>b</sup>	0.62 ± 0.04 <sup>b</sup>	0.60 ± 0.03 <sup>b</sup>
Diabetic + FM	0.77 ± 0.04 <sup>a</sup>	1.38 ± 0.06 <sup>a</sup>	0.79 ± 0.06 <sup>a</sup>

Values are expressed as  $\bar{X} \pm S.D.$  for six animals in each individual experiment. Within a column, values without a common letter (for each parameter) are significantly different from the control group at  $P < 0.05$  as determined by ANOVA.

### 2.11. Determination of skin lipid peroxides (TBARS)

The lipid peroxidation products (as malondialdehyde) were determined by the TBA reaction as described earlier by Draper and Hadley [28]. The lipid peroxide content was expressed as nanomoles of MDA per 100 mg of protein. To 0.5 ml of skin homogenate, 2.0 ml of 20% TCA was added. The contents were mixed well and centrifuged at 4000 rpm for 20 min. Two milliliters of the supernatant was mixed with 2.0 ml of TBA reagent. Reagent blank and standards (5–20 nmol) were also treated similarly. The contents were heated for 20 min in a boiling water bath. The tubes were cooled to room

temperature and the absorbance was read at 532 nm in a Unicam UV–visible double beam spectrophotometer.

### 2.12. Immunocytochemical evaluation

On day 7 after wound creation, newly epithelialized tissues of 0.2–0.5-mm dia were obtained randomly from three rats ( $n=3$ ) in each group. The tissues were gently smoothed and flattened on a piece of thick filter, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 12 h at 4 °C. Then the tissues were embedded in paraffin and 4- $\mu$ m-thick sections were made and placed on gelatin-coated glass slides. The tissues were deparaffinized, rehydrated and washed in PBS (pH 7.4). After pretreatment with a solution of PBS supplemented with 0.3% hydrogen peroxide and 0.1% sodium azide for 10 min, to inhibit endogenous peroxidase, the preparations were washed in PBS twice and then incubated with blocking medium (10% normal goat serum in PBS) for 10 min. Monoclonal anti-NGF diluted 1:4000 in PBS supplemented with 1% BSA was applied and left overnight at 4 °C. After washing in PBS twice, peroxidase conjugated goat-anti rabbit IgG Ab diluted 1:100 in PBS was incubated for 30 min. Visualization of the reaction products was performed with 0.2 mg/ml 3',3' diaminobenzidine tetrahydrochloride in PBS supplied with 0.003% hydrogen peroxide. The tissues were then counterstained with hematoxylin after immunoreaction.

### 2.13. Histological studies

Skin tissues from the wounded site of individual rat was removed immediately after sacrifice. They were then fixed

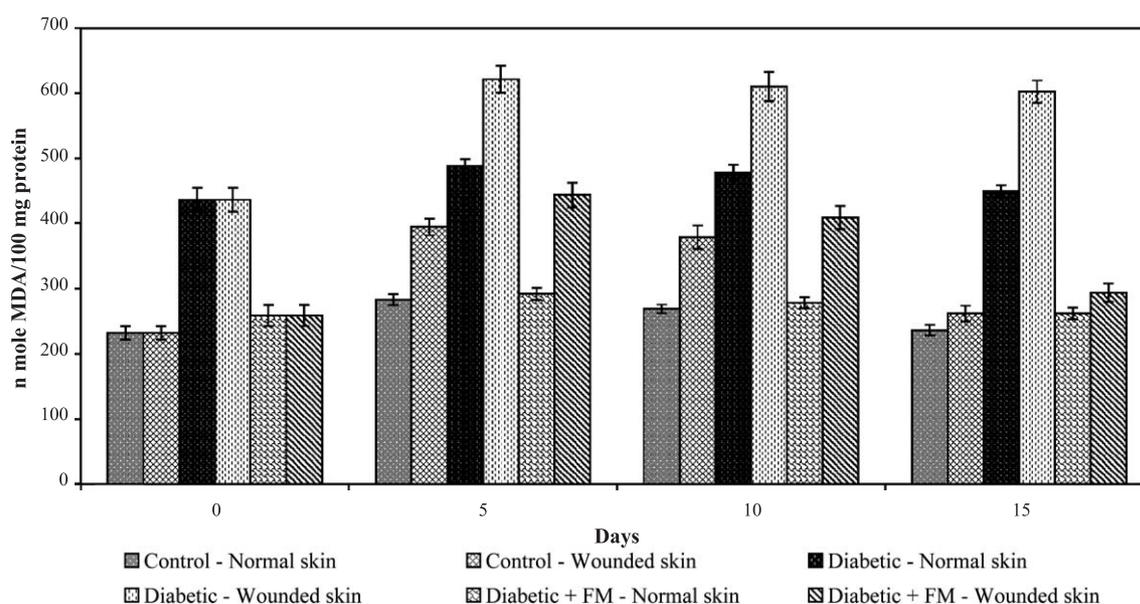


Fig. 1. Levels of TBARS in the skin tissue of control and experimental rats. Values are expressed as  $\bar{X} \pm S.D.$  for six animals in each individual experiment. Variations are significantly different from the control group at  $P < 0.05$  as determined by ANOVA.

