Effects of chitin and sepia ink hybrid sponge on the healing of burning wound rats and its impact on macrophages *in vitro*[1]

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ABSTRACT

PURPOSE: To detect whether chitin and sepia ink sponge (CS) can promote wound healing and elevate impact of CS on phagocytosis ability of macrophages.

METHODS: Forty-eight rats were assigned to four groups: Normal group (Normal), negative control group (Con), chitin and sepia ink sponge group (CS) and positive control Surgicel Gauze® group (SG). Deep second-degree burn model was created in rats. Wound area was recorded by digital imaging and determined using Image J software. Samples were collected and kept at -80ºC on 3d, 7d, 14d and 21d for cytokines detecting. Transforming growth factor (TGF)-β_1, interleukin (IL)-6, matrix metalloproteinase (MMP)-1, hydroxyproline (Hyp) and macrophage activity reflected by tumor necrosis factor (TNF)-α were determined by enzyme-linked immunosorbent assay (ELISA).

RESULTS: Comparing to Con and SG, scabs in CS group fell off and basically healed on 21 day. TGF-β_1, IL-6, MMP-1 and Hyp were significantly increased by CS and SG comparing to Con ($p < 0.05$), CS had more apparently adjustment on TGF-β_1 and MMP-1 compared to SG; results *in vitro* indicated CS significantly promoted phagocytosis ability of macrophages reflected in TNF-α ($p < 0.05$).

CONCLUSION: CS improved wound healing through exerting significant influences on secretion of kinds of cytokines and activating macrophages.

Key words: Chitin. Sepia. Cytokines. Macrophages. Rats.

Introduction

Wound dressing is one of the most promising medical applications for chitin. The adhesive nature of chitin, together with the antifungal and bactericidal properties, and the permeability to oxygen, is a very important property associated with the treatment of wounds and burns[1]. Some studies have showed chitin-based dressing can improve repair of different tissues facilitate contraction of wounds, and regulate secretion of inflammatory mediators, such as interleukin8, prostaglandin E, interleukin 1β and others[2]. It has also been reported that chitin or its derivatives can activate macrophage to express a number of pro-inflammatory cytokines, chemokine and other mediators, by which modulate the wound healing process[3].

Sepia ink contains higher trace elements of vanadium, iron, cobalt, nickel, copper, silver, cadmium, lead, and bismuth, especially calcium and strontium. It has been confirmed that trace elements are essential nutrients for tissue repair, immune, digestive function and other aspects of the role[4]. Researchers have founded that sepia ink has anti-radiation activity, antitumor activity, immunomodulatory activity, procoagulant function, etc[5]. Chen *et al.*[6] reported that sepia ink can raise the leukocyte content of blood, by which can promote the phagocytic activity of macrophages.

Currently, kinds of healing agents and sponge have existed, such as gelatin hydrogel, microcrystalline collagen and collagen sponges, gelatin sponge, cyanoacrylate adhesives, fibringlue and chitosan sponge[7]. But they basically have certain disadvatages. Collagen, fibrinogen or thrombin are expensive and include potential risk of viral infections, because they are mainly derived from animal or human blood. The adhesive properties of collagen and gelatin sponges to tissues are poor, and chitosan sponge is easy to become fragmented and dissolved away *in vivo* and *in vitro*. Water-soluble collagen has some toxicity, which may cause inflammatory responses in human body[8]. So, it makes sense to study a novel and safe biological agent for clinical use with better healing properties, adhesion and less tissue response. In previous work, our laboratory had prepared chitin and sepia ink sponge (CS sponge) and it exhibited good biological properities[9]. Compared to most other biomaterials, CS sponge is a pure biological product, which only contains chitin and sepia. During the wound healing process, CS sponge can effectively absorb the exudates slowly and can well maintain its basic sponge shape. It would adhere to the wound surface and provide a moist microenvironment for wound healing, so the wound bed would be well prevented the friction from outside, like clothing[9].

In this paper, a deep second degree burn model of rat was used to evaluate the wound healing effects of CS sponge *in vivo* and also determined its impact on macrophages *in vitro*, from which this experiment tried to reveal its basic healing mechanism.

