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Research Article
Protective Effect of Kushenlu on Lipopolysaccharide-induced Small Intestinal Inflammation in Rats

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Abstract

Background and Objective: Kushenlu (KSL) is a new type of Traditional Chinese Medicine (TCM) which is made based on the theory of TCM. The purpose of this study was to investigate the protective effect of KSL on lipopolysaccharide (LPS)-induced small intestinal inflammation in rats. **Materials and Methods:** Rats received spray inhalation with KSL daily for five days prior to the LPS challenge. Additionally, rat small Intestinal Epithelial Cells (IEC-6) and Rat small Intestinal Microvascular Endothelial Cells (RIMVEC) were pretreated with various concentrations of KSL for 24 h, then treated with lipopolysaccharide for 4 h. All statistical comparisons were made by one-way ANOVA followed by least significant difference test. **Results:** Kushenlu (KSL) pretreatment effectively prevented weight loss and attenuated LPS-induced diarrhea, reduction of red blood cells and white blood cells and the generation of proinflammatory cytokines including interleukin (IL)-1β, IL-6, IL-10 and Tumor Necrosis Factor (TNF)-α *in vivo* and *in vitro*. Furthermore, it was demonstrated that KSL reduced Toll-Like Receptor 4 (TLR4) expression and NF-κB activation in small intestinal tissues, IEC-6 cells and RIMVEC cells. **Conclusion:** All of the results indicated that KSL may be a potential anti-inflammatory drug for the clinical therapy of LPS-induced small intestinal inflammation.

Key words: *Sophora flavescens* Ait, distillation product, kushenlu, inflammation, lipopolysaccharide

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lipopolysaccharide (LPS) is a toxic component of the outer membrane of Gram-negative bacteria. As a potent initiator of inflammation, LPS induces systemic inflammatory injury and various kinds of pathological changes[1]. The small intestine, a portal of nutrient absorption, exposed to many antigens derived from food, toxins and bacteria, is also one of the target organs of LPS[2]. By inducing inflammatory injury of intestinal epithelium, administration of LPS can lead to damage of the integrity of the mucosal barrier, which plays an important role against the penetration of a variety of antigens from the gut lumen to the blood steam[3]. Furthermore, LPS activates endothelia in the rich capillary network of the small intestine to secrete high levels of cytokines[4]. This leads to an overwhelming inflammatory response and results in life-threatening multiple organ damage[5]. Therefore, inhibition or amelioration of this inflammatory response would allow small intestinal epithelial cells to resist the damage induced by LPS.

Recent studies have indicated that methods used in TCM can achieve beneficial effects in the treatment of inflammation induced by LPS[6]. According to the TCM theory, the treatment of small intestinal inflammation caused by LPS should be mainly based on heat-damp clearing and detoxifying.

Kushenlu (KSL), composed of the distillation product from *Sophora flavescens* Ait root (known as kushen in TCM), is a new formula made by following the principles found in TCM theory and could clear damp-heat and not hurt Wei-qi. The authors hypothesized that it could attenuate the inflammatory response caused by LPS.

The aims of this study were to investigate the effect of KSL on LPS-induced intestinal inflammation and elucidate its mechanisms using an LPS-induced small intestinal inflammation model and two epithelial cells, IEC-6 and RIMVEC.

MATERIALS AND METHODS

Plant materials and reagents: *Sophora flavescens* (kushen) was purchased from the Tong Ren Tang Company (Beijing, China). Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Gibco (Grand Island, NY, USA). The LPS (L2880, Sigma, USA) was diluted in normal saline to a final concentration of 2 mg mL^{-1} for animal tests. It was diluted in DMEM to a final concentration of 1 mg mL^{-1} for cell tests.

Preparation of Kushenlu: Sixty grams *Sophora flavescens* (kushen) were immersed in 600 mL distilled water for 30 min at room temperature and then heated in steam distillation apparatus. Distillation product (400 mL) was collected after 2.5 h, filtered through a 0.22 μm filter (Pall, PN4612, USA) and stored at -20°C before use.

