RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Effect of collagen sponge incorporated with *Macrotyloma uniflorum* in full thickness wound healing by down regulating of MMPs and inflammatory markers

Thangavelu Muthukumar, PhD,[1]*; Dharmalingam Prakash, PhD [2]; Kannan Anbarasu, PhD [2]; Baskar Santhosh Kumar, PhD[1]; Thotapalli Parvathaleswara Sastry PhD [1]*.

[1]Bio-Products Laboratory, CSIR-Central Leather Research Institute, Adyar, Chennai 600 020. Tamilnadu, India.

[2]Department of Biochemistry, School of Life Sciences, University of Madras, Guindy Campus, Chennai-600025, Tamilnadu, India.

***Corresponding author**
Dr. T.P Sastry
Senior Principal Scientist & Head,
Bioproducts Laboratory, CSIR-Central Leather Research Institute, Adyar, Chennai 600 020.
Telephone.: 91 44 24420709, Fax: 91 44 24911589
Email: sastrytp@hotmail.com; sastrytp56@gmail.com
T. Muthukumar, auromuthu@gmail.com

Abstract

In the present study, collagen sponge (CS) is prepared using a fish scale which is a biological waste in marine food industry. This CS was incorporated separately with the drug, mupirocin (CSM) and *Macrotyloma uniflorum* plant extract (CSPE). CS, CSM and CSPE were applied on the experimental wounds of rats and the healing pattern was observed using various biological and physicochemical techniques. CSPE enhanced wound healing and involved in the upregulation of growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and transforming growth factor-beta (TGF-β). Increased levels of hydroxyproline, hexosamine, and uronic acid were observed in CSPE treated group compared to other groups. CSPE treatment reduced the inflammation, MMPs expressions and scar formation, thereby helping faster wound healing.

Keywords: Wound healing, Collagen sponge, Cyclooxygenase, Nitric oxide synthases, Matrix metalloproteinase, Transforming growth factor.

1 Introduction

Collagen is reported to be the most abundant and ubiquitous protein representing nearly 30% of the total protein present in the animal body.[1] Due to the low immunogenicity nature of

collagen, it has been widely used in many pharmaceutical applications, e.g., wound dressings, shields, injectable dispersions, sponges, moisturiser, and microparticles etc.[2,3] Collagen induces the fibroblasts by its three-dimensional structure which is essential for the granular tissue, cell–cell and cell–matrix interactions, deposition of new fibres, and re-epitalization.[4] Collagen is now available in many different forms i.e., sheet/film, gels, sponges, sprays etc. Among them, collagen sponge is widely preferred for the burn/wound dressing due to its stability, porosity, adsorbtion of large quantities of tissue exudates, adherence to wet wound, maintenance of moist environment, and enhancement of new granulation tissue formation and epithelium on the wound surface.[5]

Wound healing is a complex process, executed and regulated by a complex signaling network, which consists of several growth factors, cytokines, and chemokines. They are essential components for the coordination of multiple cell types during the wound healing process. Among the known growth factors, vascular endothelial growth factor (VEGF) is thought to be the most ubiquitous, efficient, and long-lasting to stimulate wound healing.[6] Healing without scar formation is particularly achieved by the growth factors like transforming growth factors-β (TGF-β), fibroblast growth factors (FGF), and epidermal growth factors (EGF). These factors play a fundamental role in wound healing by recruiting fibroblasts to the site of injury and thereby stimulating connective tissues such as collagen and fibronectin.[7]

Matrix Metalloproteinases (MMPs) are capable of degrading structural components of extracellular matrix (ECM). MMPs participate in many biological processes namely development, regeneration, morphogenesis, and wound healing.[8] Inflammatory mediators such as COX-2 (Cyclooxygenase-2) and iNOS (Inducible nitric oxide synthases) are reported to be predominantly expressed during the early inflammatory process and found to be

reduced as the healing process continues. They are induced by different factors including cytokine, growth factors, and inflammatory stimuli.[9,10]

Collagen in combination with *M. uniflorum* plant extract (MPE) enhances the wound healing, as the plant extract exhibits antibacterial and anti-inflammatory properties.[11] The aim of the present study is to evaluate the feasibility of collagen sponge incorporated with the MPE in *in vivo* wound healing and its effect on growth factors, MMPs expression, COX-2, iNOs, and biochemical parameters.

2 Materials and Methods

2.1 Preparation of the dressing material

The isolation of fish scale collagen and the extraction of following MPE were done using the procedure explained in our earlier studies.[12] The collagen scaffolds were prepared using the slight modification of the method explained in reference.[13] Briefly, the collagen solution of 1% wt/vol in 0.5 M acetic acid (20ml) was prepared, this solution was continuously stirred under IKA T25 using homogenizer at 13,500 rpm to generate uniform foam. A drop of Triton X-100 was added to the mixture as a frothing agent and 0.25% glutaraldehyde (0.25ml) solution was added as a cross linking agent. The formed collagen foam was poured in to teflon trays and frozen at -80 °C for 24 h followed by freeze drying for 48 h lyophilizer using (Operon Co., Korea). The completely dried scaffolds were stored 4°C in airtight plastic containers and denoted as collagen sponge (CS). To prepare CSM, 20mg M was added to 20mg collagen solution (1% wt/vol in 0.5M acetic acid) and other steps were followed as in the case of preparing CS. To prepare CSPE 2ml (23mg dry weight) of MPE were added to 20 ml collagen solution (1% wt/vol in 0.5M acetic acid) and further steps were followed as in the case of preparation of CS. The characterization, and *in vitro* studies of collagen foam impregnated with *M. uniflorum* (PE) and mupirocin (M) were briefly studied and reported in

our previous paper.[12] The materials prepared include collagen sponge (CS), CS incorporated with M (CSM), and CS incorporated with MPE (CSPE). All the materials prepared were sterilized using ethylene oxide.[14]

2.2 *In vivo* studies

All the experiments were performed according to the Institutional Animal Care and Use Committee (IACUC) approval and guidelines [466/01a/CPCSEA]. Male albino Wistar rats between 180 and 200 g were divided into four groups and the details are given in Table 1. The rats in each group were acclimatized for 1 week prior to the study and housed individually later in 12 h light/dark cycle at 25±1°C and were provided standard rodent feed procured from M/s Hindustan Level Ltd. Feeds, Mumbai, India and water *ad libitum*.

