Original Article

The water extract of Anoectochilus formosanus protects mice from concanavalin A-induced hepatitis

Shangwen Luo¹, Yanyan Wang¹, Shishi Han¹,², Xuenong Zhang¹, Yang Zhang², Yihong Xia², Changxing Qi², Weiguang Sun², Yongbo Xue², Jianping Wang², Jinwen Zhang³, Yonghui Zhang²

¹The First College of Clinical Medical Science, China Three Gorges University and Yichang Central People’s Hospital, Yichang, China; ²Hubei Key Laboratory of Natural Medicinal Chemistry and Resource Evaluation, School of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China; ³Tongji Hospital Affiliated to Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

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Abstract: Anoectochilus formosanus, a famous traditional Chinese medicine, has been used as a source of folk medicine alternative liver injury therapy. Here, we evaluate the hepatoprotective potential of water extract of Anoectochilus formosanus Hayata (WEAF). The cultured splenocytes and concanavalin A (ConA)-induced hepatitis model in mice were established to investigate the immunoregulation and hepatoprotection of WEAF. In vitro, WEAF inhibited the production of TNF-α, IFN-γ and IL-4 in ConA-stimulated splenocytes. In vivo, mice pretreated with WEAF followed by ConA challenge showed reduction of liver damage. Pre-treatment with WEAF significantly decreased transaminase levels and hepatocyte apoptosis in ConA-injected mice. WEAF possessed potent anti-inflammatory and hepatoprotective activity. WEAF attenuated hepatic inflammation via up-regulation of IL-10 and down-regulation of TNF-α, IFN-γ, IL-4 and IL-6. Furthermore, WEAF ameliorated the intrahepatic infiltration of CD4⁰ T and CD8⁰ T cells, decreased the iNOS expression, as well as inhibited nuclear factor kappa B (NFκB) activation and Toll-like receptor 4 (TLR4) expression. Our findings provided evidences that WEAF had great potential to develop new immunomodulatory agent to treat immune-mediated hepatitis.

Keywords: Anoectochilus formosanus, concanavalin A, hepatitis, NFκB

Introduction

Hepatitis is a significant global health problem, which can be caused by alcohol, viruses, drugs, chemicals and immune disorder [1]. Immune-mediated liver diseases may develop into very serious liver injury and then cause death. Human inflammatory liver disease represents a ubiquitous health problem, but only a few cases can be administered pharmacologically. Clinically, some glucocorticoids such as dexamethasone (DEXA) and immunosuppression are often used as anti-inflammatory agent to treat these diseases but the efficacy is limit. And they all have many side effects, including endocrine system disorder and impaired immunity [2], especially in patients with long-term use. Effective drugs with precise mechanisms and limited side effects continue to be lacking. To develop new hepatoprotective agents, various animal models of liver injury have been established to facilitate functional studies on the mechanisms of hepatotoxicity. Concanavalin A (ConA)-induced hepatitis in mice is a well-established model for immune-mediated liver injury resembling viral and autoimmune hepatitis in humans [3]. After ConA administration, acute liver failure occurs with an increase in plasma transaminases, accumulation of CD4⁰ T cells and infiltration of neutrophils and macrophages in liver, and the activities of T cells and Kupffer cells (macrophages) induced by ConA produce various proinflammatory cytokines, including IFN-γ, TNF-α, IL-1β, IL-4, and IL-6, all of which play essential roles in liver injury [4-6].

Anoectochilus formosanus Hayata has been used as a source of folk medicine alternative liver injury therapy in southern-east of China for a long time. As a famous medicinal plant, it has received increased scientific attention. In the
last decade, the effects of A. formosanus have been widely reported, including effects against liver disease [7], hyperglycaemia [8], osteoporosis [8], tumours [9], hypertension [10], and so on. As the active substances of A. formosanus, some flavonoids and polysaccharides showed the protective effect on the liver [11]. Researchers found arabinogalactan from A. formosanus may be potentially effective immunomodulator [12, 13]. Hsiao et al reported kinsenoside isolated from A. formosanus effectively suppressed the production of inflammatory mediator [14].

