Glucose Oxidase Incorporated Collagen Matrices for Dermal Wound Repair in Diabetic Rat Models: A Biochemical Study

V. ARUL,[1,2] J. G. MASILAMONI,[1,3] E. P. JESUDASON,[1] P. J. JAJI,[1] M. INAYATHULLAH,[1,4] D. G. DICKY JOHN,[1,5] S. VIGNESH[1] AND R. JAYAKUMAR[1,6,*]

[1]*Bio-Organic and Neurochemistry Laboratory, Central Leather Research Institute, Adyar, Chennai 600020, Tamil Nadu, India*

[2]*Department of Pediatrics, University of Alberta, Edmonton, AB, Canada*

[3]*Department of Neurology, Yerkes National Primate Research Center Emory University, 954 Gatewood Rd., Atlanta, GA 30329, USA*

[4]*Department of Neurology, David Geffen School of Medicine University of California, Los Angeles, CA 90095, USA*

[5]*Department of Bioinformatics, Sri Ramachandra University Chennai 600116, India*

[6]*Department of Neurology and Neurological Sciences, Stanford University School of Medicine, Hagey Laboratory of Regenerative Medicine, 257 Campus Dr, Stanford, CA 94305, USA*

ABSTRACT: Impaired wound healing in diabetes is a well-documented phenomenon. Emerging data favor the involvement of free radicals in the pathogenesis of diabetic wound healing. We investigated the beneficial role of the sustained release of reactive oxygen species (ROS) in diabetic dermal wound healing. In order to achieve the sustained delivery of ROS in the wound bed, we have incorporated glucose oxidase in the collagen matrix (GOIC), which is applied to the healing diabetic wound. Our *in vitro* proteolysis studies on incorporated GOIC show increased stability against the proteases in the collagen matrix. In this study, GOIC film and collagen film (CF) are used as dressing material on the wound of streptozotocin-induced diabetic rats. A significant increase in ROS ($p < 0.05$) was observed in the fibroblast of GOIC group during the inflammation period compared to the CF and control groups. This elevated level up regulated the antioxidant status in the granulation tissue and improved cellular proliferation in the GOIC group. Interestingly, our biochemical parameters nitric oxide, hydroxyproline, uronic acid, protein, and DNA content in the healing wound showed that there is an increase in proliferation of cells in GOIC when compared to the control and CF groups. In addition, evidence from wound contraction and histology reveals faster healing in the GOIC group. Our observations document that GOIC matrices could be effectively used for diabetic wound healing therapy.

KEY WORDS: wound healing, collagen, glucose oxidase, ROS, NO, antioxidant, proteolysis.

*Author to whom correspondence should be addressed.
E-mail: jayraja@stanford.edu
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INTRODUCTION

Wound healing is a process consisting of an orderly sequence of events that reconstruct the structural and functional integrity of the damaged tissue [1]. The initial wound goes through a chain of programmed, interdependent responses to the injury, including inflammation, epithelialization, angiogenesis, and accumulation of extracellular matrix. In most of the cases, the complication in wound healing is due to inflammation-related cellular damage in the healing site. Because diabetic wounds have noticeably lower levels of several growth factors, these factors have been a focus to enhancing the repair of the wounds [2]. These growth factors are mostly produced during the inflammatory response.

During the early inflammatory phase [3], neutrophils and macrophages are attracted to the injured tissue by various chemotactic factors and reactive oxygen species (ROS) are released to kill bacteria and prevent infection [4]. The oxidants serve as redox regulators of transcription factors such as p53, Ap1, NF-κB [5], and SP1 [6]. Emerging evidence suggest that ROS at optimum concentrations may function as signaling intermediates of cellular response [6] and stimulate the release of transforming growth factors [7–9], epidermal growth factor (EGF) and basic fibroblast growth factors [7], and G protein-coupled receptors such as angiotensis II [10] and lysophospatidic acid [11,12]. On the other hand at higher concentrations, ROS can induce severe tissue damage and even lead to neoplastic transformation [13]. In order to safe guard normal cellular function, survival cells have

developed a serious of defensive mechanisms that regulate this redox state. This includes antioxidant enzymes superoxide dismutase (SOD), catalase, glutathione (GSH) peroxidase, GSH reductase, and other endogenous free radical scavengers like GSH, ascorbic acid, and α-tocopherol [14]. Hence, the improved quality of wound healing could be achieved by properly orchestrating the conflicting effects of ROS [15] in the healing bed [16,17].

Diabetes causes delayed wound healing through several proposed mechanisms [18], mostly related to defects in the inflammatory response [19] leading to inadequate and uncoordinated local production of growth factors and cytokines [20] and collagen synthesis [21]. Several groups of researchers are focused on the development of therapy that will promote healing in diabetic complications and thereby reduce the cost of hospitalization and save the patient from amputation or other severe complications. Limited literature, however, exists on the role of ROS in modulating the inflammatory response during the early or late phases of acute inflammation [22–27].

