ORIGINAL ARTICLE

A Comparative Study on the Efficacy of Chitosan Gel Formulation and Conventional Silver Sulfadiazine Treatment in Healing Burn Wound Injury at Molecular Level

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Abstract

Objective: Comparison of the efficacy of a novel 5% chitosan gel formulation in healing burn wound with respect to conventional silver sulfadiazine (SSD) treatment at molecular level. **Materials and Methods:** Different concentrations of chitosan gel were formulated and optimized with respect to spreadability, extrudability, and viscosity. Burn wounds were created on 18 rats with three groups of six animals each. Group I served as control, Group II animals were treated with standard SSD ointment, and Group III animals were treated with optimized chitosan gel till 16 days. Wound healing efficacy was compared by calculating the percentage of wound contraction, and any difference in wound healing property at the molecular level was ascertained by the levels of hydroxyproline, hexosamine, collagen, and antioxidant analysis. **Results:** On the basis of viscosity, spreadability and extrudability, 5% chitosan gel was selected as the optimized test formulation. Group III animals treated with chitosan gel showed faster contraction in wound area in comparison with control and standard treatment groups. At the end of 16th day, 95.5 ± 2.4%, 84.14 ± 2.1%, and 34.9 ± 2.1% healing was observed in case of chitosan gel, standard, and control treatments, respectively. Topical application of 5% chitosan gel increased collagen synthesis and stabilization at the wound site, as evidenced by increase in hydroxyproline and hexosamine levels, and up-regulated expression of collagen Type I. Furthermore, there was a significant increase in levels of endogenous enzymatic and nonenzymatic antioxidants and decrease in lipid peroxide levels in chitosan gel treated burn wound granulation tissue. Histological examination also confirmed the better healing efficacy of 5% chitosan gel in comparison to standard treatment. **Conclusion:** Results indicate that 5% chitosan gel has the potential to be developed as an effective burn wound healing agent in comparison to existing treatment option.

Key words: Burn wound, chitosan, collagen, hydroxyproline, silver sulfadiazine

INTRODUCTION

Burn wound injuries are one of the most common and destructive forms of trauma.[1] A variety of agents such as heat, electricity, sunlight, chemical, or nuclear radiations causes mild or severe burn injuries. The majority of these injuries require specific medical intervention as they may otherwise lead to severe morbidity and mortality. Burn injuries are generally classified as, (a) superficial burns, which heal within 2-3 weeks with minimal scaring and do not normally require any surgical intervention, and (b) deep burns, which take substantially long time to heal, are characterized by severe scarring and generally require surgical intervention.[2,3]

Management of burn wounds is a complex process that poses critical challenges such as increased susceptibility of the body to infections, antigen challenge, and repeated additional trauma caused by wound cleaning.[4,5] Burn management

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entails significant duration of hospital stay, expensive medication, multiple operative procedures, and prolonged period of rehabilitation.[6]

Topical application of antibacterial ointments and disinfectants is the first line of treatment in such cases, which focuses only on preventing any secondary infection. However, without any suitable therapeutic intervention, skin regeneration and overall recovery time increase considerably. Recombinant growth factors and tissue-engineered wound dressing may be used, but they are highly expensive and generally beyond the reach of most of the patients.[7] Silver sulfadiazine (SSD) is the topical agent of choice in severe burns and is used almost universally today in preference to compounds such as silver nitrate and mafenide acetate. SSD cream, while being effective, causes some systemic complications including neutropenia, erythema multiforme, crystalluria, and methemoglobinemia.[8-10] In view of this limitation, there has been a growing concern regarding the development of a suitable biological wound care dressing, which may provide the right microenvironment to promote healing and protect the wound throughout the healing process.

Chitosan is a cationic natural polysaccharide obtained by deacetylization of chitin and composed of units of glucosamine and N-acetylglucosamine. Chitosan is used in numerous areas of biopharmaceutical research because it is safe, biodegradable and biocompatible molecule.[11] It acts as a wound healing agent by enhancing the level of natural hyaluronic acid synthesis at wound site. It also accelerates wound re-epithelization and rejuvenation of nerve with in vascular dermis.[12] Apart from wound healing, it also activates host defense to prevent infection as it possesses antimicrobial, antibacterial and antifungal properties, which are particularly useful in wound treatment.[13] Thus, chitosan is being investigated by several research groups worldwide as an ideal material for wound healing as it possesses specific homeostatic, granulation and epithelization properties.[14]

Our laboratory has also developed a novel chitosan gel formulation for the management of wounds, including burn wounds. Aim of the present work was to assess and compare the efficacy of this chitosan gel formulation *vis-à-vis* conventionally used SSD treatment in treating full thickness burn wound in experimental rats.

