Anti-bacterial and anti-inflammatory effects of Tanshinone breast filler in mice

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The purpose of this study was to investigate the antibacterial and anti-inflammatory activities of Tanshinone breast filler (TBF) against cow mastitis. TBF on bacterial activity in vitro by the method of Oxford cup and the mice were treated by TBF before given the mixed bacterial by intraperitoneal injection in vivo. The effect of TBF against acute inflammation was studied by xylene-induced ear edema and egg white-induced paw edema in mice. The activity of TBF against chronic inflammation was assessed by the cotton pellet. TBF significantly inhibited staphylococcus aureus, staphylococcus epidermidis, streptococcus dysgalactiae and escherichia coli in vitro and vivo. TBF showed significant activity against acute inflammation on ear edema induced by xylene and paw swelling induced by egg white in mice. In chronic inflammation, TBF inhibited significant effects on cotton-induced mice granuloma. In conclusion, TBF had obvious effect against acute inflammation and chronic inflammation in mice and suppressed pathogenic bacteria in vitro and vivo.

Key words: Anti-bacterial; anti-inflammatory, TBF, cotton ball, granuloma.

INTRODUCTION

Salvia miltiorrhiza Bge (Danshen) is a perennial herbal medicine that belongs to family Lamiaceae (XW et al., 1977). The active ingredients of Danshen were Tanshinone (I, IIA and IIB), cryptotanshinone, isocryptotanshinone, miltirone, tanshinol (I and II) and salviol (Chinese Veterinary Pharmacopoeia Committees, 2010; Hao et al., 2010). Danshen has a variety of pharmacological properties including anti-oxidant, anti-bacterial, anti-inflammatory and anti-neoplastic activity. The main components of TBF contain C. tanshinone and tanshinone IIA, and it has been reported that they have antibacterial effects on pathogen.

Anti-inflammatory mechanism of Danshen have many advantages, such as inhibiting of inflammatory mediators, improving the inflammation state and circulation, protecting vascular, inhibiting and scavenging oxygen free radicals, increasing C reaction protein decrease and antibacterial effect (Liu et al., 2015). Tanshinone IIA played an anti-inflammatory role in OVX atherosclerotic apoE (-/-) mice by activating the estrogen receptor

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through the ERK signaling pathway (Dong et al., 2009). Tanshinone IIA exerts anti-inflammatory properties by suppressing the transcription of pro-inflammatory cytokine genes that might be associated with the NF-kappabeta signaling pathway (Chang-qin et al., 2012). C. tanshinone has been reported to inhibit inflammatory cytokines production in RAW264.7 cells. Recently, it has been reported that the C. tanshinone and Tanshinone IIA has been reported to exhibit antibacterial and anti-inflammatory. In this study, we investigated that TBF could inhibit the antibacterial activities in vitro and vivo. We also used xylene-induced mice ear edema, egg white-induced mouse paw edema and cotton pellet-induced chronic inflammation to evaluate the anti-inflammatory activities of TBF in vivo.

MATERIALS and METHODS

Chemicals and regents

D-D injection was purchased from the Hebei Yuanzheng Pharmaceutical Company Limited production, China. For use as the positive control, dexamethasone (DEX) were supplied by Xian Lijun Pharmaceutical Company Limited, China. Xylene was supplied from the Xi’an Fine Chemical Research Institute, China. Formaldehyde was purchased from the Xi’an Chemical Reagent Factory. Bacterial strains used were: S. aureus, S.epidermidis, Str. dysgalactiae, E. coli, according to the standard of Veterinary Microbiology (Yi-ting, 2011) isolation and identification. The culture medium: Makanke medium was provided by Beijing aoboxing bio-tech co. Ltd, China. The nutrient broth used were: ordinary plate culture medium, 5% sheep blood agar medium, according to the practical medicine standard preparation.

Animals

Male and female mice (about 22 ± 2 g) were obtained from the Experimental Animal Center at Xi’an Jiaotong University, China. Animals were housed under standard conditions with a 12/12 h light/dark cycle. And the animals were acclimatized to their environment for a week prior to the start of experiments. All experiments were performed in accordance with the guidelines of the National Institutes of Health guidelines.

Tanshinone breast filler (TBF)

TBF was provided by the Key Laboratory of Veterinary Pharmaceutics Discovery, Ministry of Agriculture, Key Laboratory of New Animal Drug Project, Lanzhou Institute of Husbandry and Pharmaceutical Science of CAAS, China. It’s the key effective content of C. tanshinone and Tanshinone IIA. The key chemical structure (Figure 1) were confirmed by nuclear magnetic resonance (NMR) spectroscopy.

