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Biopolymer gel matrix as acellular scaffold for enhanced dermal tissue regeneration

Rangasamy Judith [a], Mariappan Nithya [a], Chellan Rose [a,*], Asit Baran Mandal [b]

[a] Department of Biotechnology, CSIR - Central Leather Research Institute, Adyar, Chennai 600 020, Tamil Nadu, India
[b] Chemical Sciences Division, CSIR - Central Leather Research Institute, Chennai 600 020, Tamil Nadu, India

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ABSTRACT

Biological grafts have drawbacks such as donor scarcity, disease transmission, tissue infection, while the scaffolds of either collagen or chitosan fabrics fail to become part of the tissue at the wound site, though they favor the formation of connective tissue matrix. This study developed a novel composite consisting of the combination of atelocollagen and chitosan in order to provide a biodegradable molecular matrix in gel form as a biomimetic surface for cell attachment, to promote the wound healing in excision wounds. We found that the topical application of biopolymer composite on the wound promoted cell proliferation, migration and collagen deposition overtime. The enhanced cellular activity in the collagen-chitosan treated wound tissue was also assed by increased levels of Platelet derived growth factor (PDGF) and Nerve growth factor (NGF) associated with elevated levels of antioxidants and decreased level of lipid peroxidation. The acellular matrix-like topical application material is designed to guide the eventual re-establishment of an anatomically normal skin. The results of this study demonstrate the feasibility of multi-cell regeneration on a molecular system that mimics tissue engineering *in vivo*.

1. Introduction

Injuries to skin are the most common in human, particularly in the young and physically active population. Associated with the problems of incomplete healing and recurrent injury, these injuries are responsible for individual morbidity. Despite many improvements in the currently available therapies involving autografts or allografts [1], these remains significant limitations in our management of these conditions. Biological grafts have drawbacks such as donor scarcity, donor-site morbidity, tissue rejection and disease transmission.

A common approach in tissue engineering involves a three dimensional (3D) porous biodegradable scaffold loaded with specific living cells to launch tissue regeneration or replacement in a natural way, with the scaffold eventually disappearing over a period of time [2]. Such a material is generally biocompatible, biodegradable porous scaffold with optimized architecture, sufficient surface area for cell attachment, growth and proliferation, favorable mechanical properties and suitable degradation rate so as to be replaced by newly formed tissue, is a prerequisite to achieve success with approach [3]. Gel system containing fibrin is likely to encounter nutrient transmission problems, and cells seeded in a 3D gel are observed to proliferate more near the surface than in the center of the gel [4].

Collagen molecules in the extracellular space provide the major biochemical scaffold for all attachment and anchorage of macromolecules. Furthermore collagen molecule is known to provide physical support for cellular proliferation [5]. Likewise, chitosan has also been reported to be used in diverse fields. It becomes an interesting material in pharmaceutical applications [6] due to its low toxicity [7], biocompatibility [8] wound healing property [9].

However, report on the combined role of these biopolymers on the formation of granulation tissue by providing a scaffold for the cell migration is scarce. Therefore the objective of this study was to develop an acellular provisional matrix, mimicking the extracellular matrix of the of the wound site, and to evaluate its potential for *in vivo* guided dermal regeneration. This study focuses on the design and *in vivo* evaluation chitosan-collagen gel with the potential to induce regeneration of dermal tissue.

2. Materials and methods

2.1. Animals

Female albino rats were purchased from Sri Ramachandra Medical College and Hospital, Chennai, India. They were maintained

* Corresponding author. Tel.: +91 44 24430273; fax: +91 44 24911589.
E-mail addresses: rsjudith@gmail.com (R. Judith), chellanrose@yahoo.co.uk (C. Rose).

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in individual metabolic cages throughout the experiment, in hygienic conditions and they were fed with commercial balanced diet and water *ad libitum*. The institutional Animal Care and Ethics Committee approved all surgical interventions and post-operative animal care. The approved number is 466/01/a/CPCSEA) dated 20.12.2001.

2.2. Chemicals

PDGF, monoclonal anti-PDGF antibody purified from mouse, goat anti-rabbit IgG, 3, 3′-diaminobenzidine (DAB), thiobarbituric acid (TBA), catalase, glutathione reductase, 5-5′-dithiobis-2-nitrobenzoic acid (DTNB), bathophenanthroline, bovine serum albumin (BSA), urea and other fine chemicals were purchased from Sigma (St. Louis, MO, USA). Solvents and other chemicals purchased were of analytical grade and obtained locally from standard chemical companies.

2.3. Preparation of collagen sample

Extraction of collagen from the Marine catfish air bladder was done according to the method reported by Rose et al. [10], followed by the trypsin treatment to remove the telopeptides. A 200 mg of lyophilized collagen was dissolved in 10 ml of 0.05 M acetic acid solution. This was subjected to gamma irradiation (1 Mrad) using Phoenix-Cobalt 60 (Best Therotronics, Ottawa, ON, Canada) for 10 min, to suppress antigenicity, if any, and imparts sterility.

2.4. Preparation of chitosan samples

One hundred milligrams of chitosan was prepared from crab shell chitin according to the method [11]. This was dissolved in 10 ml of 0.05 M acetic acid and subjected to gamma irradiation (1 Mrad) for 10 min, as stated above.

2.5. Preparation of chitosan - collagen blend

The collagen and chitosan preparations mentioned above were mixed in the ratio of 1:1 and stored on ice for topical application in the wounds of experimental rats.