The streptozotocin-induced diabetic rat model mimics this condition and is useful for investigating impaired wound healing in the diabetic state [28]. Glucose oxidase (GO) is an admirable enzyme for generating H_2O_2 [29] while catalyzing glucose. Honey which contains GO in moderate amounts is used for wound healing activities by generating H_2O_2 [30]. Inspired by this, we have decided to use GO for the healing of diabetic wound. Glucose, which is present in excess during a diabetic condition, is used as substrate.

However, the topical application of GO directly on the wound site in the form of a solution rapidly diffuses and becomes toxic to the wound by causing sudden glucose deprivation and the release of a ROS burst. Further, the GO will be degraded quickly due to the endoproteases and peptidases in the healing tissue even before any beneficial effect could be realized. To circumvent this problem, we have developed a novel drug delivery system for sustained release of GO using collagen as a biocompatible and biodegradable drug carrier. In this study, we have focused on the role of sustained release of ROS on oxidant, antioxidant status, collagen synthesis, and cellular proliferation in the various phases of healing of full thickness wounds in diabetic rats.

MATERIALS AND METHODS

Dithiobisnitrobenzoic acid (DTNB) and 2′7′-diacetyl dichlorofluorescein (DCFH-DA) were obtained from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were of analytical grade or

the highest purity available. Male Wistar rats weighing approximately 130–140 g, were used for the study ($n = 8$ animals). The experimental rats were fed commercial rat feed and water *ad libitum*. Diabetes was induced in rats by single intraperitoneal injection of streptozotocin (80 mg/kg body weight, Sigma, St. Louis, MO, USA) in 0.89% autoclaved saline. Fasting blood glucose levels were checked 3 days after streptozotocin injection, and animals with glucose levels greater than 200 mg/dL were used for the study. Wounds were created on the 7th day after induction of diabetes. The fur on the back of the rats was shaved under anesthesia by the intraperitoneal injection of thiopentone sodium 40 mg/kg. Subsequently, full thickness wounds of standard size (2 cm^2) were made to the depth of loose subcutaneous tissues. Rats were divided into three groups, each group comprising of eight animals. The Ethical Committee of CLRI approved this study.

(1) Group I: (Control) Topically applied with 0.1 M PBS, pH 7.4, immediately after wound creation.
(2) Group II: (CF) Wounds were treated with collagen matrix at the concentration of 4 mg/cm^2 wound area, immediately after wound creation.
(3) Group III: Glucose oxidase incorporated collagen matrix (GOIC) Wounds were treated with GOIC at the concentration of 1 μg/4 mg collagen/cm^2 wound area, immediately after wound creation.

The progress of wound healing was evaluated periodically by monitoring the biochemical and histopathological parameters from granulation tissue obtained at 3rd, 7th, 14th, and 21st day after wound creation.

Preparation of Collagen Films

Collagen was isolated from Bovine Achilles tendon, using 0.5 M acetic acid 5% NaCl by following the reported procedure [31]. The purity of collagen was established by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and triple helical content was monitored by circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy. Purified collagen was placed in Teflon trays and air dried under a laminar flow hood as indicated in a previous article from our lab [32].

Preparation of GO Incorporated CFs

GO (Sigma, St. Louis, MO, USA) was added to the collagen solution at a concentration of 1 μg/4 mg collagen/cm^2. The concentration of collagen in the solution was estimated following the method described by

Neuman and Logan [33]. The solution was placed in Teflon trays and allowed to air dry under a laminar flow hood protected from light. The collagen was tested for sterility by the direct inoculation method. If no evidence of microbial growth was found, the preparation being examined was considered to have passed the test for sterility.

In Vitro *Proteolysis Assay*

FLUORESCENT LABELING OF GO

Fluorescein 5′-isothiocyanate (FITC) labeling of GO was carried out with slight modification of the procedure described by Twining [34]. Briefly, 8 mg of GO was dissolved in 1 mL of 100 mM Tris buffer, pH 7.2. To this solution, 3 mg FITC was added and the mixture was gently stirred for 1 h at room temperature. Free FITC was removed by dialyzing twice against suspensions of charcoal in 2 L water at 4°C, then against 50 mM Tris buffer, pH 8.5, followed by 50 mM Tris buffer, pH 7.2. The FITC–GO was stored in 0.1 mL aliquots at 4°C.

PROTEASE ASSAYS USING FITC–GO

Trypsin proteolysis measured as a change in FITC fluorescence was based on the method described elsewhere [34]. Samples containing FITC–GO were proteolysed with trypsin, progress of the reaction was monitored at room temperature for 1 h in the presence and absence of collagen. The reaction mixture contained aliquots of 10 μL of trypsin (100 ng), 1.8 mL assay buffer and 20 μL 0.3% FITC–GO preincubated with 10% (w/v) collagen at room temperature for 1 h. The assay buffer for trypsin was 100 mM Tris buffer, pH 7.8, containing 10 mM $CaCl_2$, or 100 mM phosphate buffer, pH 7.8 containing 150 mM NaCl. The solution was further mixed to ensure the entire sample was at the proper pH. The samples with FITC were excited at 490 nm, and the emission wavelength was measured at 525 nm in a cuvette with 1 cm path length and 5 nm slit width. Fluorescence spectra were recorded at room temperature using a cary-50-Bio spectrofluorometer. FITC–GO incubated without collagen and processed similarly was used as the control.