Bacteriostatic test

Bacteriostatic test in vitro

The cultured bacteria liquid was diluted to 0.5 McIntosh turbidity standard with physiological saline and 0.5 ml bacteria liquid dilution to 4.0 ml. Sterile Straw absorbed 0.1 ml into the culture medium and used a sterile cotton swab to bacteria liquid evenly. The culture was allowed to adhere to the plate for 3 to 4 min. After the culture was absorbed, an Oxford cup that had roasted in fire was placed on either side of the bacterial plate using sterile forceps, and labelled plates, then using the pipette, respectively, 200 μl liquid was added to the Oxford cup and cover the dish.

After adding sample placed on the 4°C refrigerator standing for one hour, and then posted in 37°C incubator to culture 18 to 24 h (Zhu et al., 2011). Inhibition diameters were measured by vernier caliper. All experiments were repeated once. The diameter of inhibition (the distance between the edge of the disk and the edge of the bacterial colony) was measured in millimeters (Calin et al., 2009).

Bacteriostatic test in vivo

A total of 40 mice were randomly divided into 5 groups (n = 8 per group): the TBF (7.67, 3.84 and 1.92 g/ml), D-D and blank normal control group. The mice were infected with 0.5 ml mix bacterial suspension (final concentration of 1 × 10^8 to 1 × 10^9 CFU/ml) by intraperitoneal injection. After selecting a concentration that can make mice mortality to be up to 100%, the death rate was observed within 24 h. The mice respectively received TBF for 7 days (twice a day) according to 0.10 ml/10 g. Normal and D-D control group were given distilled water and D-D injection of the same volume. On the eighth day, the mice were infected with 0.5 ml mix bacterial suspension; determined by pre-test and by intraperitoneal injection and observed the death rate of mice in 24 h (Yu et al., 2005).

Anti-inflammatory effect

Xylene-induced ear edema in mice

The mice were divided into five groups of eight. The TBF was administered orally for 6 days at the dose of 0.1 ml/10 g. In addition, dexamethasone (50 mg/kg, i.p.) was as a positive control and the negative control group received distilled water. The seventh day, 60 min after the tested mice were orally administered, Xylene (about 0.03 ml) was applied to the inner and outer surfaces of the right ear by a cotton swab and the left ear was considered as a blank control group.

Ear swellings were measured by vernier caliper (0.02 mm) at 1 h and 4 h intervals in mice, respectively. The edematous response was measured as the ear thickness difference between the right and left. The edema degree was used as the index of inflammation and the anti-inflammatory activity was evaluated by a percentage of the inhibition of edema in treated mice compared with the control mice (Hossein et al., 2000).

Egg white-induced paw swelling in mice

Edema was expressed as the difference between the control and received paws in mm. Kunming mice (n = 8/group) were treated with TBF (7.67, 3.84 and 1.92 g/ml), DEX (50 mg/kg, i.p.) and saline. The TBF was administered orally for 7 days at the dose of 0.1 ml/10 g, twice a day (morning and night). After the last oral administration, the right hind paws received 0.1 ml (20%) (v/v) egg white by subcutaneous injection in mice. The thickness (mm) of the paw was measured at 2, 3, 4 and 11 h interval after the administration (Yu et al., 2013). Mean increase in paw swelling was measured:

Paw swelling = V_t - V_0

Where V_0 is the swelling before fresh egg white injection (mm);
Figure 1. The chemical structure of cryptotanshinone and Tanshinone A.

Figure 2. The effect of TBF and D-D injection on pathogenic bacteria. When compared to the D-D control group; \#P < 0.05.

V_t is the swelling at t (h) after fresh egg white injection (mm).

Cotton pellet granuloma in mice

The mice were randomly divided into 4 groups (n = 10/group): the TBF (7.67, 3.84 and 1.92 g/ml) and control group. The cotton weighing 32 mg were sterilized in vertical pressure steam sterilizer at 121°C for 21 min and impregnated with 0.6 ml of an aqueous solution penicillin and streptomycin. Under ether anesthesia, the cotton pellets were implanted subcutaneously in the back region of the mice. TBF was received twice daily according to 0.10 ml/10 g for 14 days.

Nonetheless, the control groups were given distilled water of the same volume. On day 15, the mice were killed and the pellets and surrounding granulation tissues were dried at 58°C for 24 h. Granuloma weight equal to the weight of the dry subtracted from cotton weight and the weight of granuloma was determined (Yu et al., 2012).

Statistical analysis

The data were expressed as mean values ± SEM. and Statistical analysis was performed using a one-way analysis of variance (ANOVA) (SPSS 20.0).

RESULTS

TBF suppress pathogenic bacteria in vitro

As shown in Figure 2, TBF had significant antibacterial effect on S. aureus, S. epidermidis and Str. Dysgalactiae (P < 0.05) and a certain effect on E. coli (P > 0.05) between TBF and D-D injection control groups. The results showed that TBF could inhibit pathogenic bacteria, including S. aureus, S. epidermidis, Str.
Table 1. Pre-experiment showed the death rate of mice (n = 5).

