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EFFECT OF CHINESE MEDICAL HERBS- BURN LINIMENT ON DEEP SECOND DEGREE BURN IN RATS

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Abstract

Background: Burn Liniment (BL) is a popular traditional Chinese medicine formula consisting five herbal medicines (Flos Lonicerae, Rhizoma Polygoni Cuspidati, Pericarpium Granati,Terminalia chebula Retz. and Galla Chinensis), that has been used in China for centuries to cure burn. This study investigated the healing effect of BL on deep second degree burn wounds in rats.

Materials and methods: The animals were divided into four groups including control group, model group,1% silver sulfadiazine (SSD) group and BL group. On days 0,3,7,14 and 21,animal weight, wound area as well as histo-pathological observations of the skin were evaluated in different groups. Serum anti-intercellular adhesion molecule 1(ICAM-1), IL-10 levels and myeloperoxidase (MPO) activity were measured on the 21st day. HPLC chromatography of BL was prepared and concentrations of active constituents were determined. Antibacterial test and toxicological test were also performed.

Results: The average wound area of BL treatment group was also significantly smaller than model control rats on days 14 and 21. Serum anti-intercellular adhesion molecule 1(ICAM-1) levels and myeloperoxidase (MPO) activity of BL group decreased significantly than in model rats on day 21 while IL-10 level of BL group increased remarkably than in model rats on the 21st day, showing that BL has strong anti-inflammatory activity on burned rats. The histological studies indicated that inflammatory cells disappeared significantly and were replaced by new granulation tissue, and epithelialization progressed quickly and was treated with BL on the 21st day. Meanwhile,

HPLC chromatography of BL was prepared and concentration of Chlorogenic acid, Polydatin and Gallic acid from BL were determined. Antibacterial test revealed that the MIC of BL on Staphyloccocus aureus, Pseudomonas aeruginosa and Escherichia coli were 1.56, 6.25 and 1.56 mg·mL^{-1} respectively. Toxicological test showed that BL does not induce skin irritation or sensitivity signs and has no acute toxicity reaction.

Conclusions: Our study revealed that BL could enhance cutaneous burn wound healing effectively. It also showed strong anti-inflammatory and antibacterial activity in rats.

Keywords: Burn Liniment; Deep second degree; Burn wound; Anti-inflammatory; Antibacterial; Toxicological test

Introduction

Every year, millions of people suffer the danger of major disability or even death from burns, caused by hot water, flame and boiling oil. People suffer from burns due to domestic and industrial accidents, which along with enormous cost of treatment, cause mortality and considerable morbidity (Wasserman D 2,000). According to the World Health Organization (WHO), there were 28,200 deaths worldwide due to burns in 1998, with 96% of these deaths occurring in developing countries. Nowadays, there are over 300,000 deaths derived from burns in the entire world. In China, 20 million people suffered from burn in different degrees every year. Burn wounds are one of the health problems in modern societies associated with irreparable harms for patients and their families (L.S.Edelman, 2007). Burns are tissue lesions from thermal origin for exposure to flames, hot surfaces and liquids extreme cold, chemicals, radiation, or friction. Even with improved prognosis (J.P.Barret, 2003) and progress in the use of biological skin substitutes (M.Ramos-E-Silva and M.C.Ribeiro, 2002), burns are an important cause of mortality (R.L.Sheridan et al., 2000). In recent years, the number of burn victims has increased considerably, emphasizing the need for stronger efforts in achieving greater diversity and effectiveness in the treatment of skin

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burns.

Many experts think that many mechanisms are involved in the process of progressive injury, including microcirculatory dysfunction, inflammatory reaction, per-oxidation injury and so on, in which microcirculatory dysfunction may be one of the important reasons for progressive deepening of the wound. Depending on the burn area, depth and site, the treatment is different. At present, there are lots of medicine for burn wound therapy such as silver sulfadiazine ointment (SSD), mafenide acetate and silver nitrate. SSD is the most used topical treatment for burn injury due to its potent anti-microbial efficacy. However, it was found that silver gets absorbed systemically posing problems on prolonged use and had systemic complications such as neutropenia, methemoglobinemia and renal toxicity (N.Shanmugasundaram et al, 2008).Therefore, finding more efficient agents with fewer side effects for the treatment of burns has always been a concern for researchers.

Burn Liniment has been used for burn treatment since ancient times in China, and achieved exact curative effect in clinical trials.The objective of this study was to investigate the pharmacodynamic and mechanism of BL in deep second degree burn model rats.

Materials and Methods

Materials

BL formula was composed of *Flos Lonicerae* 25g, *Rhizoma Polygoni Cuspidati* 25g, *Pericarpium Granati* 12.5g, *Terminalia chebula Retz.*12.5g and *Galla Chinensis* 12.5g. The concentration of BL was equivalent to 200 mg·mL^{-1}(total herb weights/Final volume). BL was provided by the third hospital of Wuhan (Wuhan, China). The compositional analysis of BL was determined on a high performance liquid chromatography (HPLC) system equipped with a SHIMADZU LC-20AT pump and a SPD-10A UV-VIS detector. The chromatic separation was achieved at 25°C on Wondasil C18 analytical column (4.6×250mm, 5um).The run time was set at 40 min and the sample injection volume was 20 μL. The mobile phase was acetonitrile-0.3% H_3PO_4 solution (gradient elution). The acetonitrile proportion was from 5% to 40% in 40 minutes. The flow rate was set at 1.0 mL/min, and samples were detected at 306 nm.

