

Original Article

Chitosan/matrine membrane preparations promote wound recovery in an in vivo animal model of ulcer trauma

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Abstract: Chitosan (CS) has been shown good properties of biocompatibility, anti-hemagglutination, would healing promotion and antiseptis. Matrine (Mat), widely expressed in *Sophora flavescens*, shows anti-inflammatory, anti-liver fibrosis, analgesic and anti-arrhythmic pharmacological effects. However, the effects of matrine could be largely affected by its instability. In the current study, by using the natural biodegradable CS as matrix material, Mat as botanical drug, we prepared a Mat/CS bio-membrane. Electron microscope scanning showed that Mat molecules were asymmetric distributed in CS membrane. The Mat/CS membrane showed good physical properties and significant antibacterial effect. The membrane was non-toxic and biocompatible with cultured NIH/3T3 and ECV304 cells, and did not affect the proliferation and cell cycle of the cells. By using an ulcer trauma animal model, implantation of the Mat/CS membrane could markedly promote wound healing, skin tissue regeneration, and would not result in significant long-last inflammation. In summary, this Mat/CS membrane exhibits good drug release property, biocompatibility, antiseptis activity and can be applied as potential material for curing clinical oral diseases.

Keywords: Recurrent aphthous ulcer, chitosan, bio-membrane, matrine, inflammation, animal model

Introduction

Recurrent Aphthous Ulcer (RAU) is one of the most common oral mucosal disease, with highest incidence [1]. Common cold, indigestion, mental stress, depressed and other factors can occasionally induce the disease, occurring in the lips, tongue edge, cheek, any part of the mucous membrane and etc. [2, 3]. However, the detailed etiology and pathogenesis have not been fully defined. Pathogenic factors of RAU include the immune system, genetic factors, digestive and gastric diseases, physical body changes and lifestyle changes (lack of sleep, menstrual cycle changes, work pressure) and so on [4, 5]. Currently, there is no spectacularly effective treatment for most RAU.

Drug membrane can carry inflammatory drug via the matrix, release the drug to the target lesion and promote wound healing. There are many kinds of matrix having identical clinical

effects, such as sodium carboxymethyl cellulose, starch, polyvinyl alcohol [6]. These materials have shown great potential in the treatment of RAU. Chitosan (CS) is the only natural polymer containing positive charge and the ultimate degradation product of CS is glucosamine [7, 8]. CS is derived from the deacetylation of chitin, showing excellent biocompatibility with human tissues. CS has a broad spectrum of antibacterial activity against Gram-negative/positive bacteria, *Candida albicans* and *Helicobacter pylori* [9, 10]. CS has strong adhesive activity and membrane-forming property. CS membranes show advantages of flexibility, breathability, non-sensitization and good drug slow-release performance [11]. Because of the characters of abundant resources, low price, safety and excellent biocompatibility, CS is gradually applied in wound dressing, artificial skin and blood vessels, surgical sutures, nerve construction, drug slow-release and food industry [12-14].

Matrine (Mat), an alkaloid extracted from the plants, dried roots and fruits of *Sophora flavescens* (a traditional Chinese herb), is a molecule with multiple functions. Matrine is a potent anti-inflammatory drug with hormone-like activity but no side effects. Matrine can inhibit not only the biosynthesis but also the bio-oxidation of bacteria. Recent studies found that matrine has many other pharmacological effects including anti-tumor, anti-virus, analgesia and sedation of the central nervous system, antipyretic and anti-hypertensive disorder effects. The pharmacological effects of matrine have promising prospects for oral medicine. However, the current matrine preparations for clinical application failed to give full play to its role, and the studies on matrine mostly stay in an *in vitro* stage.

In the current study, we selected the biodegradable material CS as matrix, matrine as botanical drug to prepare a matrine loaded CS membrane (Mat/CS membrane). By *in vitro* cell culture and *in vivo* animal model, we found that this membrane showed good sustained slow-release behavior, good biocompatibility and antibacterial activity. The bio-membrane material sheds light on the potential clinical application in the treatment of RAU.

