Leigongteng (Radix et Rhizoma Tripterygii) via compatibility with Jinqiancao (Herba Lysimachiae): its toxicity-reduced efficacy in H22-bearing mice

Wang Junming, Li Jinhua, Cai Hong, Zhang Yueyue, Li Jinyang, Cui Ying

Abstract

OBJECTIVE: To observe toxicity-reduced effects of Leigongteng (Radix et Rhizoma Tripterygii) (LGT) via compatibility with Jinqiancao (Herba Lysimachiae) (JQC) in H22-bearing mice and investigate the possible underlying mechanism, and further explore whether JQC can enhance LGT-evoked anti-tumor effect.

METHODS: H22-bearing mice were orally administered with LGT alone and its compatibility with JQC, and tumors, serum, livers and kidneys were collected to evaluate the toxicity-reduced efficacy and the possible mechanism.

RESULTS: LGT evoked significantly elevated biochemical indicators including serum alanine/ aspartate transaminase (ALT/AST), creatinine (Cr) and urea nitrogen (BUN) as well as pathological damage in mice, which were all obviously reversed by JQC via compatibility at the ratios from 4/1 to 1/4. Further analysis indicated that pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α), and malondialdehyde (MDA) levels significantly decreased, while anti-inflammatory cytokine interleukin (IL)-10, and glutathione (GSH), GSH-s transferase (GST), GSH peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT) levels all increased in livers and kidneys of mice. Besides, after compatibility with JQC at the ratios of 4/1, 2/1, 1/1, 1/2 and 1/4, LGT-decreased tumor weight was further decreased by 48.4%, 57.3%, 54.0%, 49.3% and 52.9%, respectively (all P < 0.01).

CONCLUSION: JQC could reduce LGT-induced hepatotoxicity and nephrotoxicity, and enhance the antitumor efficacy via compatibility with JQC, and the toxicity-reduced mechanism could involve inhibiting hepatic and kidney oxidative stress and inflammation.

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Keywords: Celastraceae; Primulaceae; Toxicity tests; Oxidative stress; Inflammation

INTRODUCTION

Leigongteng (Radix et Rhizoma Tripterygii) (LGT) is widely used in Traditional Chinese Medicine (TCM) as a medicinal plant with prominent clinical efficacy such as anti-cancers, anti-diabetic nephropathy, anti-rheumatoid arthritis, etc. However, it can also often
cause seriously toxic side effects reported during used in clinic.\(^7\) Compatibility is one of the characteristics of TCM, meanwhile also the main form of TCM.\(^8\) TCM theory thinks that it, through appropriate compatibility, can play a role in reducing toxicity or enhancing efficacy.\(^9\) In TCM, that LGT is combined with Jinqiancao (Herba Lysimachiae) (JQC), which has diverse and broad bioactivities including hepatoprotection,\(^10\) anti-oxidation,\(^11\) anti-inflammation,\(^12\) etc., belongs to compatibility of mutual suppression of Chinese medicinal, and JQC can reduce the toxicity induced by LGT via proper compatibility.\(^13\) In folk medicine, it has been reported that JQC can alleviate toxicity when poisoning occurs due to the application of LGT.\(^14\) However, until now, the toxicity-reduced efficacy of LGT via compatibility with JQC and its possible mechanism are still unknown. Furthermore, whether JQC augments LGT-induced anti-tumor activity is unclear as well. This study is carried out to observe the toxicity-reduced efficacy of LGT by JQC via compatibility and the potential mechanism, and further explore whether JQC can enhance LGT-inhibited tumor growth.

**MATERIALS AND METHODS**

**Experimental animals**

Kunming (KM) male mice (18-22 g) were obtained from Experimental Animal Center of Henan Province (Zhengzhou, China). Animals were given rodent laboratory chow and water ad libitum and maintained under controlled conditions with a temperature of \((22 \pm 1) ^\circ C\), relative humidity of \(60\% \pm 10\%\), and a 12/12 h light/dark cycle (lights on at 7:00 AM). All the procedures were in strict accordance with the P.R. China legislation on the use and care of laboratory animals and guidelines formulated by the Institute for Experimental Animals of Henan University of Chinese Medicine, and were approved by the university committee for animal experiments.

