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Differential Effects on Fibroblast Cells Proliferation and Migration by Bismuth Subgallate and Borneol Individual and Combination

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ABSTRACT
Bismuth subgallate (BSG) is a haemostatic agent and may reduce inflammation. Borneol (BO) usually exists in many essential oil which effects on antimicrobial and anti-oxidation. Besides, combination of BSG and BO stimulated wound closure rate and granulation tissue formation in a wound-thickness model and enhanced acute wound healing in the human forearm biopsy model. However, the mechanism of BSG combined with BO on wound healing is still unclear. From previous report, proposing BSG/BO alone or combination may have beneficial effect on fibroblasts proliferation and migration. Thus, this study focused on BSG and BO alone or combination on fibroblasts proliferation and migration influence, key role in wound repair. The results showed suitable specific ratio of BSG combined with BO have more efficiently on fibroblasts proliferation and migration than BSG/BO alone. In addition, BSG combined with BO induced migration and filopodia formation through PI3K pathway. Id1 mRNA expression was increased by optimal ratio of BSG combined with BO. This investigation indicates that suitable combination of BSG and BO stimulated fibroblastic proliferation and migration which suggests these two traditional medicines also have efficiency on wound therapy by suitable combination.

Key words: Bismuth subgallate, borneol, proliferation, migration, wound healing, PI3K, Id1

INTRODUCTION

Bismuth subgallate (BSG) and Borneol (BO) are traditional used medicine. BSG firstly was used in encephalopathy and tonsillectomy as an outpatient procedure for post-operative haemorrhages to reduce clotting time (Friedland *et al*., 1993; De Hombre and Penate, 2006) and as a topical hemostatic agent on palatal wounds recently (Kim *et al*., 2012, 2010). Additionally, BSG had an anti-inflammatory effect through inhibiting NO and PGE_2 production and had synergistic effect on wound healing with BO in animal wound model (Lin *et al*., 2004; Mai *et al*., 2003). Bornel (BO), primary isolated from *Dryobalanops aromatica*, is a bicyclic monoterpene used for analgesia in traditional Chinese and Japanese medicine and is found in the essential oil and exerts antimicrobial and anti-oxidation functions (Horvathova *et al*., 2014). Recently, BO had an anti-cerebral ischemia effect and may be a promising potential preventive agent for acute lung injury treatment (Kong *et al*., 2013; Zhong *et al*., 2014).

Cutaneous wound healing is a complicated and dynamic series events including clotting, inflammation, granulation formation, reepithelialization, neovascularization, collagen synthesis and wound contraction. These events overlap in time and involve a complex array of cytokines secreted by platelets, macrophages, neutrophils, fibroblasts and epidermal cells (Pazyar *et al*., 2014; Wilkins and Unverdorben, 2013). Since wound repair is a multi-complex process, drugs targeted to every phase of wound healing process may perfectly to therapy wounds. To achieve this goal, combination of many drugs to

activate sequentially or synergistically in wound healing offers a good way for wound repair research. The BSG have a beneficial effect on coagulation, one of an essential phase of wound healing process and combination of BSG and BO could stimulate granulation tissue formation and reepithelialization in a full-thickness wound model (Mai *et al*., 2003). Besides, combination of BSG and BO (suile) stimulates wound healing in clinical study such as treatment for biopsy acute wound (Serena *et al*., 2007) which the mechanism on would healing is unclear. Therefore, suggesting that BSG and/or BO alone or combination these two drugs might modulate fibroblast proliferation and/or migration, since these are critical steps in wound repair. This study indicates BSG combined with BO at suitable ratio has better effect on fibroblasts proliferation and migration stimulation than by BSG/BO alone.