In China, decoction of A. Formosanus is a traditional use for liver injury. Preliminary works have reported protective effects of A. formosanus on liver fibrosis induced by carbon tetrachloride or thioacetamide [7, 15]. However, its hepatoprotective effects on immunological liver damage have still not been clarified. In attempting to analyses whether WEAF is able to ameliorate liver injury in the murine fulminant hepatitis model induced by ConA and explore its possible mechanism, we prepared the water soluble fraction of A. formosanus and demonstrated the role of WEAF in ameliorating liver injury.

Materials and methods

Reagents

ConA type IV for intravenous injection and the positive control dexamethasone (DEXA) were purchased from Sigma Corporation. Sterilised water for dissolving WEAF was produced by Jiangsu Tianhe Disainuo Pharmaceutical Co., Ltd. (Nanjing, Jiangsu, China). Normal saline for injection was produced by Sinopharm Group Hubei Pharmaceutical Co. Ltd. (Wuhan, Hubei, China). AST and ALT assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). The ELISA kits of TNF-α, IFN-γ, IL-4, IL-6 and IL-10 were purchased from R&D Systems. RIPA lysis buffer, BCA protein assay kit, ECL plus chemiluminescence kit were purchased from BeyotimeInc. (Shanghai, China). Antibodies against CD4+ T, CD8+ T, iNOS, NFκB p65, phospho-NFκB p65, β-Actin and related secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against TLR4 was obtained from proteintech Group Inc. (Chicago, IL, USA). The TUNEL kit was purchased from BioGenex (San Ramon, USA). All other chemicals were of analytical grade.

Herb and sample preparation

The whole plants of A. formosanus were collected from Yong’an, Fujian Province, China in September 2011 and identified by Prof. Jianping Wang of School of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology. A voucher specimen (No. 2011-0910) has been deposited at the Hubei Key Laboratory of Natural Medicinal Chemistry and Resource Evaluation, School of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology.

Air-dried whole plants of A. formosanus (2 kg) were extracted three times with 20 L water for 4 h at 100°C yielding 0.66 kg of water extract of A. formosanus (WEAF) residue.

Inhibition activity of WEAF on cytokine production in ConA-stimulated splenocytes

Spleen cells were isolated from a 6-8 week-old Balb/c mouse using conventional procedures and were suspended in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated foetal bovine serum (Gibco Laboratories) and 1% penicillin-streptomycin solution (Sigma Chemical Co.). The cells were seeded in a 96-well-plate (10⁶ cells/well). ConA was added into relevant wells at a final concentration of 5 μg/mL. WEAF was dissolved in PBS and diluted with the RPMI-1640 medium to desire concentrations (1, 10, and 100 μg/ml), and then ConA was added 1 h later. They were maintained in a 37°C incubator at 5% CO₂. After 24 h incubation, the culture supernatant was collected to detect TNF-α, IFN-γ and IL-4 concentrations with specific enzyme-linked immunosorbent assay kits (ELISA) (R&D Systems) according to the manufacturer’s instructions.

Animal experiment

Male 6-week-old Balb/c mice were purchased from Tongji Medical College, Huazhong University of Science and Technology (Hubei, China. No. 420006000002 89). All experimental animals received care in compliance with the guidelines proposed by Institutional Animal Care and Use Committee (IACUC). All animal experiments were approved by the Institutional
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Animal Ethical Committee, Huazhong University of Science and Technology, China.

Liver injury was elicited by injecting ConA type IV (15 mg/kg). WEAF (100, 200 and 400 mg/kg) and DEXA (1 mg/kg) were orally administered once a day for 7 days. Among the therapies, a high dose of WEAF alone without ConA was administered to estimate whether WEAF itself could induce any liver damage. One hour after the final administration, normal saline or ConA was injected. Mice were sacrificed in batches, and blood samples were collected from the heart at various time points under ether anaesthesia. Four or twelve hours after injection, the livers were rapidly harvested. The largest lobe of the liver was divided into two sections: one section was submerged in 10% neutral formalin for the preparation of pathological sections, and the other part was stored at -80°C for TUNEL, Western blotting and lymphocytes infiltration analysis of liver tissue.