**Cell lines**

Mouse H22 tumor cells were collected from H22 tumor-bearing mice which were purchased from the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences (Beijing, China). Mouse H22 tumor cells were maintained in the peritoneal cavities of male KM mice in the Laboratory of Experimental Animals of Henan University of Chinese Medicine (Zhengzhou, China).

**Reagents**

Kits including alanine / aspartate transaminase (ALT/AST), creatinine (Cr), urea nitrogen (BUN), malondialdehyde (MDA), glutathione-s transferase (GST), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT) were all purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Mouse tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-10 enzyme-linked immunosorbent assay (ELISA) kits were provided by Boster Biological Technology (Wuhan, China).

**Plant material and Preparations of extracts**

LGT were obtained from Taining County of Fujian province (Taining, China). JQC origins in Sichuan Province and was purchased from a traditional drug store, Henan Materia Medica Chain Co., Ltd. (Zhengzhou, Henan province, P.R. China). LGT and JQC were both identified by Prof. Sui-Qing Chen (Pharmacognosy Department, Henan University of Chinese Medicine, Zhengzhou, Henan province, P.R. China). The preparations of the ethyl acetate extracts from LGT and its compatibility with JQC were described as follows.

Ethyl acetate extracts (EAEs) from LGT and its compatibility with JQC (mass ratios from 1/4 to 4/1) were obtained by reflux extraction once 2 h for repeated three times, and the ethyl acetate was recovered under reduced pressure and dried in vacuo (60 °C) to obtain dry extract. The EAEs yields of LGT and its different ratios combined with JQC (LGT/JQC; 4/1, 2/1, 1/1, 1/2 and 1/4) were 29.70, 27.25, 42.95, 39.28, 34.03 and 44.31 mg/g raw medicinal materials, respectively. In order to make the quality of the prepared extracts controllable, we determined the contents of triptolide and celastrol as the main active and toxic chemical components\(^14\) of LGT by high-performance liquid chromatography (HPLC). The contents of triptolide in above extracts were 10.605, 2.013, 0.783, 2.031, 2.827, and 0.167 mg/g, and those of celastrol were 3.995, 0.722, 0.118, 0.060, 0.345 and 0.219 mg/g, respectively, as assayed by the analysis of high-performance liquid chromatography (HPLC).

**Treatment protocol**

Ascerts of the mouse H22 were drawn out from mice under aseptic conditions. Cells were promptly diluted 2 times with aseptic saline. The diluted H22 cell suspension was inoculated subcutaneously (0.2 mL per mouse) into the right armpit.

One day after inoculation, mice, except for the normal (non-tumor-inoculated) animals, were randomly divided into eight groups of ten mice each. The control (tumor-inoculated) groups of mice received daily oral administration of 0.5% (5 g/L) sodium carboxyl methyl cellulose (CMC-Na; 0.2 mL/10 g). The other six groups received LGT and different ratios of LGT vs JQC (4/1, 2/1, 1/1, 1/2 and 1/4) by intragastric administration (ig) for 13 d starting from 24 h after tumor inoculation. The doses of LGT and its each compatibility with JQC were all 60 mg/kg according to LGT at the same equivalent. After treatment, mice were sacrificed by cervical dislocation after peripheral blood samples, livers, kidneys, and tumors were collected at 24 h.
after the last administration. Serum samples were collected for the analysis of biochemical indicators including ALT, AST, Cr and BUN, and liver and kidney tissues were used for the analysis of the histological observation, lipid peroxide (LPO), glutathione (GSH), tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-10 levels, determination of glutathione-related and anti-oxidant enzymes. The tumors were weighed, arrayed in line on paper, and taken pictures. The tumor inhibition ratio (IR) was calculated by the formula of IR = [(C - T)/C] × 100%, where C and T are the mean tumor weights of the control group (CMC-Na) and the treated group, respectively.