MATERIALS AND METHODS

Cell culture: The experimental tissue was foreskin from young boy (mean age 7.5 years). Each sample was cut into roughly 2 mm pieces, lightly pressed using a glass slide (24×24 mm), dispersed on a 25 cm^2 culture flask (Falcon; Becton Dicknison., Frankin lakes, NJ) and then immersed in 2 mL of Dulbecco's modified Eagle's medium (DMEM) with high glucose (Gibco BRL), containing 20% Fetal Bovine Serum (FBS) (Gibco BRL Co), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Then, the flasks were incubated for three days at 37 in a humidified 5% CO_2 incubator. The medium was then replaced with new medium containing 10% FBS and was changed every three days. The dermal pieces were removed when fibroblasts grown from the dermal pieces formed a confluent layer. Subsequent subcultures were performed usual procedures. Fibroblasts from passages 5-8 were used in the experiments. Tested substances Bismuth subgallate (BSG) was purchased from Iwaki Seiyaka Co (Japan). Borneol (BO) was obtained from Tekho Camphor Co (Taiwan). LY294002 was purchased from Calbiochem Novabiochem Corp.

Measurement of cell proliferation: Counting cell number assessed cell proliferation. Cells were seeded into (Shukla and Hilgenfeld, 2014) 6 well plates (2×10^4 cells $well^{-1}$) for 24 h, then incubated with test substances for 48 h at 37 in 5% CO_2. Finally, cells were counted at light microscopy.

Chemotaxis assay: The 3×10^4 Fibroblasts cells were seeded to a Transwell upper layer (8 μm pore size, Becton and Dickinson) containing only DMEM medium and tested substances. The lower chamber included 5% FBS/DMEM. Then, cells were cultured at 37 in 5% CO_2 for 6 h. Following drugs incubation, the cells above the filter were removed by scratching and fixed in methanol (Merck) for 15 min, then stained with hematoxylin (sigma) for 30 min and finally mounted on a glass microscope slide. Cells migration was assessed by counting the number of cells in five fields under a light microscope.

***In vitro* scratch assay:** Fibroblasts (8×10^4/well) were plated in six-well plates (precoding 10 μg mL^{-1} collagen I) and cultured 24 h for confluent. The cells were incubated with 10 μg mL^{-1} of mitomycin-C for 2 h to inhibit cell proliferation and the confluent monolayer of cells was scratched in a standardized manner with using a plastic apparatus to create a cell free zone in each well, with width 2 mm. The cells were incubated for 12 h at 37 treated with drug. *In vitro* cell migration was documented by photography and the residual gap between the migration cells was measured and expressed as a percentage of area of the 0 h scratch area.

Immunofluorescence detection: Cells were seeded to coverslips (NUNC) in medium containing tested drugs for 6 h, fixed in 4% paraformaldehyde (Sigma) in PBS for 15 min and then permealized by 0.5% Triton X-100 for 15 min. Actin structure was determined by staining with Rhodamine-phalloidin (Molecular Probe) for 30 min. Finally, actin structure formation observed by immunofluorescence microscopy.

RT-PCR: Total RNA was extracted from the cells using Trizol reagent (Molecular Research Center) according to the manufacture's instructions. Single-strand cDNA was synthesized from 1 μg of total RNA. PCR amplification was subsequently performed in a reaction volume of 30 μL containing 1 μL of the appropriate cDNA, 1 μL of each set of primers at a concentration 1 mM. The RT-PCR products were electrophoresed on agarose gel and visualized via staining with ethidium bromide. Amplification was run for 25 cycles at 95°C for 2 min, 95°C for 2 min, 46°C for 1 min, 72°C for 2 min and finally extended at 72°C for 5 min. The sequences of the primers used were: ID1, forward: 5-AAACGTGCTGCTCTACGAA-3 and reverse, 5-GGAACGCATGCCGCCT-3; ISG15, forward: 5-ATTTCCGGCCCTTGATCCTG-3 and reverse, 5-GGTGGACAAATGCGACGAAC-3; IFIT1, forward: 5-AAAAGCCCACATTTGAGGTG-3 and reverse, 5-CACCTCAAATGTGGGCTTTT-3; LRP8, forward: 5-CCTCCACTGACTTCCTGAGC-3 and reverse, 5-GCTCAGGAAGTCAGTGGAGG-3; FUBULIN 1 forward: 5-CCTACCTCTGCAGCTGTTCC-3 and reverse, 5-GGAACAGCTGCAGAGGTAGG-3 GAPDH, forward: 5-ATCCCATCACCATCTTCCAG-3 and reverse, 5-CCATCACGCCACAGTTTCC-3.