Assessment of liver functions

Four or twelve hours after ConA injection [16], blood samples were gathered, and plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using ALT and AST assay kits (Nanjing Jiancheng Bioengineering Institute).

Quantification of plasma cytokine concentrations

The plasma concentrations of cytokines were determined using ELISA kits. After injected ConA for four or twelve hours, blood samples were collected, and the concentrations of TNF-α, IFN-γ, IL-4, IL-6 and IL-10 in the plasma were determined.

Histology

The livers were quickly harvested at 12 h after ConA injection. Liver specimens were fixed in 4% formalin and embedded in paraffin, and tissue sections (4 μm) were stained with haematoxylin/eosin (H&E) using a standard protocol. Sections were examined by light microscopy for histological analysis of liver tissue.

TUNEL staining

Paraffin embedded liver tissues were assayed using a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) reaction for hepatocyte apoptosis analysis. All operations were performed according to the manufacturer's instructions (Roche Molecular Biochemicals).
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Immunohistochemistry analysis

The expression and localisations of CD4+ T, CD8+ T and iNOS in the liver were detected using immunohistochemical staining. One part of liver tissues was frozen at -80°C, then frozen-sectioned into liver sections. The specific staining was visualised using an immunodetection kit (Super Sensitive link-label IHC detection system, BioGenex, San Ramon, USA) and 3,3'-diaminobenzidine. The positive reaction of the labelled CD4+ T, CD8+ T cells and iNOS protein was observed under a light microscope.

Western blotting analysis

The specific protein expressions in liver were measured by Western blotting. Briefly, total protein of liver tissue was extracted using RIPA lysis containing 1% cocktail and 1% phenylmethanesulfonyl fluoride (PMSF) on ice. The proteins were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. Then the membrane was blocked by 5% non-fat milk for 1 h and incubated with the specific primary antibodies, NFkB p65 (1:1000), phospho-NFkB p65 (1:500), TLR4 (1:1000), β-Actin (1:1000), at 4°C overnight. After washed three times, the membrane was incubated with corresponding secondary antibodies for 2 h. Finally, the membrane was washed and the specific protein-antibodies complex was visualized using ECL plus chemiluminescence kit. The intensity of the bands was quantitated by Quantity One software (Bio-Rad).

Statistical analysis

All experimental data were presented as mean ± SD and evaluated by one-way analysis of variance (ANOVA). P<0.05 was considered statistically significant.

Results

Inhibition effects of WEAF on the production of cytokines in splenocytes

We first examined the direct effect of WEAF on activation of leukocytes by ConA. Primary spleen cells were stimulated with ConA for 1 h and then treated with different concentrations of WEAF (100, 10, 1 μg/ml). Levels of some cytokine in splenocyte supernatant were assessed. DEXA was used as a positive control in this experiment. As shown in Figure 1, WEAF significantly attenuated (P<0.05) the production of TNF-α, IFN-γ and IL-4 in ConA stimulated splenocytes. WEAF itself did not affect the level of these cytokines compared with the control group. These results suggested that WEAF might have a protective potential in the T cell-dependent ConA hepatitis model.
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Table 1. Effects of WEAF on ConA-induced plasma cytokines levels in mice (n = 10)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pretreatment (mg/kg)</th>
<th>Concentration of cytokines (pg/ml)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TNF-α</td>
</tr>
<tr>
<td>Normal</td>
<td>Vehicle</td>
<td>139.0±8.53</td>
</tr>
<tr>
<td>Model</td>
<td>Vehicle</td>
<td>569.4±22.62***</td>
</tr>
<tr>
<td>DEXA</td>
<td>1</td>
<td>242.9±15.31</td>
</tr>
<tr>
<td>Low-dose</td>
<td>100</td>
<td>408.7±36.51***</td>
</tr>
<tr>
<td>Middle-dose</td>
<td>200</td>
<td>305.9±25.30***</td>
</tr>
<tr>
<td>High-dose</td>
<td>400</td>
<td>222.8±17.18***</td>
</tr>
</tbody>
</table>

*P<0.01 compared with normal group; **P<0.01 compared with model group. Normal group: treated with vehicle and without any inducement; Model group: treated with vehicle and induced by ConA; Treatment group: treated with different concentrations of WEAF or DEXA and induced by ConA.