CRUDE FIBROBLAST CELL ISOLATION

Crude fibroblast cells were dissociated as described in the reported procedure by Kosir et al. [35]. In brief, 250 mg granulation tissue was removed and minced with scalpel in a solution containing 20 μg/mL DNase and 0.3% bovine serum albumin (BSA) in Hank's buffered salt solution (HBSS). This was centrifuged and the pellet incubated in a

solution containing 0.025% trypsin, 60 μg/mL DNase, and 0.3% BSA in HBSS in a water bath at 37°C. After 15 min, the resulting mixture was centrifuged and the pellet agitated five times with a fire-polished Pasteur pipette. This was allowed to settle for 4 min to collect supernatant. These crude fibroblasts were used for the following ROS assay.

ROS Assay

This assay uses a nonfluorescent probe, DCFH-DA that is able to penetrate into the intracellular matrix that becomes oxidized by ROS to fluorescent dichlorofluorescein (DCF) [36]. Briefly, an aliquot of crude fibroblast (100 μL) with a cell count of 8.5×10^6 cells/mL was made up to a final volume of 2 mL in normal phosphate buffered saline (pH 7.4). 500 μL aliquots of fibroblast were taken to which 100 μL aliquots of DCFH-DA (200 μM) were added and the samples incubated at 37°C for 30 min. Fluorescent measurements were made with excitation and emission filters set at 485 ± 10 nm and 530 ± 12.5 nm, respectively. All initial fluorescent values (time 0) were found to differ from each other by less than 5%. Results were expressed as percentage increase in fluorescence calculated using the following formula $[(Ft_{30} - Ft_0)/(Ft_0 \times 100)]$, where t_0 and t_{30} are the fluorescence intensities at 0 and 30 min, respectively.

SUPEROXIDE DISMUTASE

SOD was assayed according to the method of Misra and Fridovich [37]. SOD catalyzes the following reaction:

$$O_2^- + O_2^- + 2H \xrightarrow{SOD} O_2 + H_2O_2$$

Superoxide O^-_2 is an intermediate in the oxidation of epinephrine. The ability of SOD to inhibit the antioxidant of epinephrine at pH 10.2 provides the basis of the assay for the enzymes. The enzymes source was prepared by homogenizing the biopsy sample in 10 volumes of cold isotonic PBS in a glass homogenizer. The homogenate was centrifuged at $4000 \times g$ for 15 min and the supernatant was used as the enzyme source for the assay. About 0.05 M sodium carbonate/bicarbonate buffer, pH 10.2 was prepared freshly. Ethylenediaminetetraacetic acid (EDTA) solution was prepared by adding 3.8 mg of EDTA in 10 mL of buffer by adding drops of dilute HCl. About 2.5 mL of buffer at pH 10.2, 0.05 mL of sample supernatant, 0.5 mL of EDTA, and 0.2 mL of epinephrine were added and the increase in absorbance at 480 nm for 120 s at every 15 s intervals was measured in a spectrophotometer (Perkin Elmer). Autooxidation of epinephrine to adrenochrome was performed in a control

tube without the enzyme. The enzyme activity is expressed in terms of unit/min/g protein in which 1 unit corresponds to quantity of enzyme required to produce 50% inhibition in epinephrine auto-oxidation.

CATALASE

The activity of catalase enzyme was determined by the method of Beers and Sizer [38]. Catalase catalyses the following reaction:

$$H_2O_2 \xrightarrow{\text{catalase}} O_2 + H_2O$$

In this method, catalase activity is estimated by the decrease in the absorption of hydrogen peroxide caused due to its decomposition in the presence of catalase at $\lambda = 240$ nm. Phosphate buffer 0.01 M at pH 7.0 was prepared freshly; 30 mM hydrogen peroxide solution was prepared in the above buffer and protected from light. The enzyme source was similar to that used in the SOD estimations. To 4 mL of the buffer, 0.04 mL of sample supernatant and 1 mL of H_2O_2 was added the decrease in absorbance at 240 nm for 180 s at every 15-s intervals was measured in a spectrophotometer. The enzyme activity is expressed in terms of micromoles of H_2O_2 consumed/min/g of protein.

TOTAL REDUCED GSH

The method of Moron et al. [39] was followed to determine the total reduced GSH. The method is based on the reaction of GSH with DTNB to give absorbance at 412 nm. Tissue homogenate (1.0 mL) was precipitated with 5% trichloroacetic acid (TCA) and centrifuged. About 0.5 mL of supernatant was added with 0.5 mL of phosphate buffer, pH 8.0, followed by 2.0 mL of DTNB reagent. The color developed was read at 412 nm in a Hitachi 320 spectrophotometer against a blank containing 5% TCA instead of sample. A series of standards were treated in a similar manner. The amount of GSH was expressed as μg/mg protein.