2.3 Surgical procedure and Dressing

Subsequent to intraperitoneal injection of standard anaesthesia (ketamine--50 mg/kg body weight and xylazine-10 mg/kg body weight), the dorsal surface of the rat below the cervical region was shaved and the skin was disinfected with 70% ethanol. 2 × 2 cm full thickness excision wound was created using scalpel blade by excising the dorsal skin. The wound area was photographed and the initial wound area was traced using transparent sheet. In control group (group 1), wounds were dressed with sterile cotton gauze. Group 2 animals were dressed with the CS, group 3 animals with CSM, and group 4 animals with CSPE scaffold and they were covered with absorbent gauze to hold the material on wound area. The dressings were changed periodically at an interval of 4 days with respective dressing materials. The wounds were cleaned with sterile distilled water before respective dressings. For control group, the wound was applied with sterile saline in addition to distilled water to facilitate injury free removal of dressing. The material was removed gently under moist condition using a sterile pair of tweezers. Wound tissues were removed by sacrificing six rats each from all groups periodically on 4th, 8th, 12th, 16th, and 21st days of post-wound

creation and the granulation tissues formed were collected and stored at –80°C until analysis. The progress of wound healing in rats was evaluated by periodical monitoring of wound contraction area and immunohistological and biochemical studies.

2.4 Planimetry: Rate of contraction and period of re-epithelialization

A visual proof of the wound healing pattern was recorded by taking digital photograph on 0^{th} day and 4th, 8th, 12th and 16th days of post w32ound creation. The time taken for full re-epithelialization of the wound biopsies was noted, the rate of contraction and surface area were measured by the standard planimetric method by tracing the wound on transparent graph sheet. The percentage of wound contraction was calculated using the following formula:

$$\%\ \text{of wound contraction} = \frac{(\text{Wound area day } 0 - \text{wound area day } (n))}{\text{Wound area day } 0} \times 100$$

where n = number of days (4th, 8th, 12th, and 16th days). The results were analyzed by one-way ANOVA at 5% error. The tensile strength of the incision wound tissues was measured at the end of the experiments.

2.5 Tensile strength measurements

The tensile strength measurements of the untreated and treated wound tissues collected at the end of the experiments were examined as described earlier.[15] The harvested tissues were trimmed into strips of 20 mm long and 2 mm wide, with the area of original wound lying lengthwise in the centre of the sample. Mechanical properties such as tensile strength (MPa) and percentage of elongation at break (%) were measured using a universal testing machine (Instron model 4501).

2.6 Biochemical analyses of the excision wounds

The excised tissue was biochemically analyzed to estimate the total amount of collagen by estimating collagen (hydroxyproline) content in defatted dried granulation tissue by the method of Woessner,[16] hexosamine was estimated by the method of Elson and Morgan,[17] Uronic acid content was determined using the method of Schiller et al.[15] and protein was determined by Lowry et al.[18] method in the treated and untreated wound tissues. Each of these experiments was repeated six times.

2.7 Histology and immunohistochemical study of granulation tissue

The regenerated skin tissues from the wound site were periodically collected along with the healthy skin of 2 mm surrounding the wound on 4th, 8th, 12th, and 16th days of post-wound creation following euthanasia. The samples were fixed in 10% buffered formalin, dehydrated with graded ethanol series, and embedded in paraffin blocks. The samples were sliced into 4-μm sections and stained with hematoxylin and eosin (H&E; Fisher Scientific) to examine the regeneration of the epidermis and dermis. Immunohistochemical expression of COX-2 and iNOs was performed as reported by Kalayarasan et al.[19] Briefly, the Paraffin-embedded tissue sections (4 μm thickness) were rehydrated first with xylene and then in series with ethanol solutions. Then the sections were blocked with 5% BSA in Trisbuffered saline [(TBS), pH 7.4] for 2 h, followed by immunostaining with primary antibody (rabbit polyclonal IgG to rat iNOS) diluted 1:500 with 5% BSA in TBS and kept overnight incubation at 4°C. After washing the slides three times with TBS, the sections were then incubated with goat anti-rabbit secondary antibody (diluted 1:2000) with 5% BSA in TBS and incubated for 2 h at room temperature, followed, by washing the sections with TBS and incubated for 5–10 min in a solution of 0.02% diamino benzidine (DAB) containing 0.01% hydrogen peroxide, counter staining was performed using hematoxylin, and the sections were examined under a microscope and photomicrographs were taken using microscope (Leica, DM5000, Germany).

2.8 Gelatin zymography

Total protein from rat wound tissues was extracted at different sampling times and the extracted protein samples (*n*=3 per treatment groups) were pooled and tested for gelatinase activities as described elsewhere.[20] Protein samples were assessed for MMP-2 and MMP-9 with 10 mg of nonreduced sample being loaded per lane, onto a 7% SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel) containing 0.5 mg/ml gelatine. They were finally stained with 1% acetic acid and 30% methanol. The MMPs were seen as a clarified band which corresponds to the zone of digestion of substrate gelatine.

2.9 Reverse transcriptase PCR

Total RNA was isolated from the regenerated skin collected on 4th, 8th, and 12th days of post-wound creation and they were trimmed off any visible fat and healthy skin. Total RNA was isolated using TRIZOL reagent (Bangalore Genei) according to manufacturer's instruction. Three micrograms of total RNA were used in the RT PCR reaction from the samples. The PCR reaction was performed in the thermal cycler (Eppendorf, Germany) using the following conditions: reverse transcription was performed at 50ºC for 50 min and activation at 95ºC for 15 min. The cDNA (2 µl) was amplified by PCR with the required primers. Primer sequences were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were synthesized by Xcelris Labs Limited (Xcelris corporate Headquarter, Bodakder, Ahmedabad, India). All the primer pairs were analysed by Primer-BLAST to ensure specificity for the intended target gene within the human genome. VEGF, Forward 5'-AGAGTGGGAGGGAAGCTCTTAG-3', Reverse 5'-CGGGATTTCTTGCGCTTTCG-3' (511); EGF, Forward 5'-TGGAAAAGATGGCTGCC ACTGGGTC-3', Reverse 5'- GTGTTCCTCTAGGACCACAAACCA-3' (430); FGF, Forward 5'- CAGGAGTACTGCAGAGCGAC-3', Reverse 5'-TCCGGTTTTGGTGCTGAT GT-3' (239); and TGF-β3, Forward 5'-CACACAGTCCGCTACTTCGT-3', Reverse 5'-

CGGGTGCTGTTGTAAAGTGC-3' (434). Amplification was followed by PCR of 35 cycles: 94ºC (denaturation) for 45 s, Variable 1 (annealing) for 45 s, and 72°C extension for 1 min. Then, the products were incubated at 72°C for 10 min to extend any incomplete single strands. Finally, PCR products (cDNA) were analyzed on 1.5% agarose gel electrophoresis, and densitometric analysis of the bands was done by image analysis software.

