International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304
Vol.8, No.6, pp 248-257, 2015

A preliminary *in vitro* study on the bovine collagen film incorporated with *Azadirachta indica* plant extract as a potential wound dressing material

Viji Chandran S, Trikkurmadom Seetharaman Amritha, Rajalekshmi G And Pandimadevi M

Department of Biotechnology, School of Bioengineering, SRM University, Kattankulathur.

Abstract: Wound healing is a natural process which is delayed in diabetic patients due to physiological changes in cells. Prolonged inflammation is one of the characteristics of diabetic wounds due to increase in induced nitric oxide synthase (iNOS). Wound dressing material with anti inflammatory effects can be used for diabetic wound healing for faster response. Collagen is a natural component in skin which has cell attachment and proliferation effects. Chrome shavings is tannery waste from which collagen can be isolated and can reduce the cost of wound dressing material. It is well known that *Azadirachta indica* (neem) has antioxidant activities and are used for curing cuts and wounds. Collagen sheet impregnated with neem extract was prepared and checked for nitric oxide reduction using chemical methods and using RAW 264.7 cell lines. Results showed more than 50% nitric oxide reduction in biosheets with 800µg/ml of neem extract in it. Biostability of the sheets were also checked using collagenase assay and the results showed a decrease in degradation in biosheets with neem extract when compared to collagen sheets without neem extract in it.
Keywords : Collagen, *Azadirachta indica*, Raw 264.7 cells, anti inflammatory, biostability.

1. Introduction

Wound healing involves a cascade of interacting phases of haemostasis, inflammation, proliferation, epithelialisation, and scar maturation, which can be affected by diabetes and its complications.[1] After injury, the changes occur in vascular flow, caliber, and permeability. The tissue fluid, proteins, and blood cells escape from the vascular system into the injured tissue in a process called exudation. With the following changes in the vascular system, include changes induced in blood and its components, starting of cellular events and characterize the inflammatory response.[2,3,4,5] It is evident that increase in blood glucose level is the cause of prolonged inflammation in diabetes and it is due to the physiological changes occurred in the cells due to diabetes. In diabetic patients impaired wound healing occurs due to prolonged inflammation[6] and production of free radicals[7,8] and subsequently the increased concentration of free radicals prevents the new tissue formation and also causes delay in wound healing.[9] It was revealed that the wound dressing material with nitric oxide scavenging capacity could be used for diabetic wound healing.[10] The healing of a wound requires a well orchestrated integration of the complex biological and molecular events of cell migration, cell proliferation, and extracellular matrix (ECM) deposition. In addition, the cellular responses to inflammatory mediators, to growth factors and cytokines, and to mechanical forces must be appropriate and precise. These fundamental processes are similar to those guiding embryogenesis, tissue and organ regeneration, and even neoplasia.[11,12,13,14] The fundamental biological and molecular events after cutaneous injury, with information mainly derived from