Statistics: In each experiment all samples were examined in triplicate and the results were expressed as Mean±SD. The level of significance was set at $p<0.05$ for evaluating the data with the Student t-test.

RESULTS

Differential effects of BSG and BO on fibroblasts proliferation: In order to determine BSG and/or BO whether affect fibroblasts proliferation, cells growth was analyzed by

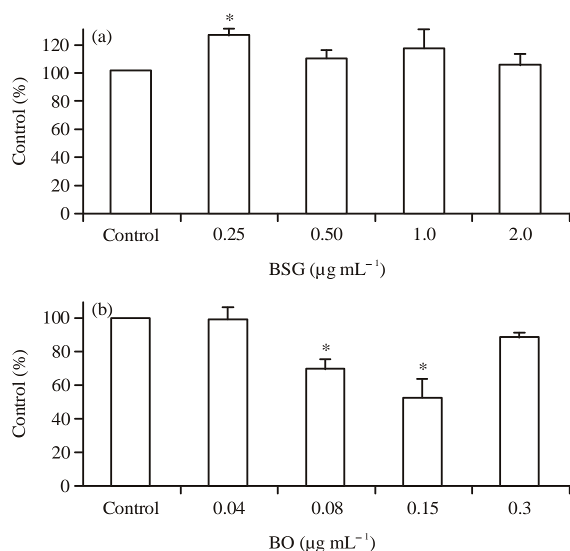

Fig. 1(a-b): Effects of BSG and BO individual on fibroblasts proliferation. (a and b) Fibroblasts were plated in 6 well plates (20000/well), allowed to attach and treated with different concentration of (a) BSG and (b) BO in 2% FBS/DMEM medium for 48 h. Then, cell numbers were determined by hemacytometer under microscopy with trypan blue staining. The significance compared to control value (*p<0.01) was calculated by the Student's t-test

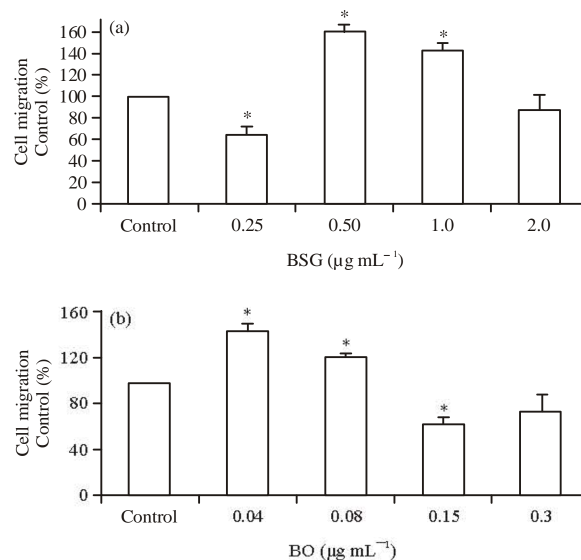

Fig. 2(a-b): Effects of BSG and BO individual on fibroblasts migration. (a and b) Fibroblast cells in serum free medium were added to the upper chamber of Transwell with the (a) BSG and (b) BO 5% FBS/DMEM added to the Transwell lower chamber as a chemoattractant. Following incubation at 37 for 6 h, migrating cells were fixed, then cells in upper chamber was scraped and stained. Finally, cell migration determined by counted cells in light microscopy. The significance compared with the control value (*p<0.01) was calculated by the Student's t-test

treat BSG and BO individually. To add BSG 0.25-2 μg mL^{-1} and 0.04-0.3 μg mL^{-1} BO to fibroblast cells for 48 h, then cells were trpsinzed and counted. The results showed 0.25 μg mL^{-1} BSG significantly increased fibroblasts proliferation by 24% ($p<0.01$) (Fig. 1a), but 0.5 and 1 μg mL^{-1} BSG only slightly increased fibroblasts proliferation. Cell cytotoxicity was not observed up to 2 μg mL^{-1} of BSG (Fig. 1a). In contrast to effects of fibroblasts proliferation by BSG, BO significantly inhibited cell growth at 0.08 μg mL^{-1} and 0.15 μg mL^{-1} BO, by 30.2 and 48%, respectively ($p<0.01$).