Table 3  
Skin antioxidants and antioxidant enzymes of control and experimental rats

Group	0 day	10th day	15th day/ final day
<b>Glutathione</b> ( $\mu\text{g}/\text{mg}$ prot.)			
Control	$2.12 \pm 0.21^a$	$2.42 \pm 0.18^a$	$2.18 \pm 0.13^a$
Diabetic	$1.02 \pm 0.11^b$	$1.12 \pm 0.13^b$	$1.08 \pm 0.09^b$
Diabetic + FM	$1.83 \pm 0.22^a$	$2.10 \pm 0.14^a$	$2.02 \pm 0.10^a$
<b>Ascorbic acid</b> ( $\mu\text{g}/\text{mg}$ prot.)			
Control	$1.84 \pm 0.11^a$	$1.73 \pm 0.10^a$	$1.78 \pm 0.12^a$
Diabetic	$0.93 \pm 0.08^b$	$0.88 \pm 0.07^b$	$0.89 \pm 0.09^b$
Diabetic + FM	$1.68 \pm 0.07^a$	$1.60 \pm 0.06^a$	$1.62 \pm 0.10^a$
<b>Vitamin E</b> ( $\mu\text{g}/\text{mg}$ prot.)			
Control	$1.08 \pm 0.06^a$	$0.93 \pm 0.04^a$	$0.98 \pm 0.03^a$
Diabetic	$0.83 \pm 0.07^b$	$0.72 \pm 0.08^b$	$0.70 \pm 0.07^b$
Diabetic + FM	$0.99 \pm 0.05^b$	$0.87 \pm 0.05^{ab}$	$0.91 \pm 0.06^a$
<b>Superoxide dismutase</b> (Units/mg protein)			
Control	$1.28 \pm 0.09^a$	$1.88 \pm 0.06^a$	$1.31 \pm 0.06^a$
Diabetic	$1.36 \pm 0.07^a$	$1.39 \pm 0.10^b$	$1.28 \pm 0.05^a$
Diabetic + FM	$1.39 \pm 0.08^a$	$1.69 \pm 0.04^a$	$1.43 \pm 0.11^a$
<b>Catalase</b> ( $\mu\text{mol H}_2\text{O}_2$ cons/min/mg prot.)			
Control	$1.43 \pm 0.12^b$	$1.86 \pm 0.10^a$	$1.58 \pm 0.11^b$
Diabetic	$1.68 \pm 0.18^a$	$1.88 \pm 0.11^a$	$1.76 \pm 0.12^a$
Diabetic + FM	$1.49 \pm 0.13^b$	$1.72 \pm 0.13^a$	$1.61 \pm 0.09^{ab}$

Values are expressed as  $\bar{X} \pm \text{S.D.}$  for six animals in each individual experiment. Within a column, values without a common letter (for each parameter) are significantly different from the control group at  $P < 0.05$  as determined by ANOVA.

in 10% buffered formalin, dehydrated through graded alcohol series (30–100%), cleared in xylene and embedded in paraffin wax (m.p.  $56^\circ\text{C}$ ). Serial sections of 5- $\mu\text{m}$  thickness were cut using microtome, and stained with

hematoxylin–eosin. Then they were examined under light microscopy.

#### 2.14. Transmission electron microscopic (TEM) studies

The healed skin tissue specimens from control and experimental rats were fixed in 3% glutaraldehyde, buffered with 0.1 M sodium carbonate, pH 8.0, containing 0.2 M sucrose. Post-fixation was performed with 1% osmium tetroxide in the same buffer for 2 h at  $4^\circ\text{C}$ . Embedding was done in a mixture based on Epon 812, after dehydration in ethanol. Ultrathin sections were poststained with 2% aqueous uranyl acetate and lead acetate. Then the specimens were examined in a Phillips 201 C Electron Microscope at 60 kV and photographed.

#### 2.15. Statistical analysis

Values are expressed as mean  $\pm$  S.D. for six animals in each group from each experimental setup. Analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple comparison test was used to determine the statistical significance.  $P$  values less than 0.05 were considered significant [29].

### 3. Results

#### 3.1. Blood glucose/body weight changes

Blood glucose content of diabetic rats was found to be  $197.08 \pm 2.78$  mg/dl, which is significantly ( $P < 0.05$ ) higher than the control group. Feeding of FM diet to the diabetic animals resulted in the reduction of glucose levels

