Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v16i3.13

Original Research Article

Therapeutic effect of Lianbeijuqin (a Chinese herbal cocktail) on periodontitis in rat

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Abstract

Purpose: *To investigate the therapeutic effect of Lianbeijuqin (LBJQ) decoction on periodontitis in rats in vivo and in vitro.*
Methods: *High performance liquid chromatography (HPLC) was used to analyze the main constituents of LBJQ. Periodontitis in rats was induced in rats by ligation with bacteria-infected silk. Gingival index (GI) and attachment loss (AL) were evaluated. Additionally, the antibacterial activity of LBJQ against Porphyromonas gingivalis, Prevotella intermedius, Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum, Streptococcus sanguis and Streptococcus mutans were evaluated using minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values. Furthermore, the inhibitory effect of LBJQ on the release of TNF-α, IL-1 β and IL-6 was also determined.*
Results: *HPLC revealed the presence of phellodendrine, baicalin, palmatine, berberine, baicalein, wogonin and obacunone in LBJQ. LBJQ decoction dose-dependently (200, 400 and 800 mg/kg) and significantly decreased GI and AL (p < 0.05) in the periodontitis model rats. It also exerted significant antibacterial effect against P. gingivalis (MIC, 0.64 mg/mL; MBC, 2.56 mg/mL), P. intermedius (MIC, 0.32 mg/m; MBC, 2.56 mg/mL), A. actinomycetemcomitans (MIC, 1.28 mg/mL; MBC, 5.12 mg/mL), F. nucleatum (MIC, 0.16 mg/mL; MBC, 0.64 mg/mL), S. sanguis (MIC, 0.64 mg/mL; MBC, 5.12 mg/mL), and S. mutans (MIC, 0.16 mg/mL; MBC, 1.28 mg/mL). Moreover, LBJQ decoction dose-dependently and significantly decreased the release of TNF-α (p < 0.01), IL-1 β (p < 0.01) and IL-6 (p < 0.01) in LPS-induced RAW 264.7 cells.*
Conclusion: *LBJQ exerts therapeutic activity against periodontitis in rats via inhibition of the release of inflammatory cytokines and growth of oral anaerobic bacteria.*

Keywords: *Lianbeijuqin decoction, Periodontitis, Phellodendrine, Baicalin, Palmatine, Berberine, Porphyromonas gingivalis, Prevotella intermedius, Inflammatory cytokines*

INTRODUCTION

Periodontitis is a chronic oral inflammatory disorder that occurs in tooth-supporting tissues such as gingiva and radices dentis. It results in significant damage to surrounding tissue structures, leading to loss of teeth [1,2]. Besides tooth loss, periodontitis is associated with other discomforts such as gomphiasis, swelling and aching of gum, fever and bromopnea [3,4].

Current treatment and periodontal tissue regeneration strategies for periodontitis involve scaling, root planning and regenerative surgeries [5,6]. However, strategies for radical treatment of periodontitis are still limited [7].

Lianbeijuqin (LBJQ) decoction is prepared from a Chinese herbal formula which consists of 7 herbal medicines (Table 1). In Chinese traditional medicinal theory system, LBJQ is used for

clearing heat, *drying dampness* and for clinical treatment of chronic periodontitis. However, although LBJQ is a traditionally used Chinese formula for periodontitis treatment, its therapeutic effects and pharmacological mechanisms of action are still not clear.

This study was carried out to evaluate the therapeutic effect of LBJQ in a rat model of periodontitis. In addition, the anti-bacterial effects of LBJQ on common oral pathogenic bacteria, and its inhibitory effects on the release of pro-inflammatory cytokines in LPS-treated RAW 264.7 cells were determined, with a view to unraveling its mechanism of action.

EXPERIMENTAL

Chemicals and reagents

Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from the Gibco Biotech. (Carlsbad, USA), lipopolysaccharide (LPS) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Co. (Shanghai, China). Tumor necrosis factor (TNF-α), interleukin (IL)-β and IL-6 ELISA kits were purchased from the Wuhan CUSABIO Biotech. (Wuhan, China); standards (phellodendrine, ferulic acid, baicalin, palmatine, berberine, baicalein, wogonin and obacunone) were obtained from the NICPBP (Beijing, China).