NITRIC OXIDE

This method is based on the conversion of NO into the S-nitroso derivatives, which yields an equivalent of nitrous acid on mercuric ion-assisted hydrolysis [40]. This nitrous acid is finally used in the formation of a brilliant azo dye from sulphanilamide and N-1-naphthylethylenediamine and measured at 545 nm.

HYDROXYPROLINE

Total collagen was estimated by measuring the hydroxyproline content as described by Neuman and Logan (1950). About 5 mg of the processed dry tissue was suspended in 5 mL of 6N hydrochloric acid, sealed and subjected to hydrolysis at 100°C for 24 h. The tube content was evaporated to dryness and resuspended in 5 mL of distilled water. L-Hydroxyproline was used as standard and measured at 540 nm.

URONIC ACID

Uronic acid was determined by Bitter and Muir [41] method using glucurolactone as standard. The reaction involves the reaction of carbazole with the unstable acid hydrolyzed dehydrated derivatives of hexouronic acid and measured at 530 nm.

PROTEIN AND DNA

Total protein was assayed using the method of Lowry et al. [42] using BSA as standard. The DNA was assayed using the method of Labarca and Paigen [43]. Total protein and total DNA were extracted in 5% TCA: 10 mL of 5% TCA was added to the tissue samples (100 mg wet weight) and heated in a 90°C water bath for 30 min to extract protein and DNA. The solution was centrifuged and the supernatant was used for the estimation.

Wound Contraction

The following formula was used to calculate the percentage of wound contraction:

$$\%\text{Wound, contraction} = (\text{Healed, area}/\text{Total, wound, area}) \times 100$$

Histological Studies

Tissue from the wound site of the individual animal was removed. These samples were then separately fixed in 10% formalin, dehydrated through graded alcohol series, cleared in xylene, and embedded in paraffin wax. Serial sections of 5 μm thicknesses were cut and stained with hematoxylin and eosin (H&E) and Masson's trichrome [44].

Statistical Analysis

Data are expressed as mean ± SD for eight animals in each group. Statistical 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. Statistical significance of the difference between wounds treated with CF and GOIC was determined by Student's t-test. The levels of significance were evaluated using the p-value.

RESULTS

The degradation of the GO enzyme, commonly used in the construction of glucose sensors has been of concern to scientists for decades. Collagen is an enzyme compatible porous matrix which is able to protect the enzyme from denaturation. This was elucidated by trypsin assay, monitoring fluorescence dequenching of the GO incorporated in the collagen [45].

Dequenching of FITC–GO on treatment with trypsin is monitored by the tryptic cleavage of FITC–GO. The addition of trypsin to FITC–GO induced a fluorescence change characterized by a rapid rise in intensity as reported in Figure 1. The rise in FITC–GO intensity was time-dependent and higher than the FITC–GO with collagen. This change in fluorescent intensity indicates that proteolysis of FITC–GO is prevented in the presence of collagen.

Streptozotocin-induced diabetic rat model indicates poor release of cytokines and growth factors in the wound site [46]. This in turn affects the proper dermal wound healing. Recently, ROS has been proved to be an essential intracellular messenger for several cytokines and growth factors [47]. Realizing the importance of ROS in the diabetic wound, we measured intracellular ROS in the crude fibroblast of the experimental animals. The ROS levels were increased to 1.9- and 3.7-fold in the CF and GOIC groups, respectively, when compared to control during the 3rd day of the inflammation period (Figure 2). However, during the 7th day, the ROS level was significantly higher in the control group when compared with the GOIC group. No significant changes were observed among the groups on the 21st day.

To determine a potential involvement of various types of ROS scavengers in the diabetic wound healing, we analyzed the major antioxidants such as SOD, catalase, GSH, and ascorbic acid (Table 1) in the healing site. SOD is one of the vital enzymes involved in antioxidant defense, which dismutases the superoxide anions to H_2O_2. Our results

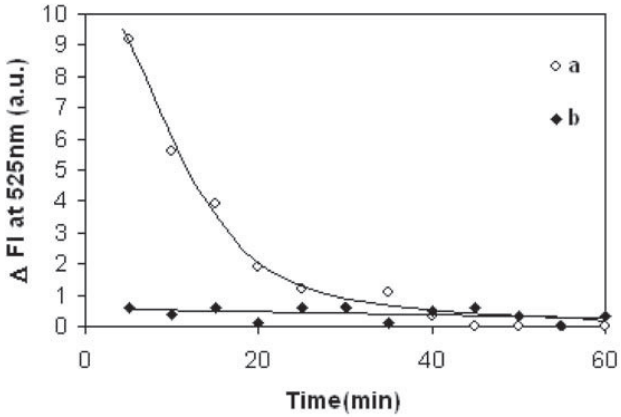

Figure 1. Difference in fluorescence intensity (ΔFI) with respect to time of the hydrolysis of FITC–GO (a) and FITC–GO with collagen (b) by trypsin. Trypsin of about 100 ng was treated with FITC–GO in 100 mM Tris buffer, pH 7.8, containing 10 mM $CaCl_2$ at 37°C for 1 h. The excitation wavelength was 490 nm and the emission wavelength 525 nm.

