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Aqueous extract of Bai-Hu-Tang, a classical Chinese herb formula, prevents excessive immune response and liver injury induced by LPS in rabbits

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\textbf{Article info}

\textbf{Article history:}
Received 6 February 2013
Received in revised form 10 May 2013
Accepted 24 June 2013
Available online 1 July 2013

\textbf{Keywords:}
Traditional Chinese medicine
Bai-Hu-Tang
LPS
Liver damage
Immunomodulation

\textbf{Abstract}

\textbf{Ethnopharmacological relevance:} Bai-Hu-Tang (BHT) was traditionally used to reduce fever heat and promote generation of body fluids.

\textbf{Aim of the study:} To investigate the effect and mechanism of BHT in the prevention of lipopolysaccharide (LPS) fever in manners of immune modulation.

\textbf{Materials and methods:} The model of fever syndrome of Chinese medicine pattern was imitated by LPS injection i.v. in rabbits, and BHT was gavaged. The serum levels of tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), interleukin (IL-6, 10) and immunoglobulin (IgG, IgA, and IgM) were determined by enzyme-linked immunosorbent assay (ELISA); alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were tested by biochemical methods. Liver tissue damage was detected by hematoxylin–eosin (H&E) stain. Subpopulation of T cells was detected by Fluorescence Activated Cell Sorter (FACS). Genes expression of Toll-like receptor 4 (TLR4) and lipopolysaccharide binding protein (LBP) in liver tissue were assayed by real-time polymerase chain reaction (RT-PCR).

\textbf{Result:} The results demonstrated that BHT prevented sudden increase of IL-10, TNF-\(\alpha\), ALT and AST, and liver damage induced by LPS. BHT also prevented significant decrease of the percentage of CD\(^8\)+ T cells since LPS injection. At the same time, BHT did not affect the gene expression of TLR4 and serum concentration of three immunoglobulins, which were increased by LPS, but made gene expression of LBP higher.

\textbf{Conclusion:} The results of this study indicated that BHT played an important role in immunity protection and anti-injury through preventing immunoinflammatory damage by LPS. The achievement thereby scientifically provided mechanism of BHT in the prevention of febrile disease, and supported its traditional use.

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1. Introduction

Fever is a reaction of homeothermic animals and humans when thermogenic substance entered the body and interacted with the cells of the immune system (Riedel and Maulik, 1999). It was a common response to infection, inflammation and trauma. Clinically, febrile response was characterized as a rise in body temperature above the normal range, and often treated with eliminating excess heat (Lisa, 2002). Although has different clinical courses, many communicable and infectious diseases can be classified into epidemic febrile disease according to therapies of traditional Chinese medical (TCM) such as severe acute respiratory syndrome (SARS) (Peng, 2003; Shen, 2003; Gu et al., 2005), avian influenza A (H5N1) (Li, 2006; Nie and Lin, 2007), and swine influenza A (H1N1) (Zhou, 2009; Liu, 2009; Zheng et al., 2010). Because of the common feature of high fever, these diseases could be treated with antipyretic prescription according to principle of febrile disease treatment in TCM.

Lipopolysaccharide (LPS), a bacterial endotoxin, is a group of heat stable molecules present in the outer membrane of Gram-negative bacteria that possesses toxic effect (Burell, 1994). The injection of LPS was a reasonable model of bacterial infection, and literatures were replete with records that LPS caused a typical
fever with intravenous or intraperitoneal injection (Shibata et al., 2005; Steiner et al., 2006; Ravaneli et al., 2007), and researchers often used LPS to induce reproducible febrile responses in animals (Roth et al., 2002). Actually, Chinese researchers believed that an injection of LPS can induce a representative fever syndrome of Chinese medicine pattern in rabbit, and the fever pattern can be used to do studies for antipyretic traditional herb medicine and ethnopharmacological research (Yang, 2002; Yu et al., 2010; Ai et al., 2011; Ni and Wei, 2012).