Animals and Model Preparation

Wistar rats weighing 220-240g were obtained from Hubei Center for Disease Control and Prevention, Wuhan, Hubei. The animals had free access to food and water, and were allowed to acclimatize for at least one week before use. All experiments conformed to the guidelines of the “Principles of Laboratory Animal Care”(NIH publication No.80-23,revised 1996) and the legislation of the People’s Republic of China for the use and care of laboratory animals. After removing the back hair of the rats, and injecting 20% ethyl carbamate solution into their abdominal cavities for anesthetic effects. The top of electrical scald instrument (manufactured by Changhai Hospital of Second Military Medical University) was pressed onto the back skin with a certain force for 15 seconds at the temperature of 75℃.A 2×2 cm^2 standard deep second degree burn wound was induced through the pathological examination of wounded skins.

Experimental Groups and Treatments

All rats were randomly divided into four groups of thirty-five rats in each: control group, model group, SSD-treated group, BL-treated group. Every animal in model, SSD and BL groups was induced into a deep second degree burn. Silver sulfadiazine cream (1%,wt / wt) was used as standard drug. In a preliminary study, the dose-response properties of BL liquid and silver sulfadiazine were examined to determine the optimal dose, and the most effective in the wound healing was 0.3 g SSD or 1mL BL liquid per wound (from our pharmacological database).The 1mL of BL liquid or 0.3 g of SSD was applied slowly to the burn wound area and extended slightly outside the wound area to ensure inclusion of the wound edges. The rats in control group were treated with 1mL of saline solution per wound. Treatments were repeated twice daily for 21 days. The first application was done directly after the wound injury.

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Body Weight and Average Wound Area Measurement

On days 0, 3,7,14 and 21 of treatment, body weights and measure of average wound areas of model control group, SSD group and BL group rats respectively were taken. The wounds were photographed with a digital camera in order to calculate the wound surface areas (WSA) with the software AUTOCAD. The change in wound surface area in a given day ($WSA_{day\text{-}x}$) was expressed in a percentage of the wound area on the second day ($WSA_{day\text{-}2}$) using the following equation:

$$WSA = \frac{(WSA_{day\text{-}2} — WSA_{day\text{-}x}) \times 100}{WSA_{day\text{-}2}}$$

Biochemical Analysis

On day 21 of treatment, serum IL-10, ICAM-1 and MPO levels of all rats were determined with ELISA Kits (XinBoSheng Biological Technology Co., Ltd., China).

Histological Study

Wound skin tissue samples were taken from model control, SSD and BL treatment group rats on days 7, 14 and 21 for histological observation. Six rats were sacrificed to obtain wound skin tissues at every point in time respectively in all group rats. The skin tissues were fixed with 10% formalin. After fixation, samples were embedded in paraffin, cut into 3 mm frozen sections with a cryostat microtome, and then stained with hematoxylin eosin reagent. Observed collagen fiber, inflammatory cell, blood vessel, fibroblast and granulation tissue of burn skin of rats with microscope.

Antibacterial Test

Agar dilution was used to determine the minimum inhibitory concentrations (MIC) of BL on Escherichia coli (ATCC25922), Staphyloccocus aureus (ATCC27217) and Pseudomonas aeruginosa (ATCC27853).The three bacteria were all diluted to 1.5×10^5 CFU·mL^{-1} with 0.9% sodium chloride solution. In a sterilization dilution orifice plate, add 3 mL sterile 0.9% sodium chloride solution into numbered holes. Then 3 mL BL was added in first hole, blended and got 3 mL into the second hole, and so forth. At last, the diluted BL concentration were 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39 and 0.20 mg·mL^{-1} in the holes respectively. Take 1.5mL different concentrations of BL onto sterile plates, and put into agar culture-medium. Cover plate cover, and spin them rapidly. After agar solidified, vaccinated 2 µL of different bacteria liquid were introduced onto them respectively. Together, a negative control was set (agar without bacterium) and a positive control (agar with bacterium and without drug) as a control. Put the plates at 37℃ constant temperature incubator to culture for 48 hr, and then observed the growth of bacterial colony. The experiment was repeated for three times to determine MIC.

Toxicological Test

Acute toxicity test

The acute toxicity test was performed on thirty rats which were divided into five groups including blank solvent control group, low-dose concentration of BL (100 mg·mL^{-1}) on intact skin group, high-dose concentration of BL (400 mg·mL^{-1}) on intact skin group, low-dose BL on damaged skin group and high-dose BL on damaged skin group. The hair on both sides of rats was shaved and **a** cut on the skin to form a wound with a needle was made on each rat. This was followed with a smear of 0.3mL high-dose or low-dose BL liquid on the intact skin or damaged skin four times every day; a smear blank solvent was applied on control rats. After 24 h, changes in weight, eye, breathing, activities of four limbs and central nervous system of rats were also noted on days 1, 2, 3 and 7. On day 7, pathological changes of heart, liver, spleen, lung, kidney of rats were analyzed .through

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standard protocols.

Skin irritation test

The skin irritation test was performed on twenty-four rats which were divided into four groups including single administration on intact skin group, multiple dosing on intact skin group, single administration on damaged skin group and multiple dosing on damaged skin group. The hair on both sides of rats was shaved. 0.3mL BL liquid was applied on the left back and blank solvent on the right back of rats as control. Single administration means that BL solution was smeared once, and multiple dosing means that BL liquid was smeared four times. The actions were performed every day for seven days. 24 hours after administration, spiloplaxia, eodema of coated parts of rats were observed. The assay was carried out according to the standard skin irritation intensity method of the "Study guide of traditional Chinese Medicine" (Ministry of Health of the People's Republic of China,1994), average scores were counted and stimulation intensities were judged. Average value of reaction = (spiloplaxia scores + eodema scores) / total animal numbers.