Animals

Sprague Dawley (SD) rats (260 ~ 280 g) were got from Shanghai Laboratory Animal Research Center (Shanghai, China). The rats were kept in a humidity/temperature-controlled room and allowed *ad libitum* access to food and water. The animal experimental protocols were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals [8], and were approved by the Animal Care and Use Committee of the Linyi People's Hospital (Linyi, China) (no. 1504A02).

Cell culture

The RAW264.7 cell line, a murine macrophage cell line, was purchased from the American Type Culture Collection (ATCC, Rockville, USA). RAW264.7 cells were cultured in DMEM containing 10 % FBS in a CO_2 incubator (5 % CO_2, 37 °C).

Preparation of aqueous extracts of LBJQ

Aqueous decocting is the primary method for preparation of LBJQ in folk medicine. The herbal contents of LBJQ were ground and extracted 6 times with water. Each extraction lasted for 30. The pooled extract was filtered, and the clear filtrate was concentrated under *in vacuum* under 50 °C with a rotary evaporator to yield the LBJQ aqueous extract (yield of LBJQ was 12.73 %).

HPLC analysis of LBJQ

The constituents of the extract were determined with HPLC using a gradient elution with a CAPCELL PAK MG II S5 C_{18} column (250 mm × 4.6 mm, 5 μm) (Shiseido, Japan).The mobile phase consisted of acetonitrile (solvent A) and 0.1 % phosphoric acid-water solution (solvent B, containing 0.02 M sodium phosphate monobasic), and the gradient program was 10 % A (0 - 10 min); 20 - 40 % A (10 - 30 min); 40 - 60 % A (20 - 40 min); 40 - 60 min (40 – 60 % A); and 60 – 80 % A (40 - 60 min). The mobile flow rate was set at 1.0 mL/min, and the detection wavelength was at 215 nm. The column temperature and sample injection volume were 30 °C and 10.0 μL, respectively.

Determination of therapeutic effect of LBJQ on rat periodontitis

Dental plaque bacteria were isolated from definite diagnosed periodontitis patients in the Linyi People's Hospital, Linyi, China, and then diluted with normal saline.

Table 1: Composition of *LBJQ*

Name	Plant origin	Part	Content (g)
Coptidis Rhizoma	*Coptis chinensis* Franch	Roots	10
Galla Chinensis	*Melaphis chinensis* (Bell) Baker	Insect gall	12
Chrysanthemi Indici Flos	*Chrysanthemum indicum* L.	Flowers	15
Scutellariae Radix	*Scutellaria baicalensis* Georgi	Roots	10
Rhei Radix et Rhizoma	*Rheum palmatum* L	Roots	8
Phellodendri Chinensis Cortex	*Phellodendrom chinense* Schneid.	Cortex	10
Glycyrrhizae Radix et Rhizoma	*Glycyrrhiza uralensis* Fisch.	Roots	5

Subsequently, the diluted dental bacteria were inoculated in anaerobic blood agar plate with sterile 5-0 silks for 5 days at 37 °C. Thereafter, the dental bacteria-infected silks were ligatured around the maxillary first molar on the left side of rats under anesthesia (pentobarbital sodium, 45 mg/kg, *ip*).The ligatures were removed 21 days after the ligature surgery. For normal (sham) rats, the same surgical protocol was given but with sterile 5-0 silks instead of dental bacteria infected 5-0 silks.

