Anti-inflammatory effects of Huang-Lian-Jie-Du decoction, its two fractions and four typical compounds

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Ethnopharmacological relevance: Huang-Lian-Jie-Du decoction (HLJDD) (Oren-gedoku-to in Japanese) as a famous traditional Chinese recipe is composed of Rhizoma coptidis, Radix scutellariae, Cortex phellodendri and Fructus gardeniae. It has been used to treat inflammation for nearly two thousand years.

Aim of the study: To explore the material base for the anti-inflammatory activity of formula HLJDD, its extract was fractionated on D101 macroporous resin to afford two fractions, HLJDD-1 and HLJDD-2. The whole formula, HLJDD-1 and HLJDD-2, and four typical component compounds were then evaluated for their effects on inflammation-related parameters using lipopolysaccharide (LPS)-induced RAW264.7 cells as a model system.

Materials and methods: The effect of HLJDD on carrageenan-induced mice paw edema was first evaluated. A series of inflammation-related parameters including malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD), prostaglandin E2 (PGE2), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10) were then measured in LPS-induced RAW264.7 cells treated with HLJDD, its two fractions, and four typical component compounds (geniposide, baicalin, berberine and baicalein).

Results: With the help of principal component analysis (PCA) technique, the data obtained revealed that the two fractions and the major group of compounds in HLJDD (iridoids, flavonoids and alkaloids) complement each other with particular emphasis to synergistically exert anti-inflammatory effects.

Conclusions: This study demonstrated that HLJDD exhibited anti-inflammatory effect as a “whole”, which justified the combined use of the four component herbs forming the compound prescription and suggested quality control of HLJDD based on its three types of components.

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1. Introduction

Huang-Lian-Jie-Du decoction (HLJDD) is a preparation consisting of Rhizoma coptidis (Coptis chinensis Franch, Ranunculaceae), Radix scutellariae (Scutellaria baicalensis Georgi, Labiatae), Cortex phellodendri (Phellodendron amurense Rupr. Rutaceae) and Fructus gardeniae (Gardeania jasminoidae Ellis, Rubiaceae) in a weight ratio of 3:2:2:3. This formula was described by Wang Tao (in the Chinese Tang Dynasty) in his treatise “Wai Tai Mi Yao”. It has been used to treat inflammation, hypertension, gastrointestinal disorders, and liver and cerebrovascular diseases (Cao et al., 1996) in the clinical practice of Traditional Chinese Medicine. Oral administration of HLJDD significantly inhibited the inflammatory responses in carrageenan injected rat air pouches, and also greatly reduced the production of leukotriene (LTB4) in vivo without any influence on the biosynthesis of cyclooxygenase (COX)-derived eicosanoids (Zeng et al., 2009). Though the main components of the whole formula and individual herb of HLJDD have been reported (Sun et al., 2006), principles in HLJDD responsible for anti-inflammatory effects remain unclear, hindering the rational use of this formula.

Inflammation involves a complex web of intercellular cytokine signals (Han and Ulevitch, 2005) and is implicated in the pathogenesis of many diseases, including cancer, diabetes, cardiovascular, neurodegenerative and other life-threatening and debilitating diseases (Lawrence et al., 2002). Macrophages play a central role in the inflammatory response, and serve as an essential interface between innate and adaptive immunity (Adams and Hamilton, 1984). In the process of inflammatory response, macrophages release nitric oxide (NO), a reactive molecule originated from the guanidino nitrogen of L-arginine, catalyzed by nitric oxide synthases enzymes (NOS) and other cytokines, e.g. interleukin-6 (IL-6) (Kock et al., 1990). Excessive NO production leads to the development of many inflammatory related diseases (Skidgel et al., 2002). Prostaglandin E2 (PGE2), generated by specific COX-2 function, was another anti-inflammatory parameter (Surh et al., 2001). Moreover, MDA and SOD, due to their contributions to the alleviation of inflammatory responses (Cherubini et al., 2005; Baskol et al., 2007), were both detected for their levels to assess the radical scavenging abilities.
of samples. Macrophages also play an important role in inflammatory diseases relating to overproduction of pro-inflammatory cytokines including interleukin-10 (IL-10), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (Holm et al., 2009). Elevated productions of these mediators have been detected in many tissues after exposure to immune stimulants including LPS (Stenvinkel et al., 2005). Thus, these inflammatory mediators, MDA, NO, SOD, PGE2, IL-6, IL-10 and TNF-α, are important targets in the treatment of inflammatory diseases.