Methods

This project was reviewed and approved by the committee of experimental animals of Lingnan Normal University and conformed to National Institutes of Health guidelines. All experimental procedures complied with the Guide for the Care and Use of Laboratory Animals, which were made to minimize suffering of animals.

Forty-eight adults male Wistar rats (180 ± 20 g, Jinan, China) were housed for one week in a room with controlled temperature at 25 ± 1°C and fed with standard laboratory diet, water and light/dark cycle of 12 hours.

Surgical procedures and groups formation

All animals were anesthetized with an intra-peritoneal injection of sodium pentobarbital (50 mg/kg) and then shaved to expose skin area by an electric razor. Deep second degree burn model was mainly prepared according to literature 10. Briefly, a 2.5 cm in diameter steel rod (24 cm in length) was heated to 100°C in boiling water for 15 min, then it was applied for 7s on the removed dorsal skin of the rats and created deep second degree burn injury. The rats were randomly divided into four groups (n = 12 per group): Normal group (Normal), negative control group (Con), chitin and sepia ink sponge group (CS) and positive control Surgicel Gauze® group (SG). But Normal group did not been treated for second degree burn, which was only taken blood for cytokine analysis at each time point; chitin and sepia ink sponge group was covered by 3 cm × 3 cm chitin and sepia ink sponge; positive control Surgicel Gauze® group was covered by 3 cm × 3 cm Surgicel Gauze®; negative control group did not be treated after disinfected. Finally, to prevent wound infecitons from external factors, Hainuo® (Qingdao, China) adhesive wound dressing was applied to cover up the burn area of each rat. Seven days later, no sterile wound dressing was applied for conveniently checking the healing process.

The gross finding of healing process was recorded using the Image J software (National Institutes of Health, USA) every three days. The rats were sacrificed on 3, 7, 14 and 21 days, and the burn sites including the surround tissues were harvested. Samples were immediately frozen in liquid nitrogen after harvest and stored at -80 °C for ELISA determining.

Observation of wound healing

The appearance of the wound was recorded by digital imaging at 1, 3, 7, 14 and 21 days. Wound areas was determined from the digital images using Image J software (National Institutes of Health, USA).

Assay of TGF-β_1, IL-6 and MMP-1 in the wounded skin

On the test day, frozen samples were weighed and allowed to thaw in ice-cold lysis buffer (Beyotime, China) at the ration of 100 mg/mL. The buffer contained 20 mmol/L Tris (PH 7.5), 150 mmol/L NaCl, 1 % Triton X-100, β-glycerophosphate, sodium pyrophosphate, EDTA, Na_3VO_4, leupeptin and 1 mM PMSF (Phenylmethanesulfonyl fluoride). Samples were homogenized with a tissue homogenizer (IKA, Germany). The homogenized were centrifuged at 4°C for 20 min (10000 r/min) before the supernatants obtained. The content of TGF-β_1, IL-6 and MMP-1 were examined by ELISA Kits (R&D Systems, USA) and normalized by total protein content of each sample. Protocols for all of these assays were followed as indicated by the manufacturer and all assays were performed in duplicate.

Detection of hydroxyproline in the wound area

The Hyp content was determined through digestion method by ELISA (Nanjing, China). 10% tissue samples in normal saline were prepared and homogenized with a tissue homogenizer (IKA, Germany) for detecting. According to instructions of ELISA, blank tube, standard tube and measuring tube were all added 0.5 ml digestive juice; then respectively added 0.25 ml of double-distilled water, 0.25 ml standard solution and 0.25 ml test sample. What is following is that 0.5 ml regent I was added and standing for 10 min after mixing; 0.5 ml regent II was added and standing for 5 min after mixing; 1 ml regent III was added and kept in water bath at 60°C for 15 min after mixing. At last, the tubes were cooled in water and centrifuged at 4°C for 10 min (3500 r/min) to obtain supernatants, then absorbance values was measured at 550 nm and the Hyp content was calculated according to the ELISA kit.

Preparation of mouse peritoneal macrophages

Each mouse was injected intraperitoneally 4% thiol-ethyl starch broth 2 ml three days in advance. Then mice were sacrificed and 5 ml PBS was injected intraperitoneally with half an hour massage. After that, cell suspension was extracted and added to the culture medium after it washed and adherent cultured for two hours. What is following is that adherent cells were trypsinized and collected. Then cells were adjusted to concentration of 2×10^6 cells per ml, inoculated to 96-well plates (100uL per well, for detection of TNF-α) and 24-well plates (500 uL per well, for neutral red uptake assay) and incubated at 37°C. Four hours later, the cells were purified by refreshing the medium.