The periodontitis model rats were divided randomly into 4 for groups (n = 10): control and 3 LBJQ treatments groups (200, 400 and 800 mg/kg). In addition, another set of 10 rats were treated as normal group. Rats in LBJQ treatments groups were orally administered LBJQ (200, 400 and 800 mg/kg, *po*), while rats in normal and control groups were orally given normal saline (10 mL/kg, *po*). At 4, 6, 8 and 10 weeks after ligature surgery, the rats were anesthetized with pentobarbital sodium (45 mg/kg, *ip*). Then gingival index (GI) and attachment loss (AL) were evaluated according to standard methods [9,10]. The GI was classified as follows: 0 (healthy gingiva; 1 (mild inflammation, i.e. mild change in gingiva color, and /or mild edema without bleeding); 2 (moderate inflammation i.e. redness in gingiva, severe edema and bleeding) and 3 (severe inflammation, i.e., visibly red and swollen; ulcerous and bleeding). Four detecting points, namely papillae gingiva, buccal margin, palatine papillae and marginal gingiva of each experimental tooth were determined, and the mean score of the four points was taken as the GI value. Furthermore, a periodontal probe was used to detect the distance between the enamel-cement junction and pocket bottom.

Antibacterial tests

The antibacterial activities of LBJQ against a number of oral pathogenic bacteria were determined. The oral bacteria were *P. gingivalis* (ATCC 33277), *P. intermedius* (ATCC25361), *A. actinomycetemcomitans* (ATCC29522), *F. nucleatum* (ATCC 23276), *S. sanguis* (ATCC 6715) and *S. mutans* (ATCC 25175). The antibacterial effects of LBJQ were measured via evaluation of MICs and MBCs according to established methods [11, 12]. The final concentrations of LBJQ in bacterial culture medium were 10.24, 5.12, 2.56, 1.28, 0.64, 0.32, 0.16, 0.08 and 0.04 mg/mL. The lowest LBJQ concentration which inhibited visible bacterial growth after 24 h incubation at 37 °C was considered as the MIC. In the evaluation of MBC, 0.01mL bacterial culture medium after the MIC

was inoculated on an agar plates for growth another 24 h at 37 °C, and MBC is the lowest concentration of LBJQ without visible bacterial growth.

Effects of LBJQ on release of pro-inflammatory cytokines in RAW 264.7 cells

Cells (1.5×10^6) were seeded in a 48-well plate and cultured for 4 h. Then, the cells were incubated separately with different concentrations of LBJQ (200, 400 and 800 µg/mL) and LPS (100 ng/mL) for another 8 h. Finally, the culture media were collected for assay of TNF-α, IL-1β and IL-6 with commercial ELISA kits.

Statistical analysis

Data are expressed as mean ± standard deviation (SD, n = 10). Differences in means between two groups were analyzed with two-tailed Student's *t*-test and were considered statistically significant at $p < 0.05$.

RESULTS

LBJQ composition

HPLC was used for the qualitative analysis of the constituents of LBJQ. As shown in Figure 1, seven (7) major compounds were identified based on their chromatographic behaviors relative to standards. These compounds were phellodendrine (1), baicalin (2), palmatine (3), berberine (4), baicalein (5), wogonin (6) and obacunone (7).

Therapeutic effect of LBJQ

Results showed that after the ligature surgery, GI was significantly increased at all the detecting time points relative to rats in normal group ($p < 0.01$, Figure 2). However, after 4-week treatment, LBJQ (400 and 800 mg/kg) significantly decreased GI ($p < 0.05$, $p < 0.01$) in the periodontitis model rats, compared to rats in control group. Interestingly, after 6 weeks' treatment (including at 6, 8 and 10 weeks), LBJQ (200, 400 and 800 mg/kg) significantly and dose-dependently decreased GI ($p < 0.01$) when compared to control rats.

In the periodontitis model rats, SAL was significantly increased when compared with normal rats ($p < 0.01$). However, the 4-week treatment with LBJQ (400 and 800 mg/kg) led to significant decreases in AL ($p < 0.05$), relative to control rats. At the end of 6-week treatment, LBJQ brought about significant and dose-

dependent decreases in AL (*p* < 0.01, *p* < 0.05) when compared with rats in the control group (Figure 3).