MATERIALS AND METHODS

Ethics statement

All animal procedures for the study were followed as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals regulations, and the experimental protocol was approved by Institutional Animal Ethics Committee.

Materials

Chitosan low Viscous was procured from Sigma Chemical Company, St. Louis, MO, USA. Glacial acetic acid and calcium chloride were purchased from Merck India Ltd., Mumbai, India. Conventionally used 1.0% w/w SSD cream (Ranbaxy Laboratories Ltd., Delhi, India) was procured from local market. All other chemicals and solvents were of analytical grade.

Optimization of calcium chloride concentration for improved blood clotting

The concentration of calcium chloride required to clot unit volume of ethylenediaminetetraacetic acid (EDTA) mixed blood was optimized by collecting EDTA mixed blood in glass tubes. 5, 10, 15, 20, and 25 mM calcium chloride solution each ranging from 100 to 500 ul was added in 1 ml of EDTA mixed blood, respectively, till clot was observed. The blood sample was collected by retro oberital venous plexus puncture from male Sprague Dawley rats. The EDTA solution was added before the collection of blood and mixed well by inverting the collecting tube. The concentration of EDTA was mixed in such a way that the amount of EDTA mixed with unit volume of blood was 1.5 mg after each addition of aliquots of calcium chloride solution and the glass tube was tiled at 45° angle to observe clot formation.

Preparation of chitosan gel

Preparation of stock solution

- Stock A: 1 g of $CaCl_2$ was dissolved in 100 ml distilled water to make 1% w/v stock solution of $CaCl_2$
- Stock B: 1 ml of glacial acetic acid was dissolved in 99 ml of distilled water and sonicate for 10 min to make 1% v/v of acetic acid solution
- Stock C: Optimized Stock A and Stock B solution was mixed in 1:3 volume ratio which contain 20 mM $CaCl_2$ and 0.75% acetic acid. Stock C is known as dispersion medium and used for gel formation.

Different concentrations (1-5%) of chitosan were dissolved separately in dispersion medium [Table 1]. The reaction mixtures were kept at 25°C under continuous stirring at 250 rpm on a magnetic stirrer, and viscous chitosan hydrogels were obtained with different viscosity.

Characterization of chitosan gel

Evaluation of pH

pH of the gel was measured using digital pH meter (Fisher Scientific, USA). The measurements of pH were done in triplicate and average value was calculated.

Table 1: Composition of chitosan gel

Batch no.	Chitosan concentrated (g)	Dispersion medium
F1	1	99 ml
F2	2	98 ml
F3	3	97 ml
F4	4	96 ml
F5	5	95 ml

Spreadability

Spreadability is the term expressed to denote the extent of area to which formulation readily spreads on application to skin or affected part. Therapeutic efficacy of a formulation also depends on its spreadability. To determine spreadability of the developed chitosan gel formulation, 0.5 g of gel was placed within 1 cm diameter pre-marked on a glass plate of 20 cm × 20 cm, over which a second glass plate was placed. A weight of 500 g was allowed to rest on the upper glass plate for 5 min. The resultant increase in diameter due to gel spreading was noted.[15]

Extrudability

A good gel extrudes optimally from a packing tube when a slight pressure is applied. The extrudability of the developed gel formulation from aluminum collapsible tubes was therefore also determined. Aluminum collapsible tubes filled with 10 g chitosan gel were held between two clamps. The tubes were compressed and extrudability of different formulation compositions was determined in terms of weight required in grams to extrude a 0.5 cm ribbon of gel in 10 s.[15]

Viscosity

Viscosity of the formulations was determined using a Brookfield digital viscometer (model DV-II, USA) equipped with spindle S27. 5 g of the gel sample was placed in the sample holder of the viscometer and allowed to settle for 5 min and the viscosity measured at a rotating speed of 50 rpm at room temperature (25-27°C).

Fourier transform infrared spectroscopy (FTIR)

Chitosan gel composition was confirmed by FTIR. Pure drug and the formulation were subjected to IR study using FTIR spectrophotometer (Perkin Elmer). The spectra were scanned over a wave range from 4000 to 400 cm^{-1} in transmittance mode.

Cryo-scanning electron microscopy (SEM)

Surface morphological study of chitosan hydrogel was carried out using SEM (Zeiss LSM 800 and LSM 880). The hydro gel samples were cryofreezid and processed under reduced pressure conditions and observed under SEM at constant 20 kV accelerating voltage.