In this study, HLJDD was first chromatographed over D101 macroporous resin eluted with aqueous ethanol in gradient to afford two fractions, the 30% (HLJDD-1) and the 100% (HLJDD-2) eluents. The two fractions and four typical compounds from HLJDD, geniposide, baicalin, berberine and baicalein (Fig. 1), were then evaluated for their effects on several inflammation-related parameters including MDA, NO, SOD, PGE2, IL-6, IL-10 and TNF-α in LPS-induced RAW264.7 cells.

2. Materials and methods

2.1. Chemicals

HPLC-grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Water was purified using a Milli-Q50 SP water purification system (Millipore, Bedford, MA, USA). The other reagents were all of analytical grade and purchased from Nanjing Chemical Company, China. Carrageenan (type IV) was bought from Sigma Chemical Company, USA.

2.2. Materials

Component herbs of HLJDD, Rhizoma coptidis, Radix scutellariae, Cortex phellodendri and Fructus gardeniae, were purchased from Kaixin Herbal Shop (Nanjing, China) and identified by Professor Mian Zhang, Department of Medicinal Plants, China Pharmaceutical University, Nanjing, China. Standards of baicalin and baicalein (2) eluents. The two fractions and four typical compounds from HLJDD, geniposide, baicalin, berberine and baicalein (Fig. 1), were then evaluated for their effects on several inflammation-related parameters including MDA, NO, SOD, PGE2, IL-6, IL-10 and TNF-α in LPS-induced RAW264.7 cells.

2.3. Preparations of HLJDD and its two fractions

Rhizoma coptidis, Radix scutellariae, Cortex phellodendri and Fructus gardeniae were mixed in a ratio of 3:2:2:3, reaching a total weight of 100 g. The mixture was decocted twice under refluxing with 70% ethanol (1:10 and then 1:5, v/w) for 2 h, the solution obtained was concentrated to dryness on a rotary vacuum evaporator, affording 29.31 g extract (yield: 29.31%). Part of the extract (5.0 g) was dissolved in water, and subjected to D101 macroporous resin, eluted with a gradient ethanol–water (30:70 and 100:0) to give two fractions: HLJDD-1 (yield: 11.20%) and HLJDD-2 (yield: 17.56%).

2.4. HPLC analysis

Chromatographic analysis was performed on a Shimadzu LC-2010 series equipped with a Shimadzu SPD-M10A photodiode array detector (Shimadzu, Kyoto, Japan). The chromatographic separation was performed on an Ultimate XB-C18 column (250 mm × 4.6 mm, ID 5 μm, Welch Materials, Inc., USA) with the column temperature at 30 ºC. Mobile phase was composed of two parts: (A) 10 mmol/L ammonium acetate in water (pH 3.0, titrated with acetic acid); (B) acetonitrile, in a gradient program: 0–4 min, 10% B; 4–15 min, 10–26% B; 15–27 min, 26–28% B; 27–35 min, 28–70% B; 35–55 min, 70–90% B; 55–60 min, 90% B. The flow rate was set at 1 mL/min and the injection volume was 5 μL.

2.5. Quantification of geniposide, baicalin, berberine and baicalein in HLJDD

The doses of compounds used in in vitro experiment were determined to maintain the same levels of compounds as in HLJDD. Therefore, the contents of geniposide, baicalin, berberine and baicalein in HLJDD were quantitatively analyzed. The extract powder was ultra-sonicated with 70% ethanol for 1 h; the suspension was then diluted 100 times. After centrifuging at 15,000 rpm for 10 min, the supernatant was analyzed by HPLC. The contents of geniposide, baicalin, berberine and baicalein in HLJDD extract were 1.65%, 4.17%, 5.12% and 0.96%, respectively.

