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ARTICLE

Protective effect of liensinine on periodontitis through its antioxidant effect in mice

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Abstract Liensinine is the most important functional compound in lotus seeds; its protective effect on periodontitis was determined and the mechanism of its antioxidant effect was also explained. During the experiment, we could observe that liensinine could reduce the gingival index in periodontitis-induced mice, and these effects were similar to that during the treatment of periodontitis with the metronidazole drug. After the treatment with liensinine, the serum Immunoglobulin G1 (IgG_1) level was found to increase and IgG_{2a} level was found to decrease compared to the periodontitis control mice. Liensinine and metronidazole could increase the superoxide dismutase (SOD) serum levels in the periodontitis-induced mice, and both the drugs at the same concentration (200 mg/kg) showed similar SOD activities, which was found to increase. Liensinine also increased the oxidation-related factor of catalase (CAT), glutathione peroxidase, and decreased nitric oxide, malondialdehyde, and endothelin in the serum of mice, compared to the periodontitis control mice. The serum cytokine levels of Interleukin-6, tumor necrosis factor-α, and Interferon-γ in liensinine-treated mice were lower than the control mice, but higher than the normal mice. The serum IL-4 cytokine level of liensinine-treated mice was reversed, i.e., higher than the control mice. In the periodontal tissue, the mRNA and protein expressions of inhibitor of kappa B-α, transforming growth factor beta 1, Interleukin-10, Mn-SOD, Gu/Zn-SOD, and CAT were increased by the treatment with liensinine compared to the control group mice, and nuclear factor kappa B expressions showed the opposite trend. Liensinine is a good functional compound in the treatment of periodontitis, similar to metronidazole drug. Higher concentration of liensinine had better effects.

Keywords Antioxidation · Cytokine · Expression · Liensinine · Periodontitis

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Introduction

Liensinine is an alkaloid compound derived from germ of *Nelumbo nucifera* Gaevth. (Lotus seed). It is the most important functional compound in lotus seed (Lv et al. 2015). Researches have shown that liensinine possesses antioxidant, anti-cancer, and anti-hypertension effects (Xiong et al. 1998; Yu et al. 2013; Yan et al. 2014).

A strong destructive oral disease, periodontitis is a kind of chronic inflammation in gingival and periodontal tissues, which can cause alveolar bone absorption and gradual loosening of teeth, and even loss of teeth (da Costa et al. 2015). Many factors can contribute to periodontitis, but living habits and nutrient deficiencies are the main reasons. In addition to partial treatment in the mouth, there are also other effective ways of relieving the periodontitis, such as the control of whole body, improvement of immunity, as well as balance of organs. In recent research, oxidative stress caused by excess reactive oxygen is considered as a risk for periodontitis (Smiley et al. 2015). The oxidative stress–antioxidant defense balance is closely associated with periodontal status of periodontitis, while its imbalance plays an important role in the nosogenesis of chronic periodontitis and tissue injuries related to periodontitis (Sağlam et al. 2015). The oxidative stress–antioxidant imbalance in the periodontal organization is closely related to the immune response between pathogenic bacteria and host. Then abnormal inflammation/immune reaction happens between microorganism plaque and the body, causing damaged periodontal tissues and inflammation reaction (Batista et al. 2002).

Damaged periodontal tissues result in the formation of lipid peroxides and inflammatory mediators and cause excessive protein oxidation, which can worsen periodontal inflammation and periodontitis (Sezer et al. 2013). Controlling the balance of oxidative stress–antioxidant defense may significantly reduce periodontitis. So, in order to determine the effect of periodontitis treatment, testing the body's antioxidant levels has been adopted. Under laboratory conditions, animal experiments have confirmed that alkaloid substances present in various plants have good inhibitory effect on periodontitis (Yu et al. 2008; Luo et al. 2010). At the same time, it has been proven that liensinine has strong antioxidant effect, and liensinine also has significant preventive effect on liver inflammation. Based on the early-stage research of liensinine, this study establishes the animal model of periodontitis to find out the preventive effect of liensinine on periodontitis in experimental animals. Through periodontitis mice tests, i.e., serum index related to immune, antioxidant and inflammation, expression of mRNA associated with inflammation and oxidation and protein of periodontic mice organizations, the preventive effect and mechanism of liensinine on periodontitis was observed. The results will promote further study of liensinine and provide scientific support for the promotion of liensinine.