Atomic force microscopy (AFM)

AFM micrograph of optimized polymeric chitosan gel was carried out with scanning probe microscopy (SPM – AFM, NTAQ13 MDT, Switzerland) in contact mode, wherein a gel sample was observed under AFM. Nitride silicon cantilevers (OMCL-TR400PSA-1) with a spring constant of 0.02 N/m and a nominal tip radius of approximately 15 nm was used. The experiments were carried out under ultra-clean conditions at room temperature, and AFM imaging was performed in air scanning frequency of 1 Hz and a vertical deflection of 0.5 V. AFM images with 512 × 512 pixels were obtained at separate locations to ensure a high degree of reproducibility of experimental data.

Animal experiments

Healthy female Sprague Dawley rats weighing (200-250 g) were obtained from the Experimental Animal Facility of Institute of Nuclear Medicine and Allied Sciences, DRDO, Delhi. All animals were housed in standard environmental conditions of temperature 21-23°C, humidity 45-50% and a 12 h light and 12 h dark cycle in polypropylene cages and were provided with standard feed (Golden Feed Ltd., Delhi, India) and water *ad libitum.*

Creation of burn wound model

The animals were anesthetized by intramuscular injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). Dorsal surface of the rats was shaved, and the underlying skin was cleaned with 70% ethanol. Full thickness burn wound was created using aluminum metal rod of 1.5 cm diameter and heated to 85°C. Temperature of the metal rod was monitored with an IR camera (Wahl heat spy(R) HSI1200). Hot rod was exposed on the shaved area of rats for 20 s, resting on its own weight of 30 g. No additional pressure was applied on the hand-leaded metal rod. After 24 h, dead tissue was excised using sterile surgical blade. Animals were allowed to recover from anesthesia and housed individually in sterile cages.

Animal grouping

18 burn wounded animals were selected randomly and divided into three groups of six animals each. Animals of Group I were taken as a negative control and left untreated to the open environment to monitor the rate of wound contraction. Group II animals were taken as standard and treated with conventional wound healing ointment of SSD. In Group III, all the six animals were treated topically with optimized 5% chitosan gel twice daily till complete healing.

Physical evaluation

Measurement of wound size

Any contraction in wound size was measured by standard method by tracing the wounded margin on a tracing paper and calculating

percentage reduction in wound area post-treatment.[16] Area of the wound at day 0 was considered as 100% for the calculations of percentage reduction in wound area. The percentage wound contraction was calculated using the following equation:

% Wound contraction = Healed area/Total area × 100

Skin irritation test

As per OECD guidelines, skin irritation is defined as the production of reversible damage of the skin following the application of a test substance. It is generally assessed by the potential of a test substance to cause erythema/eschar and/or edema after a single topical application. For this study, area around the wound of rats embedded with chitosan gel was observed for any kind of edema or erythema on skin during the period of healing.

Biological evaluation

Antioxidant analysis

Oxygen free radicals are harmful to wound healing process and account for the delay in healing due to their damaging effects on cells and tissues. Oxidative stress markers were, therefore, detected in the resultant supernatant of skin homogenate of burn wound. Appropriate kits (Bio-vision, USA) were used for determination of the activity of glutathione (GSH), GSH-S-transferase (GST), catalase (CAT) activity, and superoxide dismutase (SOD). Lipid peroxidation in skin homogenates was estimated spectrophotometrically by estimating malondialdehyde (MDA) levels.

Estimation of hydroxyproline and hexosamine

Hydroxyproline and hexosamine levels were also estimated as they have a crucial role in collagen stability. The granulation tissue was collected from the healed wound area, and hydroxyproline content was analyzed using hydroxyproline assay kit (Sigma-Aldrich, USA). Levels of hexosamine in granulated tissues were determined as per previously reported method of Morgan and Elson[17] and expressed as mg/g dry weight of the tissue.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and estimation of total protein content

Fractionation of collagen was performed by SDS-PAGE as per the method reported by Laemmli.[18] Samples for SDS-PAGE were prepared by adding 100 µl of Laemmli sample buffer to 50 µg of protein and heating for 4 min at 100°C. An aliquot (10 µl) of this mixture was applied to each well in 7% polyacrylamide resolving gel and subjected to electrophoresis at 15 mA using a Mini-protean II cell (Bio-Rad Laboratories, Hercules, CA, USA). Following electrophoresis, the gels were stained with 0.04% coomassie blue in 25% v/v ethanol and 8% v/v acetic acid for 30 min at 60°C. Excess stain was removed with several washes of destaining solvent (25% v/v ethanol, 8% v/v acetic acid). Molecular weight was determined using standard protein markers (broad range 200-6.9 kDa). Total protein content in granulation tissue was measured by the method of Lowry *et al.*[19]