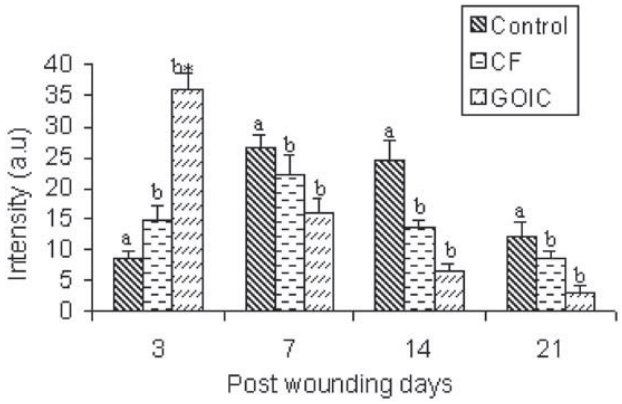

Figure 2. Concentration of ROS in the granulation tissue of control and experimental rats. Values are expressed as mean ± SD for eight animals in each individual experiment. Different superscripts indicate significant difference between groups ($p < 0.05$: ANOVA). Statistical significance of difference between wounds treated with CF and GOIC was determined by Student's t-test. Results are indicated as $^*p < 0.05$.

Table 1. The antioxidant status and NO levels of the wound granulated tissue in the control and experimental rats.

Parameters	Post-wounding days	Control	CF	GOIC
SOD (units/mg of protein)	3rd day	1.19 ± 0.26^{b}	2.3 ± 0.46^{a}	$3.75 \pm 0.67^{a*}$
	7th day	1.62 ± 0.40^{b}	3.01 ± 0.52^{a}	$3.27 \pm 0.70^{a*}$
	14th day	2.19 ± 0.56^{a}	2.38 ± 0.63^{a}	2.41 ± 0.51^{a}
	21st day	2.79 ± 0.44^{a}	2.27 ± 0.65^{a}	2.73 ± 0.48^{a}
Catalase ($\mu m H_2O_2$/min/mg protein)	3rd day	0.94 ± 0.14^{b}	2.24 ± 0.27^{a}	$3.39 \pm 0.31^{a*}$
	7th day	1.42 ± 0.18^{b}	2.88 ± 0.21^{a}	$4.05 \pm 0.26^{a*}$
	14th day	1.56 ± 0.13^{b}	3.38 ± 0.26^{a}	3.24 ± 0.23^{a}
	21st day	2.84 ± 0.18^{a}	2.39 ± 0.16^{a}	2.61 ± 0.4^{a}
GSH (μg/mg protein)	3rd day	8.76 ± 1.14^{b}	12.16 ± 2.27^{a}	$18.03 \pm 2.64^{a*}$
	7th day	11.10 ± 2.18^{b}	16.10 ± 3.21^{a}	$20.47 \pm 2.26^{a*}$
	14th day	13.69 ± 3.13^{b}	14.69 ± 1.26^{b}	$16.52 \pm 1.23^{a*}$
	21st day	12.29 ± 2.18^{a}	8.68 ± 1.16^{b}	9.14 ± 1.14^{b}
Ascorbic acid (μg/mg protein)	3rd day	7.49 ± 1.35^{b}	$10.42 \pm 2.68^{a*}$	$12.95 \pm 2.16^{a*}$
	7th day	11.4 ± 2.02^{b}	15.92 ± 1.5^{a}	$17.65 \pm 2.07^{a*}$
	14th day	9.83 ± 2.44^{b}	10.38 ± 1.8^{b}	$12.48 \pm 1.85^{a*}$
	21st day	8.29 ± 2.13^{a}	8.86 ± 1.93^{a}	8.39 ± 1.55^{a}
NO (μg/mg protein)	3rd day	3.24 ± 1.26^{b}	5.41 ± 1.53^{a}	$7.32 \pm 1.92^{a*}$
	7th day	3.86 ± 0.88^{b}	5.10 ± 1.09^{a}	$7.23 \pm 2.08^{a*}$
	14th day	3.76 ± 1.08^{a}	3.8 ± 0.89^{a}	4.12 ± 1.76^{a}
	21st day	4.59 ± 1.33^{a}	4.21 ± 1.76^{a}	3.85 ± 0.94^{b}

Values are expressed as mean ± SD for eight animals in each individual experiment.Statistical significance of difference between wounds treated with CF and GOIC were determined by Student's *t*-test.
[a]Significant difference between groups ($p < 0.05$) within a row.
[b]Nonsignificant difference as determined by ANOVA.
*Results are indicated as $p < 0.05$.