Materials and methods

Preparation of periodontitis bacterial liquid

Plaque under the gum was collected from lesions in patients with chronic periodontitis. Plaque was blended with 1.0 mL thioglycollate and diluted with saline to its 1×10^{-3} times. BHI blood agar culture medium was cultivated anaerobically in 1.0 mL bacterial dilution for 5 days and eluted and collected centrifugally (4000 r/min, 5 min) with phosphate-buffered solution (PBS). For further use, the concentration of bacterial suspension was adjusted to 1×10^{8} CFU/mL through McIntosh turbidimetric method.

Animal experiment

KM mice for experiments were divided into 5 groups, including the normal group, periodontitis group, the medical treatment (metronidazole) group (standard, Shanghai Yuanye Biological Technology Co., Ltd., China), liensinine (standard, Shanghai Yuanye Biological Technology Co., Ltd.) low-concentration treatment group (100 mg/kg), and liensinine high-concentration treatment group (200 mg/kg). Each group has 10 mice. At 10 weeks, the normal group was not processed. In the medical treatment group, liensinine low-concentration treatment group and liensinine high-concentration treatment group, each mouse was lavaged orally with 200 mg/kg metronidazole and 100–200 mg/kg liensinine daily. Two weeks later, periodontitis started. All except mice in the normal group were sutured near gum by 5/0 non-absorbable silk embedded into the neck of the first molar tooth. 0.2 mL periodontal pathogens of bacteria suspension were added in the ligation parts of each mouse, every 48 h 3 times. At 0, 2, 4, 6, and 8 weeks, blood of mice was taken through orbital sampling method, and serum was centrifugally separated (4000 r/min, 5 min) for determination. After 10 weeks, the neck of the mice was dissected to collect serum and periodontal tissues for the analyses (Rao et al. 2011). The animal protocol used in this study was reviewed by the Animal Ethics Committee of Chongqing Medical University.

Determination of gingival index

The mice were anesthetized by ether inhalation and were held in the supine position with fixed legs and head. The mice were detected using a blunt periodontal probe for gingival index according to Löe method (Löe 1967), which was divided into four levels: 0, healthy gum; 1, mild inflammation—mild change in color of gums and edema but without clinical hemorrhage; 2, medium gingival inflammation—gum turns red and edema is light and the probe will bleed; 3, severe gum inflammation—swollen; even ulceration appears in gum with automatic bleeding tendency. Each experimental tooth was measured at four points, including near the gingival papilla, the median buccal margin, the distal palatine papilla, and the lingual

gingival margin. And the final score was the average of the four points.

Serum levels

The serum ImmunoglobulinG_1 (IgG_1) and IgG_{2a} levels were measured with experiment kits (Shanghai Yanhui Biological Technology Co., Ltd.); serum superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), nitric oxide (NO), and malondialdehyde (MDA) were determined with experiment kits (Nanjing Jiancheng Bioengineering Institute, China), and endothelin (ET) was measured with kit (Beijing Puer Weiye Biotechnology Co., Ltd., China) according to the manufacturer's protocol. IgG_1 and IgG_{2a} levels were measured at 450 nm after 37 °C water bath treatment. After 37 °C water bath treatment, SOD, CAT, GSH-Px, and NO serum levels were determined at 550, 405, 412, and 550 nm, respectively, and MDA level was determined at 532 nm in 95 °C water bath. ET level was determined by the kit instruction manual at 4 °C, and the depositional cpm numbers were recorded; specification curve was made, and the levels were measured by this specification curve.

Cytokine levels

Serum levels of Interleukin-4 (IL-4), IL-6, tumor necrosis factor-α, (TNF-α), and Interferon-γ (IFN-γ) were measured at 450 nm at room temperature with a commercial ELISA kit (ELISA MAX, Biolegend, USA) according to the manufacturer's protocol.