The traditional Chinese medicine had cured over 2000 years of sage advice, making it one of the oldest and most widely used systems of medicine in the world. Herbal medicine is one of the most essential elements of TCM, and Bai-Hu-Tang (or White Tiger Decoction) is a classical natural Chinese herbal formula with a long history of use. The formula originated from the treatise on febrile disease of Shang Han Lun (or Treatise on Cold-Attack) compiled by Zhong-Jing Zhang who lived in Eastern Han Dynasty in China. It was formulated with four herbs of Liquorice, Anemarrhena Rhizome, Gypsum and Rice, and mainly used to reduce fever and promote generation of body fluids (Charles, 2000; Zhu, 2007). It was also useful in treating diabetes mellitus (Chen et al., 2008), eczema, pruritus, some anxiety and emotional disorders (Herb Toxicities and Drug Interactions: A Formula Approach, 2004). The formula has caused wide public concern over the recent years and practiced in many countries (European Herbal and Traditional Medicine Practitioners Association, 2007), and even some international press also published works for user guide (Herb Toxicities and Drug Interactions: A Formula Approach, 2004). Therefore, the BHT is an important traditional Chinese formula that has been extensively used to prevent febrile disease in the world. But there was little basic theory report on its mechanism and effect.

In this study, a febrigenic dosage of LPS (15 μg/kg i.v.) was injected into rabbits to form an animal febrile model, then the model animals were gavaged with BHT at the same time, and the injection of LPS can induce a representative fever syndrome of Chinese medicine pattern in rabbit, and the fever pattern can be used to do studies for antipyretic traditional herb medicine and ethnopharmacological research (Yang, 2002; Yu et al., 2010; Ai et al., 2011; Ni and Wei, 2012).

The herbs of Liquorice (sliced root of Glycyrrhiza glabra L. in Leguminosae), Anemarrhena Rhizome (sliced root of Anemarrhena asphodeloides Bunge in Liliaceae), Gypsum (crystal of calcium sulfate), and Rice (nonglutinous rice, polished seed of Oryza sativa L. in Gramineae) were purchased from Antaitang Pharmaceutical Co., Ltd., China, and identified by Dr. Zuoqing Yan from Lanzhou Institute of Animal & Veterinary Pharmaceutics of Chinese Academy of Agricultural Sciences. As shown in Fig. 1, all of the herbs sample were deposited in a TCM Specimen Room with voucher numbers No. 100720 for Liquorice, No. 101208 for Anemarrhena Rhizome, No. 100906 for Gypsum, and No. 101202 for Rice.

Glycyrrhizae Radix 7.2 g, Anemarrhena Rhizome 21.8 g, Gypsum 60.2 g and Rice 10.8 g were extracted together by refluxing with boiling water twice. They were boiling water (1:10, w/v) for 2 h, and water (1:5, w/v) for 1 h, respectively. Two-time juice was blended together, filtered with three-layer gauzes, and concentrated to 100 ml experimental decoction. The experimental juice was autoclaved at 121 °C for 15 min, and kept in airtight containers at 4 °C until used.

2. Materials and methods

2.1. Materials

2.1.1. Herbal materials and BHT extract

The herbs of Liquorice (sliced root of Glycyrrhiza glabra L. in Leguminosae), Anemarrhena Rhizome (sliced root of Anemarrhena asphodeloides Bunge in Liliaceae), Gypsum (crystal of calcium sulfate), and Rice (nonglutinous rice, polished seed of Oryza sativa L. in Gramineae) were purchased from Antaitang Pharmaceutical Co., Ltd., China, and identified by Dr. Zuoqing Yan from Lanzhou Institute of Animal & Veterinary Pharmaceutics of Chinese Academy of Agricultural Sciences. As shown in Fig. 1, all of the herbs sample were deposited in a TCM Specimen Room with voucher numbers No. 100720 for Liquorice, No. 101208 for Anemarrhena Rhizome, No. 100906 for Gypsum, and No. 101202 for Rice.

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2.1.2. Chemicals and reagents

LPS (Escherichia coli O55:B5) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and dissolved in saline (0.9% NaCl) at clean benches. Both of IL and Ig serum active ELISA kits were purchased from R&D Techno Co. (Minneapolis, MN, USA). Monoclonal antibodies of mouse anti-rabbit CD4+ and CD8+ FITC were obtained from ABD Serotec (Kidlington, OX5 1GE, UK). Both of one-step RT-PCR kit (DRR055A) and SYBR Premix Taq kit (DRR081A) for real-time PCR were provided by TaKaRa Biotech Co. (Dalian, China). RNA fixer was purchased from Aidlab Biotechnologies Co. (Beijing, China). Primers for target genes were synthesized by Beijing
Genomics Ins. (Beijing, China). Trizol Reagent was purchased from Invitrogen Corporation (Carlsbad CA, USA).