Materials and methods

The preparation of Mat/CS membrane

Five copies of 10 g CS solution were added with 0.2, 0.24, 0.28, 0.32 and 0.36 g glycerol. After that the solutions were dried at room temperature to make the CS membrane. CS of relative molecular mass of 31, 48 and 65 million were chosen, adding proper weight of matrine to make the 6%, 8%, 10%, 12% and 14% Mat/CS solution, CS: glycerol stayed in 1:1.4, later dry the solutions at room temperature to make Mat/CS membrane.

Physical property test of the membrane

For mechanical test, Mat/CS membranes were cut into 16 × 20 mm pieces, then membranes were stretched at rate of 1 mm/min for the tensile test. Each kind of membrane were tested 3 times, the values of modulus and elongation at break were recorded. For micro-morphological analysis, Mat/CS membranes were scanned

under a scanning electron microscope (SEM) machine (JEOL USA, Peabody, MA) at 5 kV.

Antibacterial activity determination

Staphylococcus aureus suspensions at 5×10^5 CFU/ml $\sim 5 \times 10^6$ CFU/ml were smeared on plates with nutrient agar medium, then the plates were closely covered with membranes. Plates were cultured at 37°C for 16-18 h. The diameters of the bacterial colonies were determined and recorded.

Cell culture and CCK-8 assay

NIH-3T3 mouse embryonic fibroblast cells and immortalized human umbilical vein EC line (ECV304) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and were cultured under conditions recommended by ATCC. The cytotoxicity (biocompatible activity) induced by Mat/CS membrane was determined by the CCK-8 experiment. Briefly, membranes were first placed carefully in the cultured dishes, then the cells were cultured and grown on the membrane at 37°C for indicated time. CCK-8 was added to each sample and incubated for another 2 h. The absorbance of solution was recorded at 450 nm with a Thermo microplate reader.

Cell cycle assay by flow cytometry

Cells plated in six-well plates were washed twice with PBS and fixed in 70% ethanol at 4°C overnight. Then, cells were incubated with propidium iodide at room temperature for 1 h and analyzed by flow cytometry using a FACS-can flow cytometer (BD Biosciences, Mountain View, CA, USA).

Traumatic ulcer model

Animal experiments were approved by the Jinan University. All animal procedures followed the humane care guidelines of the Chinese National Institute of Health, and the protocols were approved by the Committee on Animal Research of Jinan University. 16 SD rat were purchased from Animal center of Jinan University. After anesthetized, two experimental points for ulcer wound were selected at 1 cm away from the spine at each side. The trauma was made by using a special punch or surgical scissors deep into the muscle layer. The mem-