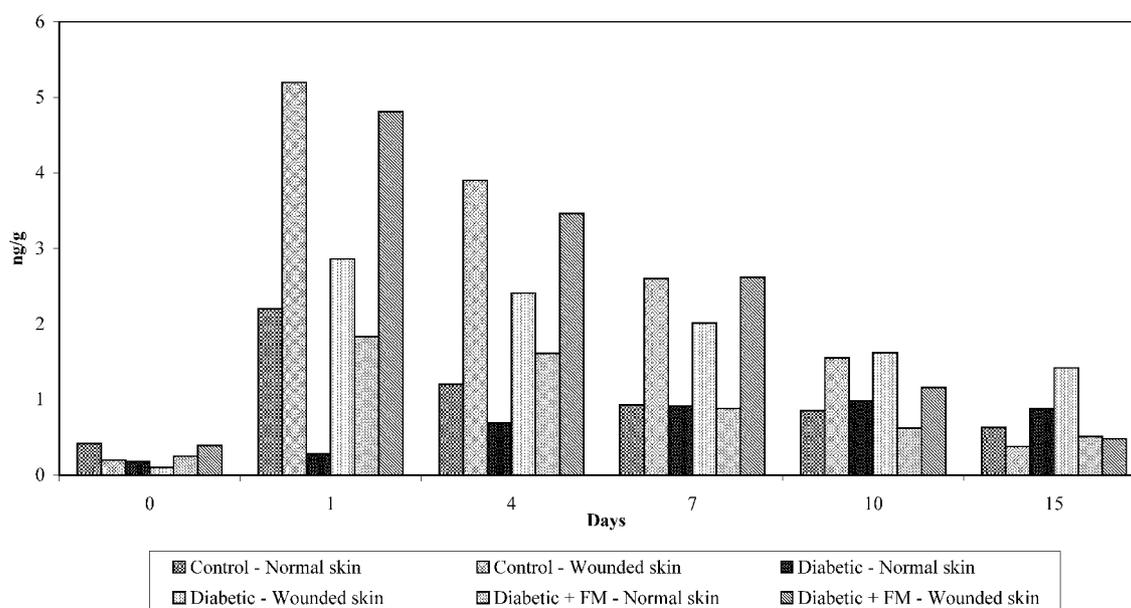


Fig. 2. Skin NGF levels determined using ELISA. The values are the average of triplicates ( $n = 3$ ).

to near control values,  $115.74 \pm 2.77$  mg/dl. It was recorded that the body weight gained by the diabetic animals was 50% less than the weight gained by the control and FM-fed animals during the study period.

### 3.2. Period of healing

The macroscopic evaluation of the wound revealed that the diabetic group required a total period of about 20 days for complete epithelialization and healing, whereas the control and FM diet-treated groups took about 14 and 15 days, respectively. At the end of 15th day, the healing was almost complete in control rats, whereas in the diabetic rats, only 60% healing was recorded ( $P < 0.05$ ). The healing was irregular in diabetic animals compared to other groups.

### 3.3. Wound contraction

Table 1 depicts the rate of wound contraction in control, diabetic and diabetic+FM diet-fed animals. Periodical monitoring of wounds during the healing process showed a significant increase ( $P < 0.05$ ) in the rate of wound contraction in control and FM diet-fed diabetic rats when compared to diabetic group. On the 15th day after wound creation, control wounds contracted completely (about 99%), whereas the diabetic wounds contracted only about 60%. Upon FM diet feeding, the diabetic animals showed an almost similar pattern of wound contraction (about 93%) as seen in the non-diabetic control animals.

### 3.4. Biochemical parameters

Table 2 depicts the collagen, hexosamine and uronic acid contents of control and experimental rats on different days after wound creation. The collagen content in control group was significantly ( $P < 0.05$ ) higher than that of diabetic group whereas the FM-fed animals were found to have near control values.

Hexosamine content (Table 2) of granulation tissue gradually decreased during the course of wound healing in all the groups. However, significant ( $P < 0.05$ ) changes between control and diabetic groups were noted.

A significant ( $P < 0.05$ ) increase in the concentration of uronic acid was noted (Table 2) on the 10th day in control and FM-fed groups, whereas diabetic animals showed no significant ( $P > 0.05$ ) change.

### 3.5. Lipid peroxides

Fig. 1 indicates the lipid peroxide levels. As the wound healing proceeds, the lipid peroxidation products were elevated in all the groups. At the day 15, levels of TBARS restored to near normal in control and FM-fed animals, whereas in diabetic animals, the TBARS level continued to be higher due to impaired healing.

### 3.6. Antioxidant status

The levels of endogenous antioxidant and antioxidant enzymes in the skin are given in Table 3. Significant ( $P < 0.05$ ) decrease in the antioxidant system was noted in diabetic animals when compared with other groups. Slight increase in the levels of GSH of control and experimental animals was noted during the process of wound healing (10th day). Wound healing process was found to be delayed in diabetic rats due to the decrease in the levels of antioxidants, when compared with control and FM-fed animals. The activities of antioxidant enzymes (SOD, CAT) were found to be increased in control and FM-fed diabetic groups, during the wound healing process. In diabetic animals, no significant change in the activity of SOD was noted, whereas the activity of CAT was found to be slightly increased.