2.2. Animals and treatment

Adult New Zealand white rabbits weighted between 2.0 and 2.5 kg were used in the experiments, and housed individually in rabbit stocks under controlled room humidity of 50 ± 5% and temperature of 25 ± 1 °C with a 12 h light and 12 h darkness cycle. Animals were fed commercial stock diet and water ad libitum, and allowed to stabilize for at least 3 d in new surroundings before any experiments. All experimental animals were obtained from the animal center of Lanzhou Institute of Biological Products (Lanzhou, China). The rabbits were divided into three groups randomly. Group I served as control, and received only physiological saline; group II was fever model, which received only LPS (15 μg/kg body weight) by intravenous injection (i.v.) into ear vein; group III was gavaged with BHT (a representative dose of 7 ml/kg body weight) at the same time of LPS intravenous injection. Rabbits then received standard diet and water ad libitum.

2.3. Collection of blood and tissue samples

At the time of 6 h after LPS injection and BHT administration, full conscious rabbits were immobilized on anchor platform through binding limbs outside body, and 6.5 mL blood was taken from heart by cardiac puncture with disposable syringe. After centrifugation (4 °C, 3000 rpm, 5 min) to get serum. 1.5 mL blood was placed into EDTA-K tubes, and mixed to form anticoagulant blood. The serum sample was used for biochemical assays of IL-6, IL-10, and TNF-α. At the same time, anti-coagulated whole blood samples from EDTA-K tubes were used to analyze T lymphocyte subpopulations of CD4+ and CD8+. Some liver tissues of rabbits were cut away, flushed in saline (0.9% NaCl), and immersed in formalin for pathology analysis. One more part of liver tissue was immersed in RNA Fixer, and stored at −80 °C for total RNA extract.

2.4. T lymphocyte subpopulations assay in blood

CD4+ T cells and CD8+ T cells were identified using directly conjugated anti-rabbit monoclonal antibodies. Briefly, 100 μl peripheral blood sample anti-coagulated by EDTA-K2 were incubated in the dark with 10 μl of the mouse anti-rabbit CD4+FITC or mouse anti-rabbit CD8+FITC antibody for 20 min at 4 °C, and washed with 500 μl of FACS (Fluorescence Activated Cell Sorter) buffer. According to manufacturer, red cells were lysed by using 2 ml lysis solution (Becton Dickinson). The remaining leukocytes were washed with PBS (pH 7.2) twice and then fixed with 0.2 ml FACS Fix solution (Becton Dickinson). Fixed leukocytes were re-suspended in 1 ml PBS, and analyzed in a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson). Fluorescence data were collected from 2 × 10^6 cells, and analyzed by using CELLQUEST software (Becton Dickinson).

2.5. Measurement of immunoglobulin and interleukin levels in serum

The serum samples were kept at −20 °C until ready for measurement. According to the manufacturer's protocol, levels of IgG, IgA, IgM, IL-6, IL-10, and TNF-α were measured by using commercially available ELISA kits.

2.6. Serum biochemical assays

The levels of ALT and AST in serum samples were determined using an automatic biochemistry analyzer and biochemical kits (Mindray Biomedical Electronics Co., Shenzhen, China), following the manufacturer's instruction.

2.7. Pathological observe in liver tissue

Liver tissue was fixed in 10% neutral-buffered formalin, and embedded in paraffin. Sections of 5-μm thickness were affixed to slides, deparaffinized, stained with hematoxylin–eosin (H&E) for general histopathology examination under a light microscope (Olympus, Japan), and assessed by a pathologist blind to the treatment groups.

2.8. Relative-quantification of gene expression by RT-PCR

A two-step process was employed to determine relative quantity of selected genes. Briefly, total RNA was isolated from the liver using the Trizol Reagent according to the manufacturer's instructions and then 2 μg total RNA was reverse transcribed to cDNAs by use of an RT-PCR kit following the manufacturer's directions. The information of PCR primers were shown in Table 1. PCR was performed for 39 cycles using the following conditions: pre- incubation at 95 °C for 30 s, denaturation at 95 °C for 5 s, annealing at 60 °C for 25 s, and elongation at 72 °C for 25 s. The PCR products were also verified by ethidium bromide-stained agarose gel electrophoresis (data not shown). For each real-time PCR sample was performed for target gene and the housekeeping gene (β-actin). Real-time PCR was performed for quantification of gene expression of TLR4 and LBP, and using the quantitative PCR Super Mix in a final reaction volume of 25 μl in 2 × SYBR green (Molecular Probes) according to the manufacturer's protocol. The data were expressed as the number of threshold cycle (Ct). The relative quantification of the target genes was determined by calculating the ratio between concentration of target gene and that of house-keeping gene. For each real-time PCR analysis, the individual experiments were performed in triplicate.