experimental wounds in animals, cannot be separated and categorised in a clear-cut way. However, it has been useful to divide the repair process into four overlapping phases of coagulation, inflammation, migration-proliferation (including matrix deposition), and remodelling. Some areas of chronic wounds are in different phases at the same time and, presumably, progression to the next phase does not occur in synchrony. These overall differences between acute and chronic wounds are not restricted to lack of progression alone. Certain events occur abnormally in the healing-impaired wound, highlighting the need to be cautious in extrapolating lessons learned from acute wounds to the situation in chronic wounds. It was proved that studies in animals have shown that isolated abnormalities can markedly modulate the healing process [15]. In a study on mice was found in impaired healing with combined deficiency of molecules that have a critical role in inflammation (E-selectins and P-selectins), and in mice without plasminogen, urokinase plasminogen activator, and tissue plasminogen activator (double knockout), fibroblast growth factor-2 (basic fibroblast growth factor), or inducible nitric oxide.[11,16] Conversely, decreased healing occurs in transgenic mice over expressing some tissue metalloproteinases (eg, matrix metalloproteinase [MMP][25] and antisense to CD44, the receptor for hyaluronic acid.[16] . Due to unexpected mutations lead to accelerated healing, was reported with Smad-3 or skn-1a knockout mice.[17] These findings offer the promise of improving healing in human beings, by manipulating growth factors, ECM, and signalling pathways.[18,19,20] Impairment of leucocyte function and proliferation occur in hyperglycaemia,[21,22] but the overall effect of the disease on healing is complex. The benefit of good blood glucose control has not been assessed, but is likely to be important, even though the rate of healing of neuropathic ulcers does not differ between people with and without diabetes.[23] It is evident that patients with diabetes, particularly with Type I, have more macrovascular disease that non diabetic people, with more distal distribution from thesuperficial femoral artery to the pedal arch and involvement of the metatarsal artery.[24] It was studied that microcirculatory deficiencies occur early in diabetes and these abnormalities include a reduction of capillary size, thickening of the basement membrane, arteriolar hyalinosis. The thickening of the basement membrane interferes with physiological exchanges and leads to altered migration of the basement membrane interferes with physiological exchanges, and leads to altered migration of leucocytes (contributing to infection) decreased maximal hyperaemia, and abnormal autoregulatory capacity.[16] The impaired endothelial function might involve a reduction of nitric oxide synthetase. Importantly, the lumen of microvessels is not decreased in diabetes. The long standing myth of small vessel disease accounted for the unfortunate and incorrect notion that revascularisation would not help diabetic patients. Nevertheless, although the luminal occlusion of small blood vessels does not occur, blood flow is maldistributed. Abnormal blood flow might also explain the development of Charcot foot, which results in dramatic changes in bone alignment and great susceptibility to pressure forces in the insensate foot. There is a clear links exist between vaculopathy and neuropathy in the disease foot, with shunts in the microcirculation, together with the presence of sympathetic nerve denervation and autonomic neuropathy, lead to the maldistribution of blood flow.[25] Some neuropathological problems have already been mentioned, as they are tied to microcirculatory defects, the motor, sensory and autonomic fibres are all affected and in fact the consequences are predictable. Because of sensory deficits, the diabetic patient does not have protective symptoms guarding against pressure and heat. Because of the absence of pain, probably combined with abnormal vasodilatory autoregulation, contributes to the pathogenesis of Charcot foot, which further impairs the ability to sustain pressure. Similarly, the addition of motor fibre abnormalities leads to undue physical stress on the insentate foot, the development of further anatomical deformities arched foot, and might play a part in the development of infection, since bacterial growth is enhanced in tissues with high compressive forces. Infection is not a stated component of the pathogenic triad for development of diabetic foot ulcers, but is an extremely important cause of morbidity and hospitalisation, amputation and impaired healing. Whether it has a role in the initial development of the ulcer, especially when combined with trauma is unclear. Depending on the extent of injury, the acute inflammation is of relatively short duration, lasting from minutes to days. The main characteristics of acute inflammation are the exudation of fluid and plasma proteins (edema) and the emigration of leukocytes (predominantly neutrophils). Neutrophils and other motile white cells emigrate or move from the blood vessels to the perivascular tissues and the injury (implant) site.[26,27,28] The accumulation of leukocytes, in particular neutrophils and monocytes, is the most important feature of the inflammatory reaction. A series of processes involves in the margination, adhesion, emigration, phagocytosis, and leucocytes accumulation including extracellular release of leukocyte products.[29] Increased leukocytic adhesion in inflammation involves specific interactions between complementary adhesion molecules present on the leukocyte and endothelial surfaces.[30] The surface expression of these adhesion molecules is modulated by inflammatory agents; mechanisms of interaction include stimulation of leukocyte adhesion molecules (C5a, LTB4), stimulation of endothelial adhesion molecules (IL-1), or both effects, i.e. tumor necrosis factor (TNF). Integrins make up a family of transmembrane glycoproteins that modulate cell-matrix and cell-cell relationships by acting as receptors to extracellular protein ligands and

also as direct adhesion molecules.[31] Diabetes is characterized by a nitric oxide deficiency at the wound site. Many collagen related products have been developed in the past years as wound dressings and few are approved by drug controlling authorities and are now commercially available.[32] Using collagen as the base material offers many advantages, which has well-documented structural, physical and chemical properties. Moreover, collagen has low antigenicity, low inflammatory, good biocompatibility and has the ability to promote cell attachment and cell proliferation. Collagen alone or collagen blend matrix materials are the most commonly used biomaterials in the skin, connective tissue and nerve tissue engineering.[33,34,35] It is a natural extracellular matrix component, posses stability and microstructure and also play a vital role in cell-cell and cell matrix interaction. Now, different forms of collagen such as gels, sheet, films, and sponges are available. Collagen film/sheet/disc is used for the treatment of tissue infection, such as infected corneal tissue or liver cancer, and as a drug carrier for antibiotics such as tetracycline.[36] Collagen film and matrix were used as gene delivery carriers for promoting bone formation.[37] Collagen film and matrix were used as gene delivery carriers for promoting bone formation. Biodegradable collagen films or matrices have served as scaffolds (give support) for survival of transfected fibroblasts. A combination of collagen and other polymers, such as atelocollagen matrix added on the surface of the polyurethane films, enhanced attachment and proliferation of fibroblasts and supported growth of cells.[38] Films made from hydrolyzed collagen have been used as a tissue adhesive for suture replacement due to its chemical resemblance to connective tissue and its tissue fluid-binding properties. Moreover, it is biodegradable, non-toxic and readily absorbed; therefore, it does not impose a hindrance to the healing process.[36,39] It has been reported that collagen has the following properties: (1) it controls the evaporation of fluid, keeping the wound pliable and flexible, (2) it promotes the development of granulation tissue, (3) it diminishes pain and (4) it provides mechanical protection against physical and bacterial insult [40].