Assay for serum ALT, AST, Cr and BUN
Blood samples were obtained from mice of all groups (ten mice per group) for the determination of serum biochemical indicators. Serum ALT, AST, Cr and BUN were assayed by the commercial kits (Boster Biological Technology, Wuhan, China) in accordance with the manufacturer’s protocols.

Assay for tissues LPO levels
Liver and kidney tissues were homogenized in cold physiological saline, respectively. LPO was determined by the previous reported method. Malondialdehyde (MDA) is an end product of LPO and serves as a means of quantifying LPO. MDA reacts with 2-thiobarbituric acid (TBA) to generate a pink-colored product, which has an absorbance at 532 nm. LPO level was expressed as micromoles of MDA per milligram of protein based on tissue protein concentration.

Assay for tissues GSH levels, GST, GSH-Px, SOD and CAT activities
Liver and kidney tissues GSH levels, GST, GSH-Px, SOD and CAT activities were determined by the commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer’s protocols, and the results were all calculated based on tissue protein concentrations.

Assay for tissues IL-10 and TNF-α levels
Liver and kidney tissues levels of IL-10 and TNF-α were determined by the commercial kits (Boster Biological Technology, Wuhan, China) in accordance with the manufacturer’s protocols, and the results were all calculated based on tissue protein concentrations.

Statistical analysis
Data were presented as mean ± standard deviation (x ± s). One-way analysis of variance followed by least significant difference was conducted to test the differences between groups. A P < 0.05 was the significant level. All data were processed by SPSS 17.0. (SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago, IL, USA).

RESULTS

JQC reduced the hepatotoxicity and nephrotoxicity induced by LGT
Compared with the normal group, serum ALT, AST, BUN and Cr levels were all found to be significantly elevated in mice of the control group and treated with LGT alone (all P < 0.01). Compared with the TG alone group, after compatibility with JQC under all combined ratios (LGT/JQC from 4/1 to 1/4) significantly prevented the LGT-induced elevation of serum biochemical markers ALT, AST and BUN levels (all P < 0.01) (Figure 1A, 1B and 1D), while that under three combined ratios (LGT/JQC: 2/1, 1/1 and 1/4) obviously inhibited the LGT-induced elevation of serum Cr levels (all P < 0.01) (Figure 1C). Further, histological evaluation of the livers and kidneys removed from mice treated with LGT demonstrated hydropic degeneration of hepatocytes and nephrocytes (Figure 2). After treatment with JQC under all combined ratios (LGT/JQC from 4/1 to 1/4), these abnormal changes obviously decreased or even disappeared (Figure 2).

JQC reverses LGT-induced LPO
As shown in Figure 3A and 3B, MDA, one of the main end-products of LPO activity, was significantly increased in the livers (P < 0.05) and kidneys (P < 0.01) of mice treated with LGT compared with control groups, while the compatibility with JQC under all combined ratios (LGT/JQC from 4/1 to 1/4) prevented these elevation (all P <0.01).

JQC reverses LGT-decreased GSH and it-related enzymes levels
In the current research, the GSH and it-related enzymes levels all decreased significantly (all P <0.01) in livers and kidneys of mice treated with LGT alone, while the compatibility with JQC under four combined ratios (LGT/JQC from 2/1 to 1/4) significantly reversed these reduction in livers and kidneys (for GSH: P <0.01, P < 0.01, P < 0.05, P < 0.05 respectively, for GST and GSH-Px: all P < 0.01) (Figure 3C-D, 4A-D), and that under the combined ratio of 4/1 also significantly reversed the reduction of GSH and GSH-Px in livers (Figure 3C, 4C) and GST and GSH-Px in kidneys (Figure 4B, 4D).

JQC reverses LGT-decreased main antioxidant enzymes SOD and CAT levels
Our results showed that LGT reduced the activities of
H22 tumor-bearing mice were orally administered with Leigongteng (Radix et Rhizoma Tripterygii) (LGT) and its compatibility with Jinqiancao (Herba Lysimachiae) (JQC) for 13 d at the same dose of 60 mg/kg. ALT: alanine transaminase; AST: aspartate transaminase; Cr: creatinine; BUN: urea nitrogen. Data are presented as mean ± standard deviation (n = 10). Significant differences compared with the normal group were designated as $P < 0.01$ and $P < 0.05$, with control as $P < 0.01$ and $P < 0.05$, and with LGT alone group as $P < 0.01$.