**Table 1**

| Gene ID | Gene symbol | Sequence | Size of PCR products |
|--------|-------------|----------|----------------------|
| XM_002712153 | β-actin | Forward primer 5'-CATGGGTGATGATCCGCTGC-3' Reverse primer 5'-GCACGGCTACGCTCAC-3' | 153-bp |
| M35534 | LBP | Forward primer 5'-GGCGTACGCTGCT-3' Reverse primer 5'-GACGAAGGT-3' | 149-bp |
| AV10394 | TLR4 | Forward primer 5'-GGGACACACCCCTGACCTC-3' Reverse primer 5'-GAAAGGTCCAGGTGCTCAAGG-3' | 153-bp |
2.9. Statistical evaluation

Data were reported as the means ± standard deviation (S.D.), and analyzed by using t-test for comparisons of significance level (P) between the control and the treated values. P < 0.05 was considered to be statistically significant, and if it was not significant then the P value was not less than 0.05. Calculations were made with the commercially available software SPSS 13.0.

3. Results

3.1. BHT prevented inflammatory cytokines sudden increase induced by LPS

Fig. 2 highlights that LPS significantly increased TNF-α, IL-6, and IL-10, especially a sudden IL-10 signaling that seems to become acute and life-threatening. After treatment with BHT, IL-10 decreased completely, and TNF-α reduced partially. Although IL-6 was still higher than control, it was lower than LPS group significantly. These data suggested that LPS led to an increase of inflammatory cytokines in rabbits, but BHT might prevent the sudden increase, and protect animals from the injury of the immoderate inflammatory response.

3.2. BHT prevented LPS-induced hepatic injury

As biochemical marker for liver function, the serum levels of hepatic enzymes AST and ALT were elevated significantly (P < 0.05) by LPS injection. Due to the work of BHT, the elevation of these marker enzymes was significantly prevented (Fig. 3A and B). Fig. 3C showed that liver pathological change of animals injected with LPS. It showed centrilobular necrosis, infiltration of immunity cells (such as macrophages and lymphocytes) into portal tract and sinusoid, hepatocytes ballooning, necrosis, and disintegration. Diffused areas of necrotic lesion, especially in the perivenular region which extends to the central zone, with collections of inflammatory cells, were observed after LPS injection when compared to control. Because of the absence of cellular necrosis and inflammatory infiltrates in the liver, it was evident that BHT prevented hepatic lesions produced by LPS, which was almost comparable to the control. These data indicated that BHT prevented liver injury and dysfunction induced by LPS in rabbits.

3.3. BHT prevent LPS-induced CD8⁺ cell decrease

The T cells subpopulation of CD4⁺ (Th cells) and CD8⁺ (Tc cells) in peripheral blood were determined by flow cytometry. The results showed that there was no change of CD4⁺ cells percentage

Fig. 2. Bai-Hu-Tang (BHT) inhibited inflammatory cytokines increase. The inflammatory cytokines of TNF-α (A), IL-6 (B) and IL-10 (C) suddenly increased by LPS injection (LPS vs. control, *p < 0.05), but decreased significantly when prevented by BHT, respectively (LPS+BHT vs. LPS, #p < 0.05).

Fig. 3. Bai-Hu-Tang (BHT) decreased significantly the serum levels of hepatic enzymes ALT (A) and AST (B), and attenuated obviously immunoinflammatory liver damage (C). As biochemical markers for evaluation of hepatic injury, the results of biochemical assays revealed that BHT has obvious protective action for liver functions (LPS vs. control, *p < 0.05; LPS+BHT vs. LPS, #p < 0.05), and H&E stain indicated that BHT also protected liver tissue from LPS injury (100 × ).
(Fig. 4A), but the percentage of $CD^8^+$ cells decreased significantly. Moreover, serum IgG, IgM, and IgA concentrations soared up after LPS injection. Due to administration of BHT, the percentage of $CD^8^+$ cells was not decreased by LPS (Fig. 4B), but enhanced immunoglobulins were still kept at a high level (Fig. 4C–E).

### 3.4. BHT prevented excessive expression of TLR4 as LPS receptor in liver tissue

The RT-PCR semi-quantitation demonstrated that mRNA of TLR4 and LBP were up-regulated significantly after LPS injection. Due to the role of BHT, the percentage of $CD^8^+$ cells was not decreased by LPS (Fig. 4B), but enhanced immunoglobulins were still kept at a high level (Fig. 4C–E).