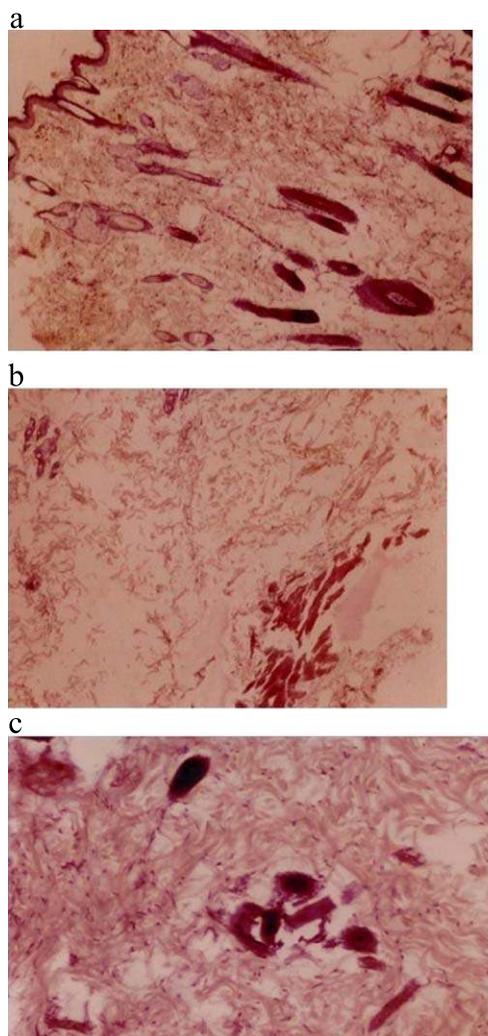


Fig. 3. Immunocytochemical expression of NGF by skin tissues of (a) control—showing intensive positive reaction (+++), (b) diabetic—showing poor reaction to NGF monoclonal antibodies (+), and (c) diabetic+FM fed—showing the positive reaction as seen in control skin ( $\times 400$ ). (For color see online version).

### 3.7. NGF levels in skin tissues

Local production of NGF by the wounded sites was evaluated on different days after wounding (Fig. 2). A very low or non-detectable level of NGF was recorded in the skin

tissues obtained during wound creation (0 h). Comparatively, low concentrations of NGF were detected in unwounded skin tissues than in the wounded tissues. However, the dermal wounding resulted in increased NGF levels upon healing. A maximum of 5.2 ng/g of NGF was recorded on