Animals: Twenty-four male Sprague-Dawley rats weighing 220±13 g were purchased from the Academy of the Military Medical Sciences (Beijing, China) and housed under controlled conditions (23-24°C; 40-60% relative humidity) with *ad libitum* access to food and tap water. The study was approved by the China Agriculture University Institutional Animal Care and Use Committee (approval number: CAU20160220-1). The rats were randomly divided into 4 groups (n = 6 per group): Control, LPS, LPS+H_2O and LPS+KSL. Rats were placed into the fog box (size: 20×30×40 cm, the front was inserted with the ultrasonic spray inhalation apparatus and the back end was provided with an air vent) for 30 min, daily for 5 days. The control and LPS group received no ultrasonic spray inhalation, whereas the LPS+H_2O and LPS+KSL group received spray inhalation with 80 mL distilled water and 80 mL KSL, respectively, while in the fog box. On the fifth day, 2 h after being taken out of the fog box, the control group rats received an intraperitoneal injection of 5 mL kg^{-1} normal saline (37°C); the other group received an intraperitoneal injection of 5 mL kg^{-1} LPS solution (previbration to suspension, 37°C). Seven hours after injection, all rats were sacrificed by decapitation and exsanguinated. Two blood samples were collected from each rat. Two centimeters length of proximal jejunum was also collected. The jejunum tissue was washed in normal saline and preserved in liquid nitrogen for western blotting. The body weight of the rats was recorded before injection (W1) and immediately before they were sacrificed (W2).

Blood analysis: Quantification of Red Blood Cells (RBC), White Blood Cells (WBC), platelets (PLT) and hemoglobin (HGB) was performed with a multispecies hematology analyzer (Celltac E, Nihon Kohden, Japan).

Cell culture: The IEC-6 and RIMVEC cells were collected from the Beijing Key Laboratory of Traditional Chinese Veterinary Medicine, Beijing Agricultural College. They were maintained in complete medium at 37°C in a humidified 5% CO_2 incubator. Formulations for culture medium are shown in Table 1. For all experiments, cells were seeded in 96-well plates (IEC-6: 1×10^4 cells/well, RIMVEC: 5×10^3 cells/well).

Viability assay: After pre-incubation in complete medium for 12 h, cells were treated with various concentrations of KSL

Table 1: Formulations for culture media for IEC-6 cells and RIMVEC cells

Medium	IEC-6	RIMVEC
Complete medium	DMEM with 10% FBS, 100 U mL^{-1} penicillin, 10 μg mL^{-1} streptomycin and 0.28 U mL^{-1} insulin	DMEM with 20% FBS, 100 U mL^{-1} penicillin and 10 μg mL^{-1} streptomycin
Control medium	DMEM with 5% FBS and 0.28 U mL^{-1} insulin	DMEM with 5% FBS
LPS medium	DMEM with 5% FBS, 0.28 U mL^{-1} insulin and 15 μg mL^{-1} LPS	DMEM with 5% FBS and 5 μg mL^{-1} LPS
5% KSL medium	DMEM with 5% FBS, 5% KSL and 0.28 U mL^{-1} insulin	-
15% KSL medium	DMEM with 5% FBS, 15% KSL and 0.28 U mL^{-1} insulin	-
20% KSL medium	-	DMEM with 5% FBS and 20% KSL
40% KSL medium	-	DMEM with 5% FBS and 40% KSL

DMEM: Dulbecco's Modified Eagle Medium, FBS: Fetal bovine serum, IEC-6: Small intestinal epithelial cells, RIMVEC: Rat small intestinal microvascular endothelial cells

or LPS in control medium for various times and then incubated in DMEM with a Cell Counting Kit-8 (CCK8) reagent (Dojindo China CO., Shanghai, China) for 1.5 h. Absorbance was measured at 450 nm in a microplate reader (MK3, Thermo, MA, USA) to determine the number of viable cells.