Effects of BSG and BO on fibroblasts migration: To analysis BSG and/or BO on the migration of skin fibroblasts, importance early process during wound healing, cells were treated by 0.25-2 μg mL^{-1} BSG and 0.04-0.3 μg mL^{-1} BO for 6 h at Transwell equipment. Figure 2a showed cells migration was significantly increased at 0.5 and 1 μg mL^{-1} BSG, by 59.6 and 40.4% of control cells, respectively ($p<0.01$). However, cell migration was significantly markedly inhibited 36% of control by 0.25 μg mL^{-1} BSG ($p<0.01$). In addition, low dose of 0.04 μg mL^{-1} BO and 0.08 μg mL^{-1} BO significantly stimulated fibroblasts migration for 45 and 22.5%, respectively. High doses of 0.15 and 0.3 μg mL^{-1} BO inhibited fibroblasts migration. These results showed that at some suitable doses of BSG and BO alone both could promote fibroblasts migration which may contribution to wound closure.

Effects of different ratio combination of BSG and BO on fibroblasts proliferation and migration: Since BSG and BO alone have differential effects on fibroblasts proliferation and migration, we interested in if there are differential or synergistically effects on fibroblasts proliferation and migration by different ratio of BSG combined with BO. Cells were treated with 0.25 μg mL^{-1} BSG and differential doses of BO for 48 h and then directly counted cells number. The results as Fig. 3a shown that 0.25 μg mL^{-1} BSG, respectively add 0.04 and 0.025 μg mL^{-1} BO significantly stimulated cell proliferation ($p<0.01$). However, cells proliferation was inhibited by 0.25 μg mL^{-1} BSG combined with 0.08 μg mL^{-1} BO. Furthermore, effects of fibroblasts migration by 0.25 μg mL^{-1} BSG combined with different doses of BO also were examined by Transwell assay. 0.25 μg mL^{-1} BSG combined with 0.04 μg mL^{-1} BO (6.25:1) significantly stimulated fibroblasts migration optimal for 110% of control cells ($p<0.01$). However, it was inhibited at 0.25 μg mL^{-1} BSG add 0.08 and 0.015 μg mL^{-1} BO by 65.7 and 75.5%, respectively (Fig. 3b).