### 4. Discussion and conclusions

Cytokines play an essential role in mediating interactions between cells of the immune system (Fletcher and Starr, 2005). A sprinkling of cytokine can be specific responses to apart from infections and get transition into recovery, but an excessive cytokine production may cause the immune system out of control, and cause the precipitation of pathological consequences (Allison and Rosenthal, 2010). Some diseases of fever, kidney dysfunction, and liver problem could be attributed to body's response to excessive cytokine production that storm physiology of the body, such as IL-6 and TNF-α (Clark, 2007). This virulent immune response with production of large amounts of inflammatory cytokines often was named as immunoinflammatory response, and played the most important role in the pathogenesis of liver damage and dysfunction (Seely and Christou, 2000; Wang and Ma, 2008; Aldridge et al., 2009). The results demonstrated that liver pathological changes were observed significantly in LPS-treated animals, and the serum concentrations of ALT and AST, which were functional readout for liver damage, increased drastically in LPS injection animals, but decreased to normal level with BHT prevention (Fig. 3). These changes were accompanied by changes of inflammatory cytokines, especially sudden change of IL-10 signaling (Fig. 2C). There were no significant changes of other cytokines of IL-2, IL-4, and IFN-γ (data not shown). This raised the intriguing possibility that excessive production of TNF-α, IL-6, and IL-10 (Fig. 2), which also caused excessive immune response, may be the direct cause of liver damage, and BHT was able to prevent the pathological process.
Because the activation of the innate immune response can be a prerequisite for triggering of acquired immunity (Akira et al., 2001), the adaptive immune system always works in synchronization with innate immunity (Abdelsalik and Trad, 2011). Depending on the combination of cytokines produced in response to the stimulus, innate and adaptive immune responses are initiated. As reported, LPS-induced proliferation of lymphoid cells is thought to be primarily restricted to B cells in acquired immune (Tough et al., 1997), and nearly all B cells require the help of Th cells before they can mature and differentiate into antibody-secreting plasma cells. The conventional T cells played a critical role in tempering the immune response recognized by innate immune system (Kim et al., 2007). In this study, the results of immunoglobulins and T cell subpopulations showed that humoral immunity mediated by B cells was activated by LPS without CD4+ cell (Th cells) increase, but cell immunity carried out by CD8+ T cells (Tc cells) was inhibited by LPS. Due to the role of BHT, the CD4+ cell decrease was prevented totally (Fig. 4B), but enhanced humoral immunity was still kept at a high level (Fig. 4C–E). Therefore, BHT did not affect the activated adaptive immunity, but prevented the cell immunity inhibition and excessive cytokines in innate immunity by LPS at the same time.

In addition to cytokines, an important role in innate immunity has been ascribed to TLR family (Lenert, 2006). Different TLR recognizes the different pattern-recognition receptors (PRRs) expressed on the effector cells of immune system, such as TLR1 recognized lipopolysaccharides, TLR3 recognized double-stranded RNA, and TLR4 recognized LPS (Akira et al., 2006; Kawai and Akira, 2011). Actually, TLRs as main PRRs of immune system played an important role in linking between rapid defense of innate immunity and delayed eradication of adaptive immunity (Wertling and Jungi, 2003; Pasare and Medzhitov, 2004). It was reported that TLR4 played an indispensable role in triggering LPS fever, and the phase of febrile response to LPS depended entirely on the TLR4 (Steiner et al., 2006). Because LPS firstly arrived at liver that is the main LPS-processing and clearance organs after intravenous administration (Li and Blatteis, 2004), it was ponderable to investigate expression of functional genes in liver. The gene expressions of TLR4 studied herein were not affected by BHT (Fig. 5A), but LBP expression was increased (Fig. 5B), which bind and transport LPS to activate cells in the immune system (Knapp et al., 2006), and inhibiting inflammatory response at higher concentration (Hamann et al., 2005). This indicated that BHT may play an important role in avoiding excessive immunity, and the elevated expression of TLR4 may be benevolent for the initiation of the following adaptive immunity linked by TLR4.

In conclusion, BHT prevented excessive cytokines increase, and protected animals from liver damage by LPS. BHT also defined CD8+ T cell immunity against LPS, but did not affect adaptive immunity mediated by B cell and linked by TLR4. Therefore, BHT played an important role in immunomodulation and anti-injury in the treatment of febrile disease.

Acknowledgments

This research was supported by the National Key Science and Technology Support Project in the 11th Five-year Plan of China (2008BADB4B01-2) and the Central Scientific Research Institutes for Basic Research Fund of China (1610322012004). Gratitude is also sent to the anonymous and editor whose suggestions greatly improved the manuscript.

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