2.6. Wound creation and treatment

The fur on the back of the rats weighing 120–150 g was shaved under mild ether anesthesia. Subsequently, open excision type of wounds of a standard size (1.5 cm^2) was made surgically. These wounded animals were divided into 4 groups with 6 animals in each group and the wounds were treated immediately after dermal excision by topical application of different preparations, once a day for 7 days, as mentioned below. The wound tissues were taken for different analyses at varying intervals of post-wounding and treatment, after euthanasia.

Control (Cont):Phosphate buffered (0.1 M) saline, pH 7.4
Collagen (Col): Collagen gel (1.5 mg/cm^2 wound area)
Chitosan (Chi): Chitosan gel (1.5 mg/cm^2 wound area)
Chitosan + Collagen (Chi + Col): Chitosan + Collagen gel, 1:1 ratio (1.5 mg/cm^2)

2.7. Wound assessment by planimetry

The contour of the wounds of control and experimental animals was measured using transparent graph sheet and the rate of epithelialization was calculated and expressed as percentage contraction [12].

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

$$\%\ \text{Wound contraction} = \frac{\text{Wound area on day '0'} - \text{Wound area on day '}n\text{'}}{\text{Wound area on day '0'}} \times 100$$

2.8. Preparation of skin homogenates

A 10% homogenate of skin tissue was prepared in 0.02 M Tris–HCl buffer, pH 7.0, using a Teflon homogenizer in ice-cold condition. The homogenate was centrifuged at 5000 rpm for 10 min. The supernatants were used for the determination of antioxidants. Apart from this melondialdehyde (MDA) the product of lipid peroxidation, was also determined using the supernatant.

2.9. Biochemical analysis

Collagen and hexosamine contents in granulation tissues were estimated by the methods of Wossner [13] and Elson and Morgan [14], respectively. The uronic acid from the tissue was carried out according to the method of Schiller et al. [15] and estimated by the method of Bitter and Muir [16], and Protein by the method of Lowry et al. [17].

2.10. Determination of skin lipid peroxidation

The lipid peroxidation was measured as thiobarbituric acid (TBA) reactive substances (TBARS) as described by Draper and Hadley [18]. Lipid peroxide content was expressed as nanomoles of MDA/mg protein. To 0.5 ml of skin homogenate, 2.0 ml of 20% TCA was added contents were mixed well and centrifuged at 4000 rpm for 20 min. Two milliliters of the supernatant was mixed with 2.0 ml of thiobarbituric acid (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.

2.11. Estimation of skin antioxidants/enzymes

Total content of reduced glutathione (GSH) was determined by the method of Moron et al. [19]. Ascorbic acid was estimated by the method of Omaye et al. [20]. Antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) were determined using the tissue homogenates by the methods of Beers & Seizer [21] and Misra & Fridovich [22] respectively. Activity of glutathione peroxidase (GPX) was determined using by the method of Rotruck et al. [23].

2.12. Transmission electron microscopy (TEM)

The healed skin tissue specimens from control and experimental rats were fixed in 3% glutaraldehyde, buffered with 0.1 M sodium

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cacodylate, pH 8.0, containing 0.2 M sucrose. Post-fixation was followed in 1% osmium tetroxide in the same buffer for 2 h at 4 °C. After dehydration in ethanol, embedding was carried out in a mixture based on Epon 812. Ultrathin sections were poststained with 2% aqueous uranyl acetate and lead citrate. Sections were examined in a Phillips 201 C Electron Microscope at 60 kV and photographed.

2.13. Histology

The healed skin tissue specimens from control and experimental rats were removed immediately after euthanatized. These samples were then separately fixed in 10% formalin, dehydrated through graded alcohol series, cleared in xylene and embedded in paraffin wax (melting point 56 °C). Serial sections of 5 μm thickness were cut, stained with haematoxylin and eosin.

2.14. Determination of PDGF and NGF by ELISA

PDGF levels in skin tissue samples were determined using monoclonal human anti-PDGF antibody according to the method of described for PDGF by Harrison et al. [24] Briefly, high protein binding polysterile microtitre plates were coated with 100 μl/well of 50 mM carbonate buffer, pH 9.6, containing 2 μg monoclonal anti-PDGF IgG and left over night at 4 °C. Non-specific binding of antibody-coated wells was blocked with 1% fetal calf serum for 1 h at 4 °C followed by incubation with 100 μl of sera samples for 12 h at 4 °C. Additional coated wells without PDGF were incubated in each assay. Monoclonal anti-PDGF antibody (2 μg) in 100 μl of PBS containing 1% fetal calf serum and 0.05% Tween-20 was added (100 μl per well) and incubated for 12 h at 4 °C. This was treated with a 100 μl of an affinity purified biotinylated goat anti-rat IgG in PBS containing 1% fetal calf serum for 12 h at 4 °C. Then the bound antibody complex is quantified with a 100 μl of peroxidase-conjugated streptovidin solution in PBS containing 1% fetal calf serum. Finally, the freshly prepared substrate solution 0.4% o-phenylene diamine dihydrochloride in 50 mM citrate-phosphate buffer, containing 0.03% H_2O_2, was added and the optical density was read at 492 nm after 30 min incubation by using an ELISA reader (Kontron SLT 210). Similarly the Nerve growth factor (NGF) levels were determined using human anti-NGF antibody according to the method Matsuda [25].

2.15. Statistical analysis

Data are expressed as mean ± S.D. Students *t*-test for small samples has been carried out to compare the mean values of various parameters between the experimental and control groups.