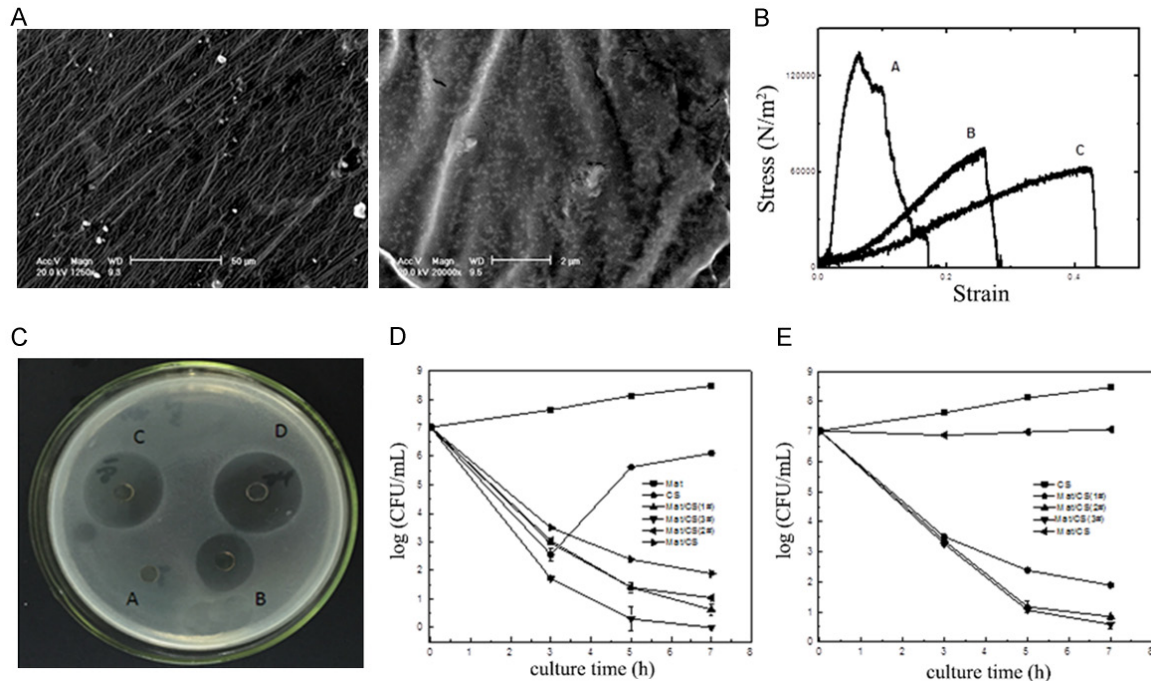


Figure 1. Physical property of the matrine/chitosan (Mat/CS) membrane. (A) Scanning electronic microscope of the Mat/CS membrane (CS molecular weight: 310,000, Mat content: 12%); left panel, fracture surface, right panel, front surface. (B) Stress-strain curves of the Mat/CS membrane with different molecular weights (A: 650000; B: 480000; C: 310000). (C) Bacteriostasis circle of the Mat/CS membrane (spot-B, 1.5% matrine; spot-C, 3% matrine; spot-D, 4.5% matrine). Inhibition activity of four bio-membrane with different molecular Mat/CS to *E. coli* (D) and *S. aureus* (E).

branes were placed into the lesion, and the other side was used as control. Then the wounds were covered with sterile gauze and sutured to normal skin. No sterilization or any antibiotics were used after the surgeries. Then the animals were generally feeding. Every 7 days, animals in each group were sacrificed and the specimens were collected for further analysis.

HE staining and immunohistochemistry

Specimens from animal model were fixed in 10% formalin and embedded in paraffin. Paraffin blocks were cut at 5 μm thickness, and sections were stained with HE. For immunohistochemistry, sections were treated with hydrogen peroxide to inactivate endogenous peroxidases. Antigen retrieval was performed in a microwave in 10 mM citrate buffer at pH 6.0. Sections were fixed with paraformaldehyde followed by permeabilization and blocking. Sections were then incubated in anti-TNF-α, IL-1β and IL-6 antibodies (Abcam, Cambridge, MA, USA). overnight at 4°C, and a secondary

antibody was used to detect protein expression. Immuno-staining was analyzed with the Super Sensitive Non-Biotin Polymer HRP Detection System according to the manufacturer's instructions (BioGenex, San Ramon, Canada).

Data analysis

All data are expressed as the means ± SD unless otherwise indicated. Differences between groups were compared by analysis of variance followed by post hoc Bonferroni tests to correct for multiple comparisons. Differences were considered to be statistically significant at $P < 0.05$.

Results

The physical and antibacterial properties of the Mat/CS membrane

After the preparation of Mat/CS membrane, we determined the physical properties. Firstly, the membranes were scanned by electron microscope to reveal the micro-morphology. As shown in **Figure 1A**, the matrine molecules