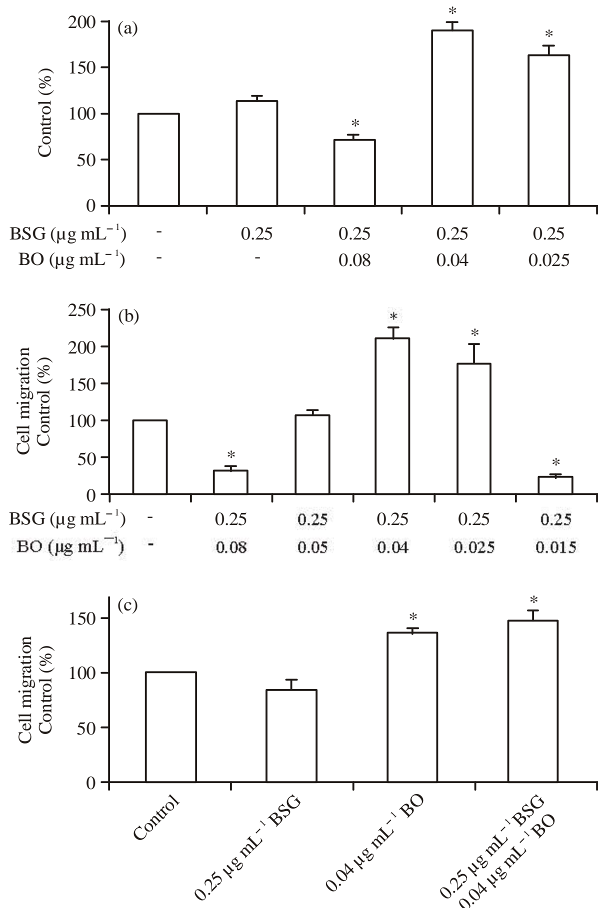

Fig. 3(a-c): Effects of BSG combined with different concentration of BO on fibroblasts proliferation and migration. (a) Effects of BSG combined with BO at different ratio on fibroblasts proliferation. Fibroblasts were treated with BSG and different concentration of BO to fibroblasts for 48 h and counted cells staining by trypan blue, (b) Effects of BSG combined with BO at different ratio on fibroblasts migration in chemotaxis assay. Fibroblasts migration was analyzed by Transwell after BSG combined with different concentration of BO for 6 h and (c) Effects of BSG combined with BO on fibroblasts migration in scratch wound assay. Fibroblasts were plated in six-well plates and cultured for confluent. The cells were incubated with 10 $\mu g\ mL^{-1}$ of mitomycin-C for 2 h to inhibit cell proliferation and the cells were incubated for 12 h treated with drug after the wounds were created. The residual gap between the migration cells was measured and expressed as a percentage of area of the 0 h. The significance compared with control value (*$p<0.01$) was calculated by the Student's t-test

BSG combined with BO stimulated fibroblasts migration *in vitro* wound scratch assay: Since it have demonstrated specific ratio of BSG combined with BO by 6.25:1 on optimal stimulation the fibroblast migration in chemotaxis assay, to further clarified the effects of fibroblast cells migration by BSG combined with BO by 6.25:1 ratio same as chemotaxis assay in would mimic scratch system. As Fig. 3c shown, 0.25 $\mu g\ mL^{-1}$ BSG combined with 0.04 and 0.04 $\mu g\ mL^{-1}$ BO alone all significantly enhanced the fibroblast cells migration in wound mimic assay. which are same with Transwell assay as Fig. 2b and 3b shown, indicating the stimulation effects on fibroblasts migration by this ratio of BSG combined with BO may major through BO alone.

BSG combined with BO stimulated fibroblasts migration and filopodia formation partially through PI3K pathway: BSG combined with BO at ratio 6.25:1 strongly stimulated fibroblasts migration, the mechanism of its action was further determined. For further analysis mechanism, PI3K inhibitor-LY294002 was added to cells incubated with 0.25 $\mu g\ mL^{-1}$ BSG and 0.04 $\mu g\ mL^{-1}$ BO fibroblast cells for 5 h. As Fig. 4a shown, fibroblasts migration was blocked 67% by inhibitor compared non-inhibitor treatment, suggesting fibroblasts migration was partly regulated by PI3K pathway at BSG combined with BO.

Effect of fibroblasts migration by combination of BSG and BO has been shown which mechanism was further been investigated. Cytoskeleton reorganization is an essential process required for cell migration. Therefore, to suggest that mechanism of BSG combined with BO stimulated fibroblasts migration whether through which induced actin cytoskeleton structure formation. Cells were treated by BSG/BO individual or combination for 5 h. Filopodia structure significantly induced by 0.04 $\mu g\ mL^{-1}$ BO ($p<0.01$). Moreover, combination of BSG and BO also induced significantly filopodia structure formation ($p<0.01$), slightly higher than that by BO alone. However, BSG induced contrary effects, inhibiting filopodia formation, suggesting filopodia formed by BSG combined with BO is major through BO influenced on cells.

The results have shown that filopodia structure formation was induced by 0.25 $\mu g\ mL^{-1}$ BSG add 0.04 $\mu g\ mL^{-1}$ BO. To treat cells LY294002 and BSG combined with BO for 5 h following determined filopodia structure formation. The results as Fig. 4b shown that LY294002 inhibited filopodia structure formation stimulated by BSG combined with BO, suggesting that filopodia formation induced by BSG combined with BO was partially through PI3K pathway.