Collagen is a natural component in skin (upto 70%). Collagen can help attach cells and enhance proliferation.[41] Thus collagen is widely used as an excellent biomaterial for wound dressings. Collagen can be prepared in forms of sheets, matrix, sponge etc.[1] Collagen in its purified form increases the cost of production and alternative natural sources such as fish scale,[1] rat tail,[42] surf smelt skin[43] etc., is used for isolation of collagen. Chrome containing leather wastes (CCLW) are the prominent solid wastes in tanning industry. Since chromium is known for its toxicity, the disposal of CCLW has been identified as a serious problem from the environmental point of view. The high concentration of trivalent chromium along with organic/inorganic compounds in CCLW discharged from leather industries causes severe groundwater contamination in land disposal and chronic air pollution during thermal incineration.[4] The CCLW mainly consists of collagen and Cr(III) complexes, which could be treated to give the potential resources of collagen protein and chromium.[44]

CCLW were utilized in the preparation of value added products to reduce the environmental pollution.[45,46] Preparation of parchment like membrane and development of leather like material using chrome shavings as the raw material was reported in earlier studies.[47] Using alkaline protease enzyme under mild conditions protein products (gelatin and collagen hydrolyte) were isolated from chrome shavings which were potentially used in cosmetics, adhesive, printing, photography, micro encapsulation and as an additive in finishing products used in leather industry.[47]. Chrome shavings are chrome containing leather waste from tannery industries and causes disposal problems due to presence of Cr(III) in it. Collagen can be isolated from chrome shavings by dechroming it.[47] Collagen alone cannot assist the healing of infected wounds because it is protein in nature and bacteria can use collagen as a substrate. Because of the imbalance between host resistance and bacterial growth, infection of the wound occurs. Treating of the wounds by systemic administration of drugs may lead to insufficient drug release to the site of infection, systemic toxicity, and drug associated side effect. This can be overcome successfully by topical application of drugs, and collagen dressings with antibiotics. Bacteria are developing resistance towards antibiotics, due to increase in the application of new antimicrobial agents.[48] Collagen can enhance normal wound healing but it does not have any nitric oxide scavenging ability. Plant extracts possessing active ingredients are incorporated into biomaterials for better performance against bacterial infection. Various plant extracts are known to have radical scavenging potential and one among them is neem leaves, which is being used for treating cuts and wounds by many tribes. From the ancient days, different crude extracts were used for preparing poultice to treat a variety of skin ailments including wounds. *Azadirachta indica* also known as Neem is one of the known plant native to India. It is considered a major component in siddha medicine, ayurvedic and Unani medicine is particularly prescribed for skin diseases.[49] The leaf extracts of neem possesses antiulcer effect, antifungal activity, antibacterial, antiviral activity, anticancer and antioxidant activity.[50] Clinically, NO deficiency is known to be associated with severe vascular disorders, especially in patients with long-term diabetes. In the present study, chrome shavings were used for the preparation of collagen, and then neem leaf extracts were incorporated separately in the

biocomposite film. Ethylene glycol was used in the film to enhance the flexibility of the dressings. The prepared biocomposite film was evaluated for its nitric oxide scavenging capacity. This study was performed to investigate whether the prepared biocomposites accelerate wound healing mainly focussing on inflammation.

2.Materials

Chrome shavings was obtained from the local tannery. Cell lines (RAW 264.7) were purchased from NCCS, Pune, India. Sodium nitroprusside, Griess Reagent, lipo polysaccharide (LPS), DPPH, potassium hexacyanoferrate, 1,10-phenanthroline, sulphanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride) were purchased from HiMedia. Dulbecco's Modified Eagle's Medium (DMEM) and fetal Bovine serum (FNS) were purchased from Sigma Aldrich. All the chemicals used were analytical grade and used without any further purification.

2. Isolation of collagen

Chrome shavings were collected from the tannery, washed twice. Later, it was soaked in 5% NaOH solution for overnight. Then it was dechromed using concentrated sulphuric acid and washed thrice using distilled water. The demineralised sample was treated with hydrogen peroxide for bleaching. The demineralised collagen was washed three times with distilled water and bleached using hydrogen peroxide. The collagen was isolated using modified method[47] The obtained collagen was lyophilised and stored at 4^oC till further use. The solid content of the solution was 14.27 ± 0.89 %.

2.2 Preparation of neem leaves extract

Fresh neem leaves were collected and washed with water and shade dried for 4 days. It was then in a domestic mixer. Dried powder weighing 10g was subjected to extraction (Soxhlet Apparatus) using 100% methanol. The extraction was continued until the powder was free of extracts. The methanol was removed by using rotary evaporator apparatus under reduced pressure at 40^0C and the dried extract obtained was lyophilised and stored at 4^oC until use.

2.3 Preparation of collagen scaffold impregnated with neem extracts

The collagen solution of 1% wt/vol in 0.05M acetic acid was prepared with continous stirring in a magnetic stirrer and it was kept for 24 hours incubation at 4^0C. The solution was then filtered and homogenised. 25µg/ml, 50µg/ml, 100µg/ml of neem leaf extract was added to 20ml of collagen solution and mixed well and 0.5ml ethylene glycol was added as a crosslinker. The collagen, neem leaf extract and ethylene glycol were mixed well and then it was poured into a plastic petri dishes. After air drying the, it was stored at 4^oC until further use.

2.4 Biomaterial Evaluation

2.4.1Collagenase assay

To assess the biological stability of the prepared biocomposite films and its degradation rate, they were exposed to Collagenase enzyme. The biocomposite films wer cut into 2*2cm size in triplicate and they were dried overnight at room temperature. All thesamples were exposed with 100U/ml solution of collagenase enzyme made in PBS. Dry weight was measured before incubation and after 3 h, 6 h, 24 h, and 48 h. Biomaterial degradation was determined gravimetrically through the weight loss based on initial weight.