3. Results and discussion

The preparation, characterization, and *in vitro* studies of CS, CSM and CSPE were reported in our previous paper.[12] According to the results obtained, SEM images of all the samples showed porous and mesh like structure with inherent interconnectivity. CS exhibited more macroporous structure and randomly oriented thin fibril like networks compared to CSM. More porous nature along with thickening of fibril like network with smooth surface was observed in the SEM picture of CSM. The pore sizes were more or less same from 30 to 70 μm in diameter for all the samples. Among the three samples prepared, CSPE showed significantly higher tensile strength (2.96 ± 0.25 MPa) and percentage elongation of (16.26 ± 1.22%) compared to those of CS (0.56 ± 0.05 MPa and 11.61 ± 0.46%) and CSM (0.57 ± 0.03 Mpa and 11.40 ± 0.04%). All the samples were biocompatible and exhibited anti-bacterial properties, hence these materials were selected for the wound healing activity *in vivo* model. Wound healing is a dynamic and continuous physiological process involving inflammation, proliferation or tissue regeneration, and tissue remodelling. There are several factors that affect the wound healing process and accelerate or retard the healing process.[21]

3.1 Planimetric, photographic, and period of epithelialization studies

Regular monitoring of the wounds and noting the wound contraction are essential for assessing the treatment effectiveness.[22] Visual evidence of wound healing was carried out by taking photographs using Sony DSLR, Camera from a constant distance for all animals and

groups. All the animals were photographed but only one respective photograph from each group was shown (Fig. 1). The photographs clearly indicated faster rate of healing in the CSPE treated groups compared to those in other groups and control. Wound assessment is a complex process which includes wound aetiology, appearance, prediction, and monitoring the rate of healing, finding the factors responsible for delaying wound healing and wound documentation. Wound size measurement is an important factor which has the potential role to provide baseline measurement and accurately helps to determine the percentage wound reduction or increase (healing/nonhealing) in the wound area over a period of time. This is also important for us to evaluate the efficacy of the prepared material.[23] The results clearly showed that the experimental groups treated along with CSPE showed faster rate of healing compared to the other groups and control animals. CSPE treated groups took only 12 days for the complete epithelialization, whereas the CS and CSM treated groups took 17 and 16 days respectively, and control group took 21 days for complete epithelialization. It has been observed that the healing pattern was regular and uniform in the CSPE, CSM, and CS treated groups compared to that in control groups. The photographic results are in support with the planimetric studies. Fig. 2a shows the rate of wound contraction in control, CS, CSM, and CSPE treated groups. The time taken for the contraction of excisional wounds was calculated from the planimetric sheet photographs using the ImageJ® software program from the wound tracing. The one-way ANOVA results showed that the groups were significantly different on the specified day. The results show the significant increase in the rate of contraction in the experimental groups compared to control over a period of time. CSPE treated groups showed complete contraction on day 12, whereas those treated with CSM and CS took 16 and 17 days; however, complete contraction was observed in control group on day 21. Increased rate of healing was observed in CSPE treated groups, which may be due to the incorporation of MPE to the CS, and the photographic results agree well with the planimetric studies.

3.2 Biochemical analyses of the excision wounds

The biochemical analyses, i.e., collagen, hexosamine, and uronic acid, were performed for the excised granulation tissue on specified days of scarification after wound creation. Wound contraction process depends on fibroblast invasion, collagen deposition, and maturation. Collagen synthesis increases in the site of injury which plays a major role in strength, haemostasis, and integrity to the newly forming matrix at the site of wound and helps in the re-epithelialization and cell–cell and cell–matrix interactions.[23] Collagen is a well-known constituent of growing cells in the healing tissues, which is measured by estimating the concentration of hydroxyproline.[24] In our experiments, hydroxyproline content (Fig. 2b) was found to be increasing in all the treated and untreated groups till the 12th day and later stabilized until complete healing. Among the groups treated, the CSPE treated groups showed higher amount of hydroxyproline content in all the days of sampling compared to those groups treated with CS and CSM and control groups. Significant increase in the hydroxyproline content in granulation tissue was seen in the group treated with CSPE, which may be due to the increased synthesis of collagen. Thus, increasing concentration of hydroxyproline indicates the faster rate of healing by improving migration of fibroblasts, increase in cellular proliferation, and re-epithelialization.[25] Compared to the treated groups, decrease in the collagen contents was observed in the control groups, which may be due to the prolonged inflammatory phase.[26] Hexosamine contents of granulation tissues were shown in Fig. 2c. The amount of hexosamine was found to be higher on 4th day in all the groups, whereas significant increase in the hexosamine was found in the group treated with CSPE compared to CSM, CS, and control. Gradual decrease in hexosamine content was observed in all the groups during the course of the study. However, significant difference between the control and the treated groups was observed. The results obtained were in agreement with the collagen contents observed. In general, decreasing trend was seen in hexosamine content in

both the experimental and the control groups in all the samples. Significant increase in the uronic acid content was observed on day 4 in all the groups. Among the groups, CSPE treated groups showed significant increase in the uronic acid content compared to CS, CSM, and control groups. Gradual decrease in uronic acid content was observed after the 4th day in all the groups, whereas similar trend among the groups was observed over the period of healing (Fig. 2d).

3.3 Tensile strength

Wound healing is a primary response to tissue injury, comprising a complex biological process of a connective tissue repair. The tensile strength and percentage elongation values of excised healed wound tissues are shown in Figs. 2e to f. It can be clearly concluded from the images that the tensile strength and elongation exhibited by the groups treated with CSPE are significantly greater than those of the other treated groups (CS and CSM) and control. Increased tensile strength values in the CSPE treated group clearly indicate the increase in collagen matrix which imparts tensile strength and elasticity to the healed skin. This is further supported by the biochemical assay results which showed increased collagen content of the wound treated with CSPE, which ultimately resulted in the increased tensile strength. Singer and Clark reported that wound tissues gain 20% of their final strength in first 3 weeks of post-wounding,[27] whereas a decreased tensile strength and percentage elongation was observed in control group compared to the CSPE treated groups, which are also further supported by the hydroxyproline assay results. Moreover, the tensile strength is directly related to the amount of collagen matrix synthesized at the wound site.