Figure 3. Effect of WEAF pre-treatment on liver histology and hepatocyte apoptosis in ConA-injected mice. A. H&E staining was performed for pathological examination of liver tissue. Model group showed gross necrosis and inflammatory infiltration around the central vein. B. TUNEL staining was executed to determine apoptosis in liver tissue. Model group showed large areas of hepatocyte apoptosis. Significant improvement could be observed in each pre-treatment group.

WEAF inhibited ConA-induced aminotransferase release

We established ConA-induced hepatitis model in mice to study the protective effect of WEAF in immune-mediated liver injury. As shown in Figure 2, the plasma concentrations of ALT and AST remarkably increased (P<0.05) in mice injected ConA. And the ALT and AST levels in mice injected WEAF had no significant difference with normal group. Pre-treatment with WEAF inhibited ConA-induced ALT and AST release after 12 h of the injection. Both 400 and 200 mg/kg of WEAF significantly reduced plasma ALT and AST levels compared with the model group, and a dose-dependent manner could be observed.

WEAF inhibited release of inflammatory cytokines in ConA-injected mice

Proinflammatory cytokines have been proven to play a critical role in triggering liver injury [17]. The concentrations of TNF-α, IFN-γ, IL-4, IL-6 and IL-10 in plasma were analysed to evaluate the protective effects of WEAF pre-treatment on the development of hepatitis. As shown in Table 1, WEAF (400 mg/kg) prominently inhibited (P<0.05) the ConA-induced production of TNF-α, IFN-γ, IL-4, IL-6 and IL-10 in plasma, whereas it augmented the level of IL-10. Low-doses of WEAF did not significantly suppress the IFN-γ and IL-4 levels.

WEAF ameliorated ConA-induced liver damage

H&E staining is one of the most straightforward methods to determine the hepatoprotective effects of WEAF. As shown in Figure 3A, ConA injection caused severe liver injury presenting as inflammatory infiltration around the
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Central veins and large areas of hepatocyte death. While liver damage in mice that was pre-treated with high and middle doses of WEAF decreased; fewer areas of interlobular necrosis were observed and the severity of inflammatory infiltration was obviously remitted.

TUNEL staining was executed to determine apoptosis in liver tissue. Substantial areas of hepatocyte apoptosis could be found in the ConA-injected group (Figure 3B). Only a few hepatocytes showed TUNEL-positive reaction, indicating that apoptosis was markedly reduced in all doses of WEAF-pretreated mice. WEAF alone did not cause any liver injury (not shown). Therefore, WEAF may efficiently protect mice from ConA-induced hepatitis by suppressing the hepatocyte apoptosis.

**Figure 4.** WEAF pre-treatment ameliorated ConA-induced CD4+ T and CD8+ T cell infiltration in the liver. The expression and localisations of CD4+ T (A) and CD8+ T (B) in the liver were detected by immunohistochemical staining. Pre-treatment with WEAF remitted CD4+ T and CD8+ T infiltration in the liver tissues on ConA-induced hepatitis.

ConA-induced hepatitis [3]. Almost no abnormal record could be discovered in the normal group (Figure 4A and 4B). However, the model group presented a considerable area of immunocytochemically positive CD4+ T and CD8+ T cells. Pre-treatment with high or middle doses of WEAF remarkably remitted CD4+ T infiltration. 400 mg/kg of WEAF slightly alleviated CD8+ T cell activation in the liver, whereas 200 and 100 mg/kg of WEAF showed no significant improvement in CD8+ T cell infiltration.