<table>
<thead>
<tr>
<th>Groups (CFU/ml)</th>
<th>Dosage (ml)</th>
<th>Mice number</th>
<th>Death number</th>
<th>Death rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1.0 \times 10^6$</td>
<td>0.50</td>
<td>5</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>$5.0 \times 10^6$</td>
<td>0.50</td>
<td>5</td>
<td>1</td>
<td>10.00</td>
</tr>
<tr>
<td>$1.0 \times 10^7$</td>
<td>0.50</td>
<td>5</td>
<td>1</td>
<td>40.00</td>
</tr>
<tr>
<td>$5.0 \times 10^7$</td>
<td>0.50</td>
<td>5</td>
<td>4</td>
<td>80.00</td>
</tr>
<tr>
<td>$1.0 \times 10^8$</td>
<td>0.50</td>
<td>5</td>
<td>5</td>
<td>100.00</td>
</tr>
</tbody>
</table>

Table 2. TBF suppressed pathogenic bacteria in vivo (mean ± SEM, n = 10).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dosage (ml)</th>
<th>Mice number</th>
<th>Death number</th>
<th>Death rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High dosage</td>
<td>0.4</td>
<td>10</td>
<td>3</td>
<td>30.00</td>
</tr>
<tr>
<td>Medium dosage</td>
<td>0.4</td>
<td>10</td>
<td>4</td>
<td>40.00</td>
</tr>
<tr>
<td>Low dosage</td>
<td>0.4</td>
<td>10</td>
<td>6</td>
<td>60.00</td>
</tr>
<tr>
<td>D-D control</td>
<td>0.4</td>
<td>10</td>
<td>10</td>
<td>100.00</td>
</tr>
<tr>
<td>Blank control</td>
<td>0.4</td>
<td>10</td>
<td>10</td>
<td>100.00</td>
</tr>
</tbody>
</table>

Figure 3. The data were expressed as the mean ± SD and the results were analyzed by a one-way ANOVA followed by a least significant difference (LSD) tests (*P < 0.05, **P < 0.01 compare to the control).

dysgalactiae and E.coli, respectively. According to the diameter of inhibition zone (Lee et al., 1999), the results showed that it was highly sensitive to Staphylococcus and Str. Dysgalactiae and sensitive to the E. coli. TBF suppress pathogenic bacteria in vivo

As shown in Table 1, pre-experiment showed that the death rate was up to 80% after receiving mixed concentration of $5.0 \times 10^7$ CFU/ml by intraperitoneal injection in mice. From the Table 2, we can know that the mortality of the control group was up to 100%. Whereas the death rate of TBF group was to 30, 40 and 60%. The results suggested that TBF could obviously decrease death rate in a dose-dependent manner.

The effect of TBF on xylene-induced mice ear edema

The clear effect of TBF on xylene-induced mice ear edema is shown in Figure 3. TBF inhibited significantly in a dose-dependent and time-dependent manner by xylene-induced ear edema in mice. After 1 h, we found that 7.67, 3.84, 1.92 g/ml of TBF and DEX (50 mg/kg, i.p.) treated mice reduced swelling by 91.43, 80.39, 73.20...
The data are expressed as the mean ± SD and the results were analysed by a one-way ANOVA followed by a least significant difference (LSD) tests (*P < 0.05, compare with the control group).

and 93.56% (P < 0.05), respectively, when compared with the control group. After 4 h, the groups of TBF and DEX (50 mg/kg, i.p.) reduced swelling by 97.69, 84.38, 76.69 and 95.34 (P < 0.01 and P < 0.05), respectively, when compared with the control group. The results suggest that TBF had obviously inhibited ear edema in mice.

The effect of TBF on egg white-induced mice paw edema

As shown in Figure 4, TBF significantly inhibited paw edema in a dose-dependent and time-dependent manner by egg white-induced in mice. We used DEX as a positive control and physiological saline as a negative control group. Treatment with TBF (7.67, 3.84, 1.92 g/ml) and DEX (50 mg/kg, i.p.) significantly reduced the paw edema by subcutaneous injection. After 4 h, treatment with TBF (7.67, 3.84, 1.92 g/ml) and DEX (50 mg/kg, i.p.) obviously reduced the paw edema by 75.62% (P < 0.05), 46.88, 41.40 and 78.76% (P < 0.05) when compared with the control group, respectively. After 11 h, we found that TBF and DEX-treated mice inhibited edema by 83.16, 80.68, 60.53 and 85.36% (P < 0.05), when compared with the control group, respectively. The results suggest that TBF had obvious effect against paw edema in mice.