3.4 Histological observation

Histological examination of wound tissue was performed using H&E staining to observe the formation of epithelium, connective tissue, inflammatory response, fibroblast proliferation, and collagen deposition (Fig. 3). On day 4, H&E-stained tissue sections showed epithelium

and connective tissue in the control and treated groups, and the connective tissue had abundance of acute and chronic inflammatory cells such as lymphocytes and neutrophils with blood vessels and extravasated RBCs. On 8th day, the H&E-stained histopathological section of CS and CSM treated groups and control showed moderate inflammatory infiltration compared to CSPE treated group. The connective tissue in the CSPE treated group is fibrous in nature with less chronic inflammatory cells such as lymphocytes and blood vessels, which clearly indicate that the MPE-incorporated scaffold helps in faster healing by preventing the prolonged inflammatory phase. Along with this, epithelialization at the wound edges was observed to be better for the CSPE treated groups. These findings indicate better initiation of the healing process. On 12th day, CSPE treated groups showed complete epithelialization with focal acanthosis and adenexal structure, whereas the connective tissue was fibrous in nature with dense collagen fibres and blood vessels which is an indication of complete healing. On the other hand, only moderate epithelialization was seen in CS and CSM treated groups. Inflammatory cells were present in 4th, 8th, and 12th day sections, but the amount of inflammatory cells was reduced in comparison to CSPE group; this showed that the healing process was slow. For the control defective, epithelialization was observed. The collagen deposition and faster epithelial regeneration seen with CSPE treated groups could significantly accelerate wound healing compared to those in the other groups. It is well established that the collagen formation is very important for tissue repair and remodelling. Additionally, faster healing and deposition of collagen did not contribute to any scar formation in the wounded areas. Hence, CSPE can be effectively tried as a wound healing material for the treatment of deep wounds.

3.5 Expression of growth factors in response to injury and MPE

The EGF expressions were found to be significantly higher in the CSPE treated groups compared to those of control and CS and CSM treated groups on fourth day (Fig. 4a). The

EGF expression pattern was found to be reducing in the treated groups, whereas the control group showed increasing expression on 8th day, and on 12th day, reduction in the expression was seen, and significant reduction was seen in the CSPE treated groups followed by CSM and CS. EGF was reported to mediate an increase in collagen content during wound repair.[28] In the CSPE treated groups, the FGF expression was found to be increasing on day 4 and elevated on day 8, later declined. On day 12, the expression was significantly reduced compared to the other CSM and CS treated groups, whereas the expression was very low on day 4 compared to other groups and started increasing on days 8 and 12 compared to treated groups. Hypoxia induces several angiogenic gene expressions at the cellular level, most particularly VEGF.[29] VEGF also followed the same pattern of expression as that of EGF and FGF. The expression in the treated groups was significantly higher compared to the control on day 4, whereas the expressions were found to be decreasing in the treated groups on day 8 and significantly reduced in the CSPE treated groups on day 12 compared to CSM and CS. On the other hand, the VEGF expressions were found to be increasing on day 8 and significant increase was found on day 12 compared to the treated groups. There are many reports supporting our results that the VEGF expressions were found to be increasing on day 3 and last till day 7 and found to be declined around 13 days[30, 31] Bao et al.[32] has reported that VEGF stimulated wound healing by involving in the multiple wound healing cascade, such as angiogenesis, epithelialization, vasodilation, endothelial cell proliferation, and promoting collagen deposition. TGF-β involves in the wound healing and tissue repair that regulates the rate and extend of the wound healing process.[33] In our study, TGF-β3 was found to be significantly reducing in the treated groups from day 4 to day 12. Among the treated groups, CSPE treated groups showed significant reduction in the expression compared to other treated groups and control group. The expression was found to be maximum in the control group on all the days (4th, 8th, and 12th days) compared to the treated groups. TGF-β3 is

reported to be involved in the reduction of scar formation by decreasing the formation of type I collagen and by degrading the type I collagen[34] and involves in processes such as inflammation, angiogenesis, proliferation of fibroblast, synthesis of collagen, deposition, and remodelling of the new ECM. This result correlated with the biochemical parameters observed for the time taken for epithelialization.

3.6 Determination of MMP-2 and -9 using Gelatin Zymography

MMP expression in the granulation tissues from all the groups in different intervals was analysed. MMPs play a vital role in the rate of wound healing process. Increasing MMP ratio leads to the ECM components degradation, growth factors, and their receptors in the wound by prolonging the rate of wound healing process.[35] In the wound healing process, fibroblasts, keratinocytes, and inflammatory cells are the major cell types that produce the MMPs. MMPs expression is regulated by the signals received from the growth factors, cytokines, cell–matrix interactions, and altered cell-cell contacts.[36] In this study, we have determined the pro- and active forms of MMP-2 and -9 expressions by the gelatin zymography from the granulation tissues (Fig. 4b). On day 4, the pro- and active forms of MMP-2 and -9 were found to be significantly high in the control groups compared to the treated groups and they were found to be reducing over a period of time. Compared to MMP-2, MMP-9 expression was significantly reduced in the treated groups on the 4th day itself and the expressions were not visible on 8th and 12th day in the control and treated groups. On day 8, significant ($P<$ 0.05) reduction in the expression of MMP-2 was found in CSPE treated groups compared to control and treated groups (CS and CSM). Compared to MMP-2, there was a significant ($P<$ 0.05) reduction in the MMP-9 expression on the fourth day itself. Expression of MMPS varies with the phases of wound healing. Increased expression of MMP-9 was reported in the inflammatory phase and later MMP-9 was found to be decreasing in its expression and MMP-2 begins to increase. This result correlated with our findings.[37] On day 12, the CSPE treated

groups showed significant reduction in the pro- and active forms of MMP-2 expression compared to the control and other treated groups. Compared to the treated groups, the expression was found to be higher in the control groups on all the days; the results obtained were correlated with the biochemical analysis, thereby supporting our investigation that the CSPE enhanced the wound healing process by reducing the MMPs synthesis and increasing the collagen synthesis.