2.5 Nitric oxide scavenging assay

2.5.1. Acellular method

Nitric oxide(NO) scavenging can be measured using Griess Illosovy reaction. Sodium nitroprusside can decompose forming NO_2 in presence of PBS of pH 7.2 [51]. NO_2 can produce nitrites and nitrates under aerobic conditions which can be measured using Griess reagent. 10mM sodium nitroprusside in PBS was mixed with different concentration of collagen and collagen with neem extract. After incubation for 2 hrs at 30°C, 0.5ml supernatant was withdrawn from each and mixed with 0.5ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride). Absorbance was taken at 540nm.

2.5.2 Determination of nitric oxide concentration using DMEM medium

RAW 264.7 cells were cultured in DMEM medium with 10% foetal bovine serum (FBS). Medium was changed every 2 days. Collagen sheets of 10mm diameter were placed on 24 well plate which is presterilized using UV sterilization for 30 min and preincubated in DMEM medium without serum for 24 hrs. $1*10^5$ cells were seeded onto each well. Once the cells reach confluency, 0.1µg of LPS was added onto each well excluding negative control. Positive control has cells with LPS and without collagen sheet and negative controls had cells alone. After 24 hrs of LPS stimulation 100µl of supernatant was taken and analysed for nitric oxide concentration in it using Griess reagent. Incubate the solution for 10 mins in dark and OD was taken at 540nm. A sodium nitrite standard was prepared using same method for finding nitric oxide concentration.[52]

2.5.3 Using conditioned medium

Raw 264.7 cells were seeded onto 24 well plates with $1*10^5$ cells/well. Once the cells reached confluency, 0.1µg LPS was added into each well except negative control, whereas, positive control had both cells and LPS. Condition medium was prepared by pre incubating collagen sheet and collagen with neem extract sheets in DMEM medium without FBS. 10µl of conditioned medium was added to wells except positive and negative control. After 24hrs of LPS stimulation, 100µl of supernatant was taken and 100µl of Griess reagent was added to it. Then, it was incubated for 10 min and absorbance was measured at 540nm. Nitric oxide concentration was determined from the standard graph.

3 Results and discussions

Collagen was isolated from collagen and incorporated with neem extract and prepared into sheets using ethylene glycol as cross linker and this ddition of cross linker can enhance tensile strength and elongation. Biostability is an important parameter for selection of a wound dressing material. Collagenase assay was performed and percentage degradation of collagen biocomposites were calculated. From the results, it was observed that with addition of neem extract in collagen increases the bio stability and increased the time required for degradation (Fig. 1).

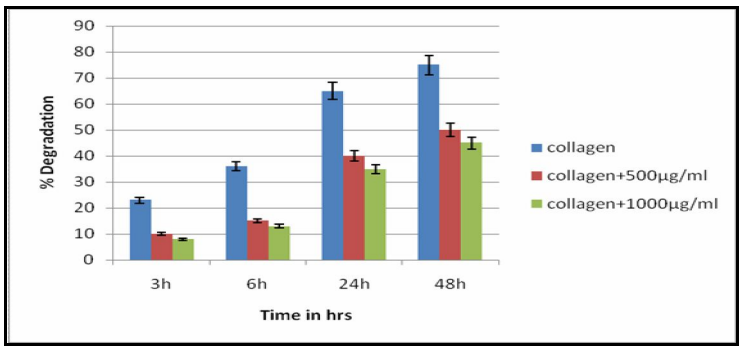

Fig. 1. *In vitro* degradation of collagen bio composites using collagenase at different time intervals

Nitric Oxide is generated by neurons, endothelial cells, macrophages etc., which regulates various physiological processes such as immune response, control blood pressure, control vasodilation, neural signal transmission etc., and also act as chemical mediator.[53] NO is a free radical since it has an unpaired electron. It becomes nitroxyl anion by accepting an electron and becomes nitrosonium cation by donating an electron.[54] Increase in concentration of nitric oxide can cause several diseases including cancer. Using nitric oxide synthesis NO is generated via metabolisation of arginine to citrulline through five electron oxidative reaction.[55]

Sodium nitroprusside when incubated with PBS produces nitrites in a time dependent manner. Nitrites thus produced were scavenged using methanolic extract of neem extract with collagen. Reduction of nitrites was found to be increasing with increase in concentration of plant extract. Determination of scavenging capacity was done using absorbance at 540nm. Collagen with 800µg/ml neem extract showed optimum reduction (Fig. 3).

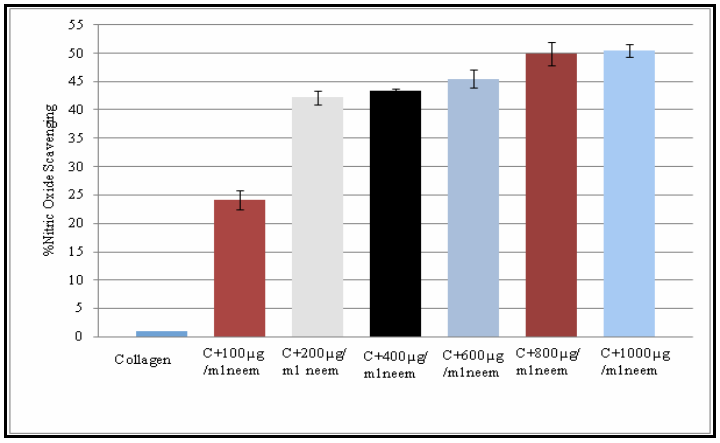

Fig. 3 Nitric oxide scavenging of collagen and collagen with different concentrations of neem extract using Griess reagent. Results were given in mean±SD.