Reverse transcription polymerase chain reaction (RT-PCR) assay

Total RNA from periodontal tissue was isolated using Trizol reagent (Invitrogen, USA) according to the manufacturer's recommendations. The periodontal tissue RNA was digested with RNase-free DNase (Roche, Switzerland) for 15 min at 37 °C and purified using the RNeasy kit (Qiagen, Germany) according to the manufacturer's protocol. cDNA was synthesized from 2 μg of total RNA by incubation at 37 °C for 1 h with avian myeloblastosis virus reverse transcriptase (GE Healthcare, UK) with random hexanucleotides according to the manufacturer's instructions. Sequences of primers used to specifically amplify the genes of nuclear factor kappa B (NF-κB, forward: 5′-CAC TTA TGG ACA ACT ATG AGG TCT CTG G-3′; reverse: 5′-CTG TCT TGT GGA CAA CGC AGT GGA ATT TTA GG-3′), inhibitor of kappa B-alpha (IκB-α, forward: 5′-GCT GAA GAA GGA GCG GCT ACT-3′; reverse: 5′-TCG TAC TCC TCG TCT TTC ATG GA-3′), transforming growth factor beta (TGF-β, forward: 5′-CTT CAG CTC CAC AGA GAA GAA CGT C-3′; reverse: 5′-CAC GAT CAT GTT GGA CAA CTG CTC-3′), IL-10 (forward: 5′-GGA CTT TAA GGG TTA CTT GGG TTG CC-3′; reverse: 5′-CAT TTT GAT CAT CAT GTA TGC TTC T-3′), Mn-SOD (forward: 5′-TTC AAT AAG GAG CAG GGA C-3′; reverse: 5′-CAG TGT AAG GCT GAC GGT TT-3′), Gu/Zn-SOD (forward: 5′-GAA GAG AGG CAT GTT GGA GA-3′; reverse: 5′-CCA ATT ACA CCA CGA GCC AA-3′), and CAT (forward: 5′-AGA TAC TCC AAG GCG AAG GTG-3′; reverse: 5′-AAA GCC ACG AGG GTC ACG AAC-3′) (Thermo Fisher Scientific, USA) were determined. Amplification was performed in a thermal cycler (Eppendorf, Germany). The polymerase chain reaction (PCR) products were separated in 1.0 % agarose gels and visualized with ethidium bromide staining (Zhao et al. 2014).

Western blot analysis

Total protein was obtained from the periodontal tissue using radio-immunoprecipitation assay buffer as previously described. The protein concentrations were determined using a Bio-Rad protein assay kit. The nitrocellulose membranes (Schleicher and Schuell, USA) were then subjected to immunoblot analysis and the proteins were visualized using an enhanced chemiluminescence (ECL) method (GE Healthcare). 50 μg of protein from treated cells was loaded onto the gels. The cell lysates were separated using 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride membrane (GE Healthcare), blocked with 5 % skim milk, and then hybridized with primary antibodies (diluted 1:1000). The antibodies against NF-κB, IκB-α, TGF-β, IL-10, Mn-SOD, Gu/Zn-SOD, and CAT were obtained from Abcam (USA). The membranes were then incubated with the HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, USA) for 1 h at room temperature. The blots were washed three times with PBS-T and developed using an ECL reagent (Amersham Life Science, USA) (Zhao et al. 2013).

Statistical analysis

Data are presented as mean ± standard deviation. Differences between the mean values for individual groups were assessed with one-way analysis of variance with Student–Neumann–Keuls post hoc test, and the data were tested for a two-sided statistical testing. $p < 0.01$ and $p < 0.05$ were considered to indicate a statistically significant difference. SPSS software 19.0 (IBM Software, USA) was used for statistical analyses.

Results

Gingival index of mice

As shown in Table 1, the gingival index in different groups had no difference at 0 week, and the normal group also showed no difference during the experiment, but the other groups showed a significant difference of gingival index after 2 weeks ($p < 0.05$). The gingival index was raised by periodontitis, and the control mice at 10 weeks showed the highest gingival index at 3.725. Liensinine could reduce the gingival index, and the high concentration of 200 mg/kg treatment showed stronger decreasing effects than 100 mg/kg liensinine treatment from 2 to 10 weeks, and these effects of 200 mg/kg treatment were close to those of metronidazole.