The effect of TBF on cotton pellet caused chronic inflammation

TBF inhibited the weight of granuloma by cotton-induced mice as shown in Table 3. The control group of granuloma was heavier than TBF groups. Treatment with TBF (7.67, 3.84, 1.92 g/ml) decreased the weight of granuloma by 49.86, 35.45 and 39.76% (P < 0.01, respectively). The weight of granuloma reduced in a dose-dependent manner by TBF treated in mice. The results suggested that TBF had obvious effect against chronic inflammation in mice.

DISCUSSION

TBF belongs to the Chinese herbal medicine, and it is natural and not only contains alkaloids, polysaccharides,
Table 3. The effect of TBF on the weight of granuloma in mice.

<table>
<thead>
<tr>
<th>Groups (g/ml)</th>
<th>Cotton (mg)</th>
<th>Granuloma (mg)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.67</td>
<td>32</td>
<td>18.12 ± 1.36*</td>
<td>49.86</td>
</tr>
<tr>
<td>3.84</td>
<td>32</td>
<td>23.33 ± 0.67*</td>
<td>35.45</td>
</tr>
<tr>
<td>1.92</td>
<td>32</td>
<td>21.77 ± 1.32*</td>
<td>39.76</td>
</tr>
<tr>
<td>Normal control</td>
<td>32</td>
<td>36.14 ± 1.52</td>
<td>-</td>
</tr>
</tbody>
</table>

The effects of TBF on cotton pellet-induced mice granuloma (n = 10/group). The data with ** mean significant difference compared with control group (P < 0.01), least significant difference (LSD) tests.

saponins, volatile oil, anthracene, effective biological activity material, but also contains minerals, vitamin and nutritional factors, which has prevention, treatment and nutrition effect. Previous studies have indicated that S. miltiorrhiza Bunge has anti-bacterial activity against Gram positive bacteria (Li et al., 2011). The main components of TBF contain C. tanshinone and Tanshinone IIA, and it has been reported that they had antibacterial effects on pathogen.

In our study, the results showed that TBF suppressed pathogenic bacteria (S. aureus, S. epidermidis, Str. dysgalactiae and E. coli) in vivo and vitro (Figure 2, Table 2). It was concluded that TBF had the potential protection to inhibit bacterial infection. For example, C. tanshinone demonstrated effective antibacterial activity against all 21 S. aureus strains tested in vitro (Haihua et al., 2009). Tanshinone IIA had antibacterial activity against a broad range of bacteria (Zhu and Luo, 2004). C. tanshinone and Tanshinone IIA had anti-bacterial effect (Mothana et al., 2009). C. tanshinone inhibited microbial activity against a broad range of Gram-positive and Gram-negative bacteria as well as other microorganisms (Tang et al., 2004; Honda et al., 1988).

In our present study, TBF effectively and significantly reduced cotton pellet-induced granuloma (Table 3), xylene-induced mice ear edema (Figure 3) and egg white-induced mice paw swelling (Figure 4), thereby suggested its activity in the proliferative phase of inflammation. Treatment with TBF (7.67, 3.84 and 1.92 g/ml) inhibited exudative inflammation in a dose-dependent manner, thus the data suggested that TBF possessed an anti-chronic inflammatory effect. Pre-experimental study found that S. miltiorrhiza has anti-inflammatory effect and not only against bacterial effect directly (Zhe et al., 2013), but can also regulate the immune function and enhance the antibacterial potential inherent in the body.

Inhibition of inflammatory mediators, improve the inflammation state and circulation, protect vascular, suppression, elimination of oxygen free radicals, the C reaction protein increased and decreased and with antibacterial function (Hao-lun et al., 2010). Pre-experimental study also found that Tanshinone IIA has anti-inflammatory (Min et al., 2015). Tanshinone IIA had a protective effect against spinal cord injury through inhibiting the inflammatory response (Yin et al., 2012). Tanshinone IIA, one of the key components of TBF, had been reported to possess the majority of Danshen’s properties with few side effects. Some studies have suggested that Tanshinone IIA inhibits the production of pro-inflammatory mediators such as TNF-α, NO, IL-1β and IL-6 (Jang et al., 2003) by the inhibition of NF-κB activity in RAW 264.7 cells stimulated with LPS, which were mediated by estrogen receptor activation (Fan et al., 2009). In conclusion, TBF had obvious effect against acute inflammation and chronic inflammation in mice and suppressed pathogenic bacteria in vitro and vivo.

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Author Contributions

WCYYB FWY designed and conceived the experiments; WCYFWYLM XHX RJZ performed the experiments; WCYJZFZ analyzed the data; WCYFWYYB LMD XHX RJZ contributed reagents/materials/analysis tools; WCYFWYYB wrote the paper.

Conflicts of interest

The authors declare that they have no competing interests.

REFERENCES