Dermal sensitivity test

The dermal sensitivity test was performed on 24 rats. The rats were divided into three groups: blank solvent control group, positive drug group and BL group. The hair on the left side of rats was shaved. 0.2mL of BL liquid, 1% 2,4-Dinitrochlorobenzene or blank solvent were smeared on the left back of rats once on day 1, 7, 14 and 28 respectively. 6 hours, 24 hours, 48 hours and 72 hours later after administration,
spiloplaxia, eodema or systemic anaphylaxis of coated parts of rats were observed. This was done according to the standard of skin irritability intensity of "Study guide of traditional Chinese Medicine", average scores and sensitization rates were counted, and degrees of sensitization were calculated .Sensitization rate = (hyperergic animal numbers / total animal numbers) ×100%.

Statistical Analysis

Values are expressed as means ± SEM. Multiple group comparisons were performed using one-way analysis of variance (ANOVA) with the SPSS 18.0 followed by Dunnett's test to detect intergroup differences. $P< 0.05$ was considered significant in all cases.

Results

HPLC Fingerprint of BL

The HPLC chromatogram of BL is shown in Figure 1.The concentration of active constituents gallic acid, chlorogenic acid and polydatin in BL is 71.883,99.917 and 20.429 μg·mL^{-1} respectively.

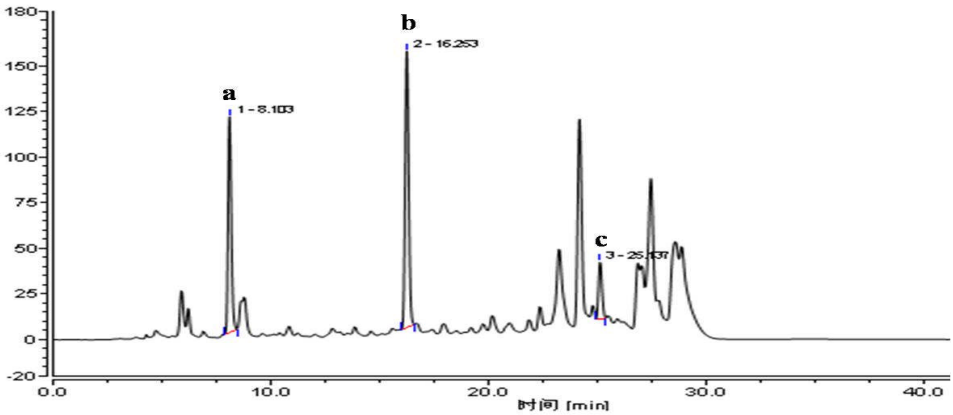

Figure 1: HPLC chromatography of BL. a:Gallic acid;b: Chlorogenic acid; c:Polydatin

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Body Weight Measurement

In all the cases, the weight of rats decreased by 3–10% during the initial period up to 7 days. After that, the weight increased gradually. The weight loss is due to the severe metabolic, hormonal, immunologic and circulatory disturbances caused by the burns(Wolfe RR,1996).Up to 7 days, the burned rats increased catecholamine, cortisol and glucagon levels and pro-inflammatory cytokines, in particular IL-8,and maintained normal or slightly elevated insulin levels(De Bandt JP et al.1994;Wilmore DW 1976). These hormonal changes promote increased proteolysis with the release of high amounts of alanine and glutamine, glycerol and free fatty acids into the systemic circulation (Cynober L 1989). The consequence of this hormonal reaction is an increase of the energy expenditure which can reach 100% in case of a severe burn. Such levels of loss can persist 7–10 days and even more, which explains that the burn is a source of major under nutrition and both muscle and visceral protein losses begin immediately after injury (Bessey P et al.1989).The average weight of model rats with BL treatment decreased more slowly than model groups between day 3($P< 0.05$) and day 7($P< 0.01$) and increased more quickly than model groups between day 14($P<0.05$) and day 21($P< 0.05$), suggesting that BL had effective resuming efficacy (Figure 2.).The average weight of model rats with SSD treatment increased more quickly than model groups between day 7($P< 0.05$) and day 21($P< 0.05$). The data are expressed as the means ± SEM. The differences between the model and the treated groups with *$P< 0.05$ and ** $P< 0.01$ were considered significantly different compared to the model.

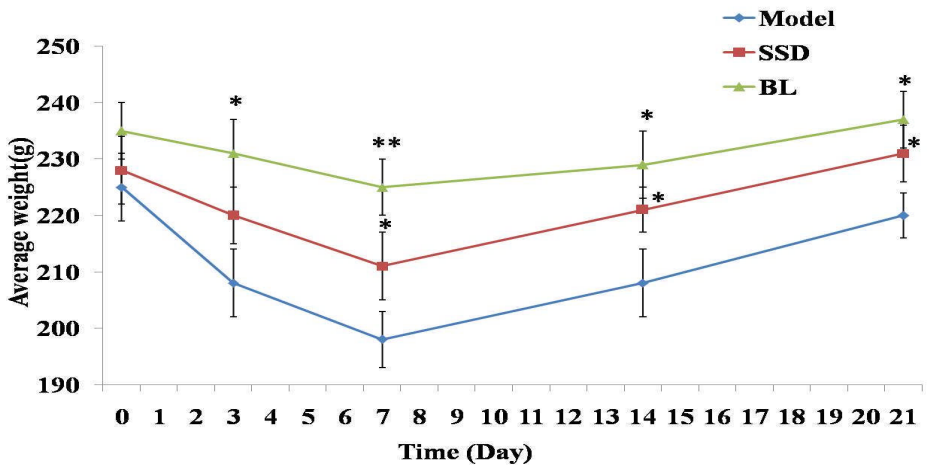

Figure 2: Body weight of rats versus healing time(n=10)