3. Results

3.1. Period of epithelialization and rate of wound contraction

The progression of wound contraction was evaluated by macroscopic observations and parallely, the contour of the wound size was monitored by planimetry at regular intervals of time. The rate of healing in terms of percent wound closure (Table 1) was observed to be in the following order: Chi + Col > Chi > Col > Cont. The macroscopic evaluation of the wounds (Fig. 1) revealed that Chi + Col treated groups required a total period of only 15 days as against 17, 18 and 20 days for chitosan, collagen treated and control animals, respectively (Table 2). At the end of day 15, the healing was almost complete in the Chi + Col treated wounds, and the healed area was very smooth with no necrotic tissue on the surface.

Table 1
Rate of wound contraction as percentage of original wound size of control and experimental rats.

Treatment	Days		
	4	8	10
Control	12.1 ± 0.90	47.95 ± 3.02	80.82 ± 1.25
Collagen	23.3 ± 0.87***	63.99 ± 8.0**	93.0 ± 2.84***
Chitosan	26.25 ± 1.55***	65.55 ± 7.60**	93.85 ± 2.7***
Chitosan + Collagen	26.85 ± 4.38***	88.18 ± 2.46**	98.93 ± 1.04***

Wounds were made surgically on the back of the rats and treated with PBS, Collagen (Col), Chitosan (Chi) and Chitosan + Collagen (Chi + Col) for 7 days as described in Materials and Methods. The individual contour of the wounds of all the animals was measured, periodically, using transparent graph sheet and the rate of healing was calculated and expressed as percentage contraction. Values are mean ± S.D. for six animals. $^{**}P < 0.01$; $^{***}P < 0.001$.

3.2. Biochemical parameters

Table 3 depicts the collagen, hexosamine and uronic acid content of control and treated animal groups on different days of post-wounding. The collagen content showed a progressive increase in all groups up to day 8, after which it declined. The different groups however, showed a distinct difference in their collagen content depending upon the composition of the topical application material used for the treatment of wound. For instance, the specimens from control animals showed a decreased level of collagen whereas the other groups that received structural support of different kind showed increased level of collagen. Hexosamine content of the granulation tissues, decreased gradually from day 4 to day 15, and the trend was observed to be opposite to that of collagen synthesis. The rate of decrease in the level of ground substratum component seems to be much faster in the Chi + Col animals than that of the other groups. The uronic acid levels in the treated groups increased slightly up to day 8 and thereafter started decreasing as it happens in the case of collagen level. But, the control animals did not record a downward trend even up to day 15.

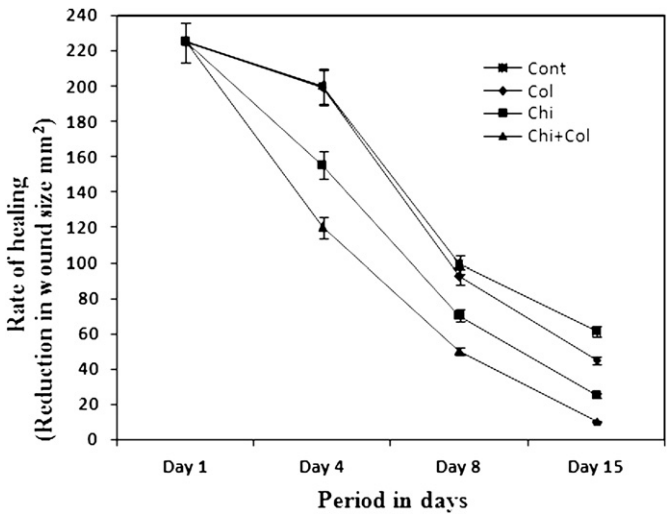

Fig. 1. Kinetics of wound closure (reduction in wound size) wounds were made surgically on the back of the rats and treated with PBS (Cont), Collagen (Col), Chitosan (Chi) and Chitosan + Collagen (Chi + Col) for 7 days. Wound closure was measured to find the extent of reduction in wound area at different post-treatment periods by planimetry. Wound area was calculated on 1st, 4th, 8th, and 15th post-wounding day by counting number of squares of retraced wound area on graph sheet. Values are expressed as mean ± S.D. for six animals in each individual experiment. In the representative diagram '*' indicates highly significant difference ($P < 0.05$) within the groups.

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Table 2
Period of wound epithelialization of control and experimental rats.

Treatment	Days
Control	22.33 ± 0.58
Collagen	18.25 ± 0.375***
Chitosan	17.66 ± 0.57***
Chitosan + Collagen	15.33 ± 0.57***

Wounds were made surgically on the back of the rats and treated with PBS, Collagen (Col), Chitosan (Chi) and Chitosan + Collagen (Chi + Col) for 7 days. The completion of wound closure in the animals of each group was evaluated by macroscopic observation. The period of epithelialization values are expressed as mean ± S.D. from 6 rats in each individual experiments *** ($P < 0.001$).

The protein level seems to be higher in Chi + Col treated animals compared to other groups (Table 4).

3.3. Histology

Fig. 2a shows the histology of control specimen wherein the inflammation process is still in progress even on day 15 after post injury. This is observed by the presence of immature collagen fibers and undifferentiated keratinocytes under the basal layer of lamina. The biopsies of collagen treated wound shows the loosely packed collagen fibers with more irregular arrangement and undifferentiated keratinocytes accumulated in the epidermis (Fig. 2b). The onset of keratinocytes differentiation and their accumulation in the basal lamina of epidermis, looser connective tissue stroma richer in capillaries, as observed in collagen group, are seen in chitosan treated wound tissue (Fig. 2c). Chi + Col treated wound tissue on day 15 shows uniform accumulation of collagen with prominent thick bundles of collagen fibers embedded with proliferative fibroblasts. The structure of hair follicle, sebaceous glands and pseudoreferous glands looked normal (Fig. 2d) with complete epithelialization.