IgG_1 and IgG_{2a} serum levels of mice

The normal mice had no difference ($p < 0.05$) in IgG_1 and IgG_{2a} serum values during the whole experiment (Tables 2, 3), but the IgG_1 serum values of control group mice decreased and IgG_{2a} serum values increased with increase in time. Liensinine and metronidazole raised the IgG_1 and reduced IgG_{2a} serum values after 2 weeks in periodontitis mice. The 200 mg/kg liensinine and 200 mg/kg metronidazole showed similar effects and the IgG_1 and IgG_{2a} serum levels of the treated mice close to those of the normal mice at 2 to 10 weeks. The 100 mg/kg liensinine also showed to have some effects in mice, but weaker than 200 mg/kg liensinine and 200 mg/kg metronidazole.

SOD level of mice

During the experiment, the normal group mice had the highest SOD levels, and the control group mice had the lowest SOD contents from the second week onwards (Table 4). Liensinine could increase the SOD levels in periodontitis mice, and high concentration (200 mg/kg) of liensinine could raise SOD contents compared to low concentration (100 mg/kg) of liensinine at 2, 4, 6, 8, and 10 weeks. Metronidazole also could raise the SOD levels compared to the control mice, and its effects were as strong as 400 mg/kg liensinine.

Serum CAT, GSH-Px, NO, MDA, and ET levels in mice

As shown in Table 5, the CAT and GSH-Px levels of normal mice were highest, and the NO, MDA, and ET levels were lowest. The control group mice had the reversal effect, i.e., these mice showed the lowest CAT and GSH-Px levels and the highest NO, MDA, ET levels. The 100 and 200 mg/kg liensinine could significantly increase the CAT, GSH-Px levels and decrease the NO, MDA, and ET levels compared to the control group ($p < 0.05$). The 200 mg/kg liensinine-treated mice and 200 mg/kg drug of metronidazole-treated mice showed similar serum CAT, GSH-Px, NO, MDA, and ET levels.

Cytokine IL-4, IL-6, TNF-α, and IFN-γ levels in mice

The levels of IL-6, TNF-α, and IFN-γ cytokine in control group mice were higher than the other group mice, and IL-4 level was lower than the other group mice (Table 6). Liensinine could make these levels near to the normal mice, and 200 mg/kg liensinine made the closest levels to normal mice. These activities were close to the drug control of metronidazole.

mRNA and protein expressions of NF-κB, IκB-α, TGF-β1, and IL-10 in periodontal tissue of mice

After the RT-PCR and Western blot determination, the results showed that the normal mice have the strongest

Table 1 Gingival index of liensinine-treated periodontitis mice

Group	0 week	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
Normal	0.962 ± 0.051^{a}	0.956 ± 0.078^{D}	0.948 ± 0.031^{d}	0.960 ± 0.036^{D}	0.975 ± 0.042^{a}	0.970 ± 0.045^{D}
Control	0.969 ± 0.052^{a}	1.715 ± 0.063^{A}	2.578 ± 0.062^{a}	2.826 ± 0.060^{A}	3.245 ± 0.059^{d}	3.725 ± 0.071^{A}
Liensinine (mg/kg)						
100	0.973 ± 0.061^{a}	1.522 ± 0.046^{B}	2.112 ± 0.058^{b}	2.220 ± 0.066^{B}	2.452 ± 0.053^{b}	2.278 ± 0.058^{B}
200	0.963 ± 0.070^{a}	1.379 ± 0.047^{C}	1.752 ± 0.051^{c}	1.792 ± 0.057^{C}	1.875 ± 0.066^{c}	1.652 ± 0.061^{C}
Metronidazole (200 mg/kg)	0.977 ± 0.071^{a}	1.368 ± 0.052^{C}	1.744 ± 0.052^{c}	1.785 ± 0.063^{C}	1.869 ± 0.052^{c}	1.650 ± 0.057^{C}

[a] Mean values with same letters in the column are not significantly different ($p < 0.05$)

A–D, *a–d*, *A–D*, **a–d**, **A–D** Mean values with different letters in the column are significantly different ($p < 0.05$) according to Duncan's multiple range test

Table 2 Serum IgG_1 levels of liensinine-treated periodontitis mice (A value)