Detection of phagocytosis ability of macrophages

CS sponge leaching liquor was added to culture medium at the concentrations from 1 to 1000 ug/mL culture medium. Incubation for 48 hours, the medium was discarded and 1000 uL of 0.07% neutral red solution was added in each well and cultured for another 1 hour. Then solution was discarded and each well was washed three times with washing liquid and blotted up with filter paper. After that, the lysing solution was added to each well and kept overnight at 4°C. Finally, the absorbance was recorded at 540 nm and saline was used for blank test.

Test of TNF-α secretion of macrophages

When the incubation of macrophages in 96-well plates at 37°C for 24 hours finished, the medium was replaced with serum free medium and cultured for another 24 hours in the same conditions. Then medium was substituted by 200 uL serum free medium which containing different concentration of CS sponge leaching liquid (1-1000 ug/mL). After 48 h incubation, upper medium was obtained and kept at -80°C for ELISA kit detection (antibodies were purchased from R&D Systems, USA).

Statistical analysis

The data were expressed as the mean ± standard deviation (S.D.) and SPSS 17.0 software was used to analyze the data of the test. The means of the different groups were using one-way Kruskal-Wallis test. Significant differences were accepted when P-values were less than 0.05.

Results

The basic properties of chitin and sepia ink sponge

Chitin and sepia ink colloidal can be prepared into different forms of CS sponge as needed, as shown in Figure 1. The deacetylation of CS sponge used in this experiment is 33.5%.

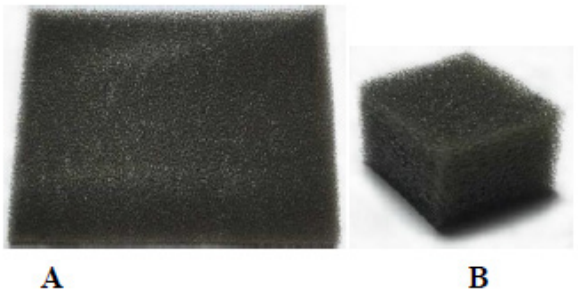

FIGURE 1 – A. Sheet of sponge. **B.** Square sponge.

Deep second degree burn cutaneous wound healing

As shown in Figure 2, results illustrated the gross determinations of wound healing process. At the beginning, the burn size of each group increased because of edema in all groups. Then the severity was reduced and healing effects occurred after 7, 14 and day 21. From day 7 to 21, wound size of CS and SG group was significantly lower than control group ($p<0.05$). On day 21, there was a significant difference between CS and SG ($p<0.05$).