Wound Healing Test

The macroscopic appearance of the deep second degree burn wound, treated with SSD and BL are shown in Figure 3.After preparing deep second degree burn,we see the formation of white vesicular membrane, of which the dermis was damaged. Three days after the burn, hard brown scab formed significantly induced by inflammatory action.Between day 3 and day 7 of treatment with BL,the brown scab became Thicker, of which the fester decreased gradually. At day 14, half of the scab failed off and the skin under it had recovered well. Compared with model and SSD groups, the rat wounds treated with BL crusted and healed more quickly. At day 21,the whole scab disappeared and the burned kin had healed up completely .It illustrated that BL had significant curative effect in curing deep second degree burn wound.

The wound area of rats increased in initial 3 days after preparing the deep second degree burn. Thereafter the wound area decreases progressively. The wounds treated with BL healed more quickly than those of model group. The average wound area of model rats with BL treatment reduced more quickly than model groups between day 14($P< 0.05$) and day 21($P< 0.01$)(Figure 4),suggesting that BL had exact effect of accelerating wound healing. Meanwhile, the average wound area of model rats with SSD treatment was smaller than model groups on day 21($P< 0.01$). On the 21st day, the contraction average of BL-treated wounds was about 95%,whereas that of the wounds treated with SSD was 79%.The differences between the model and the treated groups with *$P< 0.05$ and ** $P< 0.01$ were considered significantly different compared to the model.

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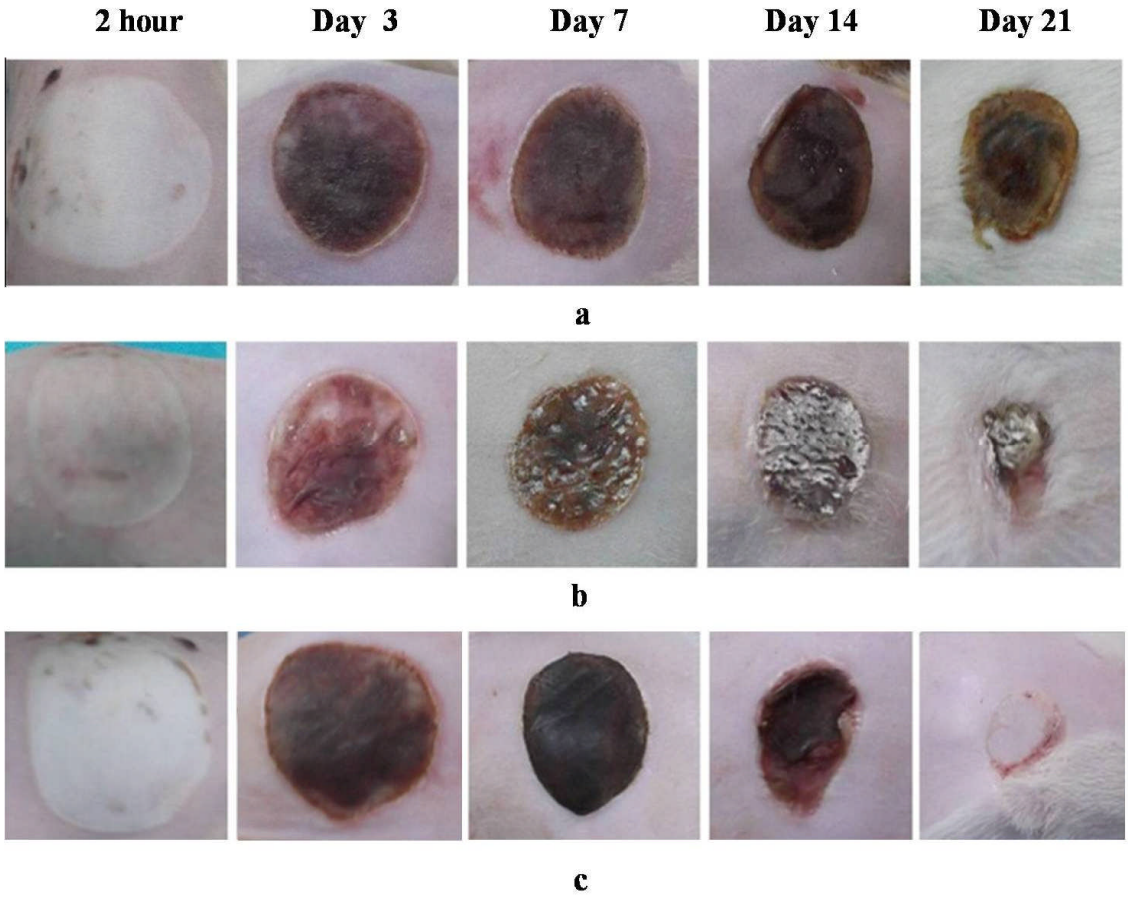

Figure 3. Photographs of macroscopic appearance of wound repair covered with (a) model, (b)SSD, and (c)BL at Hour 2,Day 3,Day 7,Day 14 and Day 21 respectively.