Inflammatory cytokine measurement: Serum was separated from whole blood using a high-speed centrifuge at 200×g for 20 min. After pre-incubation in complete medium for 12 h, cells were first treated with various concentrations of KSL (IEC-6: 5 and 15% KSL medium; RIMVEC: 20 and 40% KSL medium) for 24 h, then with LPS or control medium for 4 h, before supernatant was collected. The levels of IL-1β, IL-6, IL-10 and TNF-α were measured using ELISA kits (R and D Systems, USA) according to the manufacturer's recommendations.

Western blotting: The IEC-6 cells, RIMVEC cells and small intestinal tissues were lysed in a radio-immunoprecipitation assay lysis buffer containing phenylmethanesulfonyl fluoride (Beyotime, Beijing, China). Protein concentrations were quantified using the bicinchoninic acid kit (Beyotime, Beijing, China). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and then hybridized with the following antibodies: TLR4 (ab30667, Abcam, USA), NF-κB p65 (YM3111, Immunoway, USA), p-NF-κB p65 (3033, CST, USA), IκBα (4814, CST, USA), all at a dilution of 1:1000 and β-actin (YM3028, Immunoway, USA; diluted 1:5000) and GAPDH (YM3029, Immunoway, USA; diluted 1:20000). The blot was developed using an electrochemiluminescence kit (Beyotime, Beijing, China). The intensity of each band was analyzed using Total Lab Quant software (Nature Gene, USA). Blots were normalized by using β-actin or GAPDH to correct for differences in loading of the proteins. Quantification of TLR4 and IκBα was normalized to β-actin or GAPDH levels; quantification of p-p65 was normalized to p65 levels.

Statistical analysis: At least 3 independent replicates were carried out for each experiment. All statistical comparisons were made by one-way ANOVA followed by Least Significant Difference (LSD) test[7]. Data are expressed as Mean±Standard Deviation and values were considered statistically significant at 5% level.

RESULTS

Effect of KSL on body weight: The body weight change was calculated according to the Eq. 1:

$$\Delta W = W1\text{-}W2 \quad (1)$$

where, W1 is the body weight before injection and W2 is the body weight before they were sacrificed.

Weight loss was greatest in groups LPS and LPS + H_2O and significantly higher than in rats in the control and rats in the LPS+ KSL group, respectively (Fig. 1).

Effect of KSL on various blood parameters: There was a significant decrease ($p<0.05$) in the levels of RBC, HGB, WBC and PLT in the LPS and the LPS+H_2O group compared to the control and the LPS+KSL group as shown in Fig. 2. No significant difference in RBC or HGB was observed between the control rats and the LPS+KSL-treated rats. The WBC and PLT decreased significantly ($p<0.05$) in the LPS+KSL-treated rats compared to control rats but remained significantly higher ($p<0.05$) than the values observed in the LPS and LPS+H_2O group.

Effect of KSL and LPS on cell viability: The CCK8 assay results indicated that KSL and LPS did not inhibit IEC-6 and RIMVEC cells proliferation at the concentrations which were used *in vitro* study.

Effect of KSL on LPS-induced expression of inflammatory cytokines in rats and cell supernatant: The LPS treatment led to a significant increase ($p<0.05$) in the concentrations of IL-1β, IL-6 and TNF-α compared to control rats. The KSL-treatment ameliorated the LPS-induced increase significantly ($p<0.05$) (Fig. 3a).

Results from IEC-6 cells are shown in Fig. 3b. The expression of IL-1β, IL-6 and TNF-α induced by LPS was significantly upregulated ($p<0.05$) and IL-10 was significantly downregulated ($p<0.05$). When IEC-6 cells were pretreated with KSL for 24 h and then stimulated with LPS for 4 h, a significant dose-dependent inhibition of IL-1β, IL-6 and TNF-α but not IL-10 expression was detected.