**WEAF inhibited ConA-induced intrahepatic iNOS expression**

In ConA-induced hepatitis model, the inducible nitric oxide synthase (iNOS) played a key role in the development of liver injury [18, 19]. Then we detected the intrahepatic induction of iNOS protein expression in mice following ConA injection by immunofluorescent staining. As shown in Figure 5A, ConA injection resulted in high expression of iNOS, while pre-treatment with WEAF significantly inhibited ConA-induced iNOS expression in the liver.

**WEAF suppressed ConA-induced NFκB activation and TLR4 expression**

In the ConA-induced hepatitis model, NFκB activation plays a central role in the production of various inflammatory mediators including cytokines mentioned above. As shown in Figure 5B, ConA injection caused significant increase of NFκB phosphorylation in livers of mice, while this increase was attenuated by WEAF pre-treatment. Meanwhile, the expression of intrahepatic TLR4 was analysed by Western blotting. After ConA injection, there was an increase in the expression of TLR4, which was reduced in WEAF pre-treatment groups (Figure 5B). These results suggested that the TLR4/NFκB signaling pathway may be associated with the protective effect of WEAF in ConA-induced hepatitis.
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Discussion

The ConA-induced hepatitis is a typical and well-established model to study T-cell-dependent liver injury, which rapidly causes severe immune-mediated hepatitis due to activation of CD4+ T cells and natural killer (NK) T cells [20, 21]. Activation of NK T cells causes large amounts of cytokines such as TNF-α, IFN-γ, IL-4 and IL-6 to be released into the plasma in this model [22, 23]. Generally, injecting ConA could cause mild to moderate liver injury. Herein, we demonstrated that WEAF significantly alleviated ConA-induced liver injury by reducing plasma aminotransferases, as well as decreasing inflammatory infiltration and hepatocyte apoptosis in the liver. The analysis of proinflammatory cytokines revealed that several disease-relevant inflammatory cytokines, such as TNF-α, IFN-γ, IL-4 and IL-6, were clearly inhibited in WEAF-treated mice, whereas augmented production of IL-10 was found in the plasma. The inhibitory effects of WEAF on TNF-α, IFN-γ, and IL-4 production were supported by ConA-stimulated primary mouse splenocytes. Therefore, pre-treatment with WEAF could protect mice from ConA-induced liver injury through inhibiting the production of several major inflammatory mediators. Moreover, mice injected with high dose WEAF only did not show any obvious pathological manifestations and characteristics. In addition, it has been reported that Kinsenoside, the main content of WEAF, is a potential immunosuppressive drug for autoimmune hepatitis [24]. Our results also support future WEAF treatment application to autoimmune hepatitis in human.

Figure 5. WEAF inhibited ConA-induced intrahepatic iNOS expression, NFκB activation and TLR4 expression. A. The expression of intrahepatic iNOS was measured by immunohistochemical staining. B. The NFκB activation and TLR4 expression was determined by Western blotting. Pre-treatment with WEAF attenuated NFκB activation and TLR4 expression increase in livers of ConA-injected mice. C and D. The quantitative analysis of Western blotting. *P<0.05 and **P<0.01 v.s. Control group.
Moreover, activation of CD4+ T helper cells and acts as a major mediator in inflammation-induced hepatocyte death, which can cause liver injury through binding to its receptor-inducing apoptosis cascade [22]. WEAF may relieve hepatocyte apoptosis in the liver by means of its highly suppressive ability on TNF-α in vivo and in vitro. Our observation of reduced TNF-α production after WEAF administration suggests that WEAF suppresses disease severity by abrogating TNF-α-induced liver injury and the inflammatory response.

The important roles of IFN-γ perhaps depend on synergistic effects with TNF-α to induce the production of several cytokines and chemokines [29]. Studies from IFN-γ-deficient mice indicate that IFN-γ is not only the major cytokine responsible for signal transducer and transcription-1 (STAT1) activation but also partially accounts for STAT3 activation [30]. Previous studies have indicated that in this model, both STAT1 and STAT3 are activated in the liver, which contributes to ConA-induced hepatitis [31]. In our study, the pre-treatment of WEAF efficiently inhibited the augmentation of IFN-γ, suppressing the development of hepatitis. However, the exact underlying mechanism of the suppression effect of WEAF on IFN-γ needs to be further investigated.