3.4. Electron microscopic studies

Collagen: In addition to the observation made in the histological study, the ultrastructural details also show the immature collagen fibers in gel-like form, apparently with more water content in the control wound (Fig. 3a). Whereas, in collagen and chitosan treated wounds (Fig. 3b and c), the collagen fibril formation is clearly seen but, in a loosely packed manner with irregular arrangements. Compared to the earlier groups, the Chitosan + Collagen treated group showed an increase in collagen content, with more aggregation of fibers to form clear bundles, as it could be seen from the cross section of the bundles (Fig. 3d).

Fibroblast: Fibroblast is the most important cell behind the synthesis of collagen. Rough endoplasmic reticulum in the fibroblast is the site of collagen synthesis. The ultrastructural details of fibroblasts of control and experimental groups are given in Fig. 4. The fibroblast in the collagen-chitosan treated groups appears to be elongated in shape without any shrinkage in its periphery (Fig. 4d), unlike it is seen in the case of Fig. 4a and b, for control & collagen treated animals. In the case of chitosan treated animals, the fibroblast showed normal shape and the matrix synthesis seems to be low as the nucleus is surrounded by loosely packed collagen (Fig. 4c). These observations are in conformity with the histological findings (Fig. 2).

Table 3
Levels of collagen, hexosamine and uronic acid content in the granulation tissue of control and experimental groups.

Groups	Days		
	4	8	15
Collagen (mg/100 mg dry weight)			
Control	1.77 ± 0.06	4.83 ± 0.74	12.28 ± 1.371.37
Collagen	3.24 ± 0.02	28.74 ± 2.94***	17.06 ± 1.24***
Chitosan	5.61 ± 0.85***	29.03 ± 3.36**	23.42 ± 2.29***
Chitosan + Collagen	5.74 ± 1.36***	32.50 ± 6.12**	29.28 ± 8.61***
Hexosamine (mg/100 mg dry weight)			
Control	1.77 ± 0.06	1.25 ± 0.15	0.63 ± 0.23
Collagen	2.34 ± 0.06***	1.85 ± 0.11***	0.65 ± 0.21
Chitosan	3.12 ± 0.03***	1.88 ± 0.14***	0.68 ± 0.01
Chitosan + Collagen	4.82 ± 0.78***	2.42 ± 0.18***	1.51 ± 0.03***
Uronic acid (mg/100 mg dry weight)			
Control	0.45 ± 0.04	1.21 ± 0.18	1.35 ± 0.17
Collagen	1.10 ± 0.12***	2.13 ± 0.14***	1.68 ± 0.16*
Chitosan	2.94 ± 0.02***	3.17 ± 0.42***	2.48 ± 0.03***
Chitosan + Collagen	3.18 ± 0.43***	3.80 ± 0.62***	2.94 ± 0.02***

Wounds were created surgically on the back of the rats and treated with PBS, Collagen (Col), Chitosan (Chi) and Chitosan + Collagen (Chi + Col) for 7 days. Tissues were collected from wounds at 4, 8 and 15 post-treatment days and were analyzed for content alues are expressed as mean ± S.D. for six animals. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 4
Protein levels of normal and wounded skin on 15th day after wounding (mg/100 mg tissue).

Groups	Normal skin	Wounded skin
Control	1.49 ± 0.18	1.74 ± 0.17
Collagen	1.75 ± 0.16*	1.94 ± 0.21
Chitosan	2.01 ± 0.11***	2.34 ± 0.21**
Chitosan + Collagen	2.03 ± 0.13***	2.42 ± 0.22***

Wounds were made surgically on the back of the rats and treated with PBS, Collagen (Col), Chitosan (Chi) and Chitosan + Collagen (Chi + Col) for 7 days. Tissues were collected from wounds at day 15 post-treatment and were analyzed for protein content. Values are expressed as mean ± S.D. for six animals. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

3.5. PDGF and NGF levels in skins

The level of growth factors, PDGF and NGF, were measured in the re-epithelialized tissues at the wound site, after 15 day post-wounding, to understand the influence of exogenous biological material on the cells that produce PDGF or other growth factors such as NGF which is essential for the skin sensory purpose. The levels of PDGF and NGF in the wound tissue are presented in Fig. 5. The treated samples showed higher values of growth factors compared to that of the control.

3.6. Antioxidants status

The levels of endogenous enzymic and non-enzymic antioxidants in the skin are given in Table 5. A relatively increased level of (GSH), ascorbic acid, CAT, SOD, and GPX is observed in the treated groups in general and more particularly in the Chi + Col treated animals. The level of antioxidants in the re-epithelialized tissue and the adjoining normal skin tissue within any particular group shows least difference except in the case of GPX.

3.7. Lipid peroxidation

Presented in Fig. 6 are the levels of lipid peroxidation products, as malondialdehyde (MDA), in the normal and wounded skin specimens. Overall, the treated group animals showed relatively lower levels of MDA compared to the control. Within each group the MDA level in wounded skin specimen was higher than that in its normal skin for obvious reasons, of tissue injury that followed the inflammatory responses.