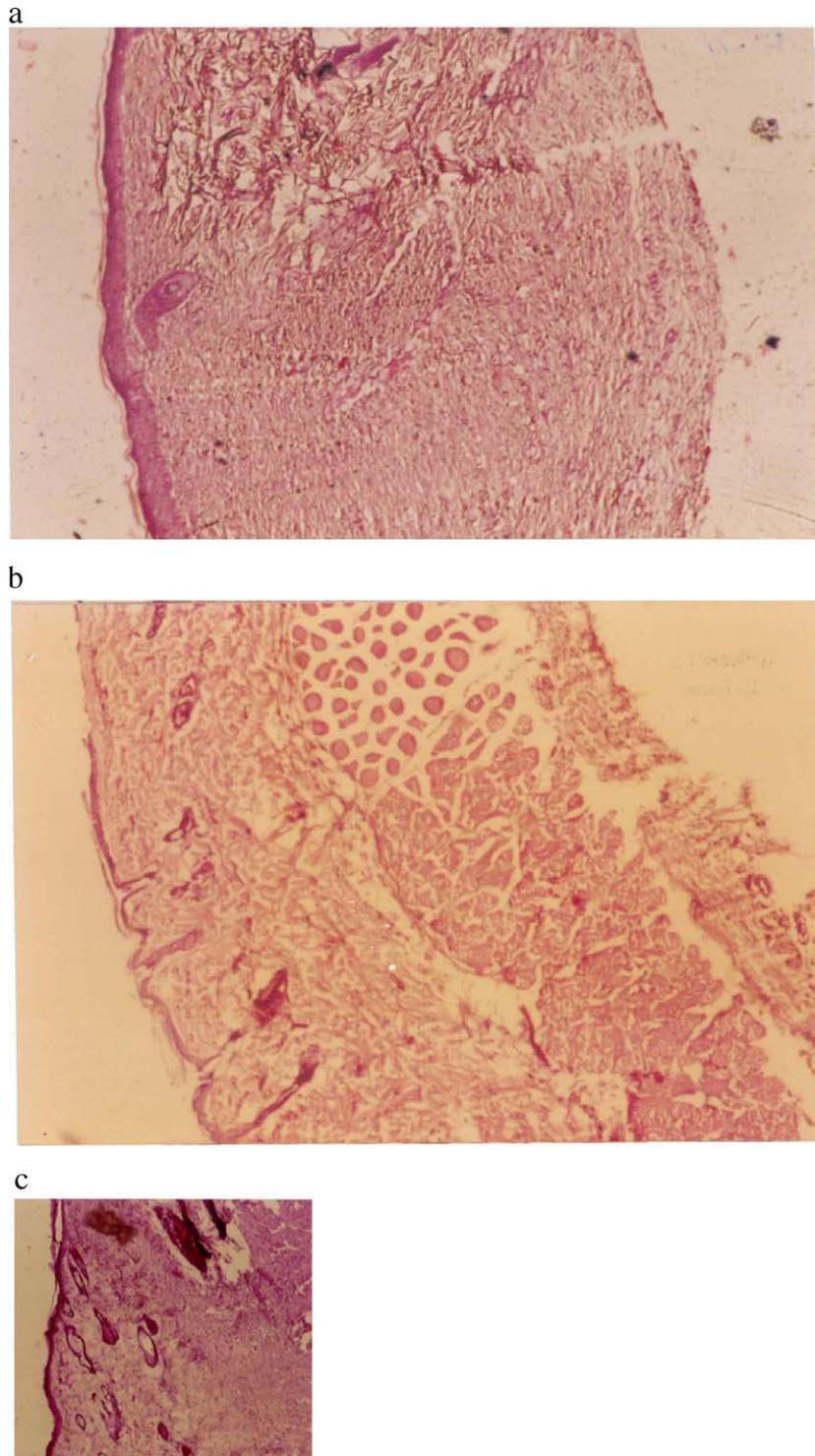


Fig. 4. Histology of wounds stained with hematoxylin–eosin ( $\times 400$ ), on 15th day after wounding. (a) Control—with collagen bundles and complete re-epithelialization, (b) diabetic—with minimal collagen formation and poor epithelialization, (c) diabetic+FM—fed with complete re-epithelialization and densely packed collagen. (For color see online version).

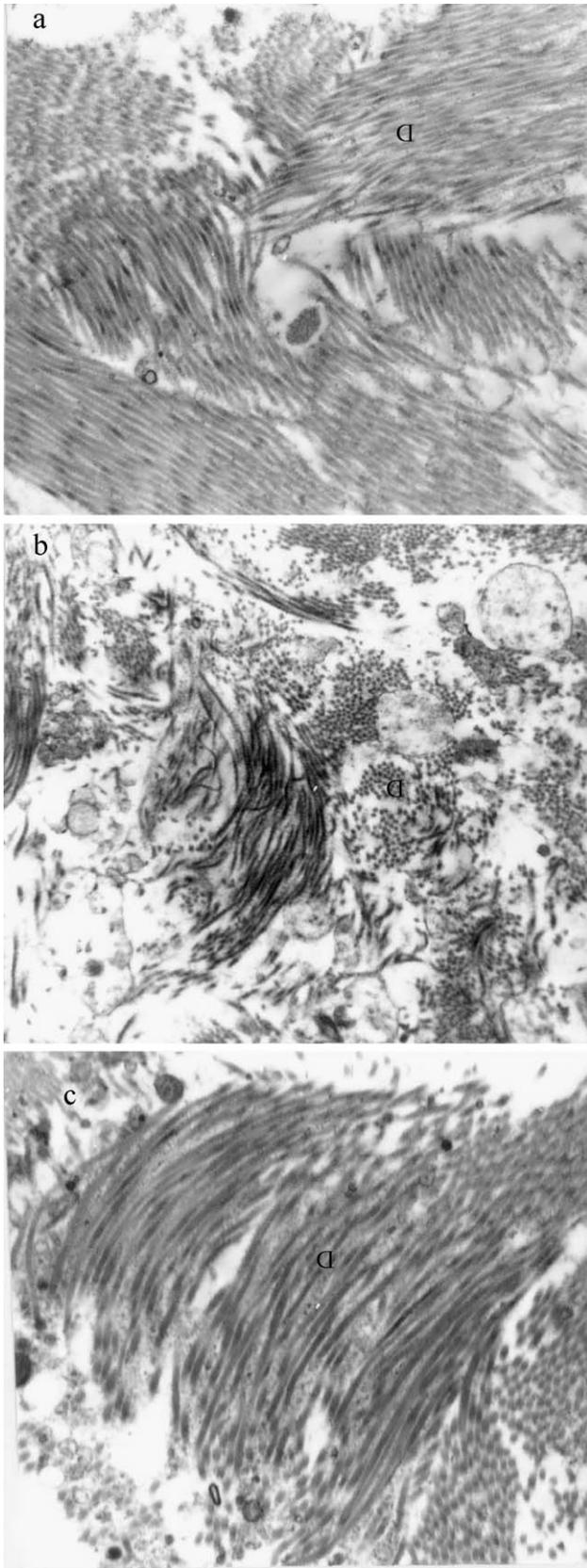


Fig. 5. Ultrastructure of dermal wounds ( $\times 10,000$ ) showing the arrangement and distribution of collagen bundles in (a) control, (b) diabetic, and (c) diabetic+FM-fed groups.

the first day after wounding in control rats. As the healing progressed, the NGF levels were found declining to near 0 h values, whereas in diabetic animals a maximum of 2.86 ng/g was reached, which was significantly ( $P < 0.05$ ) lower as compared to the other groups.