3.7 Immunohistochemical analyses of inflammatory markers

In vivo animal experiments have revealed that COX-2 inhibition reduces initial inflammatory phase of wound healing and thereby reducing the scar formation without disrupting re-epithelialization and reducing tensile strength.[38] COX-2 is normally expressed under abnormal situations such as inflammation or tumor.[39] Fig. 5 shows the COX-2 expression on different days. On day 4, among the groups, COX-2 expressions were found to be significantly (P<0.05) higher in the control group followed by the CS and CSM. At the same time, significant (P<0.05) reduction in the COX-2 expression was seen in the CSPE treated groups which may be due to the formation of new epidermal cells at the edges of wound. The same trend was seen on all the days. Compared to day 4, marked reduction in the COX-2 expression was seen on eighth day in all the groups, but significant (P<0.05) reduction was observed on the CSPE treated groups. On the 12th day, significant reduction in the COS-2 expression was seen in control and CS and CSM treated groups. From our results, we believe that the reduction in the COX-2 expression enhances the wound healing. Similar results were reported previously by Futagami et al.[9] The same expression pattern profile was seen in the iNOS (Fig. 6). During the experimental period, the iNOS expression was found to be significantly higher in the control groups on all the days compared to the treated groups. Compared to day 4, iNOS reduction was observed in the treated groups, but significant (P<0.05) reduction was observed in the CSPE-treated groups. Frank et al.[40] have reported

that the maximum effect of NOS activity will occur during the early phase of wound healing. The expression was found between 6 and 24 h and last up to 5 days. Later the expression slowly decreases over the next 10 days.[40] On 12th day, compared to the CS and CSM treated groups, the expression was found to be significantly higher in the CSPE treated group. The result clearly infers the effect of MPE on the reduction of iNOS expression by favouring the wound healing process. Witte and Barbul,[41] have reported that NO regulates cell proliferation, collagen formation, and wound contraction.[41]

4 Conclusion

The results obtained from this study indicate that the presence of MPE in the collagen sponge favours the wound healing process by upregulating growth factors and reducing inflammatory markers. CSPE treated group has shown increased levels of ground substances such as hydroxylproline, uronic acid, and hexosamine synthesis and decreased levels of MMPs. Reduced inflammation, scar formation and enhanced wound healing process was observed in CSPE treated rats. Further, CSPE treated wounds have shown improved tensile strength in the healed tissue. However, studies with purified constituents of MPE are warranted to understand the complete mechanism of wound healing activity of MPE.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgment: T. Muthukumar thanks the Council of Scientific and Industrial Research, New Delhi, India, for providing the funds and infrastructure to carry out this study. The authors would like to thank Dr. T. Gopalakrishnan (Department of Oral Pathology and Microbiology, Sree Balaji Dental College & Hospital, Bharath University, Pallikaranai, Chennai) for H&E slide interpretations.

Reference

1 C. M. Kielty, I. Hoplinson, M. E. Grant. Part I: Connective tissue and its heritable disorders. In: Royce PM, Steinmann B. (Eds.), Wiley-Liss, Inc, New York. 1993, 103-147.

2 W. Friess, *Eur. J. Pharm. Biopharm.,* 1998, **45**, 113-136.

3 P. Elsner, E. Berardesca, H. Maibach, Bioengineering of the Skin: Water and the Stratum Corneum, CRC Press LLC, Boca Raton, 1994.

4 G. S. Schultz, R. G. Sibbald, V. Falanga, E. A. Ayello, C. Dowsett, K. Harding, M. Romanelli, M. C. Stacey, L. Teot and W. Vanscheidt, *Wound Repair Regen.,* 2003, **11**, S1-S28.

5 M. Chvapil, *J. Biomed. Mater. Res.,* 1982, **16**, 245-263.

6 N. N. Nissen, P. J. Polverini, A. E. Koch, M. V. Volin, R. L. Gamelli, L. A. DiPietro, *Am. J. Pathol.,* 1998, **152**, 1445-1452.

7 H. P. Ehrlich, T. M. Krummel, *Wound Repair Regen.,* 1996, **4**, 203-210.

8 W. C. Parks, *Wound Repair Regen.,* 1999, **7,** 423-432.

9 A. Futagami, M. Ishizaki, Y. Fukuda, S. Kawana, N. Yamanaka, *Lab. Invest.,* 2002, **82**, 1503-1513.

10 M. M. Cals-Grierson, A. D. Ormerod, *Nitric Oxide,* 2004, **10**, 179-193.

11 A. Ghani, Medicinal Plants of Bangladesh*:* Chemical. Constituents and Uses. 2nd Edn. Asiatic Society *of* Bangladesh*,* Dhaka*,* ISBN: 2003; 9-845123481*:5.*

12 T. Muthukumar, P. Prabua, K. Ghoshb, T. P. Sastry, *Colloids Surf. B Biointerfaces,* 2014, **113**, 207-212.

13 R. Sripriya, M. Senthil Kumar, P. K. Sehgal, *J. Biomed. Mater. Res. Part B Appl. Biomater.,* 2004, 70, 389-396.

14 S. D. Gorham, S. Srivastava, D. A. French, R. Scott, *J. Mater. Sci. Mater. Med.,* 1993, **4**, 40-49.

15 S. Schiller, G. A. Slover, A. A. Dorfma, *J. Biol. Chem.,* 1961, 236, 983-987.

16 J. F. Jr. Woessner, *Arch. Biochem. Biophys.,* 1961, **93**, 440-447.

17 L. A. Elson, W. T. Morgan, *Biochem. J.,* 1933, **27**, 1824-1828.

18 O. H. Lowry, N. J. Rosebroug, A. L. Farr, R. J. Randall, *J. Biol. Chem.,* 1951, **193**, 265-275.

19 S. Kalayarasan, N. Sriram, G. Sudhandiran, *Life Sci.,* 2008, **82**, 1142-1153.

20 C. Heussen, E. B. Dowdle, *Anal. Biochem.,* 1980, **102**, 196-202.

21 G. C. Gurtner, S. Werner, Y. Barrandon, M. T. Longaker, *Nature,* 2008, **453**, 314-321.

22 G. Gethin, *Wounds UK.,* 2006, **2,** 60-68.

23 R. Raghow. *FASEB. J.,* 1994, **8**, 823-831.

24 Z. Q. Lin, T. Kondo, Y. Ishida, T. Takayasu, N. Mukaida, *J. Leukoc. Biol.,* 2003, **73**, 713-721.

25 R. Bernabei, F. Landi, S. Bonini, G. Onder, A. Lambiase, R. Pola and L. Aloe, *Lancet* 1999, **354,** 307.

26 M. S. Kumar, S. Kirubanandan, R. Sripriya, P. K. Sehgal, *J. Surg. Res.,* 2008, **144**, 94-101.

27 A. J. Singer, R. A. Clark, *N. Engl. J. Med.,* 1999, **341**, 738-746.

28 M. Hiramatsu, K. Hatakeyama, N. Minami, M. Kumegawa, *Jpn. J. Pharmacol.,* 1982, **32**, 198-201.

29 S. Yla-Herttuala, T. T. Rissanen, I. Vajanto, J. Hartikainen, *J. Am. Coll. Cardiol.,* 2007, **49**, 1015-1026.

30 N. J. Brown, E. A. Smyth, S. S. Cross, M. W. Reed, *Wound. Repair. Regen.,* 2002, **10,** 245-251.

31 L. F. Brown, K. T. Yeo, B. Berse, T. K. Yeo, D. R. Senger, H. F. Dvorak and L. van de Water, *J. Exp. Med.,* 1992, **176**, 1375-1379.