show a significant increase in activity of SOD in the GOIC group at 3rd and 7th day (Table 1). However, the expression of this enzyme subsequently reduced to basal levels at 14th and 21st day. In our previous article, we clearly showed that catalase protects the system by promoting the reaction involved in the decomposition of H_2O_2 to water and oxygen [48]. Elevated activity of catalase was observed up to day 7 after wounding (Table 1), but no significant changes were observed after the 14th day. GSH is also essential for cellular proliferation and cells fail to enter the S phase when GSH levels are depressed [49]. GSH depletion [50] leads to decreased collagen and proteoglycans synthesis in human chondrocyte cells [51]. We observed a significant increase in the GSH level during the 3rd and 7th day in the GOIC group when compared to

control and CF ($p > 0.05$), no significant difference was found on the 14th and 21st day (Table 1).

The NO level of the control and treated group has been given in Table 1. NO level was significantly higher ($p > 0.05$) in the GOIC group when compared with control and CF treated groups on the 3rd and 7th day, no significant changes were observed in the later days of wound healing.

The hydroxyproline content in the GOIC animal group was significantly ($p < 0.05$) higher than that of other groups throughout the experimental period (Table 2). On the 7th day, hydroxyproline content was found to be elevated about 1.95- and 3.3-fold in the CF and GOIC groups, respectively, when compared to the control. Uronic acid is a matrix molecule, which acts as ground substance for the synthesis of new extracellular matrix. The amount of hyaluronic acid content of the granulation tissue was estimated in the form of uronic acid. Results show that wounds treated with GOIC have a significant increase

Table 2. Levels of hydroxyproline, uronic acid, protein and DNA in the granulation tissue of control and experimental rats.

Parameters	Post-wounding days	Control	CF	GOIC
Hydroxyproline (mg/100 mg tissue)	3rd day	0.82 ± 0.23^{b}	3.35 ± 0.32^{a}	3.5 ± 0.36^{a}
	7th day	1.86 ± 0.18^{b}	4.65 ± 0.43^{a}	$6.23 \pm 0.45^{a*}$
	14th day	2.26 ± 0.34^{b}	3.8 ± 0.26^{a}	$4.12 \pm 0.75^{a*}$
	21st day	1.23 ± 0.32^{b}	1.11 ± 0.54	$2.8 \pm 0.19^{a*}$
Uronic acid (mg/100 mg wet weight of tissue)	3rd day	58.64 ± 5.36^{b}	62.05 ± 2.43^{a}	59.43 ± 1.65^{b}
	7th day	92.45 ± 6.32^{b}	113.08 ± 7.54^{a}	$137.45 \pm 6.78^{a*}$
	14th day	71.34 ± 8.43^{b}	105.74 ± 8.23^{a}	$116.82 \pm 5.87^{a*}$
	21st day	62.82 ± 4.67^{b}	$81.54 \pm 3.12^{a*}$	$88.73 \pm 2.76^{a*}$
Protein (mg/100 mg tissue)	3rd day	1.31 ± 0.23^{b}	4.1 ± 0.32^{a}	$4.82 \pm 0.12^{a*}$
	7th day	4.52 ± 1.04^{b}	8.42 ± 0.34^{a}	$9.12 \pm 0.07^{a*}$
	14th day	3.82 ± 0.74^{b}	7.32 ± 0.09^{a}	7.8 ± 0.20^{a}
	21st day	1.78 ± 0.34^{a}	2.32 ± 0.12^{a}	2.19 ± 0.04^{a}
DNA (mg/g wet weight of tissue)	3rd day	2.1 ± 0.69^{b}	3.64 ± 0.36^{a}	$4.12 \pm 0.54^{a*}$
	7th day	4.22 ± 0.74^{b}	6.84 ± 0.48^{a}	$7.54 \pm 0.47^{a*}$
	14th day	3.64 ± 0.43^{b}	6.42 ± 0.45^{a}	6.5 ± 0.43^{a}
	21st day	4.34 ± 0.35^{a}	4.24 ± 0.37^{a}	3.82 ± 0.61^{a}

Values are expressed as mean ± SD for eight animals in each individual experiment.Statistical significance of difference between wounds treated with CF and GOIC were determined by Student's *t*-test.
[a]Significant difference between groups ($p < 0.05$) within a row.
[b]Nonsignificant difference as determined by ANOVA.*Results are indicated as $p < 0.05$.

in hyaluronic acid (Table 2), when compared to the CF and control groups. The total protein and DNA contents were used to assess the level of fibroplasia in the granulation tissue. This study shows that there was a significant increase in the levels of protein and DNA content in the GOIC group when compared to the control and CF groups on the 7th day and no significant increase was observed thereafter in the diabetic rats (Table 2).