H22 tumor-bearing mice were orally administered with Leigongteng (Radix et Rhizoma Tripterygii) (LGT) and its compatibility with Jinqiancao (Herba Lysimachiae) (JQC) for 13 d at the same dose of 60 mg/kg. Histological sections were stained with hematoxylin and eosin (HE) for the observation under the 200 times light microscopy.
Our results indicated that LGT significantly elevated liver and kidney pro-inflammatory cytokine TNF-α (both P < 0.01) and decreased anti-inflammatory cytokine IL-10 (both P < 0.01) levels in mice, while the compatibility with JQC under four combined ratios (LGT/JQC from 2/1 to 1/4) prevented such significant reduction (all P < 0.01) (Figure 6A-D), and that under combined ratio of 4/1 also conspicuously inhibited LGEVoked abnormality in hepatic IL-10, kidney TNF-α and IL-10 levels (all P < 0.01) (Figure 6B-D).

**Effects of JQC on TNF-α and IL-10 levels of livers and kidneys**

Our results indicated that LGT significantly elevated liver and kidney pro-inflammatory cytokine TNF-α (both P < 0.01) and decreased anti-inflammatory cytokine IL-10 (both P < 0.01) levels in mice, while the compatibility with JQC under four combined ratios (LGT/JQC from 2/1 to 1/4) prevented such significant reduction (all P < 0.01) (Figure 6A-D), and that under combined ratio of 4/1 also conspicuously inhibited LGEVoked abnormality in hepatic IL-10, kidney TNF-α and IL-10 levels (all P < 0.01) (Figure 6B-D).

**JQC enhances LGE-induced anti-tumor efficacy**

The effect of JQC on the anti-tumor activity of LGT
in transplanted H22 is shown in Figure 7. Our results showed that LGT decreased tumor weight of tumor-bearing mice \((P < 0.01)\) by 39.7\%. After treatment with JQC at the ratios of 4/1, 2/1, 1/1, 1/2 and 1/4, compared with LGT alone, LGT-decreased tumor weight was further decreased by 48.4\%, 57.3\%, 54.0\%, 49.3\% and 52.9\%, respectively \((P < 0.05 \text{ or } P < 0.01)\).

**DISCUSSION**

In the study, LGT-evoked significant elevation of serum ALT, AST, BUN and Cr levels, while after compatibility with JQC their elevation was obviously reversed. The result indicated that LGT-induced hepatotoxicity and nephrotoxicity were attenuated by JQC via compatibility, which was further confirmed by the histopathological analysis. Actually, JQC-contained bioactive chemical compounds including quercetin, chlorogenic acid and rutin all had hepatoprotective and renal-protective properties.\(^{26-30}\) The attenuation in this study may be related to the combined effects of quercetin, chlorogenic acid and rutin contained in JQC and triptolide, the major toxic compound of LGT. As for how
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Figure 7 Effects of compatibility with JQC on the antitumor activity of LGT in H22-bearing mice

H22 tumor-bearing mice were orally administered with Leigongteng (Radix et Rhizoma Tripterygi) (LGT) and its compatibility with Jinqiancao (Herba Lysimachiae) (JQC) for 13 d at the same dose of 60 mg/kg. Data are presented as mean ± standard deviation (n = 10). Significant differences compared with the control group were designated as *P < 0.01, and with LGT alone group as **P < 0.05 and ***P < 0.01.

they work, it needs further study. Cellular oxidative stress often appears in the imbalance between oxidants and antioxidants, moreover the levels of many antioxidant-related enzymes and non-enzymatic antioxidants may be changed in the process of the pathogenesis of liver and (or) kidney injury. Among them, LPO is a free radical-mediated process. MDA, as one of the main end products of LPO, has the characteristics of cross-linking cellular macromolecules including protein or DNA and causes widespread cellular damage. The results in Figure 3A and 3B demonstrated that JQC significantly attenuated excessive levels of MDA in livers and kidneys evoked by LGT, indicating that JQC can prevent LGT-evoked hepatic and kidney LPO injury in H22-bearing mice.