32 P. Bao, A. Kodra, M. Tomic-Canic, M. S. Golinko, H. P. Ehrlich, H. Brem, *J. Surg. Res.,* 2009, **153**, 347-358.

33 A. Shukla, N. Meisler, K. R. Cutroneo, *Wound Repair Regen.,* 1999, **7**, 133-140.

34 R. Hosokawa, K. Nonaka, M. Morifuji, L. Shum, M. Ohishi, *J. Dent. Res.,* 2003, **82**, 558-564.

35 S. E. Gill, W. C. Parks, *Int. J. Biochem. Cell Biol.,* 2008, **40**, 1334-1347.

36 M. Egeblad, Z. Werb, *Nat. Rev. Cancer,* 2002, **2,** 161-174.

37 T. Salo, M. Makela, M. Kylmaniemi, H. Autio-Harmainen, H. Larjava, *Lab Invest.,* 1994, **70**, 176-182.

38 T. D. Warner, J. A. Mitchell, *FASEB. J.,* 2004, **18**, 790-804.

39 M. M. Hardy, E. A. Blomme, A. Lisowski, K. S. Chinn, A. Jones, J. M. Harmon, A. Opsahl, R. L. Ornberg and C. S. Tripp, *J. Pharmacol. Exp. Ther.,* 2003, **304**, 959-967.

40 S. Frank, M. Madlener, J. Pfeilschifter, S. Werner. *J. Invest. Dermatol.,* 1998, **111**, 1058-1064.

41 M. B. Witte, A. Barbul, *Am. J. Surg.,* 2002, **183**, 406-412.

Table 1. Animal grouping

Sample collection days	No. of animals used for gross, biochemical and histological analysis			
	Group I	Group II	Group III	Group IV
	Control	CS	CSM	CSPE
4th	6	6	6	6
8th	6	6	6	6
12th	6	6	6	6
16th	6	6	6	-
20th	6	6	-	-

Figure Legends:

Fig. 1 Photographic representation of wound healing pattern on different days.

Fig. 2 (a) Showing the percentage wound contrition, (b) amount of hydroxyproline, (c) amount of hexosamine, (d) showing the amount of uronic acid contents present in the excised granulation tissue, (e and f) The tensile strength and percentage elongation values of excised healed wound tissues.

Fig. 3 H & E staining of control and treated groups on 4th, 8th and 12th day.

Fig. 4 (a) Shows the RT-PCR, mRNA expression pattern of VEGF, FGF, EGF and TGF-β in fold changes, (b) differential expression of MMP2 and 9 expressions in the granulation tissues.

Fig. 5 Representative images of immunohistochemistry staining of COX-2 on day 4, 8 and 12.

Fig. 6 Representative images of immunohistochemistry staining of iNOS on day 4, 8 and 12.

Fig. 1 Photographic representation of wound healing pattern on different days.

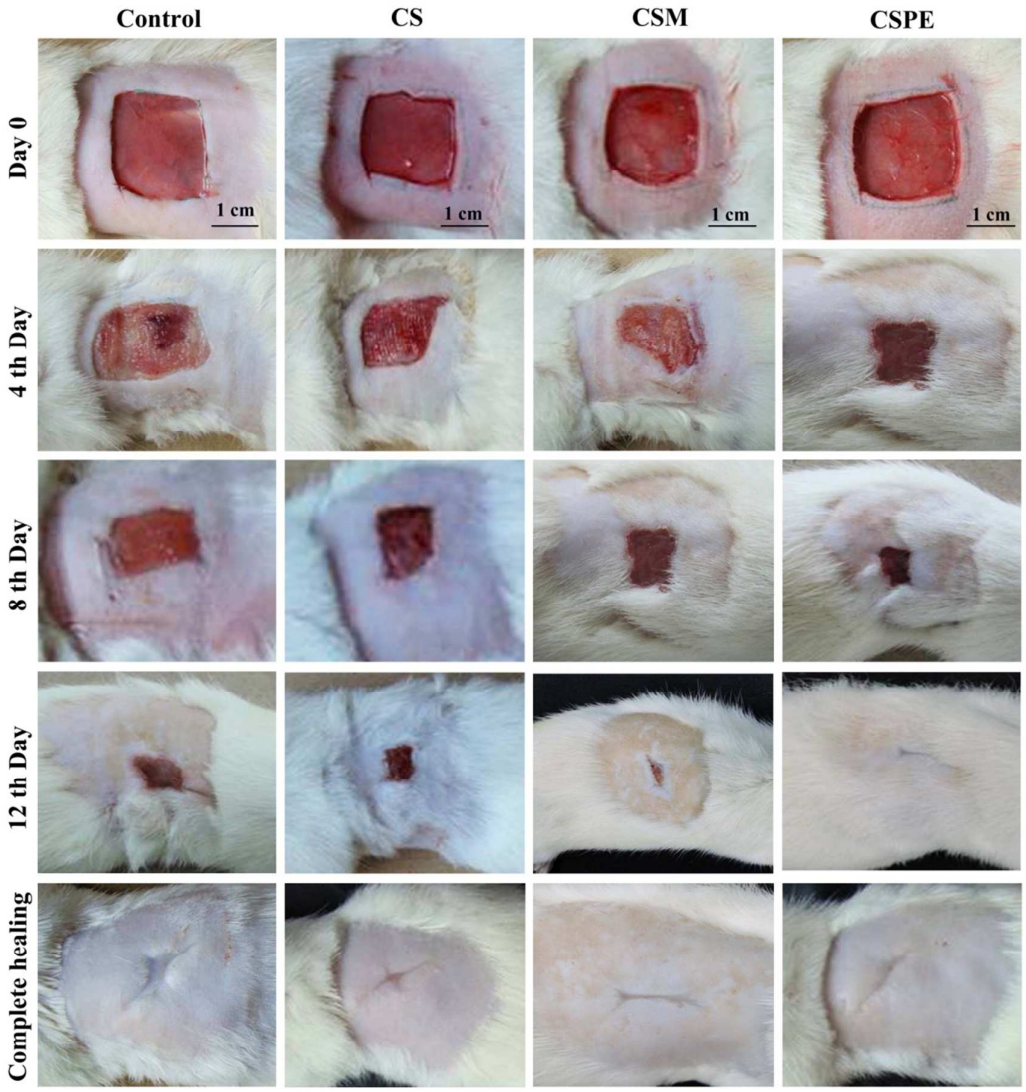

Fig. 2 (a) Showing the percentage wound contrition, (b) amount of hydroxyproline, (c) amount of hexosamine, (d) showing the amount of uronic acid contents present in the excised granulation tissue, (e and f) The tensile strength and percentage elongation values of excised healed wound tissues.