### 3.8. Immunocytochemistry

Impaired wound healing in diabetic rats was associated with low levels of NGF expression. Newly epithelialized skin tissues obtained from the control rats on the 7th day after wounding showed intensive (+++) immunoreaction against human monoclonal anti NGF-antibody (Fig. 3a), whereas in diabetic wounded skin, the reaction was found to be reduced (+) due to the impaired production of NGF by the cells with hyperglycemic and oxidative stress environment (Fig. 3b). FM feeding to the diabetic rats resulted in an elevated expression of NGF as observed in controls (Fig. 3c).

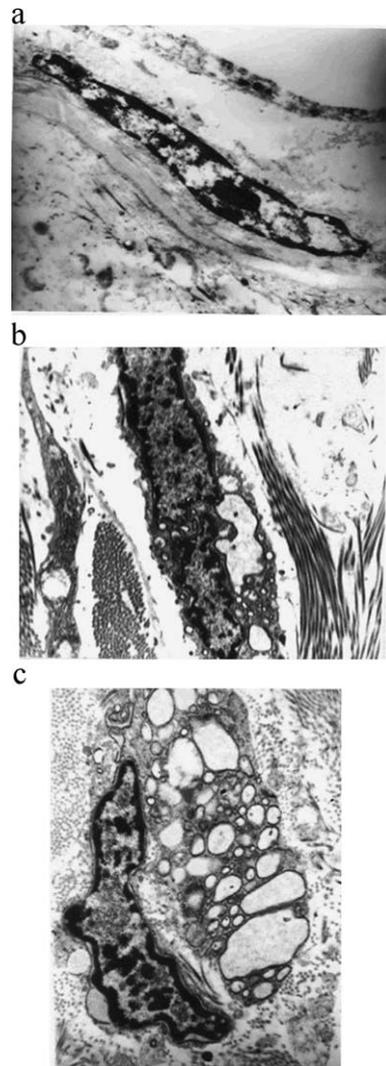


Fig. 6. TEM pictures show ( $\times 4500$ ) the fibroblast of (a) control, (b) diabetic, and (c) diabetic+FM-fed rat wound.

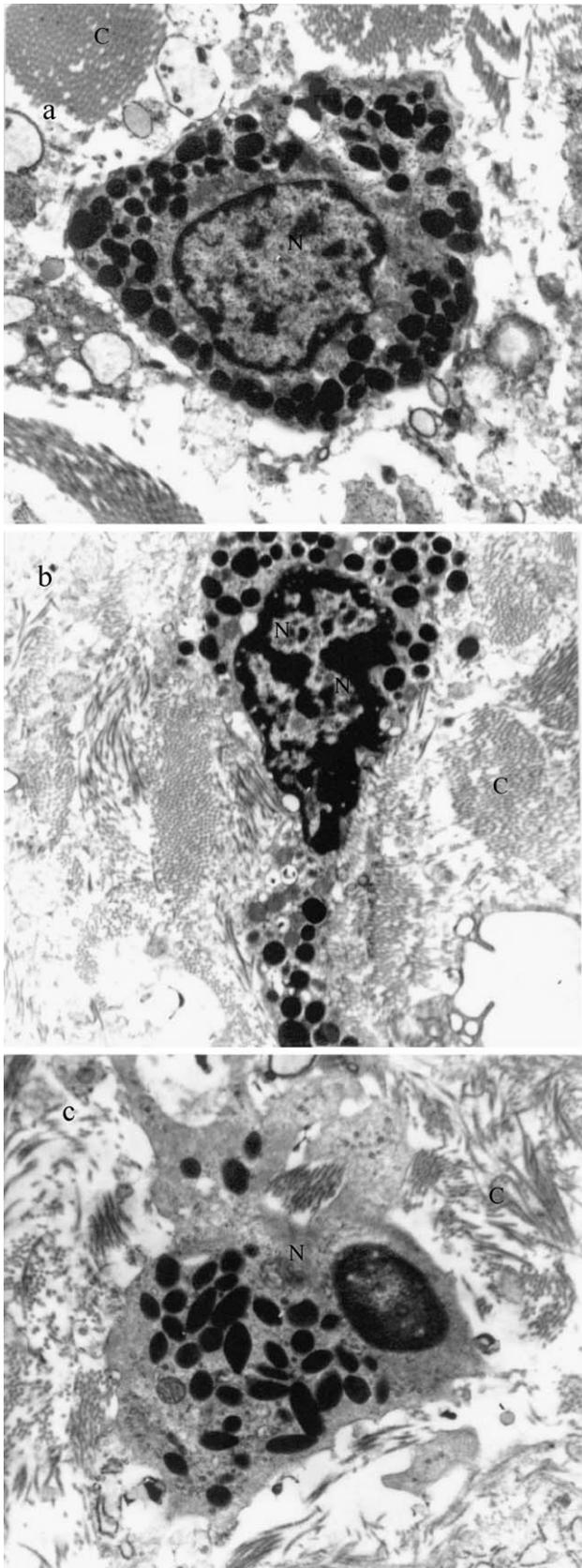


Fig. 7. Ultrastructure of the mast cells ( $\times 4500$ ) in dermal wounds of (a) control—showing normal architecture, (b) diabetic—with degranulation, and (c) diabetic + FM-fed—showing normal mast cell with intact granules. C—Collagen fibers N—Nucleus.