Histopathological analysis

At the end of experimental period, burned skin tissue samples were processed by standard histopathological procedure and microscopic changes in skin were observed. Briefly, skin tissue sections were embedded in paraffin and 5 µm size sections were cut separately. The sections were deparaffinized using xylene and ethanol and then washed with phosphate buffer saline and with permeabilization solution (0.1 M citrate, 0.1% triton X-100). The deparaffinized sections were stained with hematoxylin and eosin and Masson's trichrome stain for collagen. Tissue histology of animals applied with chitosan gel was observed under a microscope (Olympus BX 60) and compared with that of control and reference standard animals.

Statistical analysis

Commercial computer packages were used for data analysis (Graph Pad prism version 5, San Diego, California, USA and Microsoft Excel version 15 (Microsoft Corporation, New York, USA). All data are presented as mean ± SEM. All statistical tests were two tailed and difference was evaluated at the 5% level of significance.

RESULTS AND DISCUSSION

Optimization of calcium chloride concentration for improved blood clotting

5, 10, 15, 20, and 25 mM calcium chloride solution each ranging from 100 to 500 ul was added in 1 ml of EDTA mixed blood, respectively. No clot formation was observed in case of 5, 10, and 15 mM calcium chloride solution in 130 s. Whole blood clot was observed at 20 mM calcium chloride in 30 ± 3 s and it was selected as optimized concentration. The effect of different concentrations of calcium chloride solution (fixed volume) on the blood flowing through capillary resulted in clot formation and the clotting time was found to be decreased with higher concentration. As shown in Table 2 no significant change in clotting time beyond the of 20 mM calcium chloride concentration were found. These observations indicate that 20 mM concentration of calcium chloride has a significant effect in rapid blood clotting and could be used in gradient in hemostatic formulation.

Optimization and characterization of gel formulation

Chitosan hydrogel was formulated by dissolving varying concentrations of chitosan powder (1-5%). Formulation

containing chitosan between 1% and 2% are less viscous and having the free flowing nature over the surface. On the other chitosan hydrogel with 3-4% are viscous in nature but liquefy after 24 h without any shear. Polymeric hydrogel containing 5% chitosan showed the highest viscosity and homogenous in nature. Gel above 5% concentration was very viscous which could not be properly spread out on applying shear and have course in nature. Thus, 5% chitosan containing was selected as the optimized gel formation.

pH of the formulation was found to be 6.4 ± 0.5 [Table 3], which indicated that the gel formulation may not cause much irritancy to the skin on application. Spreadability of the formulation decreased with increasing concentration of chitosan. Figure 1 shows a plot of viscosity of varying conc. of chitosan gel (1-5%) against shear rate. Gel with 5% chitosan concentration (F5) showed higher viscosity at zero shear rate. Viscosity of gels decreased with an increase in the shear rate and became steady at higher shear rate indicating a pseudo plastic or shear thinning behavior. This can be advantageous, since the gel would become more fluid while it is being spread over the injured surface, leading to an easier and less painful application. The value of spreadability for optimized gel (5%) was found out to be 9.9 cm indicating that the lotion is easily spreadable by small amount of shear. This assures that the formulation maintains a good wet contact time when applied to the site of application.

Chemical stability of chitosan gel was confirmed using FTIR as shown in Figure 2. All the major peaks in chitosan gel samples matched with the standard chitosan samples, which confirmed the chemical stability and integrity of chitosan in gel. As can be seen from Figure 2, characteristic peaks of chitosan are found at 3253, 2873 (O-H stretching), 1407, 896, 656 (C-H bending), 1633, 1547 (C=C stretching), 1069, and 1073 cm^{-1} (C-O stretching). Almost similar peaks were observed in 5% chitosan gel formulation. Uniformity in the major peaks confirmed chemical stability of chitosan in chitosan gel samples independent of its concentration.

After processing through cryofreezing, hydro gel samples showed the highly porous structure with varying pore size. In addition, fibrous network was observed having the diameter of 40-45 nm which shown in Figure 3.