Various researches has shown the presence of phenolic compounds and flavanoids in neem leaves extract.[56]. Flavanoids and phenolic compounds has antioxidant potential which can scavenge singlet oxygen and free radicals.[57,58] Nitric oxide scavenging of neem extract is due to the presence of phenolic and flavanoid compounds in it. Sodium nitroprusside incubated with PBS without adding any extract was taken as control and its value was set as 100%. With respect to control the decrease in absorbance was measured at 540nm. From the results it was observed that an optimum of 50% scavenging was present in collagen sheets incorporated with 800µg/ml neem extract and more.

Intensity and duration of inflammatory phase is controlled by the concentration of chemical mediators like cytokines and eicosanoids, which is released by inflammatory cells such as macrophages and tumor necrosis factor at the time of injury.[56,60] Pro-inflammatory cytokines are required for normal wound healing process, since it regulates the cellular and molecular processes during inflammatory phase. Prolonged and chronic inflammation can delay the wound healing process.

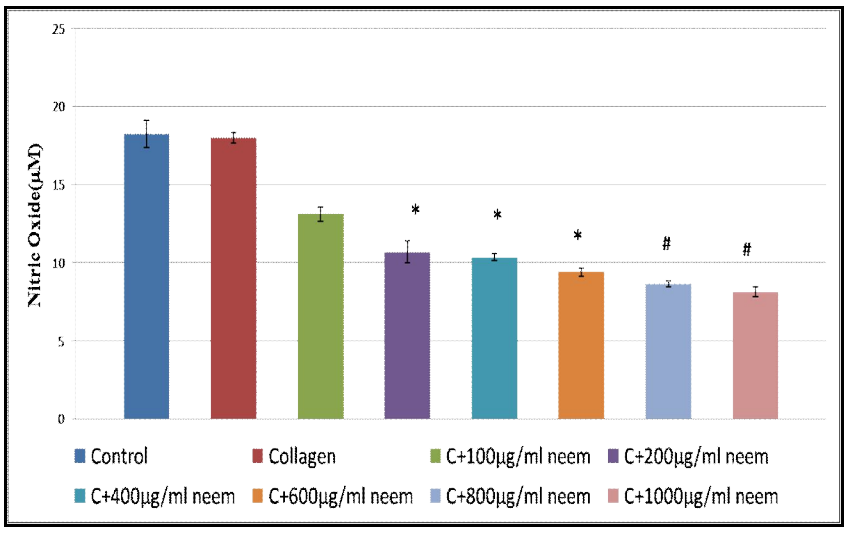

Fig 4: Amount of Nitric Oxide released from LPS stimulated RAW 264.7 cells after exposure to collagen impregnated with neem biocomposites. Positive control (cells with LPS). All results given in mean±SD. (* p<0.05 , # p<0.01 vs control)

LPS induced production of nitric oxide has been done with positive control as cells with LPS and negative control with cells alone. After 24hours of induction, in positive control wells, amount of nitric oxide produced was found to be 18.233µM. In 24 well plates with scaffolds, production of nitric oxide has been inhibited. Collagen scaffold wells showed minimal reduction of nitric oxide, whereas in wells with scaffold containing neem in collagen showed increase in reduction of nitric oxide with increase in concentration of neem extract in collagen sheets. A optimum scavenging of 55% was found for collagen sheet with 1000µg/ml of neem extract in it. (fig. 4).

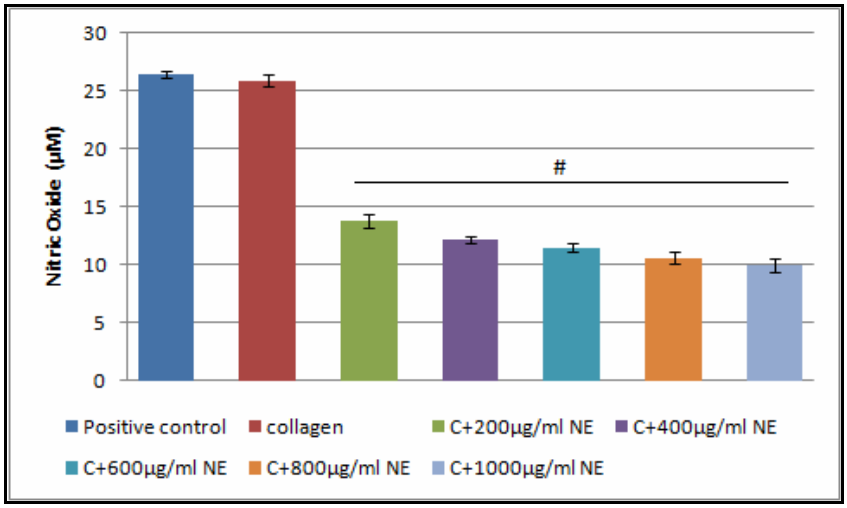

Fig. 5: Amount of Nitric Oxide released from LPS stimulated RAW 264.7 cells after exposure to collagen impregnated with neem biocomposites (conditioned medium). Positive control (cells with LPS). All results given in mean±SD. (* $p<0.05$, # $p<0.01$ vs control)