Periodical observation of animals for up to 21 days showed significant increase ($p < 0.05$) in the rate of contraction of wound in experimental groups, when compared to control (Figure 3). Complete closure of wound contraction was seen on day 21 in the GOIC group, whereas, in CF and control groups it was 84% and 69%, respectively. The histological architecture of the 14th day post-wounding granulation tissue sections of control, CF, and GOIC subjected to H&E stains is presented in Figure 4. The control section (Figure 4(a)) shows incomplete epithelialization and undifferentiated keratinocytes with highly covered inflammatory cells. Figure 4(b) shows the CF treated specimen, where the collagen fibers are loosely packed with irregular arrangement, lesser amounts of fibrous tissue and keratinocytes are also seen, whereas the GOIC group has fully matured granulation tissue with compact and

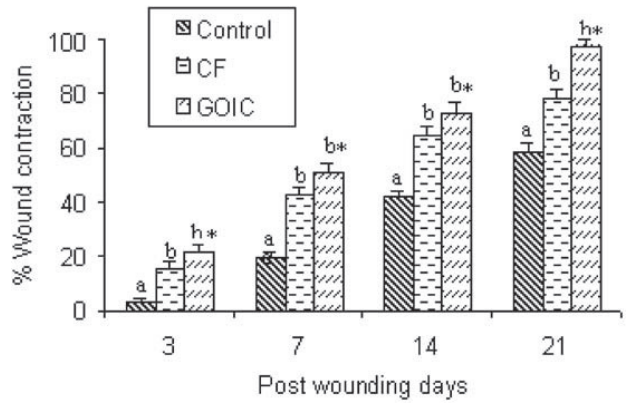

Figure 3. Percentage of wound contraction of control and experimental rats. Values are expressed as mean ± SD for eight animals in each individual experiment. Different superscripts indicate significant difference between groups ($p<0.05$: ANOVA). Statistical significance of difference between wounds treated with CF and GOIC was determined by Student's *t*-test. Results are indicated as $^*p<0.05$.

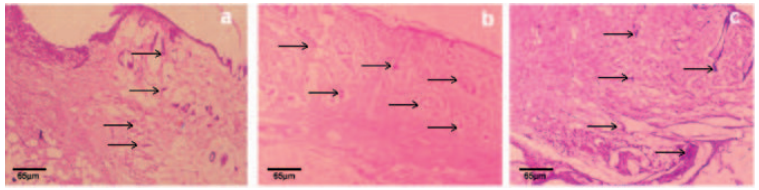

Figure 4. Wound histology of 14th day: sections are stained with H&E (40×). (a) Control rat shows incomplete epithelialization and undifferentiated keratinocytes with highly covered inflammatory cells, (b) CF treated rat shows loosely packed irregular collagen fibers, lesser amount of fibrous tissue and keratinocytes, and (c) GOIC treated rat has fully matured granulation tissue with compact and well aligned collagen fibers. Keratinocytes are clearly differentiated with more accumulation of collagen fibers in the extracellular region. Scale bar = 65 μm.

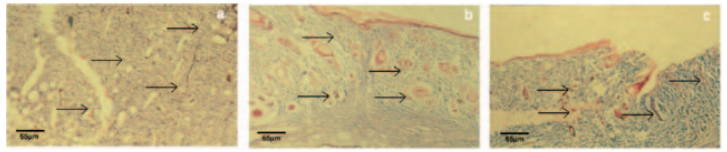

Figure 5. Sections are stained with Masson's trichrome (40×). (a) Wound histology of control rat on day 14 with loosely packed and minimal collagen formation, (b) wound histology of CF alone treated rat on day 14 with irregular arrangement of collagen bundles and moderate amount of collagen formation, and (c) wound histology of GOIC treated rat on day 14 with densely packed collagen tissue showing complete re-epithelialization. Each arrowhead indicates the collagen bundle accumulation. Scale bar = 65 μm.

well-aligned collagen fibers. Keratinocytes are clearly differentiated with more accumulation of collagen fibers in the extracellular region (Figure 4(c)). Examination of Masson's trichrome wound sections revealed dense and thick stromal tissue formation in the GOIC group (Figure 5(c)), whereas these changes were negligible in the control (Figure 5(a)) and CF groups (Figure 5(b)).

DISCUSSION

Studies in human and animal models have revealed abnormalities in various phases of wound healing process like inflammation, collagen deposition, and differentiation of the extracellular matrix, fibroplasia,

and contraction in the diabetic wound. Oxidants are now studied as secondary messenger molecules [6,52]. Burst release of H_2O_2 in the wound is potentially toxic [13,53]. Our approach in topical delivery of GO to the healing site offers two advantages to the healing process. One is the *in situ* production of H_2O_2, which is a signal molecule for the macrophage. The second is the supportive matrix with collagen. Our *in vitro* study reveals FITC–GO was not proteolysed in the presence of collagen, evidenced from unaltered fluorescence. This indicates the presence of collagen in tryptic cleavage sites of FITC–GO shielded from proteolysis inside the collagen matrix. Since GO contains multiple lysine residue, many FITC moieties are incorporated in the GO. This leads to self-dequenching. Proteolysis results in fragmentation thereby, hindering the dequenching of FITC. During 1 h of trypsin proteolysis the intact FITC–GO was rapidly digested and appeared with increased intensity, indicating proteolytic degradation. However, the collagen protected FITC–GO against rapid proteolysis by trypsin throughout the entire 1-h time course (Figure 1). The results are constant that the FITC–GO cleavage sites for trypsin were less accessible to proteolysis in the presence of collagen. Thus the *in vitro* experiment confirms the decreased proteolysis and persistent self-dequenching, indicating the sustained release of GO from the collagen matrix. In order to prevent the toxicity in topical application of GO, we have developed a novel drug delivery system for sustained release of GO using collagen as a biocompatible and biodegradable drug carrier.