Atomic force micrograph showed that the chitosan hydrogel does not contain any particle like structure and revealed that

Table 2: Optimization of calcium chloride concentration for improved blood clotting

Concentration of $CaCl_2$ (500 ul)	Time for clot formation (s)	Observation (for 130 s)
0 mM	125±5	No clot formation
5 mM	94±4	No clot formation
10 mM	70±3	No clot formation
15 mM	44±6	No clot formation
20 mM	30±3	Clot formation
25 mM	30.1±2	Clot formation

Table 3: Evaluation of different chitosan hydrogels

Formulation code	pH	Spreadability* (g.cm/s)	Extrudability (g/cm^2)
F1	6±0.5	13±0.3	#
F2	6.2±0.2	11.6±0.2	#
F3	6.6±0.3	10.3±0.3	20.79±0.2
F4	6.4±0.1	9.9±0.1	19.50±0.5
F5	6.3±0.2	9.5±0.2	18±0.3

*Spreadability is determined within 1 h of preparation of gel.
#Sample liquefies before analysis

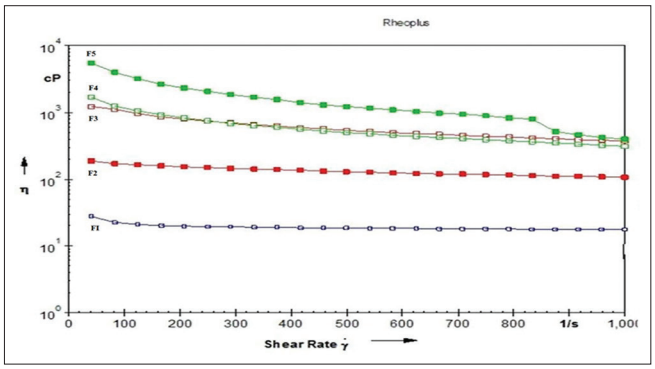

Figure 1: Viscosity of different chitosan gel test formulations (F1) 1% chitosan gel, (F2) 2% chitosan gel, (F3) 3% chitosan gel, (F4) 4% chitosan gel, and (F5) 5% chitosan gel

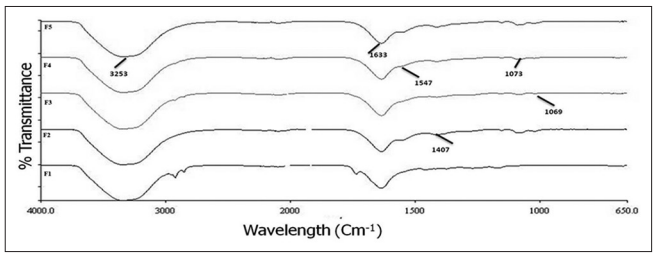

Figure 2: Fourier transform infrared spectroscopy spectra of different chitosan gel (F1) 1% chitosan gel, (F2) 2% chitosan gel, (F3) 3% chitosan gel, (F4) 4% chitosan gel and (F5) 5% chitosan gel

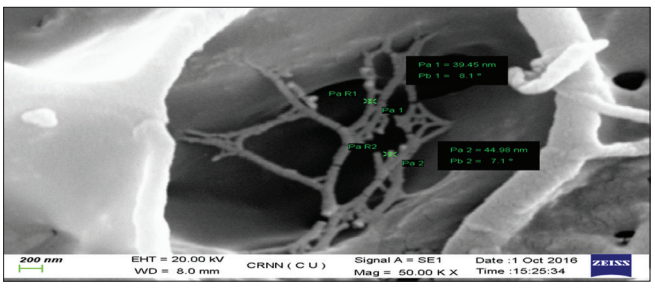

Figure 3: Scanning electron microscope micrograph of chitosan gel synthesized using 5% of chitosan, at 20.00 kV

chitosan hydrogel is homogeneous in nature. Figure 4 shows two-dimensional AFM of chitosan hydro gel.

Skin irritation test

None of animal showed any hypersensitivity reactions on the skin on topical application of the test formulation. There was no sign of erythema/Escher and or edema on the skin of rats, which indicates biocompatibility of chitosan gel with the skin.

Wound healing property of optimized chitosan gel

Animals treated with 5% chitosan gel (Group III) showed a significant faster reduction in wound area on day 8 and day 16 post wounding in comparison to control (Group I) and standard treatment (Groups II) groups as shown in Figure 5a In animals of Group III, 95.5 ± 2.4% healing was observed by 16th day in comparison to standard treatment and control group animals, which showed 84.14 ± 2.1% and 34.9 ± 2.1% healing by 16th day, respectively, as shown in Figure 5b. This indicates that 5% chitosan gel was better in promoting wound healing in experimental burn wounds as compared to standard SSD treatment. Application of chitosan gel significantly induced wound contraction and accelerated wound closure.

