Regulatory Action of Charred Gossamer Urocteae on the Functions of Mouse Oral Fibroblasts

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Objective: To explore the influence of charred Gossamer urocteae (CGU) on the functions of primary cultured mouse oral fibroblasts and reveal its mechanism in wound healing. Methods: CGU was extracted with different solvents and ethanol extract (EE), ethyl acetate fraction (EF), n-butanol fraction (BF) and aqueous fraction (AF) were obtained. The effects of different fractions on the proliferation, matrix metalloproteinase-2,9 (MMP-2,9) activities, synthesis of collagen and tissue inhibitor of metalloproteinase 1 (TIMP-1) in the mouse oral fibroblasts were determined by MTT, gelatin zymography, chloramine-T method, and enzyme-linked immunosorbent assay (ELISA) respectively. Results: EE, EF and BF at high concentrations could significantly inhibit proliferation of fibroblasts ($P<0.05$ or $P<0.01$), and at low concentrations EF and BF could promote proliferation of fibroblasts, and BF and AF could significantly inhibit collagen synthesis ($P<0.05$ or $P<0.01$). EE, EF and AF at high concentrations could significantly increase the MMP-9 activity, and BF and AF could significantly inhibit synthesis of TIMP-1. Conclusion: CGU at high concentrations can inhibit the proliferations of fibroblasts and synthesis of collagen, and in healing of wound, CGU at high concentrations possibly has the functions of anti-fibrosis and anti-scar, and the mechanism to promote degradation of collagen is possibly related to the increase in MMP-9 activity and the inhibition of TIMP-1 synthesis.

Key words: collagen synthesis; mouse oral fibroblast; matrix metalloproteinase-2,9 (MMP-2,9); tissue inhibitor of metalloproteinase 1 (TIMP-1)

Chinese drug charred Gossamer urocteae is a processed product of the whole worm and egg capsule of *Uroctea compactitis* Koch. Or *Uroctea lesserti* Schenkel. It is recorded in medical books that gossamer urocteae is salty in taste, cold in property, acts on the Lung Channel and the Liver Channel, has effects of clearing heat and toxic material, arresting bleeding and promoting tissue regeneration, and is indicated for swelling and soreness of throat, hemorrhoid with bleeding, sore, wound and ulcer, etc.

Some classical recipes composed of gossamer urocteae, such as *Bifan Powder* (壁矾散) in *Bencao Gangmu* (本草纲目 Compendium of Materia Medica) compiled by LI Shi-zhen (李时珍) in the Qing Dynasty, *Xilei Powder* (锡类散) in *Jin Kui Yi* (金匮翼 Supplements to Commentaries on Synopsis of the Golden Chamber) compiled by YOU Zai-jing (尤在泾) in the Qing Dynasty, and *Bigian Mufu Fang* in *Invaluable Prescriptions* (千金要方) compiled by SUN Si-miao (孙思邈), can effectively cure many kinds of wound and chronic ulcer. Modern clinical application also proves that these recipes have very good therapeutic effects on various wound and ulcer. But, up to now, no research report about its pharmacological mechanism has been found. The present experiment indicates its role in wound healing through investigation on effects of different fractions of gossamer urocteae carbon on the proliferation, matrix metalloproteinases-2,9 (MMP-2,9) activities, synthesis of collagen and tissue
inhibitor of metalloproteinase-1 (TIMP-1) in the cultured mouse oral fibroblasts in vitro.

MATERIALS AND METHODS

Materials
Gossamer urocteae carbon was purchased from No.707 Jiangsu Natural Pharmaceutical Co. Ltd (Zhenjiang, Jiangsu), batch number: 072, and identified by Doctor HAN Ban-xing (韩邦兴) of College of Pharmacy, Jiansu University, and the sample was kept in this laboratory. DMEM culture medium, trypsin, hydroxyproline control sample, MTT, etc. were purchased from AMRSCO company, USA, chloramines-T and SDS were purchased from the Chemical Reagents Co., Ltd, China National Medicine Group, TIMP-1 ELISA kit was purchased from Wuhan Boster Bioengineering Co., Ltd, China. All of other reagents were analytically pure.

Extraction
The 50 g of charred gossamer urocteae was extracted with 70% alcohol at 85°C (3 × 500 ml, 1.5 h each time), and after filtration, they were divided into two parts and concentrated into the extracts. One part was dissolved into the solution of crude drug 1g/ml with deionized water, which was extracted with 200 ml petroleum ether, ethyl ether, ethyl acetate and n-butanol respectively in a 500 ml-separatory funnel, extracting 5 times with each solvent and then they were concentrated as soft extracts. Thus, ethanol extract (EE), petroleum fraction (PF), ether fraction, ethyl acetate fraction (EF), n-butanol fraction (BF) and aqueous fraction (AF) were obtained, but only EE, EF, BF and AF were used for the following experiments and PF and ether fraction could not used for this experiment due to too little.

