Original Research Article

Wound healing and Metabolite profiling in Collagen-Chitosan Biomaterial-treated Chronic Wounds of Hansen's Disease Patients

ABSTRACT

Aims: Impaired wound healing causes chronic ulcers in Hansen's disease (HD) patients which are an unrecognized clinical manifestation and requires utmost care and attention for wound management. Collagen and chitosan biopolymers when synergistically combined produce a biologically active biomaterial for wound dressings. Hence, the aim was to prepare a collagen/chitosan composite and characterize for wound healing potential in HD patients.

Place and Duration of Study: CSIR Central Leather Research Institute, Sardar Patel Road, Adyar, Chennai 600021, Southern Railway Headquarters Hospital, Constable Road, Ayanavaram, Chennai 600023, and Gremaltes Hospital, India between June 2013 and July 2020.

Methodology: The HD wounds were measured by Planimetry and were also assessed for morphological structure of epidermis and collagen fiber arrangement by HR-SEM. ^{1}H-NMR spectroscopy for metabolite identification was studied in blood plasma samples of unwounded, untreated and treated HD patients

Results: Size D of the wounds were appreciably lower than Size 0 demonstrating efficient wound healing by the biomaterial. The morphological structure of the HD wounds showed healthy epidermal layer and thick fibers of collagen matrix in the treated wounds when compared to the controls. Key metabolites of metabolic pathways such as TCA cycle, creatine cycle and protein metabolism were identified by ^{1}H-NMR spectroscopy

Conclusion: The COL/CS wound dressing is a promising biomaterial for management of chronic wounds in Hansen's disease.

Keywords: Chitosan; Chronic ulcers; Collagen; Hansen's disease; metabolite profiling;

NMR spectroscopy; wound healing

1. INTRODUCTION

Skin is the largest and vital organ of the human body and acts as a protective barrier against mechanical, thermal and physical injury. Skin is a sensory organ which prevents loss of

moisture and reduces damage by harmful UV rays. Any rupture of skin by surgery, abrasion, cuts, bruises, burns, infection and diseases causes wounds which destroy the skin tissue organization and may lead to chronic conditions imposing economic burden and social concerns. Wounds may be acute or chronic depending upon the rate of healing and the reconstruction of damaged skin. Acute and chronic wounds cause microbial infection, loss of body fluids, electrolytes and nutrients [1]. Normal wound healing is an orchestrated event with four highly integrated and overlapping phases; namely, hemostasis, inflammation, proliferation and remodeling. These phases must occur sequentially in a specific manner, time and duration. In delayed acute wounds and chronic wounds, healing process is impaired and uncoordinated resulting in pathologic inflammation [2]. Wound management or treatment of wounds is required to restore the normal skin structure. Wound healing materials or wound dressings should ideally be anti-infective, hemostatic and exhibit histocompatibility, reduce wound healing time, side effects of drugs and improve bioavailability [3]. Further, the wound dressing should be flexible, biodegradable, keep wounds moist and adsorb exudates [4]. Traditional wound dressings such as cotton bandage or gauze absorb most of the moisture leaving the wound dry and decreasing the healing rate [5]. Natural biopolymers from animal origin such as collagen and chitosan are excellent biomaterials suited for wound dressings. These biopolymers are biocompatible, biodegradable, bioresorbable and promote cell adhesion and growth and tissue regeneration [6]. Zhang et al [7] and Vojtova et al [6] have discussed the advantages of collagen and chitosan as wound dressing biomaterials. Apart from their advantages, collagen, in synergistic combination with chitosan produces a biologically active material for wound dressings [8] which can promote cell proliferation [9] and angiogenesis [10] at the wound site.

Hansen's disease (HD) or leprosy as known earlier, is a debilitating illness and a public health problem. The wounds are hard-to-heal chronic plantar ulcers which require appropriate wound management. Wound dressings should be affordable and capable of

reducing the healing time because long-term wound management could seriously affect the HD patients. Upadya and Govindarajan [11] have reported a case study in an effort to treat the chronic ulcers in HD. Miyashiro et al [12] have reported that chronic ulcers in HD patients are an unrecognized clinical manifestation directing more attention on the treatment of HD ulcers. Upputuri et al [13] have studied that comorbid conditions are risk factors of delayed healing of plantar ulcers in HD patients. Thus, there is a need to fabricate or develop suitable wound dressings for the treatment of chronic wounds in HD patients. In this context, we have prepared a collagen/chitosan powder [14] and have studied its wound healing efficacy in chronic wounds of HD patients and the metabolite profiling of the patients' blood plasma levels by NMR spectroscopy.