LPS induced RAW cells were treated with conditioned medium and incubated for 24 hours. Nitric oxide concentration was determined after 24 hrs. Positive control and collagen wells showed 26µg/ml of nitric oxide whereas in wells with collagen incorporated with neem extract showed a decrease in amount of nitric oxide concentration. Amount of nitric oxide concentration was 10µg/ml in wells with collagen incorporated with 1000µg/ml of neem extract in it. Although biomaterials are not generally phagocytosed by neutrophils or macrophages because of the size disparity (i.e. the surface of the biomaterial is greater than the size of the cell), certain events in phagocytosis may occur. Within one day following implantation of a biomaterial (i.e. injury), the healing response is initiated by the action of monocytes and macrophages, followed by proliferation of fibroblasts and vascular endothelial cells at the implant site, leading to the formation of granulation tissue, the hallmark of healing inflammation. Depending on the extent of injury, granulation tissue which indicates proliferation of new blood vessels and fibroblasts may be seen as early as three to five days following implantation of a biomaterial. The new, small blood vessels are formed by budding or sprouting of pre existing vessels in a process known as neovascularization or angiogenesis.[61,62,63] This process involves proliferation, maturation, and organization of endothelial cells into capillary tubes. Fibroblasts also proliferate in developing granulation tissue and are active in synthesizing collagen and proteoglycans. In the early stages of granulation tissue development, proteoglycans predominate; later, however, collagen, especially type I collagen, predominates and forms the fibrous capsule. Some fibroblasts in developing granulation tissue may have features of smooth muscle cells. These cells are called myofibroblasts and are considered to be responsible for the wound contraction seen during the development of granulation tissue. From the results obtained it is proved that neem extract incorporation has imparted a nitric oxide scavenging capacity to collagen biocomposites. Thus it can be considered as a potential wound dressing material for diabetic wound healing.

4 Conclusion

The biocomposite film incorporated with neem extract has shown an increase in biostability with addition of neem extract into collagen sheets. Incorporation of neem extract also influence the nitric oxide scavenging capacity to the biocomposite films. These results shows that the biocomposite film has anti inflammatory properties and can also treated clinically for the wounds of animals. Hence, in order to prevent the infections, reduce the trauma, enhance the tissue regeneration, the collagen and neem extract biocomposite film is a potential candidate for wound dressing. The biocomposite films also posses flexibility, durability, gas permeability and enhanced bio stability and good biocompatibility.

Acknowledgements

The authors thank Dean, Dr.M.Vairamani, Prof. S.Thyagarajan and Prof. N.Selvamurugan for their valuable suggestions. The work was supported by the management of SRM University.