IL-4 and IL-6 have also been proven to play critical roles in the pathogenesis of ConA-induced liver injury [32, 33]. We also observed that WEAF pre-treatment significantly diminished the plasma levels of IL-4 and IL-6 in ConA-injected mice. These findings indicate that WEAF could suppress ConA-induced T-cell activation via inhibition of TNF-α and IFN-γ production, thereby alleviating liver injury. In addition, iNOS is induced in hepatocytes by TNF-α and IFN-γ, also play an important role in the development of liver injury [18, 34]. It was reported that iNOS induction has hepatotoxic effects in ConA-induced hepatitis [18]. In this study, we found that WEAF decreased iNOS expression in liver, suggesting its hepatoprotective effect.

Moreover, activation of CD4+ T and CD8+ T cells in the liver was suppressed. After injection of ConA, the plasma level of IL-4 and IFN-γ both increased dramatically, suggesting that the CD4+ T helper cell was involved in liver damage [35]. The target cell lysis by cytotoxic CD8+ T lymphocytes was also determined to contribute to liver injury but not as the major factor [36]. In our research, WEAF administration exhibited remarkable suppression on CD4+ T activation and a slight inhibition on CD8+ T cells. As reported, kinsenoside disrupts dendritic cell-induced cross-priming of CD8+ T cell responses [24], which may be a secondary mechanism of WEAF.

In the ConA-induced hepatitis model, NFκB is the critical transcriptional factor that mediates production of inflammatory cytokine. Many reports have demonstrated that some compounds could protect mice from ConA-induced liver injury through inhibiting NFκB signalling [37, 38]. Here, we found that WEAF pre-treatment significantly inhibited ConA-induced NFκB activation in ConA challenged mice. In addition, TLRs signalling, another important mediator also plays a key role in ConA-induced liver injury [39]. TLR4 signalling pathway has a contribution to activating TLR-expressing liver macrophages in ConA-induced hepatitis model [40]. In this paper, we detected expression of TLR4 in the liver tissue of ConA-injected mice. Our results indicated that WEAF pre-treatment inhibited the increase of TLR4 protein expression induced by ConA. Taking together, inhibition NFκB activation and TLR4 expression may be the mechanism of WEAF for its protective effect in ConA-induced hepatitis. Therefore, WEAF appears to effectively exert its immune-modulating effects on cytokine levels to alleviate liver damage, with a low toxicity compared to dexamethasone, a glucocorticoid with evident side effects for long-term therapies. As a pre-treatment, WEAF showed effective liver protection.

In summary, we have observed the hepatoprotective activity of WEAF in a model of T-cell dependent liver injury resembling autoimmune hepatitis. The major mechanism of this hepatoprotective activity appears to prevent the subsequent synthesis of TNF-α, IFN-γ, IL-4 and IL-6, suppress the activation of CD4+ T and CD8+ T cells, and down-regulate iNOS expression. Furthermore, the TLR4/NFκB signaling pathway may be associated with the protective effect of WEAF in ConA-induced hepatitis. Although the detailed mechanisms still need to be further investigated, the results from the study suggest new perspectives for hepatoprotective activity of Anoectochilus formosanus on ConA-induced autoimmune hepatitis. Consistent with this, our study provided evidence to use A. formosanus and develop new candidate drug in the treatment of immune-mediated hepatitis.
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Disclosure of conflict of interest

None.

Address correspondence to: Yanyan Wang, Yichang Central People’s Hospital, 183 # Yiling Avenue, Yichang 443003, China. Tel: +86-717-6486827; +86-151-71763193; E-mail: wangyy1001@163.com; Yonghui Zhang, School of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology, 13# Hangkong Road, Wuhan 430030, China. Tel: +86-27-8369 2311; E-mail: zhangyh@mails.tjmu.edu.cn

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