### 3.9. Histology

Fig. 4 shows the wound histology of healed skin tissue on day 15. In control specimen, completely epithelialized, matured keratinocytes were seen with densely packed collagen fibers arranged in parallel fashion (Fig. 4a). Uniform distribution of fibers was also noted. Diabetic wound tissues appeared with incomplete epithelialization and irregularly arranged, less dense collagen fibers. Also noted were the inflammation and reduced fibroblast cell population in these tissues (Fig. 4b). In the case of FM diet supplemented group, the above structural abnormalities were much reduced and the architecture of control skin tissue is mimicked (Fig. 4c).

### 3.10. Electron microscopic studies

#### 3.10.1. Collagen

Collagen fibers from control specimens (Fig. 5a) appeared more packed and presumably better aligned, which suggests the beginning of macromolecular organization of granulation tissue. In the granulation tissue of diabetic animals, collagen content was reduced, and collagen fibers were loosely packed and irregularly arranged. Collagen was sparse and immature (Fig. 5b). The granulation tissue of FM-fed animals was found to have an increased-collagen content, even though fibers were still immature (Fig. 5c).

#### 3.10.2. Fibroblast

In addition to the histological examinations, the ultrastructural details of fibroblasts from control, diabetic and FM supplemented groups are given in Fig. 6. Control wounds show intact fibroblasts with regular shape and densely packed materials (Fig. 6a). In the case of diabetic wounds, fibroblast were irregular in shape with loosely packed matrix and they were found to be inactive (Fig. 6b). The granulation tissue obtained from the FM-fed group showed similar fibrillar arrangement as seen in the non-diabetic controls (Fig. 6c).

#### 3.10.3. Mast cells

Mast cells from the control specimens contained numerous homogeneous electron-dense granules (Fig. 7a), but mast cells of diabetic wounded skin specimens possessed few dense granules with cytoplasmic vacuoles containing flocculent materials. It was also noted that plasma membrane disintegrated and the electron-dense granules were discharged from the mast cell of diabetic skin tissue (Fig. 7b). In the case of FM-fed animals, the mast cell contains similar architecture with homogeneous electron dense granules (Fig. 7c).

## 4. Discussion

Oxidants are involved in many pathological conditions and ageing processes. These free radicals are highly toxic

to tissues and may also result in the generation of other free radicals. It has been postulated that free oxygen radicals contribute to the cellular damage in the zone of stasis, which leads to necrosis and conversion of superficial wound into a deeper wound [30]. We examined the oxidative stress-mediated impaired cutaneous wound healing in mild hyperglycemic rats and its reversal by FM diet supplementation.

In general, cutaneous wound healing involves a complex series of physiological processes that include inflammation, proliferation and migration of different cell types, angiogenesis, matrix synthesis, collagen deposition leading to re-epithelialization, neovascularization and formation of granulation tissues [9,31].

In the current study, the healing of skin wounds ( $1.5 \times 1.5 \text{ cm}^2$ ) was delayed significantly in diabetic rats, which may be correlated with the elevated glucose levels ( $\geq 200 \text{ mg/dl}$ ) measured in the blood plasma of these animals. As expected, the diabetic conditions had a deleterious influence on the wound healing process through abnormal physiological response. Significant decrease in the body weight gained by the diabetic animals indicates impaired metabolic activities and may also be due to irregular absorption and elimination of wastes. The hyperglycemic status triggered the animals to become catabolic, breaking down protein and fat stores for energy, as glucose is not accessible to cell nutrition, which had not supported the actual healing process. Involvement of oxidative stress is implicated in the progression of complications of DM, which may also lead to secondary complications such as hepatotoxicity, nephropathy, neuropathy and coronary heart disease [32–34].

Deep skin wounds in non-diabetic cases heal by contraction and granulation tissue formation [35]. In contrast, diabetic wound closure is predominantly due to the formation of granulation tissue and re-epithelialization [36]. It is evident from this study that the rate of wound contraction in diabetic animals was slow when compared with non-diabetics and diabetics fed with FM diet (Table 1) and this can be attributed to the delayed healing as reported earlier [35,36]. One of the reasons for this could be the difference in the levels of NGF found in the injured tissues of diabetic and non-diabetic individuals (Fig. 2). After injury, non-diabetics initially showing much higher NGF levels came down significantly by day 7 to allow contraction of epithelial gap. In the diabetic animals, levels of NGF initially remained low and then rose slightly (Fig. 2). These results are well correlated with the levels of PDGF recorded in the non-diabetic and diabetic injured human subjects [37,38]. In the present investigation, NGF production in the wounded skin was found to be diminished in alloxan-induced diabetic rats. In agreement with the previous studies [9], impaired NGF production is attributed to the mild hyperglycemic condition and elevated production of ROS and other  $\text{OH}^\cdot$ , which might have caused damage to the NGF producing

cells. It could be suggested that the deprived defense mechanisms in the skin of diabetic rats would have reflected in the fibroblast, keratinocytes and macrophages to facilitate poor synthesis of NGF and its release. Our recent study [39] and several other studies [9,40,41] reveal the vital role of NGF in wound healing process. FM feeding to the rats was found to normalize the NGF production through controlling the blood sugar levels and balancing the antioxidant status.