Antibacterial activity

As shown in Table 2, LBJQ produced significant antibacterial activities against all the tested bacterial strains (*P. gingivalis*, *P. intermedius*, *A. actinomycetemcomitans*, *F.nucleatum*, *S. sanguis* and *S. mutans*), with MIC values below 2 mg/mL (the MICs were 0.64, 0.32, 1.28, 0.16, 0.64 and 0.16 mg/mL, respectively). The MBC values were less than 6 mg/mL (MICs were 2.56,

2.56, 5.12, 0.64, 5.12 and 1.28 mg/mL, respectively).

**Effect of LBJQ on release of pro-inflammatory cytokines**

After LPS treatment, there were significant increases in the release of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) from RAW 264.7 cells (*p* < 0.01, Figure 4). However LBJQ exposure led to significant and dose-dependent decreases in the levels of these cytokines (*p* < 0.01).
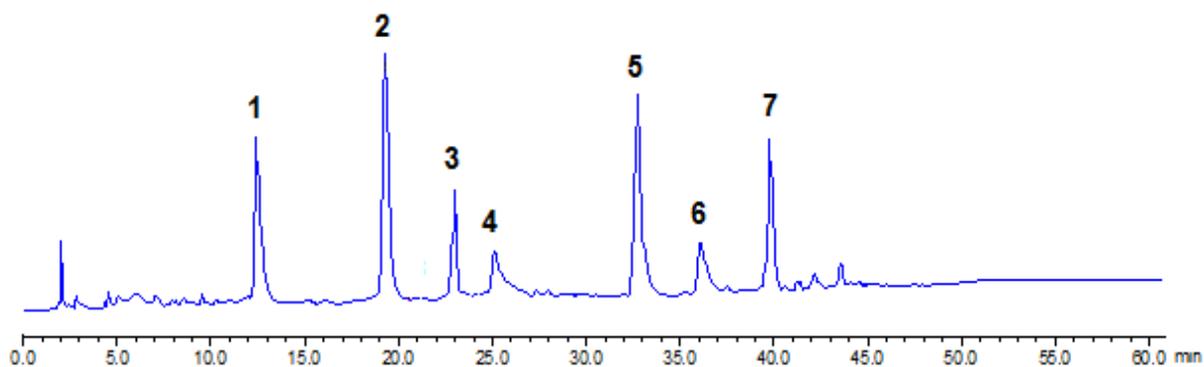
Figure 1: HPLC analysis of the LBJQ. 1-7 represented the phellodendrine (1), baicalin (2), palmatine (3), berberine (4), baicalein (5), wogonin (6) and obacunone (7), respectively
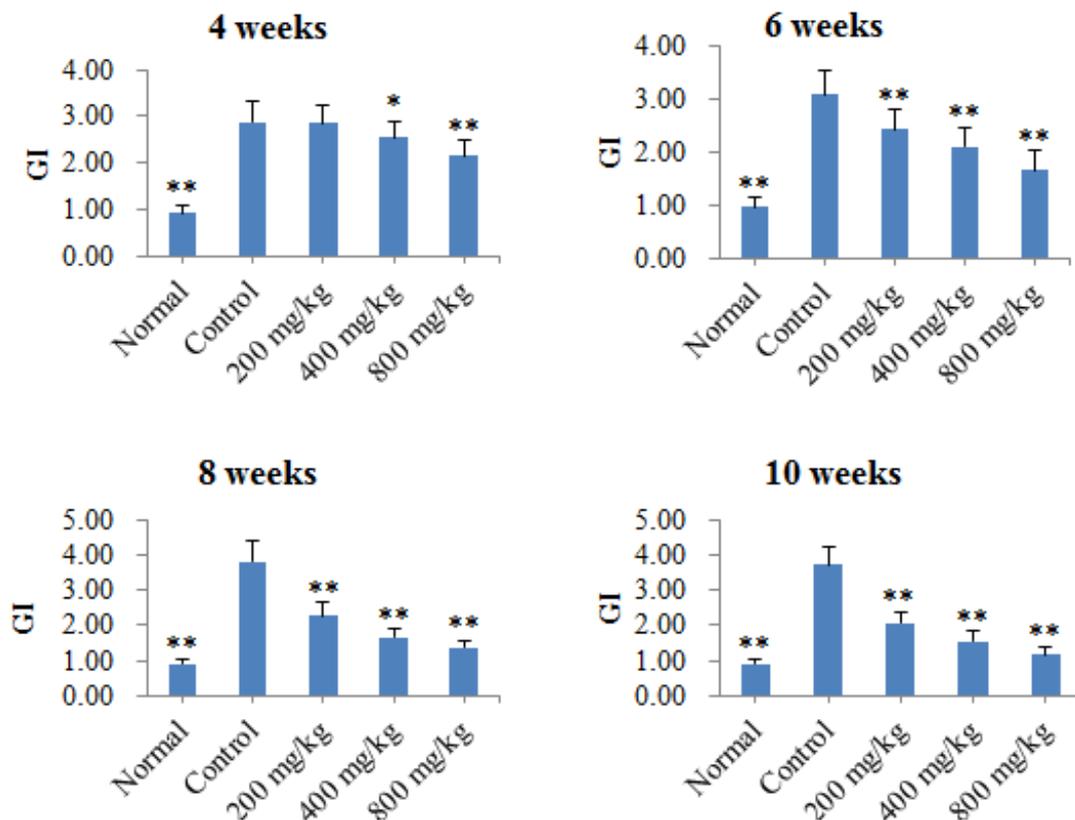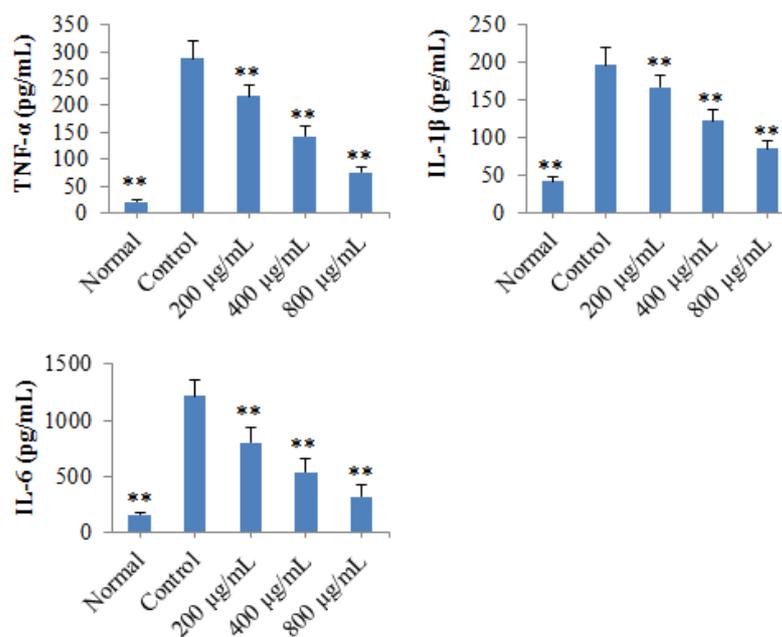