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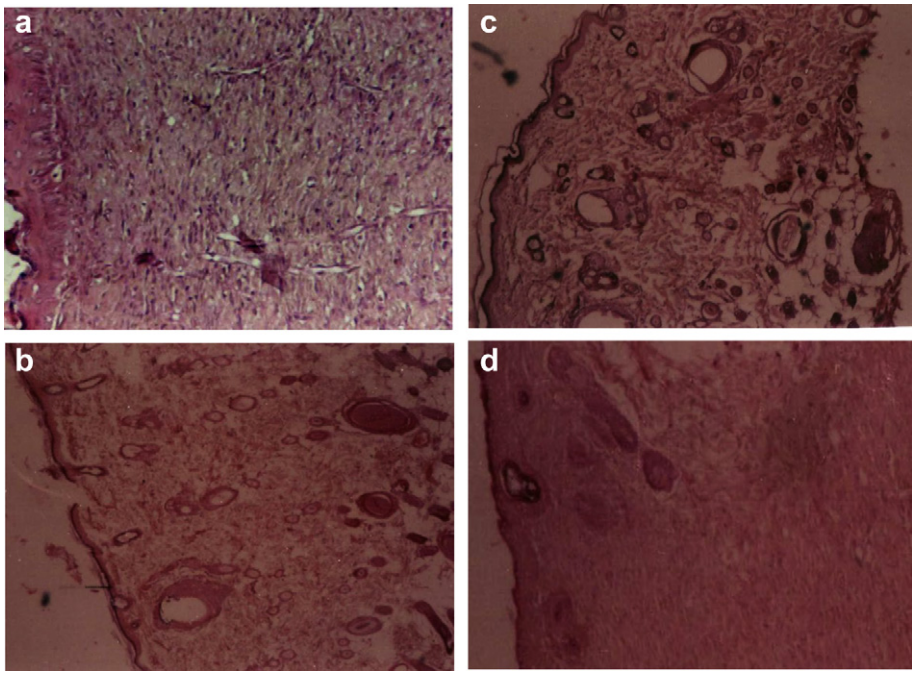

Fig. 2. Wounds were created surgically on the back of the rats and treated with PBS, Collagen (Col), Chitosan (Chi) and Chitosan + Collagen (Chi + Col) for 7 days. H & E staining of wound specimens from control and experimental rats on 15th day after wounding.

4. Discussion

The focus of this study was to test our hypothesis that the Chitosan + Collagen biopolymer can be a scaffold on excision wound surface, to mimic the ECM of natural tissue. Since the ECM is a gel-like substance containing collagen Type I and glycosaminoglycans [26] the use of monomeric collagen and chitosan oligomer blend in gel form as ECM substitute was investigated in this study.

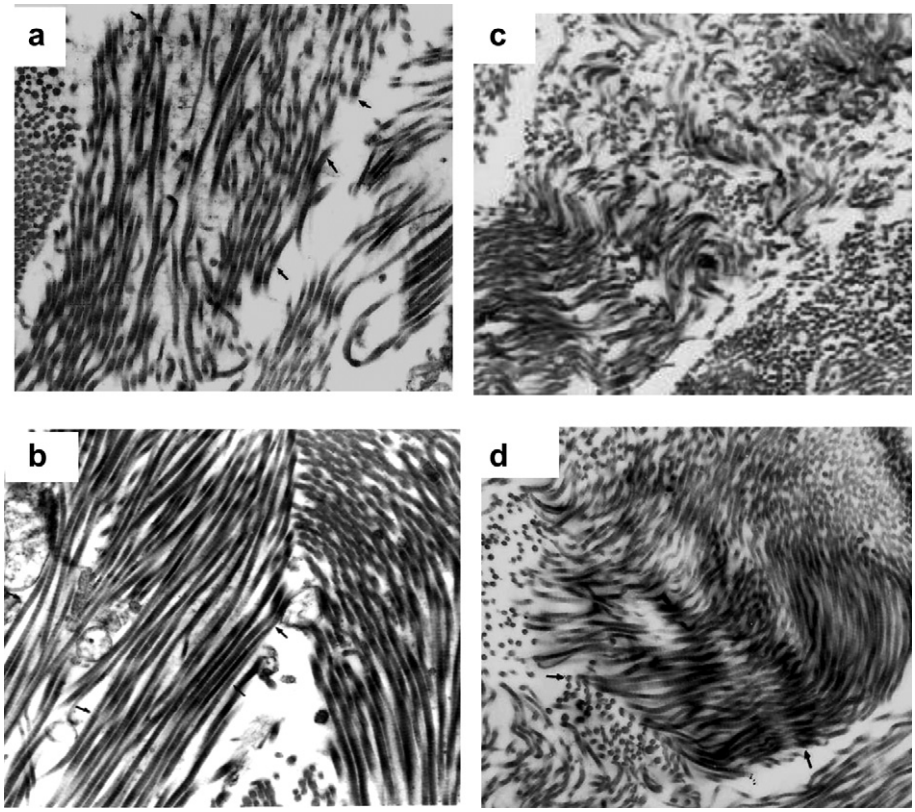

Fig. 3. Ultrastructure of dermal wounds showing the arrangement and distribution of collagen bundles wounds were made surgically on the back of the rats and treated with PBS, Collagen (Col), Chitosan (Chi) and Chitosan + Collagen (Chi + Col) for 7 days. The healed skin specimens from control and experimental rats on 15th day after wounding are fixed, sectioned, examined and photographed under Phillips 201 C Electron Microscope. (a) Control (b) Collagen (c) Chitosan (d) Chitosan + Collagen (Original magnification × 10,000).