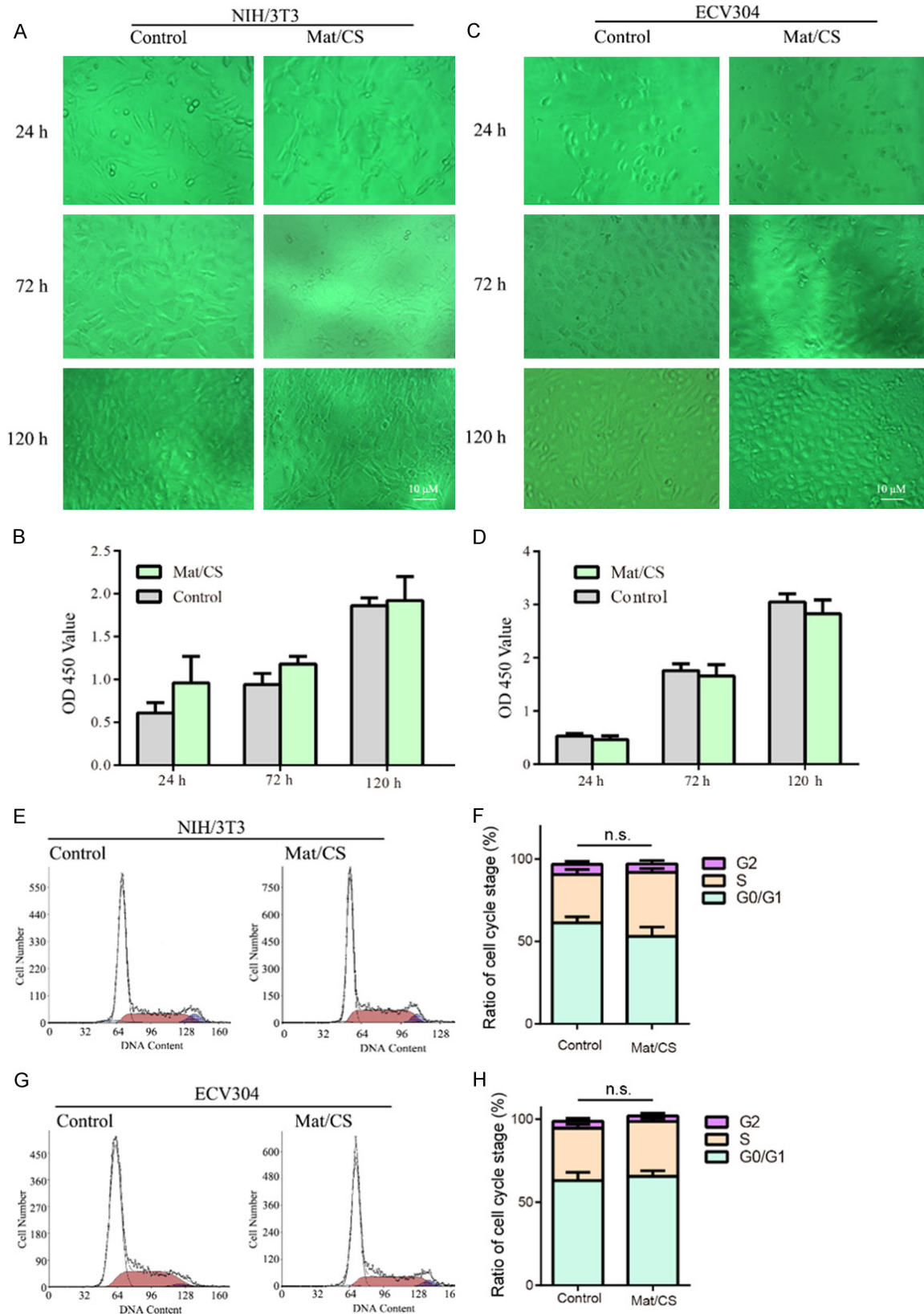


Figure 2. Biocompatibility performance of Mat/CS membrane with cultured cells. Cells of NIH3T3 (A) and ECV304 (C) were cultured on Mat/CS membranes with different time points (24 h, 72 h and 120 h). Representative white

filed images were shown. Then cells were harvested for the CCK-8 assay. The OD values of each treatment in every group were shown in (B, NIH3T3) and (D, ECV304). Cells cultured on Mat/CS membrane with 72 h were subjected for flow cytometry to analysis cell cycle. Representative images and percentage of cell cycle stages were shown for NIH3T3 (E, F) and ECV304 (G, H).