2.6. Animals

The animal studies were approved by the Animal Ethics Committee of China Pharmaceutical University. Male Kunming mice (25–30 g) were obtained from Animal Multiplication Centre of Qionglong Mountain (Nanjing, China). The mice were housed in an air-conditioned room at 22–24 °C with a 12 h dark/light cycle and were allowed food and water spontaneously. Mice were fasted for 16 h prior to their use for the assay (Ruben et al., 2009). The animals were randomly assigned to two groups: control and HLJDD, each 12 mice.

2.7. Carrageenan-induced mice paw edema

Pedal inflammation in mice was produced as described previously (Winter et al., 1962). Mice were administered orally with 20 mL/kg distilled water or 400 mg/kg HLJDD (Wang and Xu, 2000) for 5 days continuously. Paw edema was induced by injecting 0.05 mL of 1% carrageenan saline solution into hind paw of each mouse 1 h after the fifth administration of distilled water or HLJDD. The volume of injected paws was measured by a plethysmometer (YLS-7B, Beijing, China) before injection (V0), and at 1, 2, 3, 4 and...
5 h \( (V_t, t = 1–5\, \text{h}) \) after the first injection \( (\Delta V = V_t - V_0) \). For each animal, the anti-inflammatory effect of the drugs was expressed as a percentage of edema inhibition (Femandez-Duenas et al., 2008):

\[
\text{% inhibition} = \frac{\Delta V_{\text{control}} - \Delta V_{\text{HLJDD}}}{\Delta V_{\text{control}}} \times 100
\]

2.8. Cell culture

RAW264.7 mouse macrophage cells were obtained from the Chinese Academy of Science Cell Bank (Shanghai, China). Cells were cultured at 37 °C in 1640 medium supplemented with 10% heated-deactivated FBS, penicillin (100 U/mL), streptomycin (15 mM) in
a humidified atmosphere of 5% CO2 and passaged when about 85% confluence were achieved with trypsinase solution [0.25%, dissolved in phosphate buffer saline (PBS)].

2.9. Drug pre-treatment

For HLJDD treated group, the doses were set at 10⁻⁴, 5 × 10⁻⁵, 10⁻⁵, and 5 × 10⁻⁶ g/mL. The doses of HLJDD-1, HLJDD-2, geniposide, baicalin, berberine and baicalein were determined according to the contents of compounds in HLJDD, adjusted to maintain the same levels as in HLJDD (Table 1).

2.10. MTT assay

Cells were cultured at a density of 1 × 10⁵ cells in a 24-well cell culture plate with 100 μL of culture medium. After 6 h, cells were treated with samples. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution in PBS (20 μL) were added to each well reaching a final concentration of 0.5 μg/mL after 48 h and the cells were further incubated at 37 °C for 4 h. The medium was then removed, 100 μL DMSO was added to each well to solubilize the MTT-formazan product. After thorough mixing with a mechanical plate mixer for 3 min, absorbance at 570 nm was measured with a microplate reader (iQuantTM, BIO-TEK Instrument Inc., Winooski, VT).

2.11. Measurements of NO, MDA, SOD, PGE2, IL-6, IL-10 and TNF-α level

RAW264.7 cells were plated at a density of 1 × 10⁵ cells in a 24-well cell culture plate with 500 μL culture medium, and incubated for 24 h. The cells were pre-treated with samples for 2 h and stimulated with LPS (2 μg/mL) for 18 h with dexamethasone (DXM) as positive control, the dose of which was decided based on our preliminary experiments and the literature (Montanher et al., 2007). The levels of NO, MDA and PGE2, and pro-inflammatory cytokines (IL-6, IL-10 and TNF-α) increased in LPS group while the activity of SOD decreased significantly as compared with the control group, demonstrating the successes of the model. Effects of HLJDD, its two fractions and four component compounds on the production of NO, MDA, PGE2, and pro-inflammatory cytokines (IL-6, IL-10 and TNF-α), and on the

2.12. Statistical analysis

Results were expressed as mean ± S.D. and all statistical comparisons were made by means of a one-way ANOVA test followed by Dunnett’s t-test.