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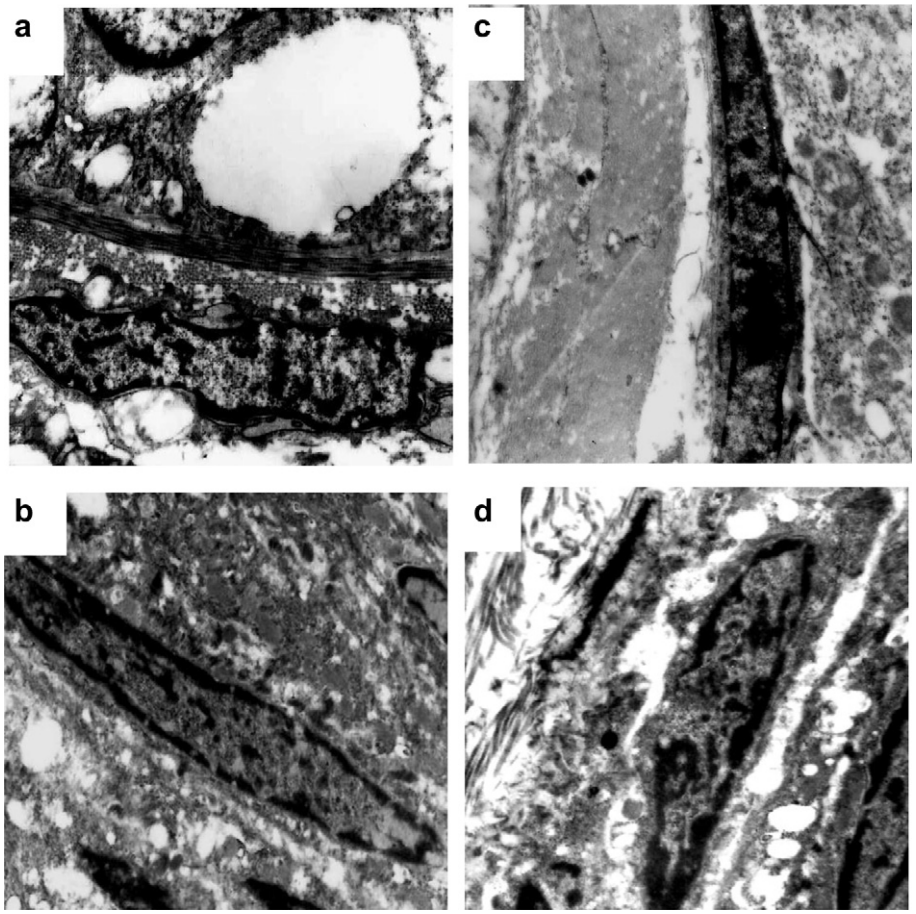

Fig. 4. Transmission electron micrographs reveal presence of fibroblasts. Wounds were made surgically on the back of the rats and treated with PBS, Collagen (Col), Chitosan (Chi) and Chitosan + Collagen (Chi + Col) for 7 days. The healed skin specimens from control and experimental rats on 15th day after wounding are fixed, sectioned, examined and photographed under Phillips 201 C Electron Microscope.(a) Control (b) Collagen (c) Chitosan (d) Chitosan + Collagen (Original magnification × 4500).

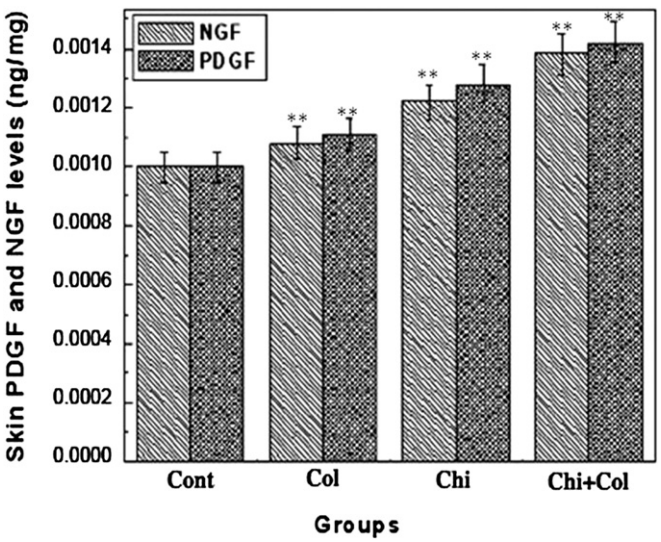

Fig. 5. Wound healing and PDGF and NGF levels. Wounds were created surgically on the back of the rats and treated with PBS, Collagen (Col), Chitosan (Chi) and Chitosan + Collagen (Chi + col) for 7 days. The levels of PDGF and NGF were determined by ELISA in skin wound specimens from control and experimental rats. Values are expressed as mean ± S.D. for six animals $^{*}P < 0.01$.

As the collagen and chitosan are in the hydrated state in a gel-like system possibly with adherence to tissue might easily provide internal spaces for cell proliferation, nutrient diffusion and neovascularization after *in vivo* application on the wound site. Our goal is to study whether the granulation tissue-like acellular scaffold containing hydrated collagen in molecular state and the chitosan, both in solubilized, hydrated form has the potential to influence the formation and remodeling of dermal tissue, after implant of the same gel in the wound site.

In the present study, the topical application of collagen or chitosan or the combination of both seem to show some advancement in the period of epithelialization compare to the control, indicating the biocompatible and bioactive nature of these biological materials. An advancement of 4 days in the healing process in collagen treated group, compare to the control, may be due to the monomeric and atelo nature of collagen as well as its gel form that is readily acceptable on the edges of the wound. This might have promoted the cellular mobility and growth to some extent.