Culture of Mouse Oral Primary Fibroblasts
BALB/c mice (20±2 g) were purchased from the Center for Laboratory Animals, Jiangsu University (Zhenjiang, Jiangsu). The mice were sacrificed by dislocation of cervical vertebra and the mucous membrane of mouth was taken under sterility. The mouse oral fibroblasts were cultured as described in the literature. 5

Test of Cell Proliferation
MTT method was used for investigation on effects of the four extracts on proliferation of the oral fibroblasts.5 The 4 extracts were first dissolved in DMSO and then diluted into the series gradient 15.625, 31.25, 62.5, 125, 250 μg/ml with DMEM culture medium containing 10% foetal bovine serum. The final concentration of DMSO was lower than 0.5% and a pilot experiment showed no effect of 0.5% DMSO on cell growth. The DMEM culture medium containing 10% foetal bovine serum (containing 0.5% DMSO) was used as blank control. Minimum concentration which showed a significant difference (P<0.05) in the effect on cell growth as compared with the blank control after culture for 48 h was regarded as minimum effective concentration, and which was used for the following test. According to test results, 62.5, 125, 125 and 31.25 μg/ml were adopted as minimum effective concentrations of EE, EF, BF and AF respectively.

Determination of Hydroxyproline
Chloramines-T method was used to test effects of the four extracts on collagen synthesis in oral fibroblasts. Fibroblasts were inoculated into a six-well culture plate (1x10^5 cells/well) and cultured in DMEM medium containing EE (62.5 μg/ml), EF (125 μg/ml), BF (125 μg/ml) and AF (31.25 μg/ml) of 10% fetal bovine serum for 72 h, with the DMEM medium containing 10% foetal bovine serum (containing 0.5% DMSO) used as blank control. Hydroxyproline was determined by the method of Edwards et al., 6 and the results were expressed as collagen amount (mg) produced by 10^6 cells based on the content of hydroxyproline in collagen being 13.5%.

Gelatin Zymography Assay
Fibroblasts (1 × 10^5) were inoculated on a 6-well culture plate with DMEM medium of 10% calf serum containing EE (62.5 μg/ml), EF (125 μg/ml), BF (125 g/ml) and AF (31.25 μg/ml, and DMEM medium of 10% fetal calf serum was used as blank control and the medium of 1% calf serum was used as negative control. After culture of 72 h, the ultrasonicated cells were collected and centrifuged at 1200 r/min for 30
minutes, and stored in a refrigerator at -70°C for gelatin zymography assay and following determination of TIMP-1. MMP-2,9 activities were determined with gelatin zymography assay and were expressed as the product of the area of electrophoretic band and the staining degree. 7

Determination of TIMP-1 with ELISA
Cellular lysate was collected with the above-mentioned method, and TIMP-1 was determined according to the guidance of the ELISA kits.

Statistical Process
SPSS13.0 statistical software was used for paired t-test or one-way ANOVA (X ±s).

RESULTS

Growth State of Primary Cells
After culture for 7 days, fibroblast began to grow up around the tissue, and after culture for 15 days, the fibroblast grown all over 80% bottom of the culture flask, and showed a long cambiform (Fig.1A). Transferring to the second generation, the cell showed a cambiform or a polygon (Fig.1B). Transferring to the third generation, most of the cells showed a cambiform and small amount of the cells showed a lamellar form (Fig.1C). The cells from the third generation to the fourth generation were used for the experiment.

Fig.1 Form characteristics of mouse oral fibroblasts
A: Primary culture (15 days); B: The second generation culture (5 days); C: The third generation culture (5 days)

Fig.2 Effects of charred gossamer uroctaeae (μg/ml) on proliferation of fibroblasts
A: Culture for 48 h; B: Culture for 72 h. (X ±s, n=5), *P<0.05, **P<0.01.
Effect on Proliferation of Fibroblasts

As showed in Fig.2, after culture for 48 h, EE (62.5 μg/ml) and EF (125 μg/ml) could significantly inhibit the growth of fibroblasts (P<0.05), and after culture for 72 h, BF (125 μg/ml) also could significantly inhibit the growth of fibroblasts (P<0.05). After culture for 48 h or 72 h, AF at the all concentrations did not have effect on the growth of fibroblasts. In addition, at low concentration, EF (from 15.625 to 31.25 μg/ml) and BF (from 15.625 to 62.5 μg/ml) could promote the proliferation of fibroblasts, but with no statistical significance. According to the results, 62.5, 125, 125 and 31.25 μg/ml were regarded as minimum effective concentration of EE, EF, BF and AF respectively.