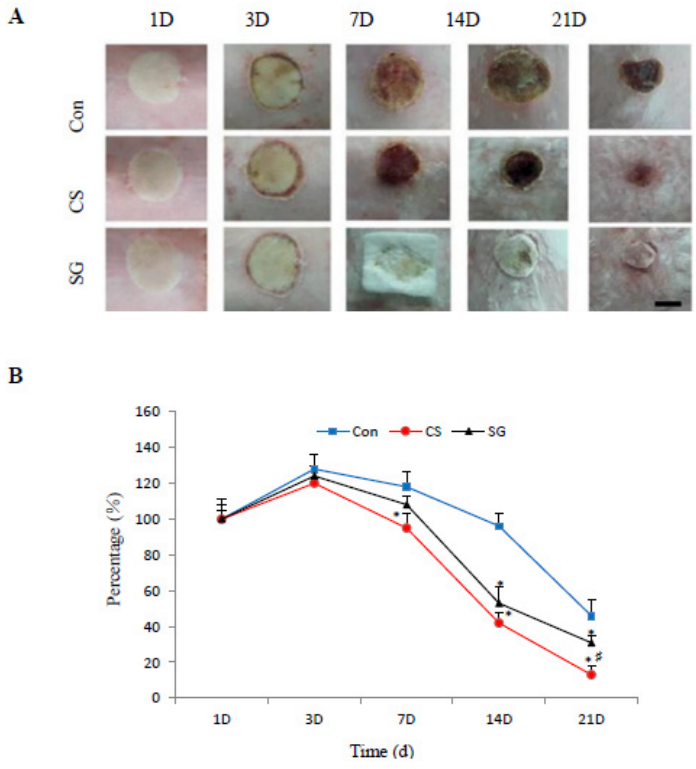

FIGURE 2 - Postburn wound size obtained from injury site. Con as control group; CS as CS sponge; SG as Surgicel Gauze®. The scale bar is 0.5 mm (**A**). Determination for the percentage of burn healing process by Image J software (**B**) (*$p<0.05$ *vs*. Con group; $^{\#}p<0.05$ *vs*. SG group).

Healing effects of CS sponge on wound site

Figure 3 showed that TGF-β_1 was significantly enhanced by CS and SG on day 3and 7 ($p<0.05$). In CS group, it nearly reduced to normal level on day 21. On day 14 and day 21, there was a significant difference between CS and SG group ($p<0.05$).

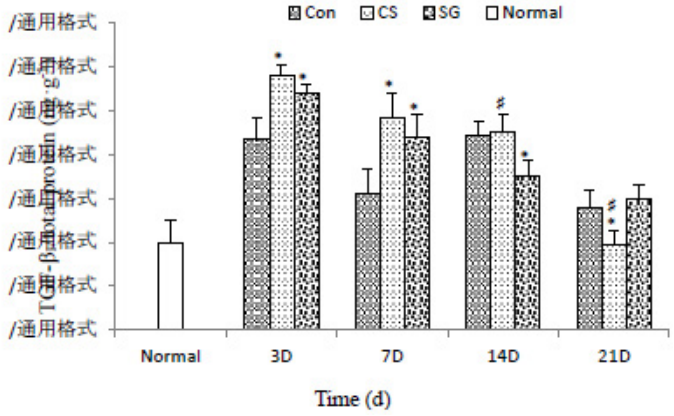

FIGURE 3 - Contents of TGF-β_1 in the wound site. Results are expressed as mean ± SD (n=3). (*$p<0.05$ *vs*. Con group; $^{\#}p<0.05$ *vs*. SG group).

Figure 4 illustrated that IL-6 was significantly elevated both in CS and SG group from three to 14 days ($p<0.05$), then it declined to normal level earlier compared to control group.

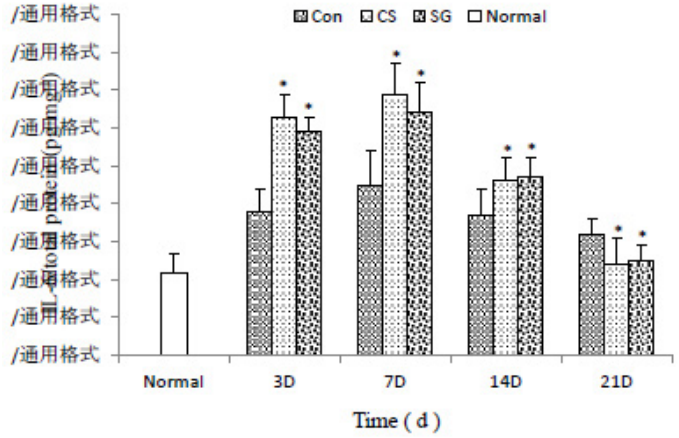

FIGURE4 - Contents of IL-6 in the wound site. Results are expressed as mean ± SD (n=3). (*$p<0.05$ *vs*. Con group).

Figure 5 depicted that MMP-1was significantly increased by CS and SG on day 3 and 7 ($p<0.05$). In CS group, it reached its peak value on day 14, and there was a significant difference compared to SG and control group ($p<0.05$).