5 References

1. Thangavelu Muthukumar., P.Prabu., Kausik Ghosh., Thotapalli Parvathaleswara Sastry. Fish scale collagen sponge incorporated with *Macrotyloma uniflorum* plant extract as a possible wound/burn dressing material, *J.Colloids and Surfaces B: Biointerfaces*. 2013, 113, 207-212.
2. Cotran RZ, Kumar V, Robbins SL, eds. Pathologic Basis of Disease, Philadelphia: Saunders. 1999. 6th. Edition, pp. 50– 112.
3. Gallin JI, Synderman R, eds. Inflammation: Basic Principles and Clinical Correlates. New York: Raven. 2nd. Edition 1999.
4. Weissman G, Smolen JE, Korchak HM. Release of inflammatory mediators from stimulated neutrophils.N. Engl. *J. Med.* 1980, 303:27– 34.
5. Salthouse TN. Cellular enzyme activity at the polymer–tissue interface: a review. *J. Biomed. Mater. Res.* 1976, 10:197–229.
6. Naguib G., Al-Mashat H., Desta T., Graves D.T. Diabetes prolongs the inflammatory response to a bacterial stimulus through cytokine dysregulation. *Journal of Investigative Dermatology,* 123, 2004, 87-92.
7. Hossam Ebaid., Osama M Ahmed., Ayman M Mahmoud., Rasha R Ahmed. Limiting prolonged inflammation during proliferation and remodelling phases of wound healing in streptozotocin-induced diabetic rats supplemented with camel undenatured whey protein. *BMC Immunology*, 2013, 14:13, doi:10.1186/1471-2172-14-31
8. Vincent A M., Russell J W., Low P., Feldman E L. Oxidative stress in pathogenesis of diabetic neuropathy. *Endocrine Reviews.* 2004, 25, 612-628.
9. Guo S., DiPietro. Factors affecting wound healing. *Journal of Dental Research.* 2010 89(3), 219-229.
10. Dias A M A., Rey-Rico A., Oliveira R A., Marceneiro S., Alvarez-Lorenzo C., Concheiro., Junior R N C., Braga M E M., de Sousa H C. Wound dressings loaded with anti-inflammatory juca (Libidibia ferrea) extract using supercritical carbon dioxide technology. *The Journal of Supercritical Fluids*. 2013, 74, 34-45.
11. Martin P, Parkhurst SM. Parallels between tissue repair and embryo morphogenesis. *Development*. 2004, 131, 3021–3034.
12. Martin P. Wound healing—aiming for perfect skin regeneration. *Science.* 1997, 276, 75–81.
13. Singer AJ, Clark RA. Cutaneous wound healing. *N Engl J Med*. 1999, 341, pp. 738– 746
14. Vascotto SG, Beug, S Liversage RA, Tsilfidis. C. Identification of cDNAs associated with late dedifferentiation in adult newt forelimb regeneration. Developmental dyanamics Dyn,2005 233, 347–355. DOI: 10.1002/dvdy.20304.
15. Mehendale FMP Martin P. The cellular and molecular events of wound healing. V Falanga (Ed.), Cutaneous wound healing, Martin Dunitz, London. 2001, pp. 15–37.
16. Dinh TL, Veves A. A review of the mechanisms implicated in the pathogenesis of the diabetic foot. *Int J Lower Extrem Wounds*. 2005;4(3):154-159.
17. Asbcroft GS, Yang X, Glick AB, Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. *Nat cell Biol ,* 1999,1:260-266.
18. Hamilton AI, Blackwood HJ. Insulin deficiency and cell proliferation in oral mucosal epitheliam of the rat. *J Anat* 1977; 124: 757–63.
19. Yue DK, Swanson B, McLellan S, et al. Abnormalities of granulation tissue and collagen formation in experimental diabetes, uraemia and malnutrition. *Diabet Med* 1986, 3: 221–25.
20. Tengrup L, Hallmans G, Agren MS. Granulation tissue formation and metabolism of zinc and copper in alloxan—diabetic rats. *Sanc J Plast Reconstr Surg Hand Surg*. 1998, 22: 41–45.
21. Delamaire M, Maugendre D, Moreno M, et al. Impaired leucocyte function in diabetic patients. *Diabet Me.d* 1997, 14: 29–34.
22. Loots MA, Lamme EN, Zeegelgar J, et al. Differences in cellular infiltrate and extracellular matrix of chronic diabetic and venous leg ulcers versus acute wounds. *J Invest Dermatol*. 1998, 111: 850–57.
23. Birke JA, Novick A, Patout CA, et al. Healing rates of plantar ulcers in leprosy and diabetes. *Lepr Re.v* 1992, 63: 365–74.
24. Ferrier TM, Comparative study of arterial disease in amputated lower limbs from diabetes and non-diabetes (with special reference to feet arteries). *Med J Aust*, 1967. 1;5-22.
25. LoGerfo FW, Coffman JD, Current concept vascular and macrovascular disease of the foot in diabetes-Implication for foot care. *N Engl J Med*. 1964, 311, 3615-19.
26. Ganz T.. Neutrophils and host defence. Ann. Intern. Med. 1988, 109:127– 42.