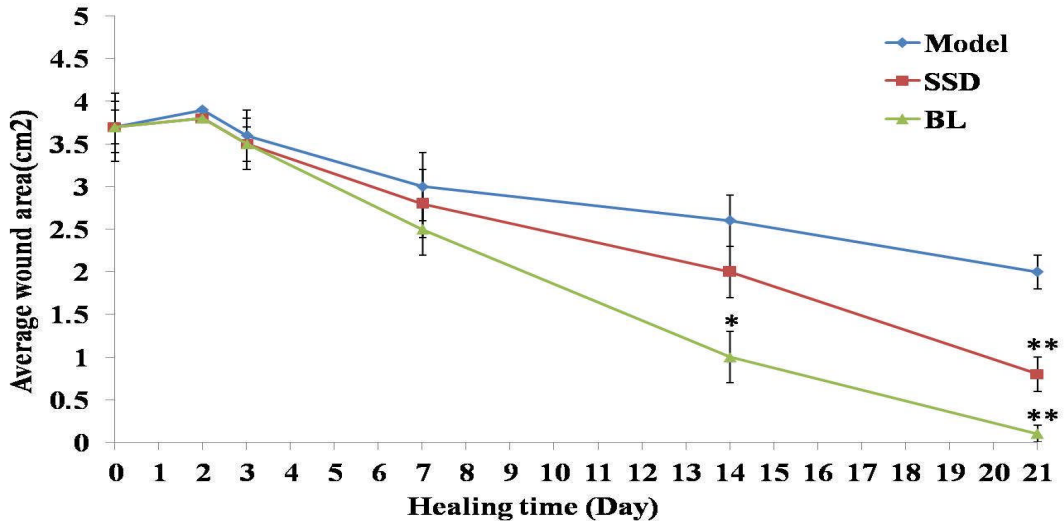

Figure 4: Evaluation of wound area versus healing time (n=10)

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Effect of BL on Serum ICAM-1,IL-10 and MPO

The ICAM-1 concentrations and MPO activity in the serum were significantly (P<0.01) higher in the burned model rats than in the control rats. The BL significantly (P<0.01) suppressed the increase of serum ICAM-1 concentrations and MPO activity in the BL-treated rats.SSD also reduced the serum ICAM-1 concentrations and MPO activity in the SSD-treated rats (P<0.05).The serum IL-10 concentrations were significantly (P<0.01) lower in the burned model rats than in the control rats. The BL or SSD significantly (P<0.01) increased serum IL-10 level to almost the control concentration (Table 1).

Table 1: Effect of BL on serum ICAM-1,IL-10 and MPO in rats(n=10)

Group	ICAM-1(ng/ml)	IL-10(pg/ml)	MPO(U/ml)
Control	0.342±0.052	163.6±10.82	0.52±0.08
Model	0.915±0.061**	67.52±5.63**	2.85±0.17**
SSD	0.514±0.092△	107.43±13.94△△	1.97±0.31△
BL	0.528±0.043△△	147.24±7.55△△	0.74±0.07△△

*P< 0.05, ** P< 0.01, compared with control group rats.; △P< 0.05, △△ P< 0.01, compared with model group rats.

Histological Analysis

The wound tissues used to prepare pathological sections have been examined to confirm the burn degree. After initial burning and elimination of necrotic tissue, a deep second degree burn showing muscular and adipose tissues formed, of which there were neither dermis nor epidermis and infection was absent (Figure 5). Histological findings of the wounded skin, treated with SSD and BL on day 7, 14 and 21 are shown in Figure 6 and Figure 7. On day 0, collagen fiber was necrotic, inflammatory cell infiltrated below striated muscles and vascular engorgement and necrosis were seen in burn skin of model rats. On day 7, inflammatory cell infiltrated severely and some fibroblast and granulation tissues were found in burn skin of model control rats. Meanwhile, burn skin of rats treated with SSD or BL showed that inflammatory cell reduced greatly and many fibroblast and granulation tissues appeared. On day 14, many inflammatory cells and some fibroblast and granulation tissue were found in burn skin of model rats. However, many new collagen fibers growed, inflammatory cell decreased heavily and a lot of fibroblast and granulation tissues appeared in burn skin of rats treated with SSD or BL. On day 21, inflammatory cells were still seen and some fibroblast and granulation tissue growed in burn skin of model rats.

However, there were fleshy buds constituted with blood vessels and fibroblasts, inflammatory cells disappeared and were replaced by new granulation tissue, and epithelialization progressed very quickly in rats treated with SSD or BL. The results suggested that BL treatment had a benefic influence on the various phases of wound healing.