Chitosan being a biocompatible polysaccharide reported to have wound healing property [9] by promoting the granulation phase in the process, was also applied topically on the wound in gel form for comparative study. On the other hand, the animals that received the blend of collagen and chitosan had the advantage of exploiting the collective bioactive properties of these polymers. For instance, the bio adhesive nature of chitosan due to its positive charges, and the poor antigenicity of collagen due to its atelo form and the free molecular state of collagen similar to tropho collagen conform to

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Table 5
Antioxidants levels in the epithelialized and unwounded skin on day 15.

Antioxidants	Normal skin	Wounded skin
Glutathione (μg/mg protein)		
Control	2.62 ± 0.18	2.68 ± 0.09
Collagen	2.65 ± 0.20	2.66 ± 0.09
Chitosan	2.65 ± 0.42	2.67 ± 0.38
Chitosan + Collagen	3.01 ± 0.25*	3.43 ± 0.43**
Ascorbic acid (μg/mg protein)		
Control	2.02 ± 0.03	2.37 ± 0.04
Collagen	2.95 ± 0.036***	3.15 ± 0.09***
Chitosan	2.83 ± 0.31***	2.96 ± 0.28**
Chitosan + Collagen	3.32 ± 0.41***	3.39 ± 0.29***
Catalase (μmol H_2O_2 cons/min/mg protein)		
Control	2.02 ± 0.03	2.37 ± 0.04
Collagen	2.95 ± 0.036***	3.15 ± 0.09***
Chitosan	3.32 ± 0.41***	3.43 ± 0.43**
Chitosan + Collagen	2.83 ± 0.31***	2.96 ± 0.28**
SOD (Units/mg protein)		
Control	1.46 ± 0.04	1.51 ± 0.03
Collagen	1.88 ± 0.09***	2.32 ± 0.16***
Chitosan	1.88 ± 0.09***	2.32 ± 0.16***
Chitosan + Collagen	1.93 ± 0.12***	2.46 ± 0.09***
GPX (nmole GSH util/min/mg/protein)		
Control	1.54 ± 0.26	2.96 ± 0.27
Collagen	2.13 ± 0.15***	3.29 ± 0.30***
Chitosan	6.12 ± 0.67***	7.83 ± 1.02***
Chitosan + Collagen	6.29 ± 1.12***	8.36 ± 1.21***

Wounds were created surgically on the back of the rats and treated with PBS, Collagen (Col), Chitosan (Chi) and Chitosan + Collagen (Chi + Col) for 7 days. Tissues were collected from wounds at day 15 post-treatment and were analyzed for glutathione, ascorbic acid, catalase, SOS, and GPX. Values are expressed as mean ± S.D. for six animals. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

the requirement of the provisional matrix that is laid down initially in the wound site.

Additionally, the collagen and chitosan in the blend interact with each other through hydrogen bond or electrostatic bonds to protect it from digestion by collagenases [27] whereas the chitosan itself is able to protect the wound through its antibacterial activity [28]. Therefore, collagen and chitosan in combined form provides stability on the wound site, and serve as scaffold to facilitate the initial events of epithelialization. Also, the exogenous material should have become part of the ECM in the form of epithelial tissue, much ahead of the process of epithelialization or, parallel to the proliferation step. Thus the exogenous application might have triggered the preponement of cellular and metabolic activities that enabled overlapping and merging of initial events, with one another, at an increased rate.

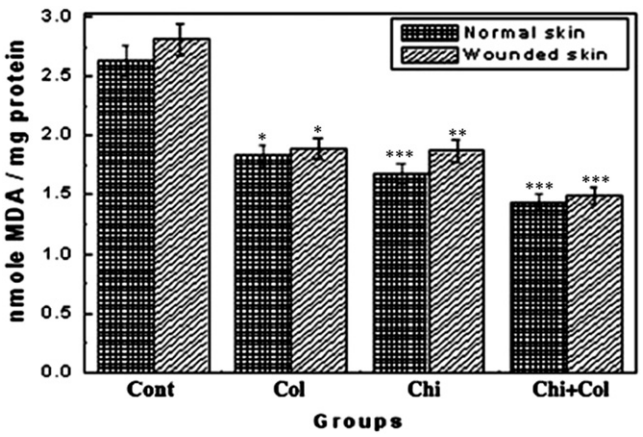

Fig. 6. Wound healing and TBARS. Wounds were created surgically on the back of the rats and treated with PBS, Collagen (Col), Chitosan (Chi) and Chitosan + Collagen (Chi + col) for 7 days. The lipid peroxidation products (as malondialdehyde) were determined by the TBA reaction in skin specimens from control and experimental rats.

Uronic acid is one of the main glycosaminoglycans (GAG) secreted by the fibroblast in the proliferative stage of wound healing, to stimulate the migration and mitosis of fibroblasts and epithelial cells [29] The increase of GAG at the wound site takes place maximally five days after wounding, and decreases thereafter in an untreated normal case [30]. Whereas in the present study, uronic acid level was decreasing from day 4 onwards suggesting the maximum production of uronic acid well in advance or immediately after the wounding, probably due to the exogenous application of different types of biomaterials on to the wounds. As could be seen from the results of Table 3, decrease of hexosamine level with increasing number of post-wounding days and the decreased production of uronic acid indicate the progression of the process of collagen synthesis followed by healing. It is therefore presumed that the presence of either uronic acid or glucosamine (in the form of chitosan) forming an extracellular matrix along with collagen, promotes wound healing at an increased rate.