2. MATERIALS AND METHODS

2.1 Subjects

Ethical clearance and approval was obtained from Human Ethics Committee, Southern Railway Head Quarter Hospital, Perambur, Chennai, India. The proposal for conducting human clinical study was scrutinized and approved with the Approval No. SRHQH/EC 08112014. Human in-patient subjects at Gremaltes Hospital, Chennai, India, were treated according to standard clinical guidelines. Appropriate controls were represented from non-Hansen disease patients (NHD). The study was performed according to the Declaration of Helsinki.

2.2 Preparation of collagen-chitosan composite powder

Chitosan (80% deacetylated), was purchased from Sigma Aldrich (St. Louis, MO, USA). Collagen powder [14] and chitosan were mixed in the ratio of 10:1 (w/w). Collagen/chitosan powder COL/CS was sterilized by Ethylene oxide 'ETO' gas and used for consecutive wound dressings (Fig.1).

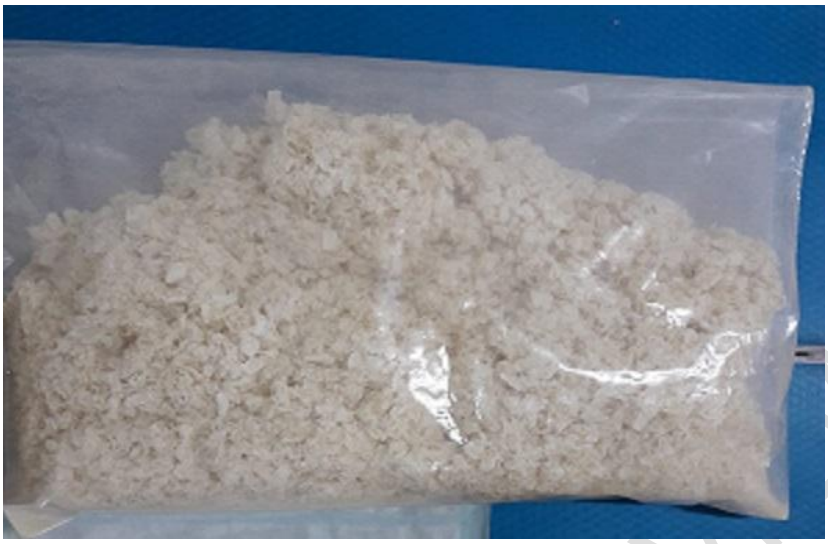


Fig.1: Collagen/chitosan composite powder

2.3 Clinical study

Patients with Hansen disease (HD) were identified and included in the study. Their personal data, clinical data and past and present history were recorded. Deep (sinus) wounds were treated with collagen cream as these wounds cannot be penetrated by COL/CS powder, while, plantar and chronic wounds (Static) were treated with the COL/CS powder. Wound cleaning and debridement was done prior to the application of biomaterial. Wound dressings were done at intervals of four days. In this study, chronic wounds including amputations were also used as subjects of treatment. Post treatment, wounds were periodically monitored and the images of the wound contour size were recorded. In a representative control, a patient was treated with T-Bact ointment. The ointment was applied on the wound and compared with the experimental wound.

2.4 Planimetry

Wound size measurements of HD patients (n=65) was done by planimetry method according to Babu et al. [14] and the size of the wound was calculated in square cm.

2.5 Morphology examination by Scanning Electron Microscopy

Samples were cut out from the wound site at the wound edges. The sample was then washed with a phosphate-buffered saline solution and freeze-dried by using lyophilizer (Lyovapor™ L-300, BUCHI, Switzerland). A small section of samples was attached to the stub, and the specimens were coated with a thin layer of gold ions in an Edwards vacuum coater with an offset rotating sample holder (for uniform coating of the irregular surface). The conducting path from the coating to the stub was done by the use of a conducting sticker. Samples were examined, and photomicrographs were recorded in the scanning electron microscope (HR-SEM, FEI; Quanta FEG 200, USA).