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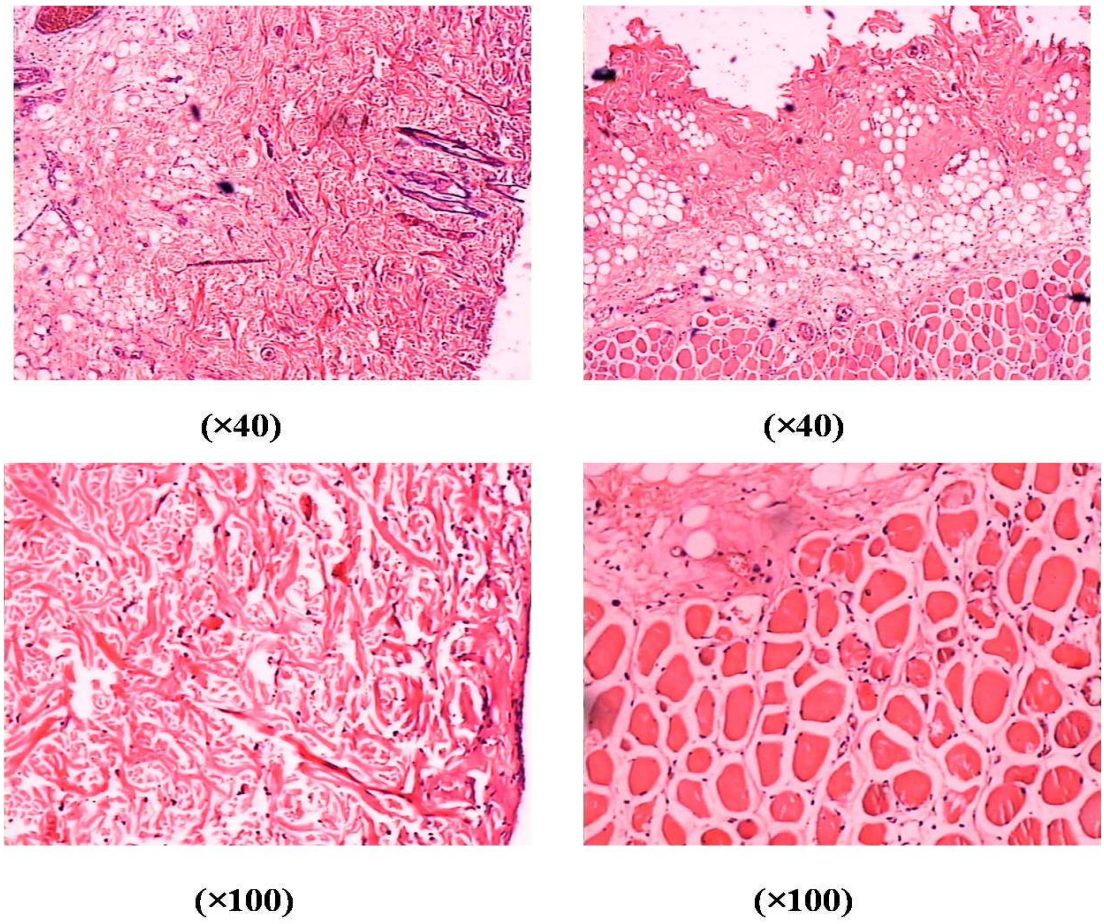

Figure 5: Pathomorphology of burn skin in deep second degree burn model rats (40× or 100×)

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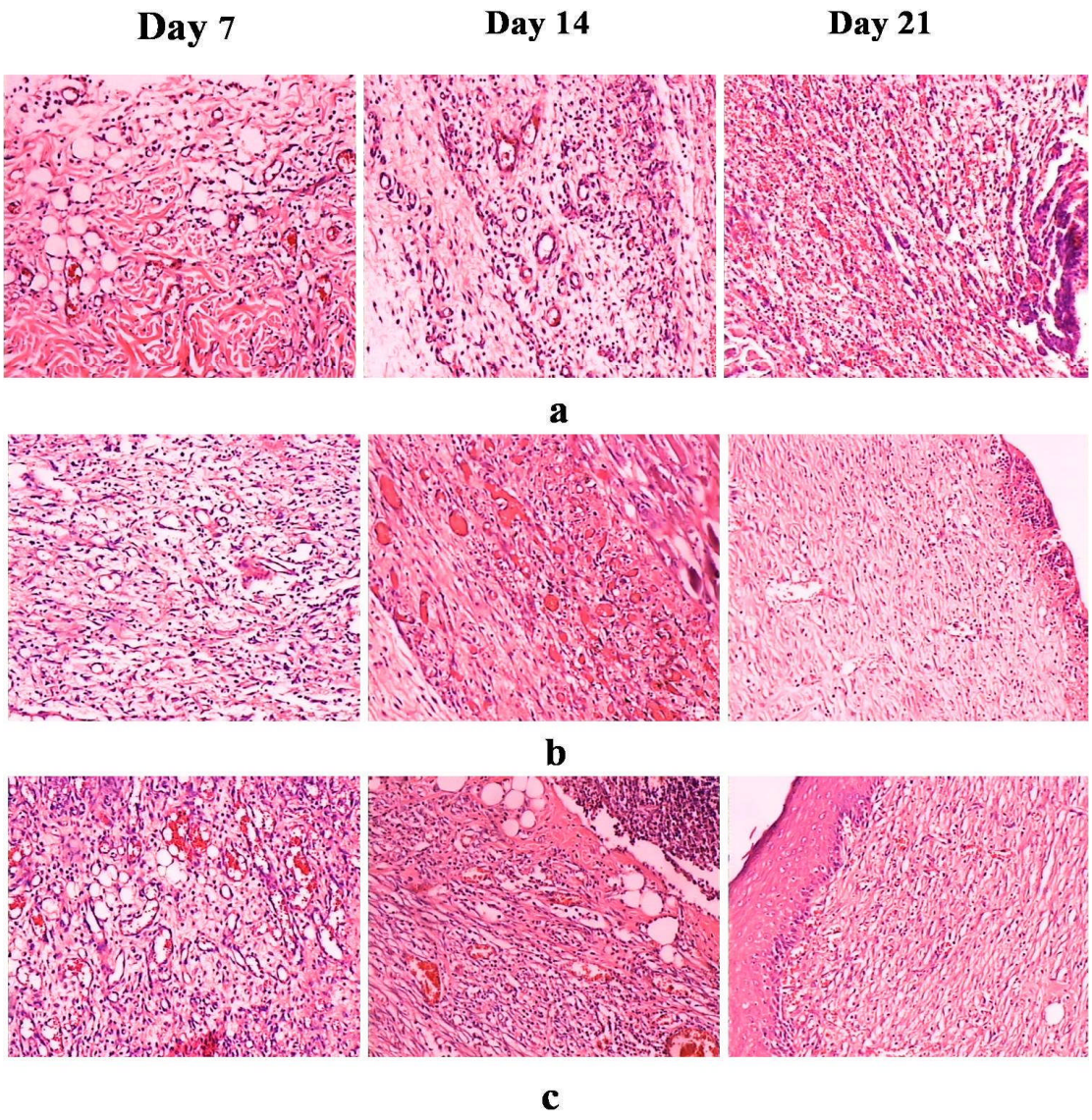