It has been demonstrated that increased levels of ROS can also have a series of negative effects. Usually at low concentration,  $\text{H}_2\text{O}_2$  and other ROS inhibit proliferation and migration of various cell types, like keratinocytes [42]. At high concentrations, ROS can induce severe tissue damage and even lead to neoplastic transformation [43]. In line with the above, our present findings show significantly increased TBARS levels, indicating the lipid peroxidation (LPO) in diabetic rats. Thiobarbituric acid reactive substances (TBARS) were elevated in the wounded tissues of all the groups (Fig. 1), which indicates the excess production of  $\text{H}_2\text{O}_2$  and ROS during inflammation process at the wound site. Following tissue injury, adequate inflammatory vascular responses are essential for the repair [2,10,44]. However, 10th and 15th day wound tissues of diabetic rats showed highly significant (Fig. 1) increase in the TBARS content when compared to control and FM-fed animals. Thus, increased levels of oxidative stress markers accompanied by decreased levels of antioxidants play a vital role in delaying wound healing in diabetic rats as previously reported by Shukla et al. [45].

Took [46] had reported that poor diabetic control increases the rate of free radical production, which contributes to the widespread damage of the vessel walls. It has also been documented that people with diabetes were found to have decreased concentrations of ascorbic acid, a well-known antioxidant that scavenges free radicals [47]. In correlation with this, the observed decreased levels of antioxidants, GSH, ascorbic acid and  $\alpha$ -tocopherol, might have contributed to diminished defense mechanisms against free radical overload in diabetic rat skin. The role of SOD and CAT during the process of wound healing has been reported [48]. The current study also examines the activities of these enzymes in modulating repair of full thickness dermal wounds in diabetic animals. Our results showed that the activities of these enzymes were increased (Table 3) upon onset of healing and found to be normalised upon complete healing in control and FM-fed rats; whereas in the diabetic rats, activities of both enzymes decreased significantly; this might have extended the inflammation, which leads to further production of ROS and delayed healing responses. It is also known that the overproduction of ROS is toxic to these enzymes [49]. Prior feeding of FM-enriched diet to the rats before induction of alloxan had prevented the above changes. It is attributed that the phenolic antioxidants present in the

FM partially protected the insulin-producing cells from alloxan-mediated cell damage, and hence promoted the healing process.

The characteristics observed in the skin specimens from diabetic animals, which were more severe in comparison with those from control and FM supplemented animals, suggested that the oxidative stress created in diabetic animals has potentiated the deleterious effects of DM on fibroplasia. A significant reduction in collagen fibers content in the granulation tissue from diabetic animals was observed by Devlin et al. [50], Tengrup et al. [51] and Spanheimer et al. [52]. Thus, it is proposed in this study that the characteristic changes noted in the histology of diabetic groups were probably in response to an altered extracellular environment, e.g., high glucose, persistent inflammation, excess H<sub>2</sub>O<sub>2</sub> production and declined anti-oxidant status. Since collagen is synthesized by fibroblasts [53], which in turn are vulnerable to the effects of free radicals, we might suppose that the decrease in reactive collagen content may be related to a reduction in the population of fibroblasts in the diabetic tissue, as well as to functional alterations in these cells. During the period in which wound healing was evaluated, it was possible to observe that DM caused a decrease in the collagen content of the granulation tissue, which was more regulated in the case of FM-fed diabetic animals (Table 2). With regard to the macromolecular organization of the granulation tissue, the initial stage of this process was delayed and extended till 22 days in the specimens obtained from diabetic animals, in relation to control and FM supplemented animals.

By this study, delayed healing of diabetic skin wounds could be attributed to the altered inflammatory and repair processes. The mechanisms underlying the anti-oxidative effects of the components of FM on diabetes and tissue repair are beyond the scope of this report and remain to be elucidated further. However, based on the current study, we can speculate that one of the underlying mechanisms could be related to modulation of antioxidant status by the FM-enriched diet. One of the reasons for the anti-oxidative property of FM could be their selenium content. Vital roles for selenium in several antioxidant enzymes functions have been reported [54].

In conclusion, our results altogether suggest that diabetic conditions have deleterious influences on the healing of skin wounds due to impaired production of growth factors and poor antioxidant status. FM feeding to the animals was found to have a protective role against alloxan induction of diabetes and its causes. The anti-oxidative activities observed here could be ascribed to several factors, including the structures of the different phenolic compounds, the anti-oxidative mechanisms exhibited by the compounds and, possibly, also due to the synergistic effects of different compounds. Further studies are underway to reveal the molecular mechanism involved in the action of dietary phenolic antioxidant compounds.

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