3. Results

3.1. HPLC profiles of HLJDD and its two fractions

The major components in HLJDD and its two fractions were analyzed with HPLC (Fig. 2). By comparison with the standard compounds or based on the LC–MS results, the main components in HLJDD were revealed to be geniposide, baicalin, baicalein, berberine, palmatine, jatrorrhizin and coptisine. These principles belong to three structure groups, iridoids, flavonoids and alkaloids, of which, iridoids and flavonoid glycosides are the main components in HLJDD-1, and alkaloids and flavonoid aglycones are the major components in HLJDD-2.

3.2. Anti-inflammatory activities evaluation

HLJDD significantly inhibited carrageenan-induced paw edema in mice (Fig. 3) in an initial experiment. A model was then established using LPS-treated RAW264.7 cells. The levels of NO, MDA and PGE2, and pro-inflammatory cytokines (IL-6, IL-10 and TNF-α) increased in LPS group while the activity of SOD decreased significantly as compared with the control group, demonstrating the successes of the model. Effects of HLJDD, its two fractions and four component compounds on the production of NO, MDA, PGE2, and pro-inflammatory cytokines (IL-6, IL-10 and TNF-α), and on the
Fig. 4. Histograms of the effects of HLJDD, its two fractions and four typical compounds on lipopolysaccharide (LPS)-treated RAW264.7 cells (mean ± S.D.). RAW264.7 cells treated with LPS (0.2 μg/mL) and four concentrations of these tested samples. All data were expressed as concentrations of NO, MDA, SOD, PGE2.

activity of SOD in LPS-treated RAW264.7 cells were then evaluated (Figs. 4 and 5).

Pretreatment with HLJDD, HLJDD-1 and HLJDD-2 oriented these parameters toward the control group to an extent comparable to the positive control, DXM, an important steroidal anti-inflammatory drug. Most of the four typical compounds showed a weaker effect on these parameters than HLJDD, HLJDD-1 and HLJDD-2, except baicalin which has the strongest ability to enhance the activity of SOD. Berberine, the main component in Rhizoma coptidis and Cortex phellodendri, had a distinguished inhibitory effect on NO among tested compounds. Baicalein, the aglycone of baicalin, and geniposide showed the weakest overall effects on the four inflammatory mediators.

4. Discussion

The inflammation response protects the host against tissue injury and microbial invasion. During the activation process induced by bacterial endotoxin and/or LPS, macrophage is a major source releasing various inflammatory mediators which contribute to the local inflammatory response, including oxidants, cytokines, and lytic enzymes that play key roles in the inflammatory response (Chang and Lee, 2001). The control of macrophage overproduction of inflammatory mediators, such as PGE2 and NO, would therefore greatly facilitate the treatment of inflammatory diseases (Quan et al., 1998; Skidgel et al., 2002). Moreover, MDA, an indicator of lipid peroxidation, together with SOD are also concerned as they evaluate the ability to scavenge reactive oxygen radical, a characteristic feature of inflammation (Lu et al., 2007). It has also been reported that alterations in the production or function of cytokines, such as IL-6, IL-10 and TNF-α, play important roles in many inflammatory lesions (Holm et al., 2009). As a result, all the samples were tested for their ability to reduce the levels of NO, MDA, PGE2, IL-6, IL-10 and TNF-α, and increase the activity of SOD in LPS-treated RAW264.7 cells.

In this study, HLJDD showed almost equivalent ability to DXM in lowering the levels of NO, MDA, PGE2, IL-6, IL-10 and TNF-α, and increase the activity of SOD in RAW264.7 cells induced by
LPS. The results also indicated that fractions of HLJDD and four representative compounds in it may have diverse activities with particular emphasis. HLJDD-1 showed higher antioxidant activity than HLJDD-2 as evidenced by decreasing the level of MDA and inhibiting the attenuation of SOD activity much more obviously, which might ascribe to its subordinate components of iridoids and flavonoid glycosides. Iridoids, e.g. geniposide (Kuo et al., 2005), and flavonoid glycosides, e.g. baicalin (Li et al., 2009), might show a more apparent involvement in antioxidant activity among the four single compounds. However, on the other side, HLJDD-2 has a much more obvious inhibition on the productions of NO and IL-6 than HLJDD-1.