Chitosan increases wound contraction as chitosan gradually depolarizes to release N-acetyl β-D glucosamine, which initiates fibroblast activation and stimulate collagen synthesis in regenerated wound tissue.[20] During wound contraction fibroblasts play an important role, where they become activated and differentiate into myofibroblast, which reduces the size of a wound by riveting the wound edges and exert tension in the extracellular matrix (ECM), actively producing ECM protein such as collagen to facilitate wound closure. Both fibroblast and myofibroblast play a key role in wound healing by generating traction and contractile forces, respectively, to enhance wound contraction.[21,22]

Biological evaluation

Antioxidant analysis

In burn wound, there is an increase in oxidative stress and reactive oxygen species (ROS) generation, which leads to lipid peroxidation damage to cellular membranes, nucleotides, proteins and lipids, thereby delaying the wound healing process. Overproduction of ROS results in an attack on not only DNA but also other cellular components including the polyunsaturated fatty acid residues of phospholipids, which are highly sensitive to oxidation. Therefore, unsaturated fatty acids in cell membranes are susceptible to free radical-mediated oxidation. In this study, the MDA levels were found to be significantly decreased after chitosan gel treatment, suggesting decreased oxidative injury, which could be due to increased quenching or scavenging of oxygen free radicals by the elevated levels of enzymatic and nonenzymatic antioxidants such as GSH, SOD, CAT, and GST as shown in Table 4.

Estimation of hydroxyproline, hexosamine, and total protein content

Collagen is the major protein of the ECM protein and a principle component of connective tissue acting as structural scaffold in tissue, which ultimately contributes to wound strength. Chitosan treated group showed significantly enhanced levels of hydroxyproline, hexosamine, and total

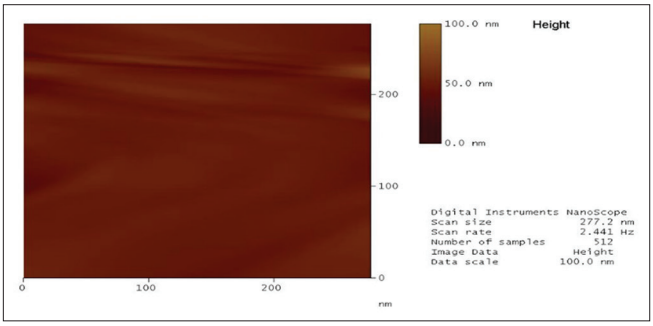

Figure 4: Atomic force micrograph of F5 (5%) of chitosan hydrogel

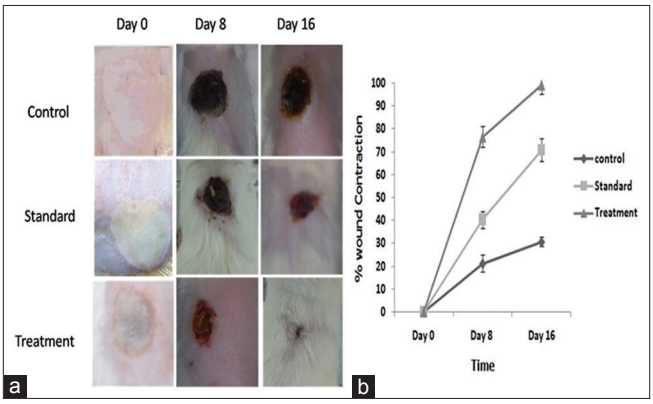

Figure 5: (a) Effect of topical application of 5% chitosan gel on wound area contraction, (b) physical examination of wound contraction at various time intervals in control, standard and treatment group

Table 4: Effect of topical application of chitosan gel for 8 days on the levels of antioxidants and lipid peroxidation in granulation tissue

Antioxidants	Burn control	Treatment
GSH (ug mg^{-1} protein)	1.28±0.05	2.13±0.04*
GST (ug mg^{-1} protein)	1.2±0.062	2.5±0.15*
CAT (ug mg^{-1} protein)	6.81±0.45	8.57±0.46*
SOD (ug mg^{-1} protein)	1.53±0.13	2.20±0.06*
MDA (nmol^{-1} mg protein)	2.24±0.083	1.38±0.021*

Values are mean±SE, n=6. *P<0.05 compared with burn control. GSH: Glutathione, GST: Glutathione-S-transferase, MDA: Malondialdehyde, CAT: Catalase, SOD: Superoxide dismutase, SE: Standard error

Table 5: Effect of topical application of chitosan gel on various biomarkers to assess wound healing

Biomarkers	Burn control	Standard	Treatment
Hydroxyproline (mg/g tissue wt)	20.90±0.98	26.24±2.54*	28.67±1.37**
Hexosamine (mg/g tissue wt)	0.53±0.03	0.63±0.05*	0.81±0.28**
Total protein (mg/g tissue wt)	59.38±1.99	106.88±2.35*	118.18±2.11**

Values are mean±SE, *n*=6. **P*<0.05 standard compared with burn control, ***P*<0.05 Treatment compared with burn control. SE: Standard error

protein as shown in Table 5. Collagen synthesis is evident by increased hydroxyproline content of granulation tissue.