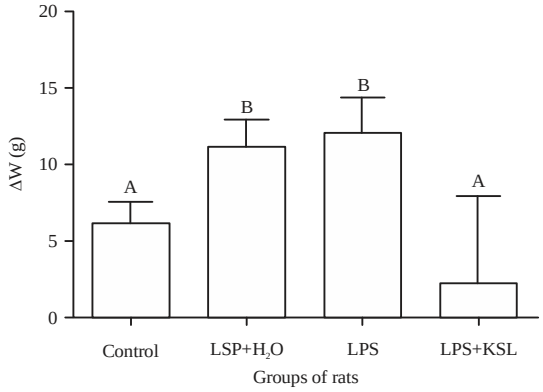

Fig. 1: Effect of kushenlu (KSL) on body weight
Means with different letters are significantly different ($p<0.05$)

Results from RIMVEC cells are shown in Fig. 3c. The expression of IL-1β, IL-6 and TNF-α induced by LPS was significantly upregulated ($p<0.05$) and IL-10 was significantly downregulated ($p<0.05$). When RIMVEC cells were pretreated with KSL for 24 h and then stimulated with LPS for 4 h, a significant dose-dependent inhibition of IL-6 and TNF-α expression was detected but no significant difference in the concentrations of IL-1β and IL-10 was observed.

Effect of KSL on LPS-induced TLR4, IκBα expression and p65 phosphorylation: The intraperitoneal injection of LPS significantly increased ($p<0.05$) the expression of TLR4, phosphorylation of p65 and degradation of IκBα in the small intestines and these changes were partially inhibited by KSL (Fig. 4a).

The LPS treatment significantly increased ($p<0.05$) the expression of TLR4, phosphorylation of p65 and degradation of IκBα in IEC-6 cells as shown in Fig. 4b. The LPS-induced TLR4 expression was reduced by pretreatment with 15% but not 5% KSL for 24 h. The phosphorylation of p65 and degradation of IκBα induced by LPS was partially inhibited by KSL in a dose-dependent manner.