were assembled on the membrane, with asymmetric distribution. Then the stretch experiment was applied for different molecule weight of CS. As shown in **Figure 1B**, as the decrease of CS molecule weight, the modulus decreased and the rate of breaking elongation increased. These data show the good physical property of the membrane. CS membrane has been reported to have antibacterial effect. Accordingly, we applied the similar experiments. As shown in **Figure 1C**, single use of matrine in spot-A did not shown any antibacterial effect. However, by the addition of CS membrane with different concentration of matrine (spot-B, 1.5% matrine; spot-C, 3% matrine; spot-D, 4.5% matrine), the antibacterial effect showed up, increasing with the concentration of matrine. Further, we determined the antibacterial effect by time points, against *E. coli* and *S. aureus*. As shown in **Figure 1D** and **1E**, the application of Mat/CS membrane revealed sustained antibacterial effect. Higher the CS molecule, much more inhibited the bacteria growth. These data suggest the combination of matrine and CS results in better antibacterial effect.

The bioactivity and biocompatibility of the Mat/CS membrane

Then we determined the bioactivity and biotoxicity of the Mat/CS membrane to cultured cells. NIH/3T3 cells seeded on Mat/CS membrane showed clear fusiform shape, normally attached on the membrane, grew gradually with the increased culture time (**Figure 2A**). Then cells were harvested for CCK-8 assay to determine the differentiation rate. As shown in **Figure 2B**, compared with control material, there was no significant difference in Mat/CS membrane group, indicating good bioactivity for cell growth and no toxicity. For ECV304 cells, this membrane showed the same effects (**Figure 2C** and **2D**). Then cells were subjected to cytometry to determine the effect on cell cycle. NIH/3T3 (**Figure 2E** and **2F**) and ECV304 (**Figure 2G** and **2H**) cells cultured on Mat/CS membrane showed no changes with that on control. These data indicate that Mat/CS membrane show excellent biocompatibility with cultured cells.

Mat/CS membrane promotes the wound healing in traumatic ulcer model

After determine the *in vitro* bioactivity of the Mat/CS membrane, we wondered whether this membrane would be effective for the traumatic recovery. To determine this, traumatic ulcer rat model was created as described in the material and method section. We first tested whether this membrane implantation would induce acute inflammatory effect. Two days and 7 days after the implantation, rats were sacrificed and the lesion species were collected, and were subjected for HE staining and immunohistochemistry for the detection of inflammatory factors, including TNF- α , IL-1 β and IL-6. As shown in **Figure 3A**, inflammatory cells could be found between the muscle fibers of the implantation lesion, but no tissue necrosis was seen, indicating the normal foreign body response, which was necessary for the traumatic recovery. Inflammatory cells were decreased 7 days after the implantation, indicating the reversible non-bacterial inflammatory response. The expression levels of TNF- α , IL-1 β and IL-6 showed no significant differences between the two groups and 7 days after the implantation, the levels of these inflammatory factors were decreased (**Figure 3B-D**). These data suggest that the application of the Mat/CS membrane would induce slight and short inflammatory response.

Further, we observed whether the implantation of the membrane would promote the traumatic recovery. As shown in **Figure 4A**, the trauma was recovered after 3 weeks, but appearance between the control and the membrane group showed no significant difference. By HE staining, we found that 2 weeks later, newly formed microvascular and neovascular, and significant fibrous tissue hyperplasia and thick dermis were seen in the membrane group (**Figure 4B**), compared with control group. The differences disappeared at 3-week time point. Moreover, the immunostaining of IL-1 β showed that inflammatory response in the two groups were slight, and showed no significant difference (**Figure 4C**). These data indicate the recovery promotion effect by the application of the Mat/CS membrane.