BSG combined with BO stimulated Id1 mRNA expression: Wound healing is a complex process including cells proliferation, migration, tissue remodeling which involves many kinds of molecules. For further understand such a complex process molecule level effects by the optimal ratio of

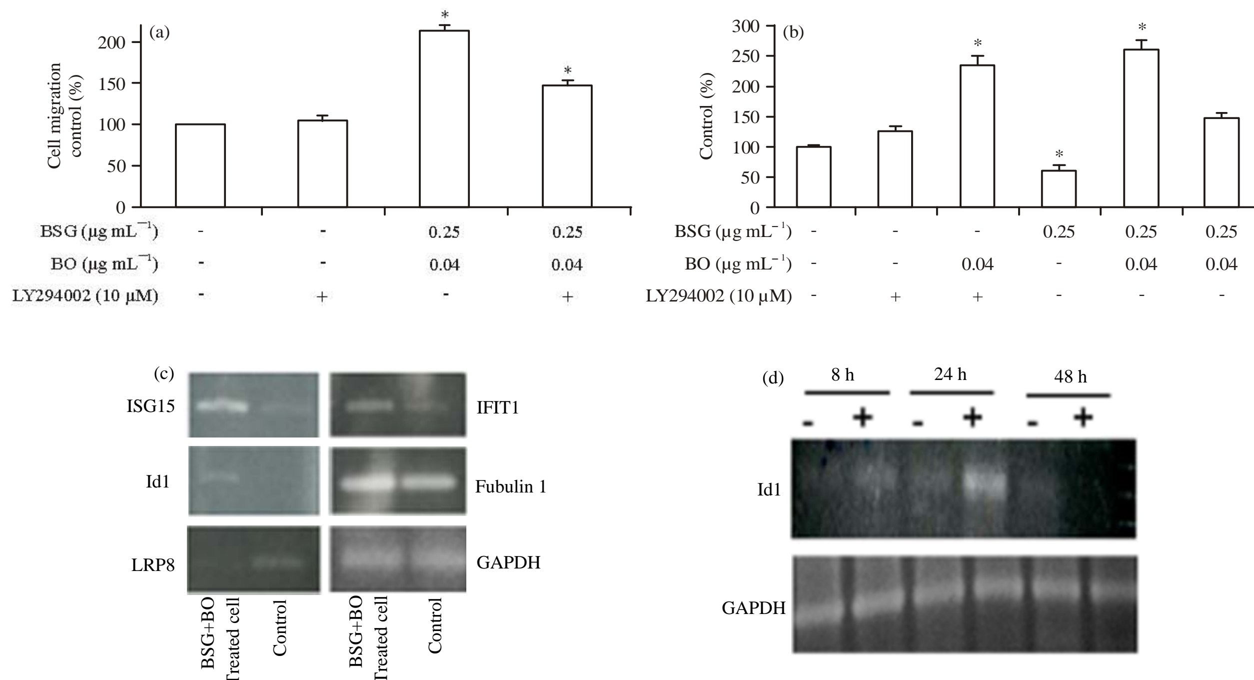

Fig. 4(a-d): PI3K pathway on fibroblasts migration, filopodia formation and genes expression treated by specific ratio of BSG combined with BO. (a) Effects of BSG combined with BO on fibroblasts migration through PI3K pathway. Cells migration were analyzed by treated with 0.25 μg mL^{-1} BSG and 0.04 μg mL^{-1} BO and which combined with 10 μM LY294002 for 6 h at Transwell equipment, (b) Effects of BSG combined with BO on filopodia formation through PI3K pathway. Cells were treated with BSG and BO individual and combination and which combined with BO for 6 h. Then, cells were analyzed under immunofluorescence microscopy following Rhodamine-Phalloidin staining. The significances (*$p<0.01$) compared to control were calculated by the Student's t-test, (c and d) Effects of BSG combined with BO on genes expression, (c) Genes including Id1, ISG15, fubulin1, IFIT1 and LRP8 expression were tested after BSG with BO by 6.25:1 treated cells for 24 h, then genes expression were analyzed by RT-PCR and (d) Time dependent effect of Id1 expression by BSG combined with BO. Cells were treated BSG with BO by ratio 6.25:1 for 8, 24, 48 h, the gene expression was analyzed by RT-PCR. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was amplified as an internal control in gene expression analysis