Figure 2: Effect of *LBJQ* on GI of periodontitis rats. Data are represented as mean ± SD (n = 10); **p* < 0.05, ***p* < 0.01, compared to rats in control group
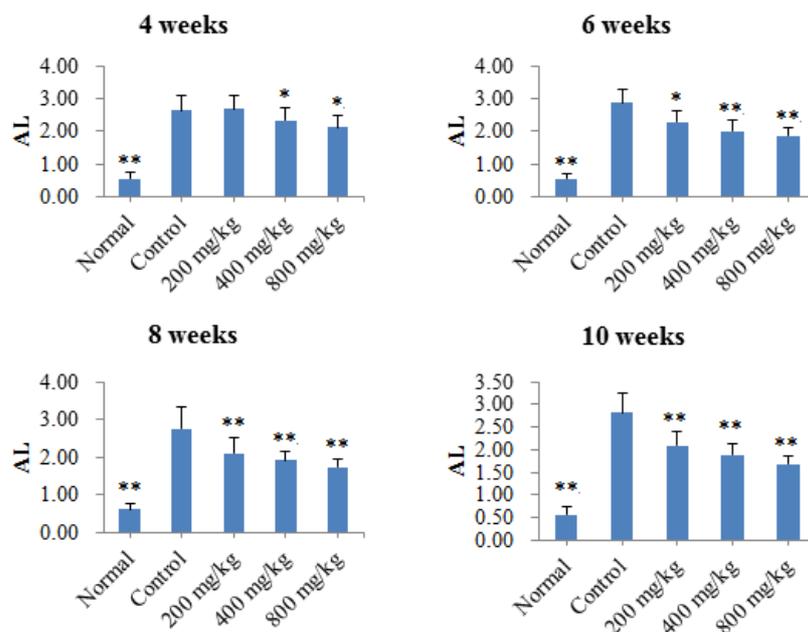
Figure 3: Effect of *LBJQ* on AL of periodontitis rats. Data are represented as mean ± SD (n = 10); *$p < 0.05$, **$p < 0.01$, compared to rats in control group