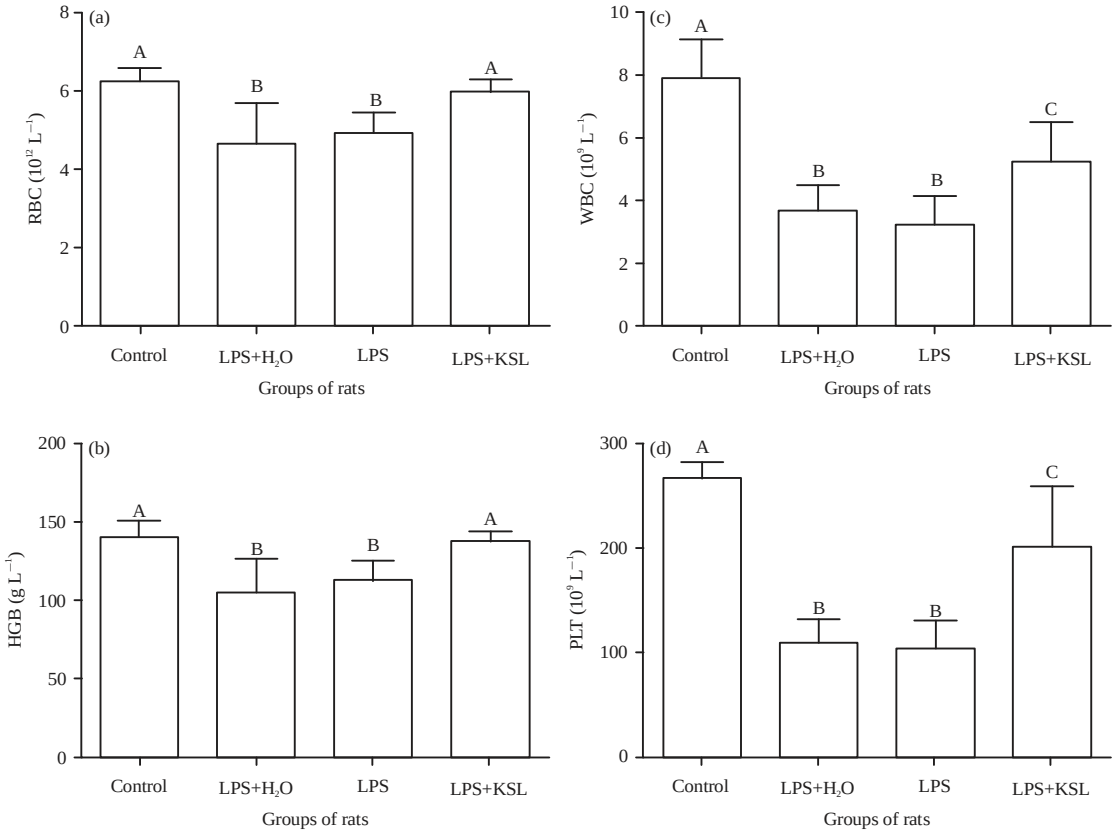

Fig. 2(a-d): Concentration of (a) Red blood cells (RBC), (b) Hemoglobin (HGB), (c) Total leukocytes (WBC) and (d) Platelets (PLT)
Means with different letters are significantly different ($p<0.05$)

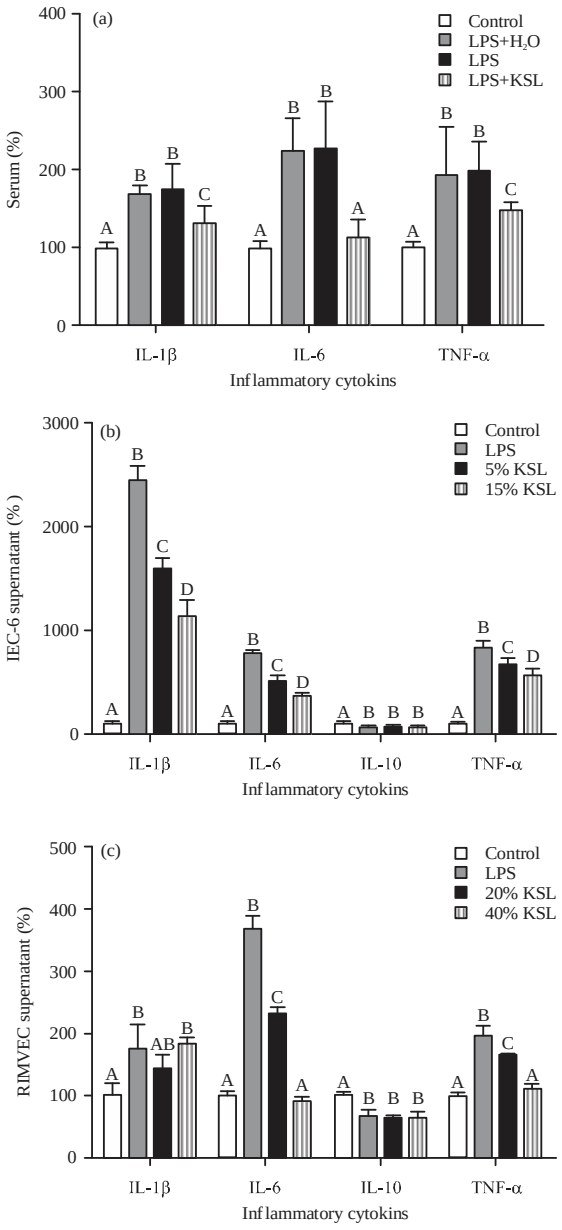

Fig. 3(a-c): Effect of KSL on LPS-induced expression of inflammatory cytokines in (a) Serum, (b) IEC-6 cells supernatant and (c) RIMVEC supernatant
Means with different letters are significantly different ($p<0.05$)

In RIMVEC cells, pretreatment with 20 and 40% KSL for 24 h reduced TLR4 expression, p65 phosphorylation and IκBα degradation induced by LPS in a dose-dependent manner as shown in Fig. 4c.