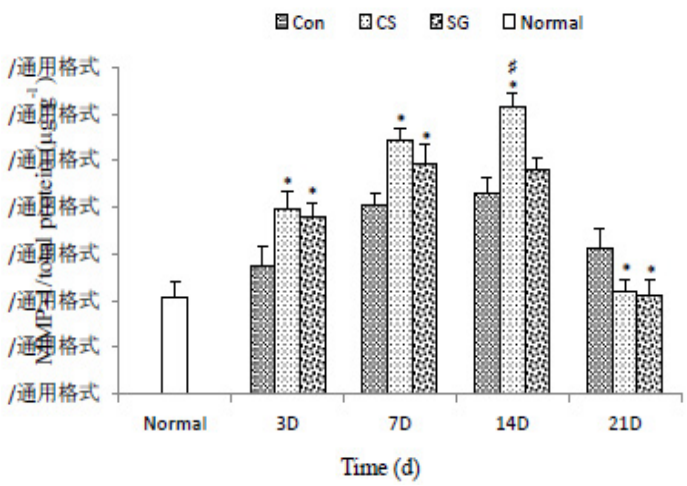

FIGURE 5 - Contents of MMP-1 in wound site. Results are expressed as mean ± SD (n=3). (*p<0.05 *vs*. Con group; ♯p <0.05 *vs*. SG group).

Figure 6 suggested that Hyp was significantly stimulated by CS and SG from 3 to 21d (p<0.05). On day 14, there was a significant difference between CS and SG group (p<0.05).

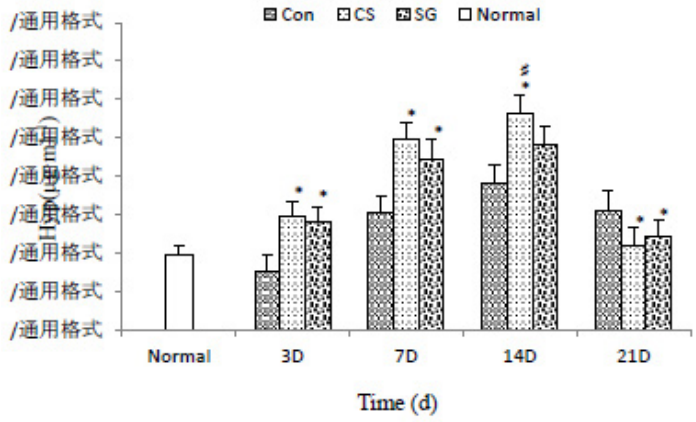

FIGURE 6 - Contents of Hyp in wound site. Results are expressed as mean ± SD (n=3). (*p<0.05 *vs*. Con group; ♯p<0.05 *vs*. SG group).

Effects of CS sponge on macrophages in vitro

Figure 7 revealed the phagocytosis ability of macrophages was significantly enhanced with the concentration by CS leaching liquid increasing (p<0.05).

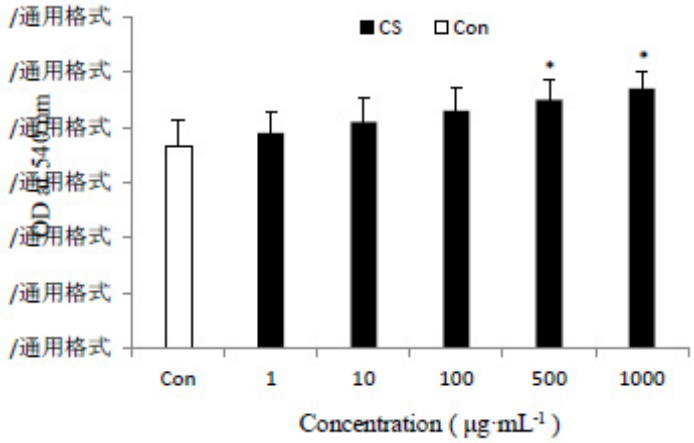

FIGURE7 - Phagocytosis ability of macrophages influenced by CS at different concentrations. Results are expressed as mean ± SD (n=3). (*p<0.05 *vs*. Con group).

Figure 8 described TNF-α production of macrophages at each testing concentration was significantly promoted by CS at concentrations of 100, 500 and 1000 μg·mL^{-1} (p<0.05).