2.6 Metabolite profiling by ^{1}H-NMR spectroscopy

Blood plasma samples of the patients were studied for metabolite profiling. About 350 µL of plasma was mixed with 150 µL of deuterium oxide (D_2O) and transferred into a 5 mm OD NMR sample tube. The ^{1}H-NMR acquisition was carried out on a 400 MHz high-resolution BRUKER-Ascend TM-400 NB-NMR spectrometer with the following test parameters: 5 mm PABBO-BB probe and zg30 pulse program with 128 scans. The FID data was processed using BRUKER Topspin (v.3.2) software.

2.7 Statistical analysis

Data were processed for Mean ± SD of 3 wound measurements of the sample and plotted by MS-Excel software v.2013.

3. RESULTS AND DISCUSSION

3.1 Wound healing studies of COL/CS

Wound size measurements were done before (Size 0) and on the day of discharge (Size D) after application of COL/CS wound dressings. The data are represented in Fig.2 which shows the marked decrease on Size D values compared to Size 0 values of 65 patients. Planimetry studies clearly indicate the efficacy of COL/CS as a wound healing material. This dressing material composed of collagen and chitosan can be considered beneficial for wound management as collagen supports cell growth and tissue growth while, chitosan has

red blood cells binding and antibacterial properties [15]. Tamer et al [16] have reported the depolymerisation of chitosan to release N-Acetyl glucosamine during the wound healing process. The N-Acetyl glucosamine has been shown to promote fibroblast proliferation, increase collagen matrix deposition and stimulate hyaluronic acid synthesis on the wound [17].

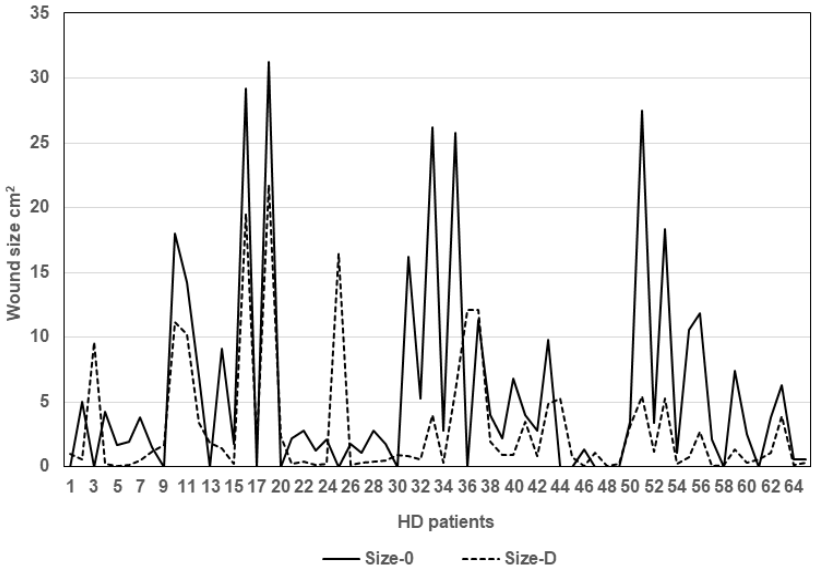


Fig.2: Wound sizes of HD patients on Day 0 and Day of discharge

The surface morphology of wound tissue by HR-SEM is depicted in Fig.3 showing the epidermal layer (A, B) and the collagen fiber arrangement (C, D). The epidermis structure is distinguished in the fibrous tissue through the thickness of the dermis. The epidermis of the skin tissue has well developed and firm structure in COL/CS biomaterial-treated wound, but it is comparatively very less in control. Nearly underlying the epidermis and following its undulating form, is the layer of fine collagen fibers. There are thicker collagen fibers found in the test sample than the control sample. The porous structure of the wound dressing imparts roughness which improves cell affinity and increases the contact area of the dressing with

the wound further accelerating hemostasis [18]. The microstructure of the dressing plays an important role in cellular attachment, migration and proliferation [19]. Our study therefore suggests the improved wound healing by COL/CS wound dressing in chronic wounds of HD patients.