BSG with BO at 6.25:1 on fibroblast cells proliferation and migration, it was analyzed that the global genes expression by cDNA microarray analysis. In addition, the gene expression of Id1, Fubulin1, IFIT1 and ISG-15 were increased and LRP8 was decreased by BSG combined with BO by ratio 6.25:1 from RT-PCR assay shown as Fig. 4c. Since Id 1 involves in cell proliferation and migration process, suggesting it was a key important molecule on fibroblast cells proliferation and migration affected by BSG combined with BO. The time expression effects on Id1 by BSG combined BO was analyzed shown as Fig. 4d. Id1 mRNA expression was increased time dependently in the fibroblast cells treated by combination of BSG with BO by 6.25:1.

DISCUSSION

This study indicates effects of BGS/BO alone and combination on fibroblasts proliferation and migration which possible to distinguish the function of BSG and BO on wound healing effect. BSG combined with BO induced better beneficial both of fibroblasts proliferation and migration than BSG/BO alone, indicating combination of these two drugs is efficiency in regulating wound repair which may explains the mechanism for the bismuth subgallate combined with borneol (Suile) treated on acute would healing (Serena *et al*., 2007; Shukla and Hilgenfeld, 2014).

Suitable doses of BSG alone and it combined with BO all increased fibroblasts proliferation. In addition, suitable ratio of BSG combined with BO at 6.25:1 and 10:1 all induced greater stimulation effects of fibroblasts proliferation than BSG alone, suggesting that BO and BSG may induce different activation mediators. Thus, suggesting that BSG combined with BO may promote wound healing through inducing granulation formation. Moreover, the mechanisms of its effects on fibroblasts proliferation need to be further determined.

Cell migration is a prominent key cellular phenomenon of wound repair which is integrated by complex processes. The initial response of a cell to a migration-promoting agent is to polarize and extend protrusions in direction of migration (Schafer *et al*., 2009; Xue *et al*., 2010). From Transwell and scratch assay, suitable ratio of BSG and BO combination induced optimal fibroblasts migration compared with BSG/BO alone. Thus, to add BSG and BO together to cells may activate different mediators to regulate this effect. According to the results of migration assay, BO alone and BSG combined with BO at ratio 6.25:1 all induced filopodia formation, suggesting the effects of BSG with BO on fibroblasts migration stimulation major through BO alone. Besides, the effects of BSG with BO on fibroblasts proliferation and migration were two drugs ratio dependently. Synergistic effects of BSG combined with BO on fibroblast cells proliferation and migration were ratio dependently, indicating BSG/BO alone may affects some molecule mediators expression by the drug dose dependently or induces some new molecules by specific ratio of BSG and BO combination. The mechanism of BO alone and it combined with BSG stimulated fibroblasts migration through filopodia formation partially by PI3K pathway could further examined and whether through promote cells extend protrusions is also further clarified.

Fibroblasts proliferation and migration are importance phases during wound healing process. Firstly, cells need to migrate from wound edge to wound site and then proliferate. From this study, BSG/BO alone stimulated fibroblasts migration at its suitable doses, indicating drugs could enhance early process of wound repair. In addition, Id 1 mRNA expression was increased by the BSG combined with BO at ratio 6.25:1 treated fibroblast cells. According to the Id 1 function including in regulating wound healing related cellular processes, promoting cell-cycle progression, delaying cellular senescence, facilitating cell migration (Viales *et al*., 2014; Sun *et al*., 2011) and wound healing rate reduced in Id1-deficient mice trauma model (Viales *et al*., 2014; Sun *et al*., 2011), suggesting Id1 may play a major role by BSG combined with BO on fibroblasts proliferation and migration. The role of Id1 on fibroblast proliferation and migration treated with BSG combined with BO could further been analyzed.

This report shows specific ratio of BSG combined with BO have optimal effects on fibroblasts proliferation and migration. This ratio matches with constitution of Sulbogin®(Suile) containing BSG with BO. This investigation indicates that combination of BSG and BO is efficiency in regulating fibroblasts events, suggesting the mechanism of Sulbogin®(Suile) on promoting acute wound healing may be through regulating fibroblasts proliferation and migration.

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