As depicted in Fig. 1, the rate of healing in terms of reduction in wound size, in Chitosan + Collagen treated animals, show a reverse sigmoid curve with more downward deviation in the foot region in phase I (1–4 days) followed by a drop in phase II (5–8 days), indicate relatively a faster reduction in the wound size. In normal process of healing the contraction is maximal between 5 and 15 days after wounding and is mediated to a great extent by the myofibroblasts [31]. But the Chitosan + Collagen treated showed a highest percentage (88%) of reduction in wound size compare to other groups, on day 8, suggesting the promoting role played by the Chitosan + Collagen combination in the process of wound healing.

An interesting observation of the present study is a significant increase in the collagen content of granulation tissue is isolated from collagen-chitosan treated animals, which may be due to an increased synthesis of collagen, and could be correlated with the effective healing of wounds [32]. Hence our findings strongly suggest that the exogenous application enhances collagen synthesis and deposition.

The fibroblast is the most important cell behind the synthesis of collagen [33]. The results of Fig. 3 reveal that excepting the control group all other groups showed clear collagen fibrillar bundles because the water is gradually reabsorbed from the scar allowing collagen fibers and other matrix components to lie closer together. However, there is a difference in the fiber density (Fig. 3) and the shape of the fibroblast (Fig. 4), among the groups that differentiate one from the other. For instance the control specimen showed an intermediary structure that appears neither amorphous nor fibril due to the premature status of the matrix, as observed in the histology. Whereas in other groups and more particularly in the Chitosan + Collagen groups, the collagen bundles appear to grow bulk and become progressively reoriented from a random pattern to like-parallel to the skin surface.

Cutaneous injury is initiated by inflammatory response during which the neutrophils and macrophages release reactive oxygen species (ROS) to destroy contaminating bacteria [34]. in addition to which the cells also follow phagocytosis. Normally, the ROS and other cytotoxic chemical species are effectively detoxified by the scavenging substances called antioxidants and thus perfect the healing of injured tissues to occur. Small antioxidant molecules such as vitamin C and glutathione provide second line of antioxidant defense [35]. The increasing levels of GSH in the epithelial tissues, starting from control through Chitosan + Collagen may be

due to the participation of the different healants, at varying degrees, in promoting the inflammatory response and thereby minimizing the tissue degeneration by combating the free radical and maintaining the antioxidants levels throughout the regenerative process (Table 5).

The results on the ascorbate levels showing the reversible oxido-reduction reaction of ascorbic acid in the process of antioxidant regeneration. An increased level of dismutation (Table 5) of superoxide radical by the inflammatory cell-derived oxidases in the treated groups compared to that of the control suggests the enhanced cellular activities and thus confirming the increased expression of SOD by the immune cells during the process of healing. A rapid detoxification of this molecule is essential and H_2O_2 is achieved by catalase and glutathione peroxidase enzymes. According to the results of Table 5, the rate of conversion of H_2O_2 into water seems to be increasing in the different Groups in the following order: Chi + Col > Chi > Col > Cont, and hence the rate of healing also lies in the same line. It is understood from this observation that the healants used in this study are very well acceptable by the wound. The results of GPX content may be correlated with the extent of epithelialization of the tissue at wound site. Because the formation of well defined active epithelium and the hair follicles, at the wound edge, are the prominent sites of expression of GPX [35] and therefore increased levels of GPX was observed in the Chi + Col treated group.

Malondialdehyde (MDA) was measured to identify the extent of lipid peroxidation, an index of oxidative stress. In the treated groups are suggestive of either decreased phagocytosis in the earlier inflammatory stage or the absence of any pathological condition in the experimental animals. For instance, diabetes is associated with increased level of ROS, which inturn depletes the antioxidants and results in increased lipid peroxidation [36]. Since, the wound healing study in present investigation was conducted by using the normal rats, the chances of pathology in the animals are more unlikely and therefore, wide variation in the MDA levels between the treated groups was not observed. Almost same level of MDA found in the unwounded normal skins of different groups as that of their corresponding epithelial tissues suggests the occurrence of LPO in the periphery of wound to an extent as same as in the wound site.

Topical application of biopolymers to the wounds was carried out upto 7 days and the epithilialized tissue was analyzed for PDGF and NGF levels with a speculation that exogenous matrix supported chemotaxis might have lead to over secretion of these growth factors and would have altered their levels at the wound site. Growth factors such as PDGF and NGF may play important roles in wound healing, mediating essential activities for normal tissue repair. PDGF, an important biochemical mediator of wound healing released by platelets, macrophages, endothelial cells and fibroblasts [37], improves dermal regeneration, promote protein and collagen synthesis, causes endothelial migration or angiogenesis [38] This inturn would have influenced the NGF synthesizing cell types such as fibroblasts, mast cells, keratinocytes, in response to inflammation, as this growth factor along with some other growth factors are essential for regulating the cellular events involved in the wound healing. More particularly, NGF is considered to be a stimulant for the regeneration of neurons, which is an important step in the remodeling of wounded tissue, in addition to its cutaneous wound healing. As expected, the biological matrix treated wound tissue showed higher levels of PDGF and PDGF-mediated NGF production. In addition to these growth factors examined, other growth factors would also have been secreted by various cell types in the process of tissue regeneration.

In conclusion, the topical application of gel comprising monomeric-atelocollagen and easily soluble chitosan, to the wound triggers the wound healing process by mimicking the extracellular matrix to facilitate enhanced cellular activities and thus show advancement in each phase of tissue repair.

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