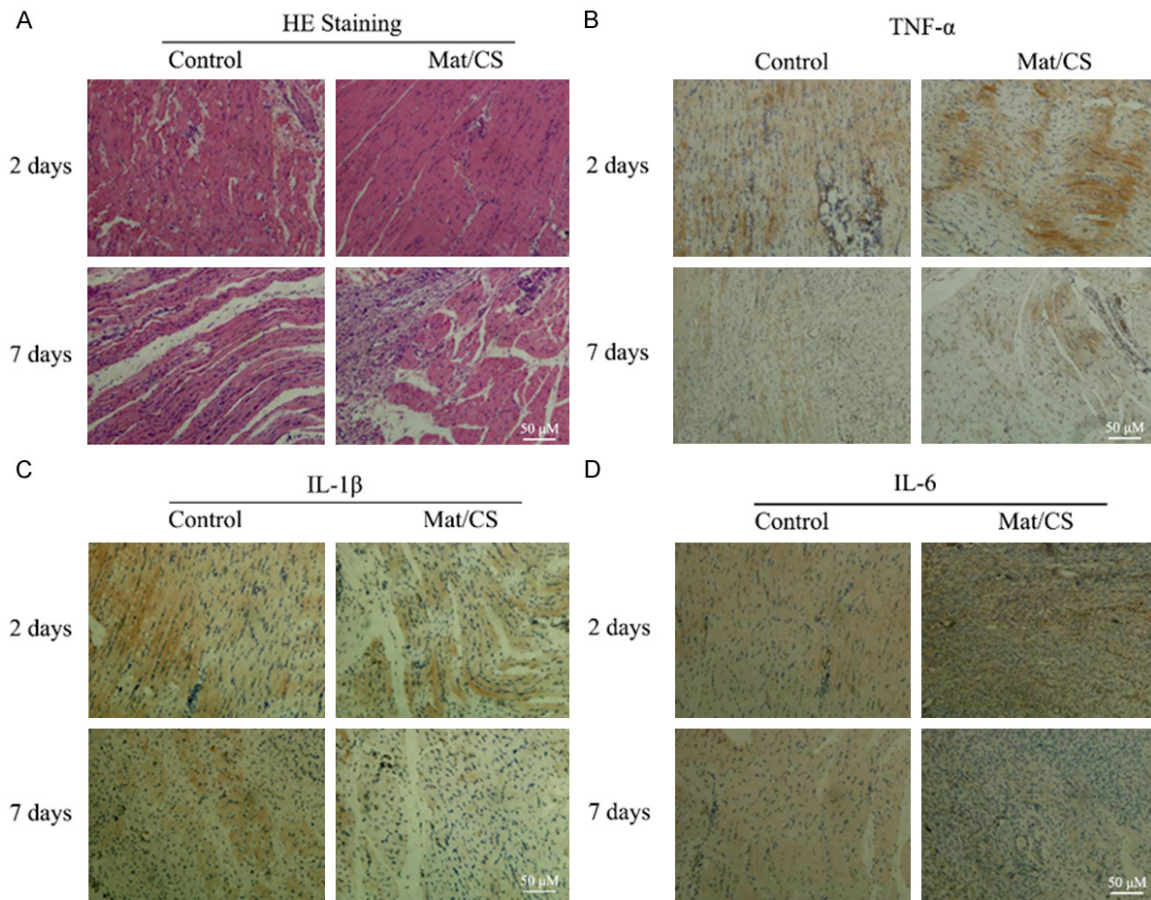


Figure 3. Implantation of Mat/CS membrane induces only slight inflammatory response in rat traumatic model. Traumatic ulcer rat model was created as mentioned in the material and method section. Mat/CS membranes were implanted same as control for 2 and 7 days. The lesion tissues were subjected for HE staining (A) and for immuno-histochemistry to detect TNF- α (B), IL-1 β (C) and IL-6 (D).

Discussion

In the current study, we selected chitosan as matrix, matrine as the loading drug, to make the flexible Mat/CS sustained release membrane. We determined the physical property of the membrane, tested the anti-bacterial activity, revealed the biocompatibility and bioactivity toward cultured cells, and further showed the traumatic recovery promotion ability via a traumatic ulcer rat model. The data suggested that this Mat/CS membrane showed excellent biocompatibility and nontoxic to cultured cells and animal tissues, indicating the potential application for the clinical use to cure oral ulcers.