Figure 6: Photographs of pathological sections of burn skin.(a) model,(b) SSD, and (c) BL at Day 7, Day 14 and Day 21 respectively (Original magnification, 100×).

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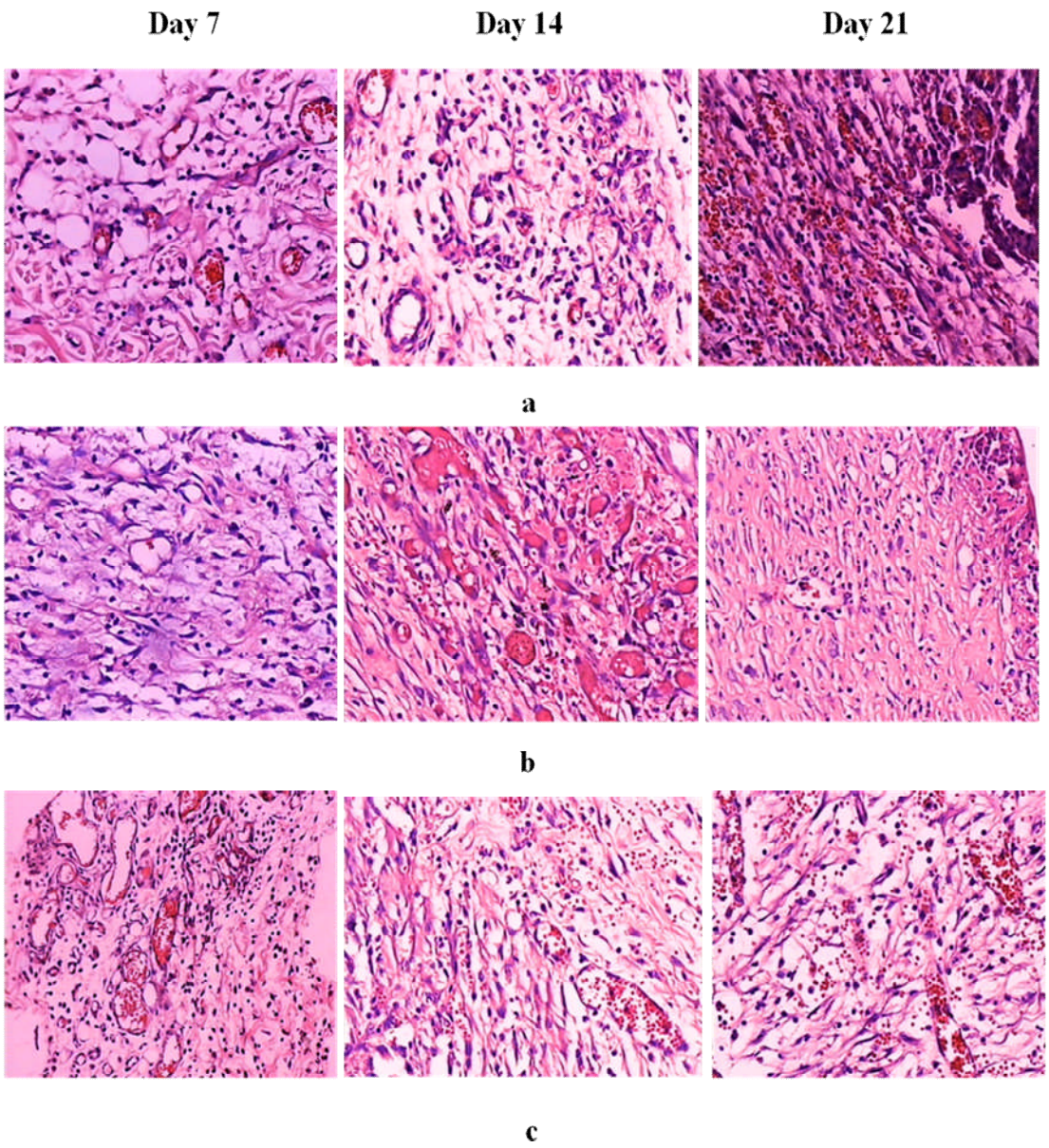

Figure 7: Photographs of pathological sections of burn skin.(a) model,(b) SSD, and (c) BL at Day 7, Day 14 and Day 21 respectively (Original magnification, 200×).