To further confirm this observation, SDS-PAGE analysis of granulation tissue post-treatment with 5% chitosan gel was carried out and it indicated a significant amount of collagen formation as compared to that in case of standard treatment and control animals. Figure 6 shows SDS-PAGE banding pattern of collagen from control, standard and chitosan gel treated wound tissue. Skin collagen has been reported to comprise two or more different chains of alpha-I (100 kDa), alpha-2 (97 kDa), and beta (200 kDa) component, which is recognized as Type I collagen. Type I collagen is the most abundant type of collagen present in normal skin.[23] From the banding pattern, a significant increase in collagen Type I was observed in chitosan gel treated group as compared to burn wounds in control and standard treatment groups. In case of chitosan treated group, bands corresponding to alpha-I subunit and beta component could be clearly seen, while they were very weak in case of standard treatment group, suggesting better wound healing property of chitosan gel.

It is generally accepted that mechanical strength of soft tissues and hydroyproline level in tissues are related to collagen formation, whose synthesis is an essential feature of wound healing.[24] Stabilization of collagen molecule is reflected by increased hexosamine content. Chitosan gel treated animals were found to possess an increased level of hexosamine content as compared to standard treatment and control groups, which could be crucial for rapid collagen production. Therefore, enhanced synthesis of hydroxyproline and hexosamine in chitosan gel treated animals may provide help to strengthen repaired tissue, and in the healing process.

Histopathalogical evaluation

Histopathological analysis revealed that on the 16th day, chitosan gel treated group showed more advanced re-epithelialization and layering with continuous basement membrane in addition to better organization of the collagen bundles. An overall early recovery and regeneration in the chitosan gel treated group were seen when compared with control and standard treated groups. Masson's trichrome stain blue showed dense, uniform, compact and regularly arranged collagen fibers in the wound tissue of chitosan treated rats, whereas untreated burn wounds and standard treatment groups had less compact and irregularly arranged collagen fibers [Figure 7]. Histological findings corroborated biochemical results.

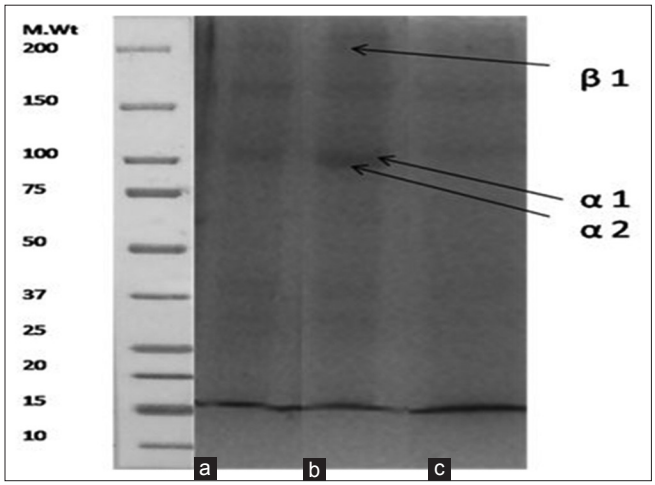

Figure 6: Expression of standard marker proteins (200-20 kDa, BIORAD, 10% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) collagen analyzed by SDS-PAGE (a) standard treatment, (b) 5% chitosan gel treated, and (c) control wound tissue of experimental rats after 16 days of topical treatment

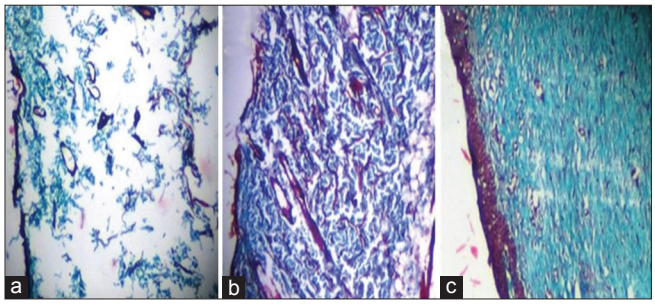

Figure 7: Histological staining of healed skin in each experimental group on 16th day postwounding in wound section of (a) control, (b) standard, and (c) treatment. Masson's trichome staining shows distribution and density of collagen protein in healed skin in various groups. Collagen protein is stained blue, nuclei are stained black and the background (muscle, cytoplasm, and keratin) are stained red

CONCLUSION

This study suggests that 5% chitosan gel possesses significant wound-healing ability in treating full-thickness burn wounds

as compared to standard SSD treatment. Topical application of chitosan gel augmented levels of hydroxyproline, hexosamine, collagen synthesis, endogenous antioxidants and prevented free-radical-mediated tissue injury.