GSH, as a non-enzymatic antioxidant, is vital in protecting hepatocytes and nephrocytes against exogenous toxins, and the exhaustion of cellular GSH is related to oxidative injury. Our results showed that JQC significantly inhibited the GSH excessive exhaustion induced by LGT, suggesting that GQC could involve the protection of JQC against LGT-evoked liver and kidney oxidative injury.

GST and GSH-Px are cellular glutathione-related antioxidant enzymes. Of them, the cytosolic GSTs exist in almost all aerobic species. It can catalyze the conjugation of electrophilic compounds formed during the oxidative stress with glutathione. GSH-Px can catalyze hydrogen peroxide decomposition to the stable form of hydroxides, specifically using GSH as the electron provider. In the current study, JQC significantly elevated the LGT-evoked decreased activities of GST and GSH-Px in livers and kidneys of H22-bearing mice, which further confirmed that GSH-related antioxidant enzymes involved the protection of JQC against LGT-evoked liver and kidney oxidative injury.

SOD and CAT both play important roles in the enzymatic defenses of the cells against oxidative stress injury. SOD, a metalloenzyme, can convert O₂⁻ generated in the process of the oxidative stress to hydrogen peroxide. CAT mainly presents in the peroxisomes of all aerobic cells and serves to protect the cells against the damage of hydrogen peroxide by catalyzing it into molecular oxygen and water without producing toxic free radicals. As peroxisomes are abundant in proteins, where oxidative stress always happens, thus CAT is a classical oxidative biomarker. Our results showed that JQC reversed LGT-reduced enzymatic levels of SOD and CAT, suggesting that JQC protect against LGT-evoked liver and kidney oxidative stress injury, while SOD and CAT both taken part in such protection.

Cytokines, generated by virtually every nucleated cell in the body, are pleiotropic, regulatory peptides and include many types of hepatocytes and nephrocytes. The cytokine families contain several subfamilies including the interleukins, the tumor necrosis factor (TNF) family, chemokines, colony-stimulating factors and others. Among them, at least two different cytokines from different cytokine families, namely the pro-inflammatory molecule TNF-α and the anti-inflammatory cytokine IL-10, have emerged as vital factors in many aspects of liver and kidney diseases. In the current study, JQC significantly reversed the LGT-evoked excessively increased TNF-α and decreased IL-10 levels in H22-bearing mice, indicating that pro- and anti-inflammatory cytokines could involve the protection of JQC against LGT-evoked liver and kidney injury.

After confirming that the toxicity of LGT can be inhibited by JQC via compatibility, we further investigated the effect of the compatibility of the two on the antitumor activity of LGT. The results showed that, after compatibility with JQC, not only could reduce the toxicity of LGT, but also enhance the anti-tumor activity of LGT. Actually, JQC-containing bioactive chemical compounds including quercetin, chlorogenic acid and rutin all had anticancer properties. The enhanced efficacy in this study could be associated with the combined effects of quercetin, chlorogenic acid and rutin contained in JQC, and triptolide and celastrin, the main anticancer active compounds of LGT. However, the effect of JQC alone on H22 tumor-bearing mice was not tested here, which is a limitation of the current study.

In conclusion, the present research demonstrates that LGT-evoked hepatotoxicity and nephrotoxicity can be attenuated by JQC via compatibility, and the mechanisms underlying can be likely to involve inhibiting LPO, enhancing GSH, GSH-related and antioxidant-related enzymes, regulating pro- and anti-inflammatory cytokine levels (at least reducing TNF-α and elevating IL-10 levels) and thus ameliorates LGT-in-
dressed toxicity. Besides, the anti-tumor activity of LGT could be also enhanced by compatibility with JQC.

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