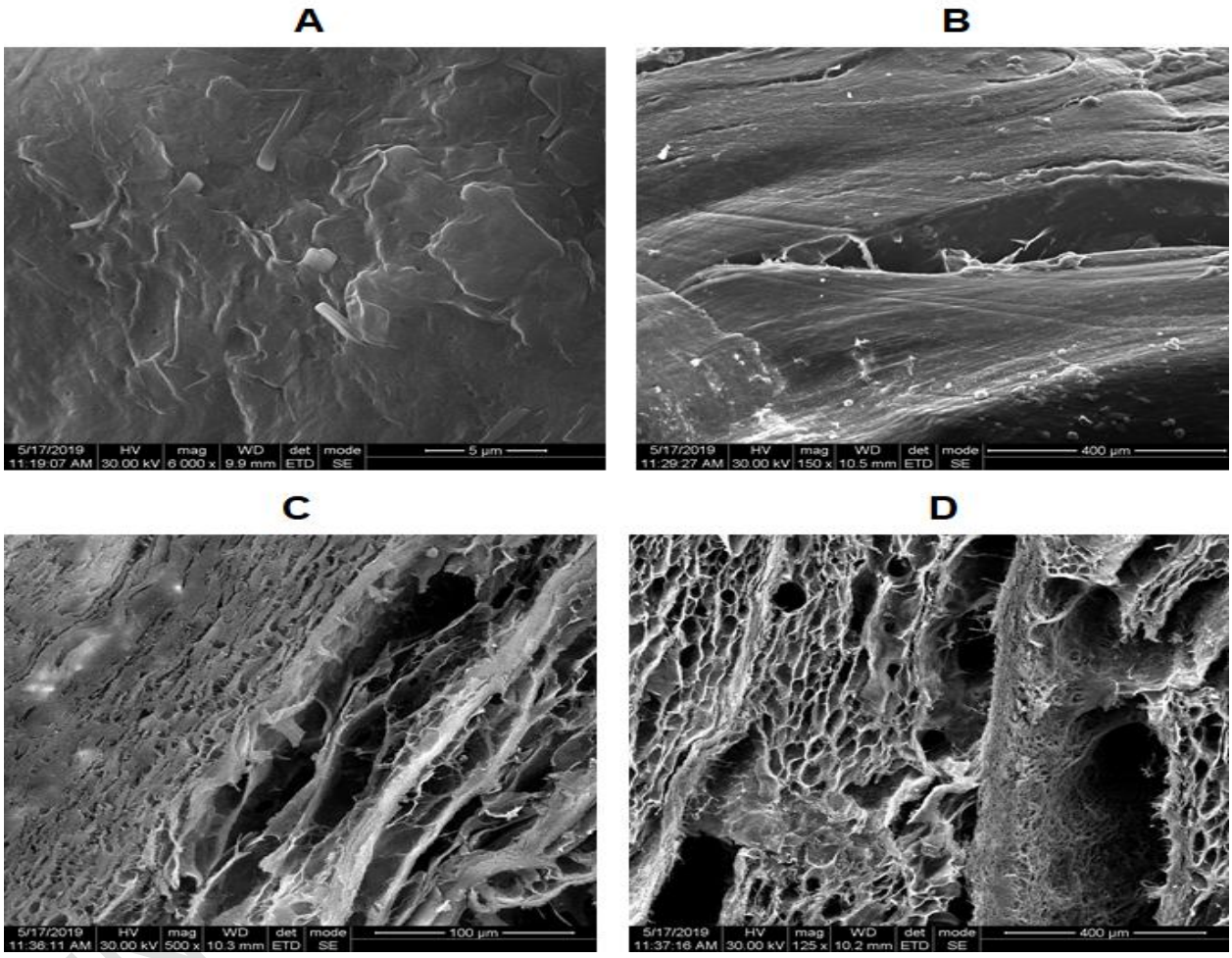


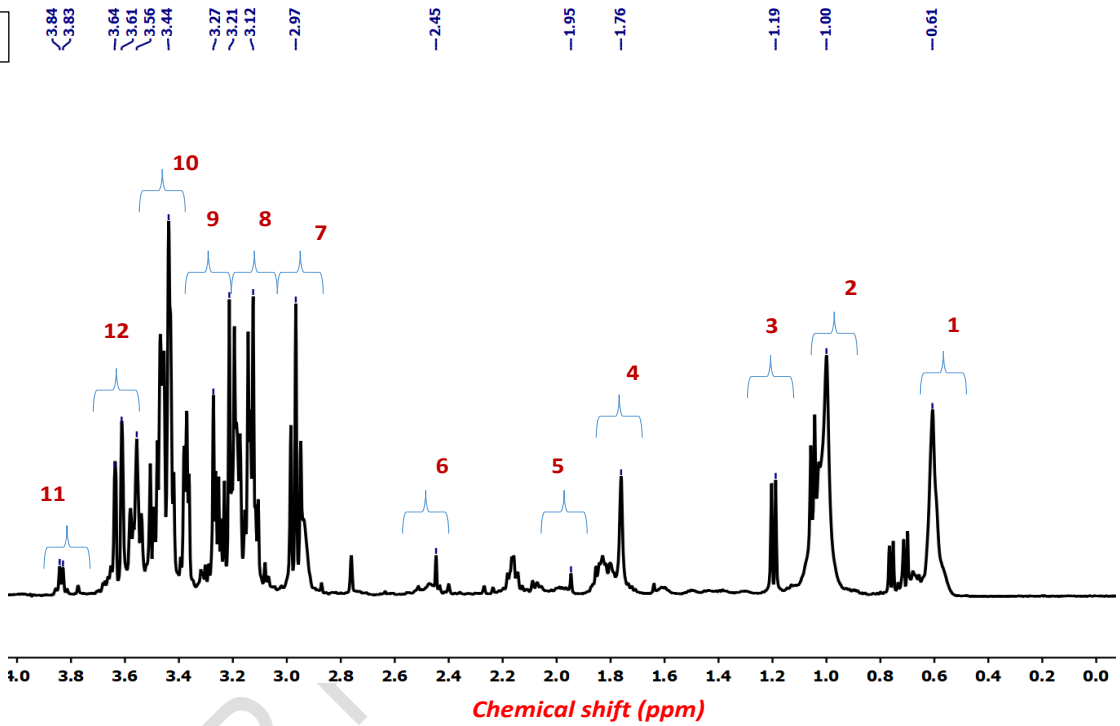
Fig.3: Epidermal layer of control (A) and test (B) group and collagen fiber arrangement in control (C) and test (D) group.

3.2 Metabolite profiling by NMR spectroscopy

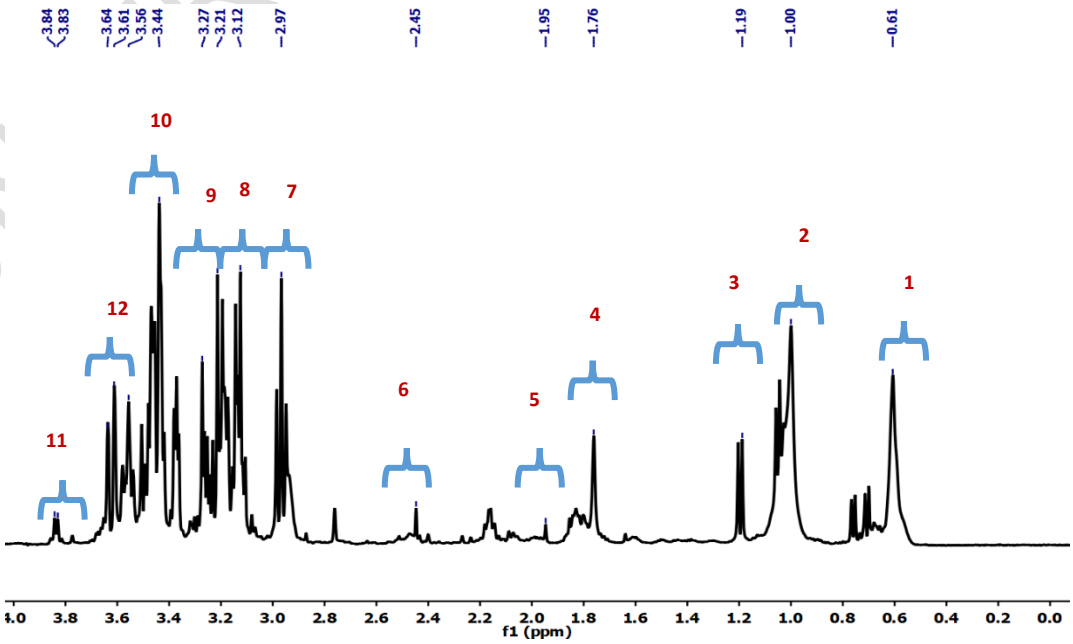
NMR spectroscopy is a highly versatile technique which shows specificity of spectra with respect to a particular metabolite and hence determines the structure of unknown compounds from biological samples [20]. By this strategy, the blood plasma samples of HD patients were processed and analysed. The unwounded patient sample was used as a