27. Henson PM, Johnston RB Jr. Tissue injury in inflammation. *J. Clin. Invest*. 1987, 79:669–74.
28. Malech HL, Gallin JI. Neutrophils in human diseases. *New England J. Med.* 1987, 317:687–94.
29. Jutila MA. Leukocyte traffic to sites of inflammation. *APMIS*. 1992, 100:191–201
30. Pober JS, Cotran RS. 1990. The role of endothelial cells in inflammation. *Transplantation 50: 537–544*.
31. Hynes, R.O. Integrins: versatility, modulation, and signalling in cell adhesion. Cell 69: 11–25.
32. Ruszczak Z. (2003). Effect of collagen matrices on dermal wound healing. *Adv. Drug Delivery Rev*. 1992. 55 (12) 1595-1611.
33. R.L. Sheridan, R.G. Tompkins, Skin substitutes in burns. *Burns*. 1999, 25, 97 -103.
34. Habermehl, J. Skopinska, J. Boccafoschi, F., Sionkowska, A., Kaczmarek, H. , Laroche, G. Mantovani. D. *Macromol. Biosci.* 2005, 5 (9) 821.
35. Ramshaw J.A.M., Peng Y.Y., Glattauer V. Werkmeister, J.A. *J. Mater. Sci. Mater.Med.* 2009, 20:S3–S8.
36. Chvapil M, Kronenthal RL, Van Winkle W. Medical and surgical applications of collagen. *Int Rev Connect Tissue Res.* 1973, 6:1–61.
37. Minabe M, Uematsu A, Nishijima K, Tomomatsu E, Tamura T, Hori T. Application of a local drug delivery system to periodontal therapy: I. Development of collagen preparations with immobilized tetracycline. *J Periodontol*. 1989, 60:113–7.
38. Park JC, Hwang YS, Lee JE, Park KD, Matsumura K, Hyon SH, et al. Type I atelocollagen grafting onto ozone-treated polyurethane films: Cell attachment, proliferation, and collagen synthesis. *J Biomed Mater Res*. 2000;52:669–77.
39. Gorham S. D. Biomaterials, D. Byrom, Ed., New York, 1991, Stockton Press, p. 55.
40. Mitchell R. A New Biological Dressing for Areas Denuded of Mucous Membrane. *Br Dent J*. 1983, 155:346–8.
41. Atefeh Solouk., Hamid Mirzadeh., Mohammad Ali Shokrgozar., Mehran Solati-Hashjin., Siamak Najarian., Alexander M. Seifalian. The study of collagen immobilization on a novel nanocomposite to enhance cell adhesion and growth. *J. Iranian Biomedical*, 2011, 15(1-2), 6-14.
42. Rajan N, Habermehl J., Coté MF., Doillon CJ., Mantovani D. Preparation of ready-to-use, storable and reconstituted type I collagen from rat tail tendon for tissue engineering applications. *Nature Protocols*, 2006, 1(6), 2753-2758.
43. Takeshi Nagai., Nobutaka Suzuki., Yasuhiro Tanoue., Norihisa Kai., Toshio Nagashima. Characterization of Acid-Soluble Collagen from Skins of Surf Smelt (*Hypomesus pretiosus japonicas* Brevoort). *Food and Nutrition Sciences*, 2010, 1, 59-66.
44. Ramnath V. Sekar S, Sankar S, Sankaranarayanan C, Sastry TP. Preparation and evaluation of biocomposites as wound dressing material. *J Mater Sci: Mater Med.* 2012, 23:3083–3095 DOI 10.1007/s10856-012-4765-5.
45. Heidemann E. 'Fundamentals of leather manufacturing', 1993, Eduard Roether KG, Darmstadt.
46. Heidemann E. and Roth W. 'Synthesis and investigation of collagen model peptides', Adv. Poly. Sci., 1982, Vol. 43, pp. 143-203.
47. Sekar S, Mohan R, Das B N, Sastry T P. Preparation and characterisation of composite boards using chrome shavings and plant fibres. *JILTA*, 2009, 29, 765-770.
48. Lee, J.E, Park, . J.C. Lee, K.H Oh . S.H., Suh H., Laminin modified infection-preventing collagen membrane containing silver sulfadiazine-hyaluronan microparticles. *Artif. Organs*. 2002, 26, 521.
49. Zillur Rahman S. Shamim Jairajpuri. M. *Neem in Unani Medicine*. Neem Research and Development Society of Pesticide Science, India, New Delhi, 1993, 208-219.
50. Debjit B, Chiranjib C, Jitender Y, Tripathi KK, Kumar KP. Herbal remedies of *Azadirachta indica* and its medical application. *J Chem Pharm Res* 2010, 2:62-72.
51. Green L C., Wagner D A., Glogowski J. Analysis of nitrate, nitrite and nitrate in biological fluids. *Analytical Biochemistry*. 1982, 126, , 131-138
52. Yoon H J, Moon M E, Park , H S, Im, S Y, Kim Y H. Chitosan oligosaccharide(COS) inhibits LPS-induced inflammatory effects in RAW 264.7 macrophage cells. *Biochemical & Biophysical Research Communications*, 2007, 358, 954-959.
53. Ganesh Chandra Jagetia, Shaival Kamalaksh Rao, Manjeshwar Shrinath Baling, Kiran S Babu. The evaluation of nitric oxide scavenging activity of certain herbal formulations in vitro: A preliminary study. *Phytotherapy Research*, 2004, 18, 561-565
54. Nathan C, Xie Q W. Nitric oxide synthases:roles tolls, and controls. *Cells,* 1994, 78, 915-918.
55. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990's. *Nature*, 1993,362, 801-809

56. Garima Pandey, KK Verma, Munna Singh. Evaluation of phytochemical, antibacterial and free radical scavenging properties of *Azadirachta indica* (neem) leaves, International Journal of Pharmacy and Pharmaceutical Sciences, 6(2), 2014
57. Rice-Evans C A., Miller J M., Paganga G. Structure-antioxidant activity relationship of flavanoids and phenolic acids. *Free radical biology and medicine,* 1996, 20, 933-956.
58. Jorgensen LV, Madsen HL, Thomsen MK, Dragsted LO and Skibsted LH . Regulation of phenolic antioxidants from phenoxyl radicals: An ESR and electrochemical study of antioxidant hierarchy. *Free Radical Research*. 1999, 30: 207-220.
59. McDaniel J C, Belury, M, Ahijevych, K,. Blakely W. Omega-3 fatty acids effect on wound healing. *Wound Repair and Regeneration*, 2008, 16, 337-345.
60. Collins N., Sulewski C.. Omega-3 fatty acids and wound healing. *Ostromy Wound Management,* 2011, 10-13.
61. Mahadevan V, Hart IR and Lewis GP Factors influencing blood supply in wound granuloma quantitated by a new in vivo technique. Cancer Res. 1979, 49, 415-419.
62. Thompson JA, Anderson KD, DiPetro JM, Zweibel JA, Zmaetta M. Site-directed neovessel formation in vivo. *Science*. 1988, 241:1349–52.
63. Ziats NP, Miller KM, Anderson JM. *In vitro* and *in vivo* interactions of cells with biomaterials. *Biomaterials.* 1988, 9:5–13.

*****