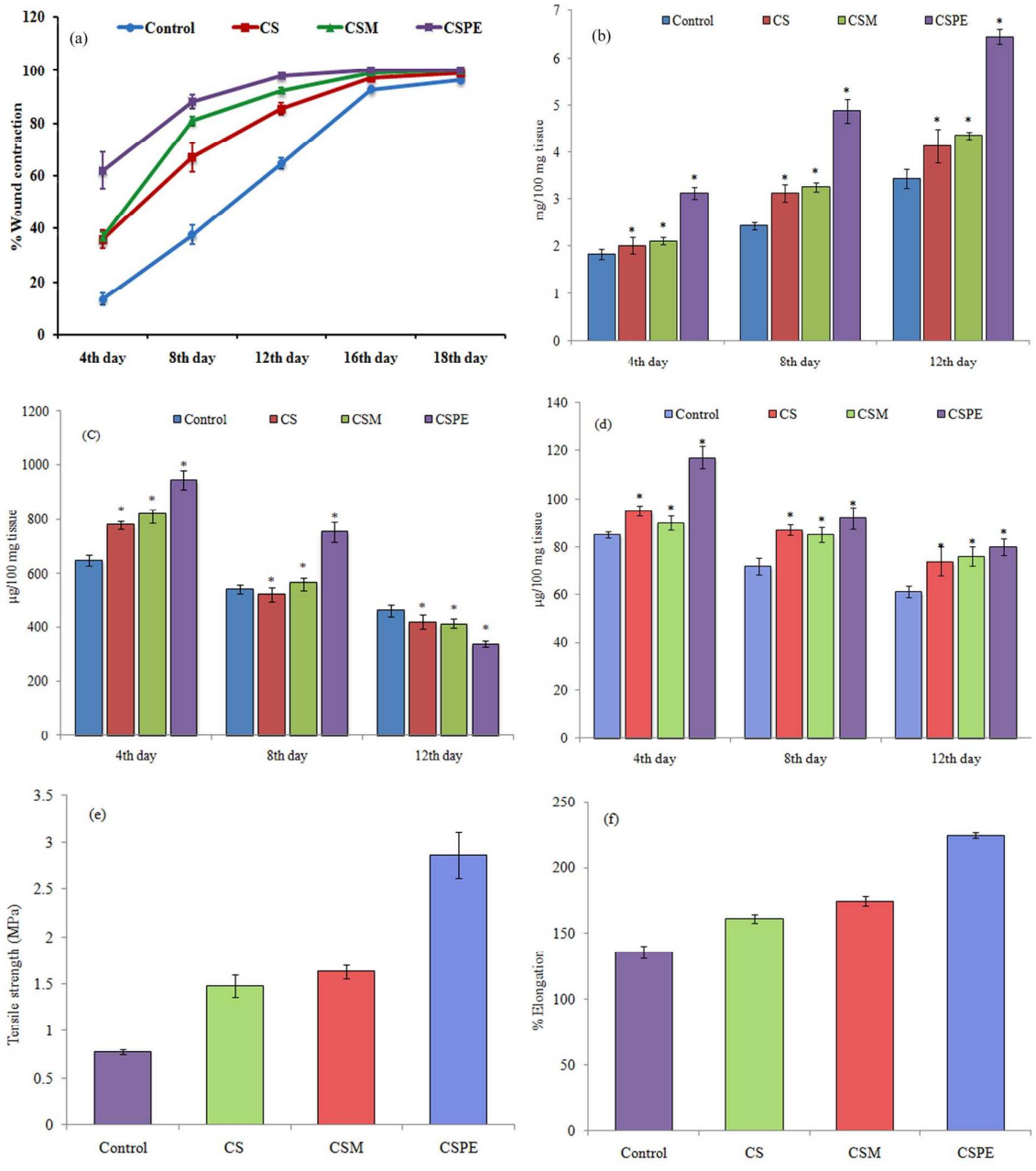

Fig. 3 H & E staining of control and treated groups on 4th, 8th and 12th day.

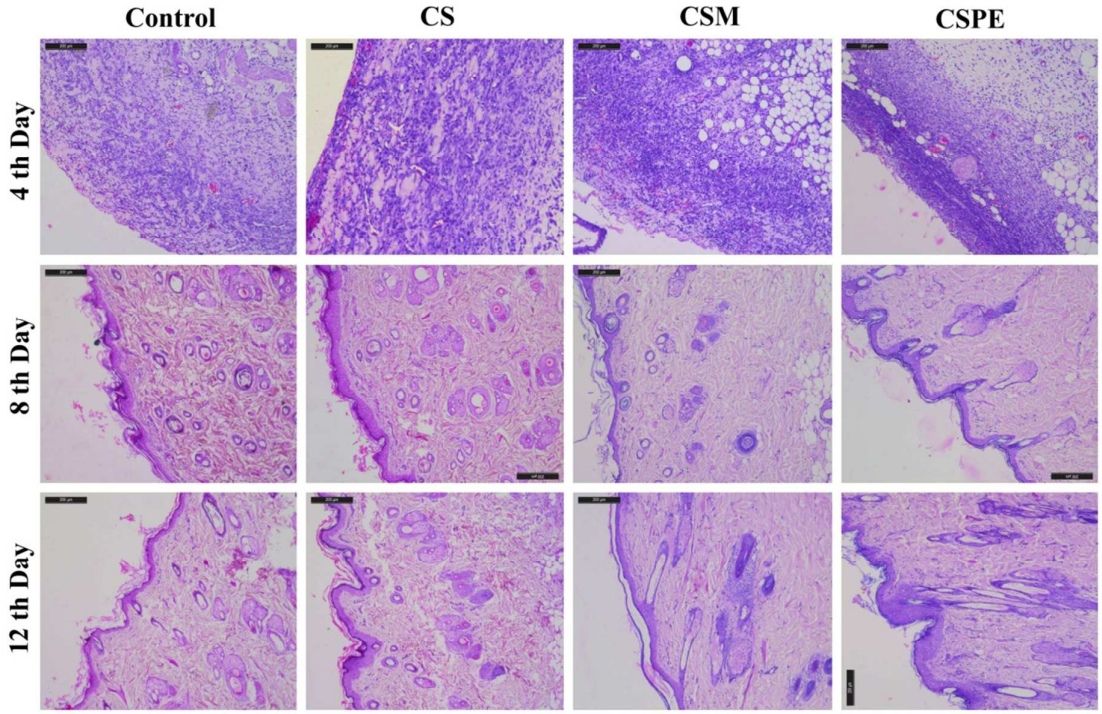

Fig. 4 (a) Shows the RT-PCR, mRNA expression pattern of VEGF, FGF, EGF and TGF-β in fold changes.