representative blank (Fig.4a) and the reference peaks of metabolites were evaluated with untreated wounds as control (Fig.4b) and COL/CS-treated HD patients as the test sample (Fig.4c). Assignments of chemical shifts of unknown metabolites in the blood plasma to those reported in literature provide valuable information for the identification of the metabolites [21]. The identification of a new metabolite without standard spectral data are often a difficult task in the study of metabolome [22]. Therefore we have used an unwounded sample for a reference spectrum. The high presence of betaine in the COL/CS-treated sample suggests that this compound was over-produced to maintain normal function in the skin. The presence of betaine helps to maintain the normal physiological functions. The peaks of each spectrum correspond to the individual metabolite present in the circulating blood during healing. Therefore, these metabolites are considered biological markers in the healing of wounds. Many metabolites such as amino acids (alanine, glutamine, glycine and/or leucine/isoleucine), lipids and other energy metabolizing molecules (citrate, lactate, α-ketoglutarate, betaine, creatine, and creatinine) were identified in the plasma of wounded sample. Several metabolites of the tricarboxylic acid cycle, creatine cycle, and protein metabolism were identified in the NMR spectrum of plasma samples. The ^{1}H-NMR spectra of the control studies were compared with the plasma of the treated sample. The plasma sample of the control showed the presence of lipid metabolites, isoleucine & leucine, N-acetyl glycoproteins, betaine, and glycine in small quantities. The origin of creatine in the circulation of wounded sample is considered an inflammatory substitute. Additionally, the appearance of α-ketoglutarate indicates enhanced utilization of glucose by the TCA cycle linked with glycolysis. Thereby, metabolic profiling by ^{1}H-NMR spectroscopy is a powerful tool to identify the total metabolites in the biological system under a given physiological condition[23].

4a


3.84
3.83
3.64
3.61
3.56
3.44
3.27
3.21
3.12
2.97
2.45
1.95
1.76
1.19
1.00
0.61
10
9
8
7
12
3
2
1
4
11
6
5
4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 0.8 0.6 0.4 0.2 0.0
Chemical shift (ppm)

4b


3.84
3.83
3.64
3.61
3.56
3.44
3.27
3.21
3.12
2.97
2.45
1.95
1.76
1.19
1.00
0.61
10
9
8
7
12
3
2
1
4
11
6
5
4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 0.8 0.6 0.4 0.2 0.0
f1 (ppm)