DISCUSSION

The present study demonstrated that KSL had *in vitro* and *in vivo* anti-inflammatory activity. Kushen has been widely used in TCM as herb for the treatment of pruritus, inflammation and gastrointestinal diseases. The traditional use of kushen in clinics is to make water decoction or pills. Recent studies have shown that kushen contains alkaloids, flavonoids and terpenoids[8], which are known to have various biological activities, e.g., anti-fungal[9], anti-bacterial[10] and anti-inflammatory[6,11,12]. However, most of these studies focused on its aqueous and alcohol extract. To the authors' knowledge, there are few reports about the biological activities of its distillation product. Therefore, this study expands the understanding of the medicinal value of kushen. Distillation product from the herb (known as Yaolu in TCM) has been widely used in TCM clinics since the Qing Dynasty. Yaolu preserves medical efficacy well and has the advantages of rapid absorption and few side effects. Thus, in this study the authors chose Yaolu (i.e. KSL) to study the potential use of kushen. Compared with its water decoction, KSL is a transparent liquid, not sticky and has no strong bitter taste, which means that it is more easily accepted by patient than its water decoction. Therefore, KSL has the potential to be used clinically as an anti-inflammatory drug.

The ultrasonic spray inhalation was chose for KSL administration in the animal tests. Drug delivery by ultrasonic spray inhalation enables the drug to enter the blood circulation directly through the rich capillary network of the lung. This would effectively avoid the first pass effect and improve the bioavailability of the drug. Yaolu has been used orally but the authors are not aware that it has ever been administered by spray inhalation, a more modern TCM application route.

This is the first study, to the authors' knowledge, which has investigated the medicinal function of KSL and tried to elucidate the mechanistic basis of its effects. The LPS can cause diarrhea, which is thought to be associated with small intestinal inflammation[13,14]. In the present study, diarrhea was assessed on the basis of weight loss, stool consistency as well as soiling of the perianal area. The results show that KSL spray inhalation significantly reduced ($p<0.05$) the weight loss of LPS treatment (Fig. 1). In fact, the weight before and after treatment was not different in the KSL group compared to the control group. Compared with the LPS and the LPS+H_2O group, KSL treatment led to solid feces and no soiling of the perineum. These results indicate that KSL treatment can prevent diarrhea. There was also a difference in the appearance of the small intestine. Small intestinal mucosal edema, intestinal wall thinning and intestinal pneumatosis were observed in the rats of the LPS and the LPS+H_2O group but not in the KSL-treated group. The western blotting results also support this finding and suggest that KSL can significantly