Table 2: Anti-bacterial activity of *LBJQ*

Bacterial species		Concentration (mg/mL)	
		MIC	MBC
Porphyromonas gingivalis	ATCC 33277	0.64	2.56
Prevotella intermedius	ATCC 25361	0.32	2.56
Actinobacillus actinomycetemcomitans	ATCC 29522	1.28	5.12
Fusobacterium nucleatum	ATCC 23276	0.16	0.64
Streptococcus sanguis	ATCC 6715	0.64	5.12
Streptococcus mutans	ATCC 25175	0.16	1.28

Figure 4: Effects of *LBJQ* on the releases of inflammatory cytokines induced by LPS in RAW 26.7 cells. Data are represented as mean ± SD (n = 10); *$p < 0.05$, **$p < 0.01$, compared to rats in control group.

DISCUSSION

Induction of periodontitis in rat model with dental bacteria-infected silk ligation is a standard experimental model for investigating therapeutic activities of candidate drugs against periodontitis [13,14]. The results of this study demonstrate for the first time, that LBJQ has potential therapeutic effect against experimental periodontitis in rats.

In recent years, studies have revealed that the pathogenesis of periodontitis is closely related to dental plaque bacteria and their metabolic products which induce local inflammatory reactions in pre-disposed hosts [15]. Thus, the search for more effective agents for suppression of microbial invasion, control of inflammation and protection of periodontal tissue are the primary objectives of periodontitis treatment [4,17]. The results of the current research revealed that LBJQ, an age-old Chinese herbal medicine formula, could significantly inhibit the growth of common oral pathogenic bacteria such as *P. gingivalis*, *P. intermedius*, *A. actinomycetem-comitans*, *F. nucleatum*, *S. sanguis* and *S. mutans*.

Pro-inflammatory cytokines play important roles in the development of periodontitis. Inflammatory response leads to leukocyte infiltration, edema, vascular dilatation and release of excess inflammatory cytokines, resulting in detachment of connective tissue, formation of periodontal pocket and resorption of alveolar bone and ultimately tooth loss [18,19]. Previous investigations revealed that some early-response pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β are released in response to external stimuli or tissue injury [20]. However, excessive levels of these pro-inflammatory cytokines activate inflammatory reaction cascades, thereby aggravating the inflammatory response that results in periodontitis [18,20]. It is also known that excess release of these pro-inflammatory cytokines induce damage to bone tissues of the teeth [2,21,22]. The results of this work demonstrate that LBJQ significantly decreased the releases of TNF-α, IL-1β and IL-6, which might be one of the potential mechanisms through which it exerts its therapeutic effect.

CONCLUSION

The findings of this study reveal that LBJQ possesses potential therapeutic effects against periodontitis *in vivo* and *in vitro*. The probable mechanism may be related to its inhibitory effects on release of inflammatory cytokines and its antibacterial activity against oral anaerobic bacteria. This finding may provide a basis for development of a new and effective drug from LBJQ for clinical treatment of periodontitis.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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