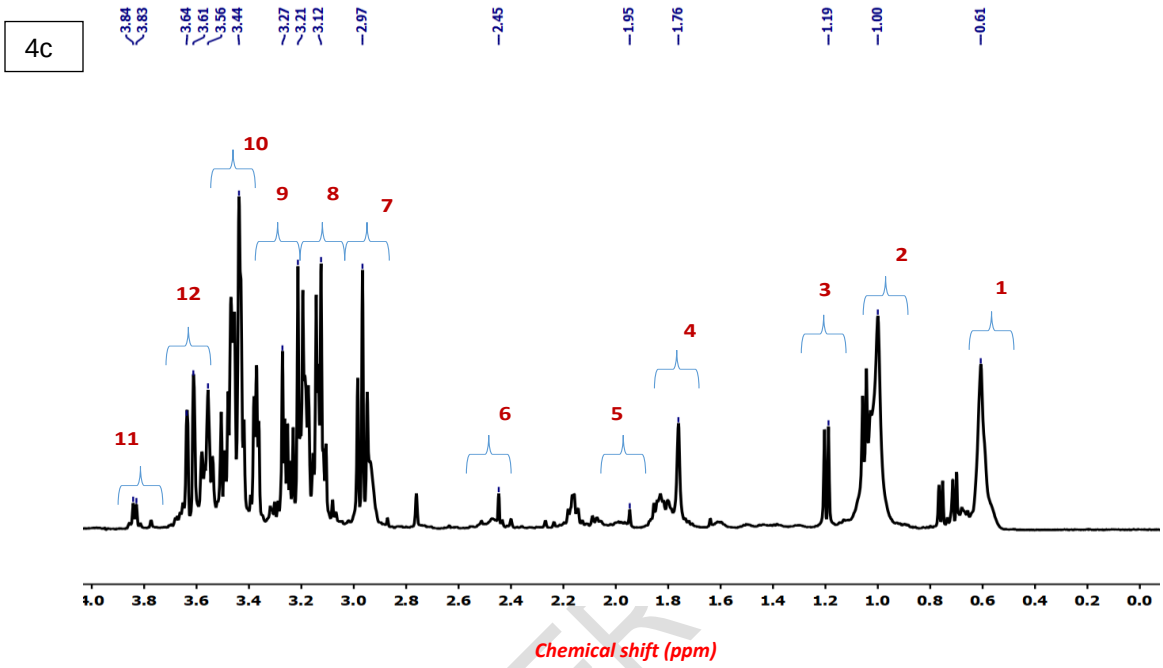


Fig. 4: A representative ^{1}H NMR 400 MHz spectrum of Unwounded (Blank) (4a); Untreated (Control) (4b) and Treated (4c) blood plasma of HD patients: (1) Lipid, Fatty acid chains-CH_3, (2) Leucine/Isoleucine, (3) Lactate, (4) Alanine, (5) *N*-acetyl glycoproteins, (6) Glutamine, (7) Citric acid, (8) α-ketoglutarate, (9) Betaine, (10) Glycine, (11) Creatine and (12) creatinine. Chemical shift assignments of metabolites are based on published literature.

4.CONCLUSION

Wound healing of chronic ulcers in Hansen's disease is a major condition to be addressed due to non-healing or long-term wound management causing alarming public health concern. We have prepared a suitable wound dressing composed of collagen/chitosan as a powder and have studied the wound healing efficacy by Planimetry and HR-SEM analyses and have further characterized the metabolic profiling of the HD patients' blood plasma by

proton NMR spectroscopy. We have obtained good healing potential of the wound dressing by a significant decrease in wound size on the day of discharge and the morphology of the wound showed healthy epidermal layer and restructuring of the collagen matrix in the treated wounds. By NMR metabolite identification, several key intermediary metabolites were found to be in association with different metabolic pathways in the plasma samples of untreated and treated HD patients. These results were obtained from previous literature based on the chemical shifts of the unknown metabolites. Thereby, an effective wound dressing has been prepared from collagen-chitosan composite for treatment of chronic ulcers of HD patients.

CONSENT

All authors declare that 'written informed consent' was obtained from the patient (or other approved parties) for publication of this case report and accompanying images. A copy of the written consent is available for review by the Editorial office/Chief Editor/Editorial Board members of this journal.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

REFERENCES

[1] Zhao X, Wu H, Guo B, Dong R, Qiu Y, Ma PX. Antibacterial, antioxidant, electroactive injectable hydrogel as self-healing wound dressing with hemostasis and adhesiveness for cutaneous wound healing. Biomaterials 2017; 122: 34-47.

[2] Guo S, DiPietro LA. Factors affecting wound healing. Journal of Dental Research 2010; 89(3):219-229.

[3] Mohammadi Z, Zak MS, Majdi H, Mostafavi E, Barati M, Lotfimehr H et al. The effect of chrysin-curcumin-loaded nanofibers on the wound healing process in male rats. Artificial Cells, Nanomedicine and Biotechnology 2019; 47:1642-1652.

[4] Abd El-Hack ME, El-Saadony MT, Shafi ME, Zabermavi NM, Arif M, Batiha GE et al. Antimicrobial and antioxidant properties of chitosan and its derivatives and their applications: a review. International Journal of Biological Macromolecules 2020; 164: 2726–2744.

[5] Ghomi ER, Khalili S, Khorasani SN, Neisiany RE, Ramakrishna S. Wound dressings: Current Advances and Future directions. Journal of Applied Polymer Science 2019; 47738.

[6] Vojtova L, Pavlinakova, V., Muchova, J. Kacvinska, K, Brtnikova J, Knoz M. et al. Healing and angiogenic properties of collagen/chitosan scaffolds enriched with hyperstable FGF2-STAB® protein: in vitro, ex ovo and in vivo comprehensive evaluation. Biomedicines, 2021; 9(6): 590.