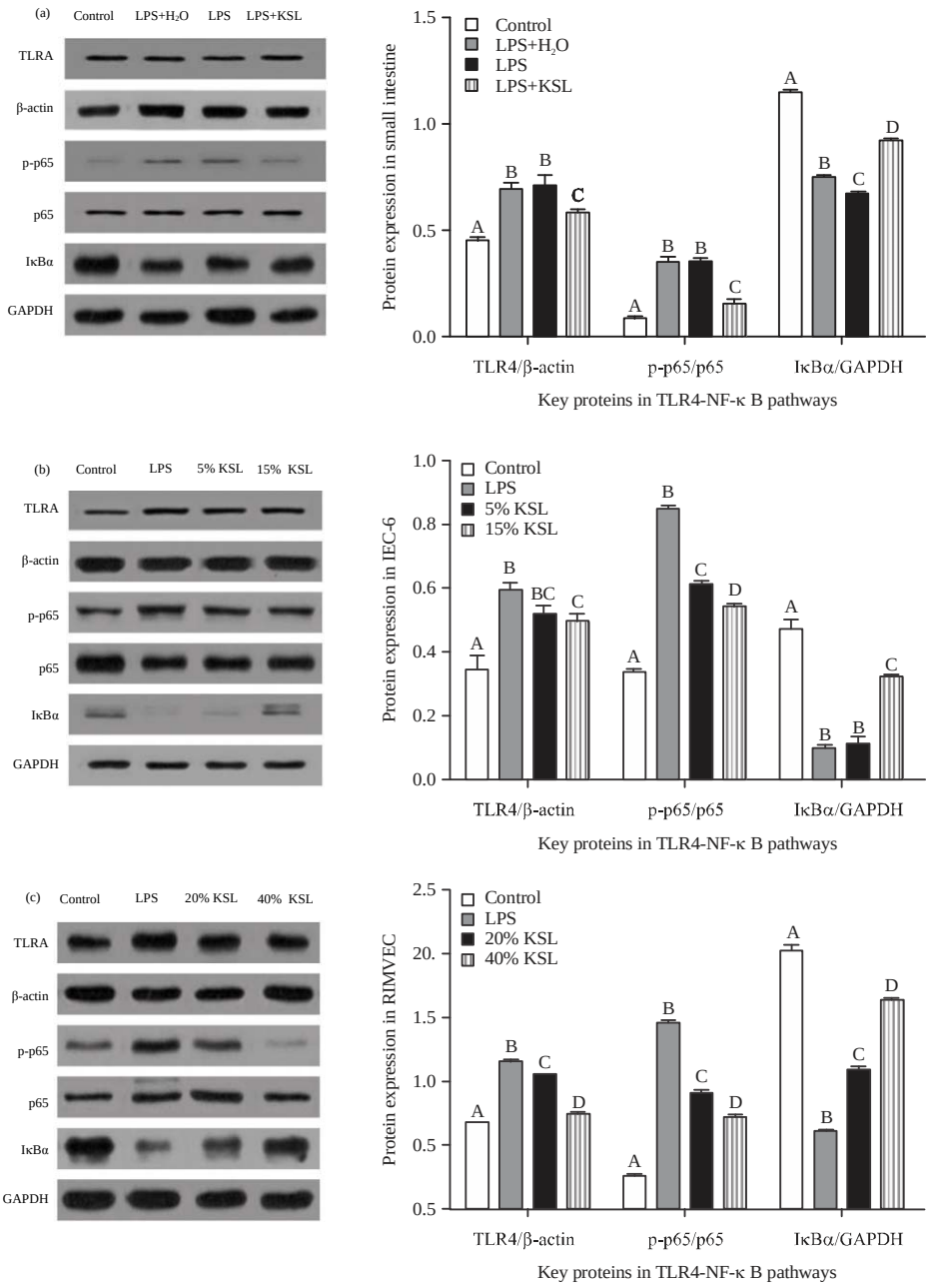

Fig. 4(a-c): Effect of KSL on LPS-induced TLR4, IκBα expression and p65 phosphorylation in (a) Rat small intestine tissues, (b) IEC-6 cells and (c) RIMVEC examined by western blotting
Means with different letters are significantly different (p<0.05)

reduce (p<0.05) the activation of LPS related inflammatory signaling pathway (Fig. 4a) and ameliorate small intestinal inflammation.

The intraperitoneal injection of LPS caused a decrease in red blood cells, white blood cells, platelets and hemoglobin concentrations. These changes may be related to LPS-induced Disseminated Intravascular Coagulation (DIC) but could be due to LPS-induced bone marrow toxicity[15-18]. Regardless of the cause, the results of this study show that KSL spray inhalation completely normalized red blood cell counts and hemoglobin concentrations and significantly improved white blood cell counts and platelet counts (Fig. 2), which suggest that KSL spray inhalation can significantly improve (p<0.05) the detrimental effect of LPS on important blood parameters.