To explore the relationships among tested physicochemical indicators (variables) of samples, the data were first auto-scaled to avoid the influences of the different range levels of the variables and concentrations and then analyzed using principal component analysis (PCA) (Fig. 6). The basic goal of PCA is to reduce the dimensionality of a data set while retaining the most possible information by replacement of the original correlated variables with new uncorrelated components called principal components (PCs). The link between the original variables and these new ones (i.e. the PCs) is well described by the correlation circle: the closer a variable is to an axis and to the circle, the higher the correlation with the corresponding PC will be. As shown in the PCA correlation circle for the variables, an obvious negative correlation between the level of MDA and the activity of SOD was observed, which is in agreement with the commonly accepted view that both of them were implicated in inflammatory oxidative stress (Lu et al., 2007). NO also showed positive correlations with the cytokines (PGE₂, IL-6, IL-10 and TNF-α), consistent with those reported in literatures (Pang and Hoult, 1997; Viviana et al., 2003).

In the scores plot (Fig. 6), samples were mapped in the space spanned by the first two principle components PC1 (variables that accounts the maximum amount of variation) versus PC2 (the next largest remaining amount of variation which is orthogonal to the first principal component) as they could describe 89.8% of the variances from the original data. Here, the first factor explained more than 69.4% of the total variability. Thus, PC1 reflects the overall anti-inflammatory effects of samples: the larger the PC1, the weaker the effect of samples. The PCA result combined with Figs. 4 and 5 revealed that HLJDD exhibited stronger effect than the two fractions at the same concentration level, which suggested that HLJDD produced a marked anti-inflammatory effect as a whole and its two fractions, HLJDD-1 and HLJDD-2, can complement to produce strong biological effects. The two fractions, on the other hand, are generally of more potency than their constituents with the only exception of baicalin in the ability to increase the activity of SOD, which might due to the antioxidant ability of baicalin (Li et al., 2009).

HPLC analysis revealed that the main components in HLJDD are iridoids (geniposide), flavonoids (baicalin, baicalein) and alkaloids (berberine, palmatine, jatrorrhizin, coptisine). Among
them, iridoids and flavonoid glycosides are the main principles in HLJDD-1, and flavonoid aglycones and alkaloids are the main constituents in HLJDD-2. The PCA could also classify the two fractions and their components into two groups. The first group including HLJDD-1, and its two constituents, baicalin and geniposide, located in the upper-left region, in general, has greater anti-inflammatory potential than the second group including HLJDD-2, and its two constituents, berberine and baicalein, located in the lower-right region. It may postulate that these compounds in the fractions had synergistic anti-inflammatory interactions against the LPS-treated RAW264.7 cells and that reducing oxidative injury may be an important therapeutic pathway for HLJDD.

5. Conclusion

The present study suggested that HLJDD has a potent anti-inflammatory activity against carrageenan-induced mice paw edema and LPS-induced RAW264.7 cells. HLJDD contains multicomponents that produce a marked anti-inflammatory effect through multi-target and multi-channel actions. Moreover, HLJDD produced a much more obvious anti-inflammatory effect as a whole prescription compared with the anti-inflammatory effects of its two fractions and four typical compounds. The chemical analysis has shown that the main active compounds in HLJDD included iridoids, flavonoids and alkaloids derived from four herbs. Therefore, the combinational use of these herbal drugs is necessary to exert the anti-inflammatory effect of HLJDD against LPS-induced RAW264.7 cells. On the basis of anti-inflammatory results, it provided convincing data supporting the potential clinical use of HLJDD, and it also revealed that iridoids, flavonoids and alkaloids should be used for the quality control of HLJDD. Further research elucidating the mode of action of these effects using component herbs and combinations of them would give an insight into the usefulness of this prescription for its anti-inflammatory effects.

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