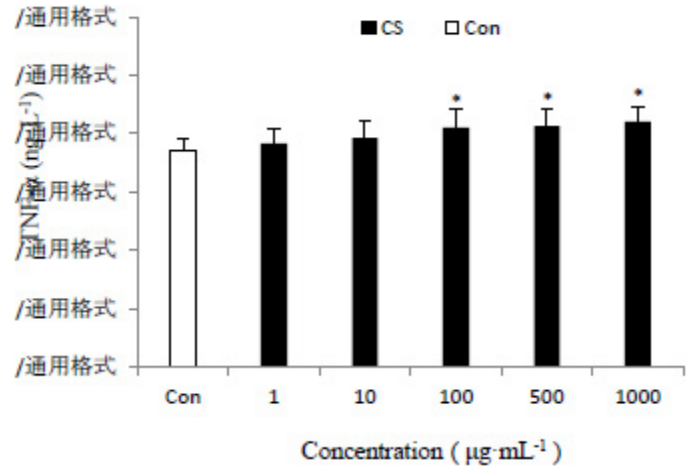

FIGURE 8 - TNF-α secretion of macrophages influenced by CS at different concentrations. Results are expressed as mean ± SD (n=3). (*p<0.05 *vs*. Con group).

Discussion

TGF-β_1 and IL-6 are two important kinds of cytokines in wound remodeling. Many authors have investigated the changes of TGF-β_1 and IL-6 during the wound healing. Gopal *et al*.[11] examined chitosan-based copper nanocomposite (CCNC) in rat wound healing and discovered TGF-β_1 was significantly increased in the CCNC-treated rats. Studies also

showed that exogenous TGF-β_1 applied in the wound area can significantly promote various types of wound repair. Li *et al*.[12] found that chitosan membrane containing TGF-β_1 promoted the proliferation of osontoblast-like cells than that without TGF. Gallucci *et al*.[13] found that granulation tissue formation was impaired in IL-6 gene knock-out rats and these animals had longer healing time than wild type control.

In the present study, results showed TGF-β_1 and IL-6 had the similar change trend compared to the results above, the contents of which were increased in some level at the early period of wound healing. TGF-β_1 and IL-6 in the CS and SG group were all significantly ($p<0.05$) enhanced from day 3, and then both of them gradually declined to normal levels. But CS sponge showed the better moderating effect, which made the content of TGF-β_1 and IL-6 fast reach a higher level and drop to a normal standard earlier compared to control group and SG group. Hence, it can be seen that CS sponge has more obviously regulating effect on the cytokines of TGF-β1 and IL-6 during the wound healing process.

Under normal conditions, collagenase, or MMP-1, is restricted to a few tissues which show intense remolding[14]. Collagen degradation is mainly regulated by collagenase. Hyp is a unique amino acid, which occupies the constant ratio in collagen fibers. Usually collagen synthesis is reflected by measuring the amount of Hyp[15]. Shin *et al*.[16] assessed the role of MMP-1 in collagen remodeling during wound healing, and inferred that increased MMP-1 enabled remodeling the extracellular matrix. Sun *et al*.[17] also examined the MMP-1 production fluctuations in the experiment of *Galla chinensis* (GAC) extracts applying on UVB-irradiated in hairless mice, but the results showed that GAC may suppress MMP-1 expression, by which can form a more smooth and elastic skin tissue.

Compared to the researches above, our present experiment found both MMP-1 and Hyp were significantly ($p<0.05$) increased in CS group and SG group and quickly reduced to normal levels, the adjustment effect was particularly obvious in CS group. It is speculated that CS sponge may have a different regulatory mechanism, through which can accelerate decomposition and regulation of the wound collagen by MMP-1 and Hyp, to achieve better healing effect.

TNF-α is mainly produced by activated macrophages, the content of

which is considered as an indicator to measure the activity of macrophages[18]. Hübner *et al*.[19] found a strong and early induction of TNF-α after cutaneous injury *in vivo* of mice and highest level of TNF-α was seen at 12-14 h after wounding. In our present research, it also showed that CS sponge promoted the activity of macrophage which was reflected by the content of TNF-α. But our experiment was conducted *in vitro*, the effect of CS sponge for TNF-α *in vivo* still need to be explored.

Present studies show that CS sponge is a relative safe and effective biomaterial, but there are still some limitations in this study. For clinical application, the sample sizes of the experimental animals are small, and the data is primary. Also, different species of animals should be applied to achieve more convincing and comprehensive results. Therefore, further investigation should be conducted and the relevant results would be reported in our following work.

Conclusions

CS sponge can effectively promote wound healing by activating macrophages and exerting significant effects on secretion of kinds of cytokines, such as TGF-β_1, IL-6, MMP-1, Hyp and TNF-α. Present study only conducted basic research of CS sponge, further investigations should be performed in future.

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