[7] Zhang M-X, Zhao W-Y, Fang Q-Q, Wang X-F, Chen C-Y, Shi B-H, Zheng B, Wang S-J, Tan W-Q, Wu, L-H. Effects of chitosan-collagen dressing on wound healing in vitro and in vivo assays. Journal of Applied Biomaterials and Functional Materials, 2021; 19: 1-10.

[8] Dorazilova J, Muchova J, Smerkova K, Kociova S, Divis P, Kopel P et al. Synergistic effect of chitosan and selenium nanoparticles on biodegradation and antibacterial properties of collagenous scaffolds designed for infected burn wounds. Nanomaterials 2020; 10(10): 1971.

[9] Peng L, Xiang RC, Jia WW, Dong XX, Wang GE. Preparation and evaluation of porous chitosan/collagen scaffolds for periodontal tissue engineering. Journal of Bioactive and Compatible Polymers 2006; 21: 207-220.

[10] Tong C, Hao H, Xia L, Liu J, Ti D, Dong L et al. Hypoxia pretreatment of bone marrow-derived mesenchymal stem cells seeded in a collagen-chitosan sponge scaffold promotes skin wound healing in diabetic rats with hindlimb ischemia. Wound Repair and Regeneration 2016; 24: 45-56.

[11] Upadya GM, Govindarajan S. Novel treatment for Hard-to-heal chronic plantar ulcers in Hansen's disease. Journal of Clinical and Diagnostic Research 2019; 13(3): WD01-WD03.

[12] Miyashiro D, Cardona C, Valente NYS, Avancini J, Benard G, Trindade MAB. Ulcers in leprosy patients, an unrecognized clinical manifestation: a report of 8 cases. BMC Infectious diseases, 2019; 19: 1013

[13] Upputuri B, Srikantam A, Mamidi RS. Comorbidities associated with non-healing associated with plantar ulcers in leprosy patients. PLOS Neglected Tropical Diseases, 2020; 14 (6): e0008393

[14] Babu PS, Srinivasan H, Dhandapani BS, Rose C. Collagen-chitosan composite powder as a novel biomaterial for chronic wounds in Hansen disease-A clinical study. Journal of Pharmaceutical Research International 2021; 33(49B): 154-165.

[15] Kim CL, Kim DE. Self-healing characteristics of collagen coatings with respect to surface abrasion. Scientific Reports 2016; 6: 20563.

[16] Tamer TM, Valachova K, Hassan MA, Omer AM, El-Shafeey M, Eldin MSM et al. Chitosan/hyaluronan/edaravone membranes for anti-inflammatory wound dressing: in vitro and in vivo evaluation studies. Materials Science and Engineering: C: Materials for Biological Applications 2018; 90: 227-235.

[17] Jayakumar R, Prabaharan M, Sudheeshkumar PT, Nair SV, Tamura H. Biomaterials based on chitin an chitosan in wound dressing applications. Biotechnology Advances 2011; 29: 322-337.

[18] Weiss L, Nessler Y, Novelli M, Laheurte P, Grosdidier T. On the use of functionally graded materials to differentiate the effects of surface severe plastic deformation, roughness and chemical composition on cell proliferation. Metals 2019; 9(12): 1344.

[19] Wang K, Qi Z, Pan S, Zheng S, Wang H, Chang YX et al. Preparation, characterization and evaluation of a new film based on chitosan, arginine and gold nanoparticle derivatives for wound-healing efficacy. RSC Advances 2020; 10: 20886.

[20] Sahoo NK, Tejaswini G, Sahu M, Muralikrishna KS. An overview on NMR spectroscopy based metabolomics. International Journal of Pharmaceutical Sciences and Development Research 2020; 6(1): 016-020.

[21] Lindon JC, Nicholson JK, Everett JR. NMR spectroscopy of biofluids. In: Webb GA, editor. Annual reports on NMR spectroscopy. Academic Press, 1999.

[22] Bharti SK, Roy R. Metabolite identification in NMR-based metabolomics. Current Metabolomics 2014; 2: 163-173.

[23] Jana SK, Dutta M, Joshi M, Srivastava S, Chakravarty B, Chaudhury K. 1H NMR based targeted metabolite profiling for understanding the complex relationship connecting oxidative stress with endometriosis. Biomed Research International 2013; 329058