RAU is a common oral mucosal disease, with the clinical characters of oral mucosa ulceration, pain and cyclical recurrent. However, the etiology and pathogenesis of RAU remain to be

elusive and no special effective drugs can be applied. To relieve the pain induced by RAU, local treatments are predominantly used, which is one of the most important subjects in the stomatological research. These are many types of local drug treatment, including gargle, tablets, powder, ointment and drug membrane. Because of the special oral environment, locally given drugs for RAU cannot stay long, making the lower efficiency and not satisfied outcome. However, membranes can load the anti-inflammatory drugs into the matrix, and can slow-release these drugs. Thus, the biological membrane become the focus of our research. The ideal artificial membrane for the treatment of RAU should meet the following standard: (1) of good affinity with mucosa, to protect the ulcer surface and to maintain a certain adhesion time; (2) to promote ulcer would healing; (3) to reduce or totally inhibit the inflammatory

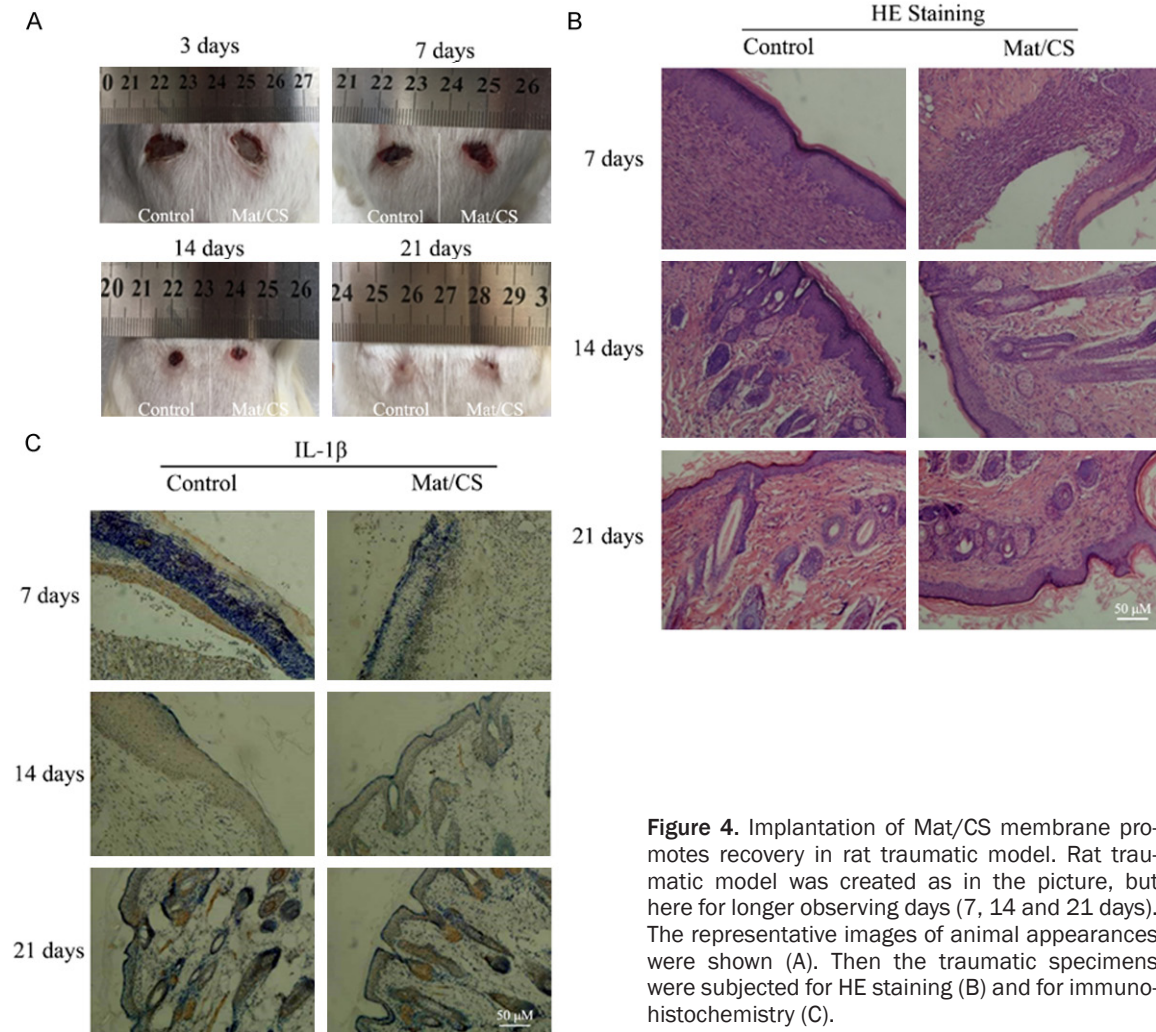


Figure 4. Implantation of Mat/CS membrane promotes recovery in rat traumatic model. Rat traumatic model was created as in the picture, but here for longer observing days (7, 14 and 21 days). The representative images of animal appearances were shown (A). Then the traumatic specimens were subjected for HE staining (B) and for immuno-histochemistry (C).

response; (4) to show good bioactivity, biocompatibility, antibacterial and hemostatic function; (5) can be prepared easily with simple formula and with excellent elasticity; (6) can be sterilized, easily stored and can prevent bacterial infections. Here, we prepared a Mat/CS membrane, this membrane meets the standard for the treatment of RAU, showing good physical property and excellent biocompatibility.

Matrine, as a treasured traditional Chinese medicine, has shown the potential in clinical application. Matrine was firstly extracted at 1958, the molecular formula is $C_{15}H_{24}N_2O$, belonging to the quaternary quinolines [15, 16]. *In vivo* animal models have determined the anti-allergic and anti-inflammatory effects of matrine. Matrine functions not via pituitary-adrenal system, but relies on its role of inhibi-