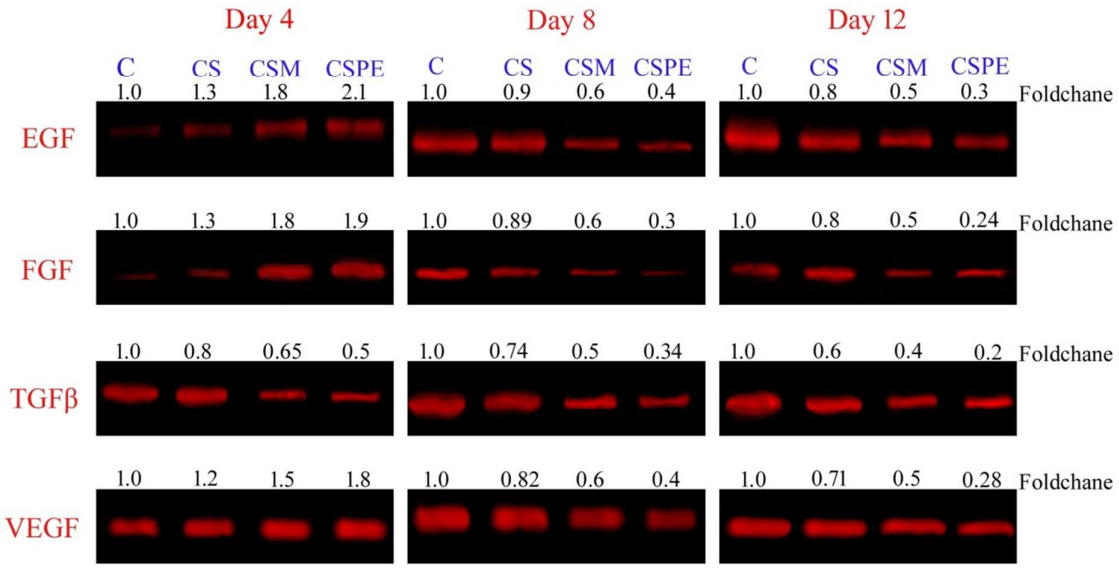

Fig. 4 (b) differential expression of MMP2 and 9 expressions in the granulation tissues.

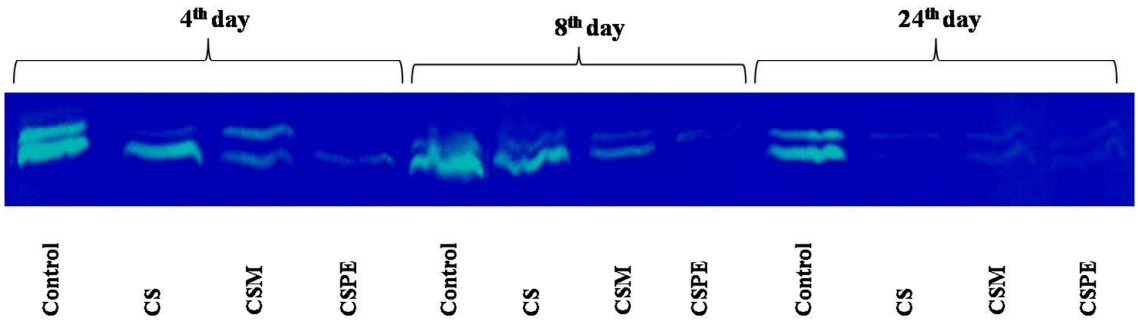

Fig. 5 Representative images of immunohistochemistry staining of COX-2 on day 4, 8 and 12.

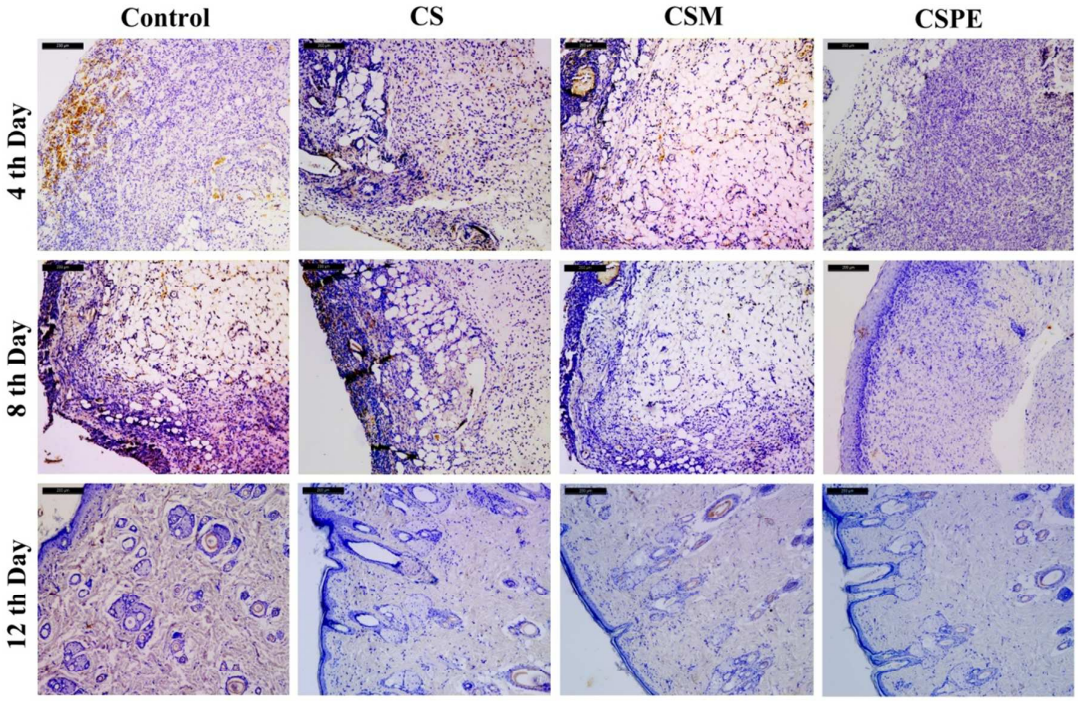

Fig. 6 Representative images of immunohistochemistry staining of iNOS on day 4, 8 and 12.