Our results demonstrate that the GOIC group has significantly higher ROS in the crude fibroblast during the inflammation period, which can be correlated with the effective wound healing. Exogenous production of ROS in the wound milieu of the GOIC group is a possible reason for higher ROS production. Poor microvasculature blood flow is a major problem in the diabetic wound, which leads to a delay in the inflammatory phase and wound healing, and makes it prone to infection. ROS regulates microvasculature blood flow [54], vascular endothelial growth factor (VEGF) expression [7], triggers tenascin expression [9], and provides defense against microbial contamination [55]. All these factors play a possible role in the enhanced wound healing process in the GOIC group.

Earlier studies have provided evidence on the beneficial roles of antioxidants [32,48] in wound healing of rat skin. In this study, we observed a significantly higher level of antioxidant enzyme SOD and catalase in the GOIC group (Table 1). Earlier studies report an increase in ROS induced gene expression of SOD through the activation of the transcription factor NF-κB and increased expression of catalase by

H_2O_2 stimulation. Current evidence shows that several cytokines and their receptors respond to ROS [56,57] and up regulate the expression of ROS scavenging enzymes in various cell types. This may be the possible reason for higher SOD and catalase activity in the GOIC group. Thus, the co-increase in the activity of catalase with the SOD could be an important mechanism to avoid the toxic effects of H_2O_2. A significant increase in the level of GSH was also noted in the GOIC group. Meister [58] reported that the oxidant production in the wound milieu might produce thiol conjugates and promote GSH synthesis. The increased activity of these antioxidants could be essential for the efficient migration and proliferation of fibroblast and keratinocytes to protect against the inhibitory effects of ROS. Thus, our results suggest that a sustained production of ROS exogenously increases antioxidants, thereby enhancing the wound healing process in diabetic rats.

Exogenous supplementation of ROS may promote the formation of other oxidant species, which suggests that the wound site may be rich in both oxygen- and nitrogen-centered reactive species along with their derivatives. Hence, in this study we measured the NO level in the granulation tissue. Decreased NO levels in the wound milieu may be one of the important factors for impaired wound healing in diabetic animals. An interesting observation in this study is a significant increase in the NO production in the granulation tissue of GOIC group, which may be due to an increase in the ROS level. Recent studies revealed that ROS serves as a redox regulator in transcription factors NF-κB and cytokines [59,60]. This, in turn, induces the *inducible nitric oxide synthase* (iNOS) expression in different cell types [61]. Ferrini et al. [62] reported that iNOS might be one of the main sources of NO production. NO promotes wound healing during the early inflammatory stages since NO donors or *nitric oxide synthase* (NOS) gene transfer favors collagen synthesis and the formation of granulation tissue [63].

The metabolism of collagen and its regulation are of vital interest in clinical situations such as tissue repair and wound healing. Loss of collagen in diabetes is due to decreased synthesis or enhanced catabolism of collagen or both. The modulated expression of collagen is required to heal the damaged tissue. The hydroxyproline content in healing wound is a known index to assess the collagen synthesis. An earlier report suggested that, exogenously added ROS are involved in TGF-β stimulated collagen production [64,65]. ROS and NO are the main mediators in the intensification of collagen synthesis [60]. The sustained production of ROS and subsequent increase in the NO production in the wound milieu hastens the wound healing process by increasing the collagen synthesis in the GOIC group. The protein and

DNA content of granulation tissues indicates protein synthesis and cellular proliferation. Increase in protein synthesis and cellular proliferation in the GOIC treated wound suggest the sustained production of ROS [66].

Earlier studies showed that [67] the accumulation of inflammatory cells at the full thickness wound area occurs 4–7 days after injury. This analysis shows incomplete epithelialization and undifferentiated keratinocytes with increased inflammatory cells at the full thickness wound area on the 14th day, confirming a delayed inflammatory response in the control group, whereas in the case of the GOIC group, a fully matured granulation tissue with compact well-aligned collagen fibers and keratinocytes is seen, indicating a faster wound healing cascade taking a course of only 21 days for complete closure of the wound [52]. This could be due to the GOIC group enhanced centripetal movement of fibroblasts that causes the wound contraction [68,69].

In conclusion, our biochemical and histological results suggest that GOIC therapy may have a beneficial influence on various phases of diabetic wound healing like inflammation, collagen synthesis, and cell proliferation. Therefore, GOIC would be a promising wound healing material for diabetic dermal wound.

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