It is believed that these beneficial effects of KSL can be attributed to a reduction of the serum concentrations of proinflammatory cytokines, IL-1β, IL-6 and TNF-α (Fig. 3a) which are the critical cytokines profoundly modulating inflammation and immunity[19,20]. Their excessive expression causes injury to cells and tissues.

In order to further study the beneficial effect of KSL, two cell types were used for *in vitro* studies: IEC-6 is a rat small intestinal epithelial cell, which can represent the epithelial barrier function of the small intestine and RIMVEC is a microvascular endothelial cell, which is a critical element in inflammation[21]. The KSL reduced the LPS-induced secretion of IL-6 and TNF-α in a dose-dependent manner in both cell types (Fig. 3b, c). A reduction in IL-1β was also observed in IEC-6, whereas the IL-1β response in RIMVEC cells was not altered. The IL-1β is one of the most powerful proinflammatory cytokines that affects virtually every organ[22]. Considering that KSL led to a decrease in IL-1β in serum, further studies are needed to investigate whether a higher concentration of KSL might be able to diminish the IL-1β response to LPS in RIMVEC cells as well. The expression of IL-10, an anti-inflammatory cytokine, which suppresses the expression of proinflammatory cytokines[23], was not affected by KSL in IEC-6 or RIMVEC cells. These results indicated that the beneficial effects of KSL is mainly achieved by reducing the secretion of proinflammatory cytokines rather than increasing the secretion of anti-inflammatory cytokine. The synthesis of proinflammatory cytokines is regulated by NF-κB and its activation has been shown to be involved in numerous inflammatory disease[24,25]. In the inflammatory response, LPS stimulation leads to the excessive expression of TLR4[11,26]. Importantly, it promotes the recruitment of adaptor proteins to trigger the phosphorylation of p65 and the degradation of IκBα which leads to activation of NF-κB[27,28]. In the present studies, KSL affected all of the key proteins (TLR4, p65, IκBα) which play a role in the activity of TLR4-NF-κB pathway in a dose-dependent manner in RIMVEC cells, whereas in IEC-6 cells, only a higher dose (15%) of KSL was able to achieve this. These results, combined with the effects of KSL on the secretion of proinflammatory cytokines, indicate that there may be other signaling pathway involved in the regulation of LPS-induced inflammation by KSL. Results of *in vitro* experiments demonstrated that the mechanism for the beneficial effect of KSL against LPS-induced small intestinal inflammation was at least partly through reduction of TLR4 expression and NF-κB activation in epithelium and capillary endothelium, which inhibits production of downstream proinflammatory cytokines.

This study only focused on the TLR4-NF-κB pathway. There are other signaling pathways associated with inflammation, e.g. Mitogen-Activated Protein Kinase (MAPK) pathway. Therefore, other possible pathways and targets related to the inflammation need to be further investigated to elucidate the mechanism of the protective effect of KSL on LPS-induced small intestinal inflammation.

CONCLUSION

This study demonstrated the protective effect of KSL on LPS-induced small intestinal inflammation both *in vivo* and *in vitro*. This effect was in part achieved by the decreased production of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) mediated by reduction in TLR4 expression and NF-κB activation. The authors suggest that KSL might be used as an anti-inflammatory drug for the clinical therapy of small intestinal inflammation.

SIGNIFICANCE STATEMENT

Kushenlu (KSL), made from the distillation product of *Sophora flavescens* Ait root, effectively attenuated lipopolysaccharide (LPS)-induced small intestinal inflammation in rats by ultrasonic spray inhalation. It is believed that this is the first study to investigate the medicinal function of KSL and its possible mechanism and expands the understanding of the medicinal value and potential use of *Sophora flavescens* Ait root.

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