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Antibacterial Test

The antibacterial results are listed in Table 2.The MIC of BL on *Staphyloccocus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* were 1.56, 6.25 and 1.56 mg•mL^{-1} respectively.

Table 2: The MIC of Burn liniment on three standard strains

Kind of bacterium	Negative control	Positive control	Concentration of BL(mg•mL^{-1})									
			100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.2
Staphyloccocus aureus	–	+	–	–	–	–	–	–	–	+	+	+
Pseudomonas aeruginosa	–	+	–	–	–	–	–	+	+	+	+	+
Escherichia coli	–	+	–	–	–	–	–	–	–	+	+	+

"-" indicates sterile growth, "+" indicates bacterial growth.

Toxicological Test

During the period of acute toxicity observation, all rats acted normally with no death. With examining the pathological tissues of heart, liver, spleen, lung, kidney, no pathological changes were found in all rats. It revealed that BL had no acute toxicity reaction. In skin irritation test, no spiloplaxia and edema of coated parts of rats were found. The average value of reaction is zero, illustrating that BL had no irritation. In dermal sensitivity test, solvent control group rats and BL group rats had no spiloplaxia and edema in skin respectively. Rats treated with positive drug 2,4-Dinitrochlorobenzene acted sever sensitization with 100% sensitization rate at 6 hr after the last administration. According to these results, the topical application of BL does not induce skin irritation or sensitivity signs and has no acute toxicity reaction.

Discussion

Thermal burn injury is still a major cause of death and disability in the world and its healing process is a challenge in modern medicine. Burn in human body may be treated by different methods depending on the extent and severity of the burn.SSD could kill a wide variety of bacteria, so it is commonly used to prevent and treat infections of the second and third degree burns. Recent studies revealed that SSD ointment has had positive effects on proliferation of fibroblasts which are the main source of collagen and fibronectin (Coelho JM et al. 2010). However, current reports suggest that silver-based products are better to be avoided due to their side effects and researchers are making efforts to seek for better topical antimicrobial products.

ICAM-1 seems to be the initial marker of inflammatory reactions and plays a crucial role in various different pathologies, including allergic rhinitis, HIV-1 infection, Malaria ,tissue or organ transplant rejection(Adams DH et al. 1989;Adams DH et al. 1993),diabetes mellitus, glomerulonephritis, asthma (Wegner CD et al.1990),
rheumatoid arthritis (Potocnik AJ et al.,1990) and atherosclerosis.ICAM-1 is expressed on many different cells and serum concentrations of ICAM-1 can be increased during immune or inflammatory disorders (Ballantyne CM et al.,1991;Seth R et al.,1991).Data also show that ICAM-1 was involved in the acute inflammatory reaction following burns (Mileski WJ et al.,2003)IL-10 is an anti-inflammatory cytokine that plays a significant role in controlling inflammation and modulating adaptive immune responses that cause tissue damage(Yao Y et al.,2013).The haem protein MPO is the most abundant protein in neutrophils and an important enzyme in innate immunity. More, MPO has been shown to elicit pro-inflammatory properties independent of its catalytic properties (A. Haegens et al.,2008).Extracellular MPO activity gives an estimate of the oxidative stress in inflammatory

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diseases, while intracellular MPO activity correlates well with tissue neutrophil content (Benjamin Pulli et al.,2013). It was reported that MPO could be as a marker enzyme for the diagnosis of wound infection (Hasmann A et al., 2012).Our study revealed that serum ICAM-1 levels and MPO activity of BL group rats decreased significantly than in model rats while IL-10 level of BL group increased remarkably than in model rats after the treatment of 21 days, revealing BL had strong anti-inflammatory activity in burned rats.

In the treatment of curing burn, the key method is to control bacterial infection.

The common and main bacteria isolated from clinical burn patients were *Staphylocco-cus aureus* ,*Pseudomonas aeruginosa* and *Escherichia coli* SSD is the most used topical treatment for burn injury due to its anti-microbial efficacy. However,it has systemic complications such as neutropenia, methaemoglobinemia and renal toxicity. Traditional Chinese medicine has the advantage of inhibiting bacterial growth with few side effect. The main constituents such as chlorogenic acid, emodin and gallic acid from BL all showed strong antibacterial activity (Zaixiang Lou et al., 2011; J.C. Chukwujekwu et al., 2006; Aijuan Li et al., 2007). Therefore, BL inhibited the bacterial growth of burn wound effectively.

In recent years, there has been a growing interest in alternative medicines and natural medicinal products for the local treatment of wounds due to the high costs of traditional drug treatments (Lee J-A et al.,2011).Wound healing includes number of stages like clotting, inflammation, granulation, fibrosis, arrangement of collagen with spasm of wound and epithelization. The time required for complete healing of deep second degree burns, without the application of specific therapeutic agents, can be three to six weeks or more and these burns will leave a scar tissue that may hypertrophy and contract itself (R.M. Johnson et al., 2003). In our study, BL accelerated the scab of deep degree burn wound and controlled infection effective for healing. Model rats with BL treatment got healing of burn wound at day 21 while model rats still showed severe inflammatory cell infiltration and no complete healing through histological tissue analysis.

In conclusion, the results presented significant therapeutic effects of BL on burn wound healing. In addition, BL showed strong anti-inflammatory and antibacterial activity which possibly contributed together to the wound healing.

Conflict of Interest: The authors declare no conflict of interest.

Acknowledgements

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