tion of leukocyte migration, promotion of free radical scavenging, stabilization of lysosomal membrane and inhibition the synthesis and release of inflammatory factors [17]. During the recent years, matrine has shown many aspects of pharmacological properties, such as anti-tumor, anti-virus, anti-fibrosis and anti-hepatitis [18, 19]. Matrine shows significant effect on skin diseases such as atopic dermatitis, eczema and gynecological disease, also on antihypertensive, antiarrhythmic, atherosclerosis and immune function regulation [20, 21]. Matrine has shown the great potential in the clinical application, however, most of the current preparations of matrine fails to give full play of matrine. Thus, because of the anti-inflammatory effect, we choose matrine as the loading drug on CS-matrix membrane. And in the current study, we revealed the slow-release prop-

erty of Mat/CS membrane and showed its traumatic recovery promotion effect.

Chitosan is widely used because of its good biocompatibility, bioactivity and membrane forming property [22]. Membrane preparations can be used as coating materials for skin burns, traumatic wounds or inflammatory surfaces. According to the medical needs, the membrane can be prepared as single-layered, multi-layered or sandwich membrane [22]. Chitosan shows abundant resources on earth and because of its safety and nontoxicity, flexibility, air-permeability, non-allergenic and non-absorptive poisoning, coating membrane made of chitosan with drugs shows great effects of anti-inflammatory, wound healing promotion and scarring prevention. In the current study, chitosan membranes loaded with matrine (Mat/CS) is prepared for the cure of RAU. This Mat/CS membrane significantly promoted the traumatic surface recovery, showing remarkable potential of topical administration.

In summary, Mat/CS membrane we prepared show good physical property, excellent biocompatibility with cultured cells and traumatic recovery promotion effect in animal ulcer model. This work provides evidences of drugs loaded Mat/CS membranes in the potential application for the cure of RAU.

Disclosure of conflict of interest

None.

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