Effects on Collagen Synthesis in Fibroblasts

Hydroxyproline production can directly reflect collagen synthetic quantity of fibroblasts. In the present experiment, the collagen synthetic quantity of fibroblasts was calculated by the determined content of hydroxyproline, and the standard curve equation was Y= 8.4193 X-0.0379 (R²=0.9994). The results were showed in Fig.3. BF (125 μg/ml) and AF (31.25 μg/ml) could significantly inhibit collagen synthesis of fibroblasts (P<0.05 or P<0.01), but EE (62.5 μg/ml) and EF (125 μg/ml) basically did not have this effect.

![Fig.3 Effects of charred gossamer urocteae on collagen synthesis in fibroblasts (X ± s, n=5), *P<0.05, **P<0.01.](image)

Effects on MMP-2,9 Activities in Fibroblasts

As showed in Fig.4A and 4B, after culture for 72 h, EE (62.5 μg/ml), EF (125 μg/ml), BF (125 μg/ml) and AF (31.25 μg/ml) almost did not have effects on MMP-2 activity, but EE (62.5 μg/ml), EF (125 μg/ml) and AF (31.25 μg/ml) could significantly enhance MMP-9 activity (P<0.05), and BF (125 μg/ml) could inhibit MMP-9 activity (showed in Fig.4C). In addition, EE (62.5 μg/ml), EF (125 μg/ml) and AF (31.25 μg/ml) could enhance pro-MMP-2 activity (showed in Fig.4A).

![Fig.4 Effects of charred gossamer urocteae on MMP-2,9 activities in fibroblasts](image)
Effects on TIMP-1 Synthesis in Fibroblast

TIMPs are endogenous inhibitors of MMPs. In the present experiment, TIMP-1 levels in the all medication groups were determined by ELISA. As showed in Fig.5, BF (125 μg/ml) and AF (31.25 μg/ml) could significantly inhibit TIMP-1 synthesis in fibroblast \( (P<0.05) \), but EE (62.5 μg/ml) and EF (125 μg/ml) basically did not have the effect.

![Fig.5 Effects of charred gossamer urocteae on tissue inhibitor of metalloproteinase-1 (TIMP-1) in fibroblasts (\( \overline{x} \pm s, n=3 \))](image)

\( *P<0.05, **P<0.01 \).

DISCUSSION

Chronic and difficultly healing ulcer is often closely related with low fibroblast division ability and disturbance of extracellular matrix synthesis, so activating the function of fibroblasts is very important for healing of ulcer.\(^8\) In this experiment, it was found that ethanol extract, ethyl acetate fraction (EF) and n-butanol fraction (BF) at high concentrations could inhibit proliferation of fibroblasts, and EF and BF at low concentration had a certain promoting action on proliferation of fibroblasts, and BF and aqueous fraction (AF) could inhibit collagen deposit. Therefore, it is held that even though charred gossamer urocteae as a traditional Chinese herb is used for treatment of wound and ulcer, it only at low concentration can play this role. In addition, it possibly has functions of anti-fibrosis and anti-scar, because it can inhibit proliferation of fibroblasts and collagen deposit.

About the mechanism of charred gossamer urocteae inhibiting the collagen deposit, the authors observed the effects of charred gossamer urocteae on MMPs and TIMPs of fibroblasts. MMPs are a proteolytic enzyme family, in which 23 members have been found at present, mainly including collagenase, gelatinase and matrilysin. Gelatinase can be divided into gelatinase A (MMP-2) and gelatinase B (MMP-9), which almost can degrade all of extracellular matrix. TIMPs are endogenous inhibitor of MMPs, and among the four kinks of TIMPs found at present, TIMP-1, TIMP-2, TIMP-3 and TIMP-4 are studied mostly. TIMP-1 widely exits in tissues and body fluid, and it non-covalently combines with activated MMPs at 1:1 to inhibit activities of most of MMPs.\(^9\) In the study, no effects of all fractions of charred gossamer urocteae on MMP-2 were found, but the ethanol extract, EF and AF could significantly enhance MMP-9 activity, and all fractions of charred gossamer urocteae could decrease synthesis of TIMP-1, so as to further strengthen the ability of accelerating degradation of collagen.

In brief, this study indicates charred gossamer urocteae at high concentrations can inhibit proliferation of fibroblasts and collagen synthesis, and at low concentrations possibly promote proliferation of fibroblasts, and in healing of wound, it at high concentrations may function in anti-fibrosis and anti-scar. The mechanism of promoting degradation of collagen is possibly related with increase of MMP-9 activity and inhibition of TIMP-1 synthesis.

REFERENCES


(Translated by WANG You-jing 王友京)

Received January 10, 2009