REFERENCES

1. Church D, Elsayed S, Reid O, Winston B, Lindsay R. Burn wound infections. Clin Microbiol Rev 2006;19:403-34.
2. Rowan MP, Cancio LC, Elster EA, Burmeister DM, Rose LF, Natesan S, *et al.* Burn wound healing and treatment: Review and advancements. Crit Care 2015;19:243.
3. Srivastava P, Durgaprasad S. Burn wound healing property of *Cocos nucifera*: An appraisal. Indian J Pharmacol 2008;40:144-6.
4. Evers LH, Bhavsar D, Mailänder P. The biology of burn injury. Exp Dermatol 2010;19:777-83.
5. Benson A, Dickson WA, Boyce DE. Burns. BMJ 2006;332:649-52.
6. Upadhyay NK, Kumar R, Siddiqui MS, Gupta A. Mechanism of wound-healing activity of *Hippophae Rhamnoides* L. Leaf extract in experimental burns. Evid Based Complement Alternat Med 2011;2011:659705.
7. Shevchenko RV, James SL, James SE. A review of tissue-engineered skin bioconstructs available for skin reconstruction. J R Soc Interface 2010;7:229-58.
8. de Gracia CG. An open study comparing topical silver sulfadiazine and topical silver sulfadiazine-cerium nitrate in the treatment of moderate and severe burns. Burns 2001;27:67-74.
9. Hosnuter M, Gürel A, Babucçu O, Armutcu F, Kargi E, Isikdemir A. The effect of CAPE on lipid peroxidation and nitric oxide levels in the plasma of rats following thermal injury. Burns 2004;30:121-5.
10. Gregory SR, Piccolo N, Piccolo MT, Piccolo MS, Heggers JP. Comparison of propolis skin cream to silver sulfadiazine: A naturopathic alternative to antibiotics in treatment of minor burns. J Altern Complement Med 2002;8:77-83.
11. Cheung RC, Ng TB, Wong JH, Chan WY. Chitosan: An update on potential biomedical and pharmaceutical applications. Mar Drugs 2015;13:5156-86.
12. Jayakumar R, Tamura H, Kumar PT, Nair SV, Furuike T, editors. Novel Chitin and Chitosan Materials in Wound Dressing. Rijeka: INTECH Open Access Publisher; 2011.
13. Azuma K, Izumi R, Osaki T, Ifuku S, Morimoto M, Saimoto H, *et al.* Chitin, chitosan, and its derivatives for wound healing: Old and new materials. J Funct Biomater 2015;6:104-42.
14. Tang F, Lv L, Lu F, Rong B, Li Z, Lu B, *et al.* Preparation and characterization of N-chitosan as a wound healing accelerator. Int J Biol Macromol 2016;93:1295-303.
15. Khan AW, Kotta S, Ansari SH, Sharma RK, Kumar A, Ali J. Formulation development, optimization and evaluation of aloe vera gel for wound healing. Pharmacogn Mag 2013;9:S6-10.
16. Rashed AN, Afifi FU, Disi AM. Simple evaluation of the wound healing activity of a crude extract of *Portulaca oleracea* L. (growing in jordan) in *Mus musculus* JVI-1. J Ethnopharmacol 2003;88:131-6.
17. Morgan WT, Elson LA. A colorimetric method for the determination of N-acetylglucosamine and N-acetylchrondrosamine. Biochem J 1934;28:988-95.
18. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265-75.
20. Paul W, Sharma CP. Chitosan and alginate wound dressings: A short review. Trends Biomater Artif Organs 2004;18:18-23.
21. Bainbridge P. Wound healing and the role of fibroblasts. J Wound Care 2013;22:407-8, 410-2.
22. Tettamanti G, Grimaldi A, Rinaldi L, Arnaboldi F, Congiu T, Valvassori R, *et al.* The multifunctional role of fibroblasts during wound healing in *Hirudo medicinalis* (*Annelida*, *Hirudinea*). Biol Cell 2004;96:443-55.
23. Shoulders MD, Raines RT. Collagen structure and stability. Annu Rev Biochem 2009;78:929-58.
24. Diegelmann RF, Evans MC. Wound healing: An overview of acute, fibrotic and delayed healing. Front Biosci 2004;9:283-9.

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