Group	0 week	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
Normal	0.389 ± 0.015[a]	0.393 ± 0.017[A]	0.395 ± 0.011[a]	0.391 ± 0.021[A]	0.395 ± 0.018[a]	0.398 ± 0.014[A]
Control	0.391 ± 0.012[a]	0.322 ± 0.015[C]	0.295 ± 0.015[d]	0.247 ± 0.018[E]	0.215 ± 0.020[d]	0.189 ± 0.009[D]
Liensinine (mg/kg)						
100	0.388 ± 0.020[a]	0.328 ± 0.021[C]	0.322 ± 0.008[c]	0.287 ± 0.016[D]	0.245 ± 0.013[c]	0.237 ± 0.018[C]
200	0.392 ± 0.009[a]	0.342 ± 0.013[BC]	0.340 ± 0.011[bc]	0.325 ± 0.007[C]	0.299 ± 0.016[b]	0.287 ± 0.011[B]
Metronidazole (200 mg/kg)	0.394 ± 0.010[a]	0.351 ± 0.016[C]	0.353 ± 0.012[b]	0.348 ± 0.010[B]	0.305 ± 0.012[b]	0.293 ± 0.017[B]

[a] Mean values with same letters in the column are not significantly different ($p < 0.05$)

A–C, *a–d*, *A–E*, **a–d**, **A–D** Mean values with different letters in the column are significantly different ($p < 0.05$) according to Duncan's multiple range test

Table 3 Serum IgG_{2a} levels of liensinine-treated periodontitis mice (A value)

Group	0 week	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
Normal	0.613 ± 0.031[a]	0.618 ± 0.027[C]	0.609 ± 0.022[d]	0.621 ± 0.033[D]	0.615 ± 0.019[d]	0.622 ± 0.025[D]
Control	0.620 ± 0.022[a]	1.058 ± 0.032[A]	1.272 ± 0.029[a]	1.365 ± 0.019[A]	1.492 ± 0.023[a]	1.648 ± 0.036[A]
Liensinine (mg/kg)						
100	0.617 ± 0.024[a]	1.021 ± 0.035[A]	1.156 ± 0.021[b]	1.188 ± 0.030[B]	1.154 ± 0.020[b]	1.012 ± 0.028[B]
200	0.610 ± 0.019[a]	0.878 ± 0.028[B]	0.972 ± 0.018[c]	0.982 ± 0.025[C]	0.910 ± 0.032[c]	0.802 ± 0.019[C]
Metronidazole (200 mg/kg)	0.615 ± 0.026[a]	0.871 ± 0.026[B]	0.968 ± 0.022[c]	0.973 ± 0.024[C]	0.902 ± 0.027[c]	0.795 ± 0.029[C]

[a] Mean values with same letters in the column are not significantly different ($p < 0.05$)

A–C, *a–d*, *A–D*, **a–d**, **A–D** Mean values with different letters in the column are significantly different ($p < 0.05$) according to Duncan's multiple range test

Table 4 Serum SOD levels of liensinine-treated periodontitis mice (U/mL)

Group	0 week	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
Normal	119.45 ± 12.37[a]	113.79 ± 11.65[A]	117.65 ± 19.52[a]	115.64 ± 12.20[A]	110.23 ± 15.51[a]	112.61 ± 14.78[A]
Control	118.54 ± 14.50[a]	94.52 ± 7.68[C]	80.39 ± 5.32[c]	71.25 ± 4.45[D]	62.33 ± 3.78[d]	54.51 ± 3.18[D]
Liensinine (mg/kg)						
100	116.52 ± 13.37[a]	103.25 ± 6.58[B]	85.21 ± 5.50[c]	84.20 ± 4.11[C]	75.61 ± 4.52[c]	72.52 ± 4.32[C]
200	121.03 ± 10.28[a]	107.62 ± 4.56[BC]	93.52 ± 6.02[b]	90.43 ± 5.36[B]	88.39 ± 5.20[b]	87.23 ± 4.71[B]
Metronidazole (200 mg/kg)	116.54 ± 15.63[a]	108.37 ± 5.06[BC]	94.27 ± 5.88[b]	91.20 ± 5.17[B]	90.22 ± 5.63[b]	89.33 ± 5.08[B]

[a] Mean values with same letters in the column are not significantly different ($p < 0.05$)

A–C, *a–c*, *A–D*, **a–d**, **A–D** Mean values with different letters in the column are significantly different ($p < 0.05$) according to Duncan's multiple range test

mRNA and protein expression of IκB-α (1.77- and 7.39-fold of mRNA and protein expressions in control), TGF-β1 (2.37- and 3.55-fold of mRNA and protein expressions in control), IL-10 (22.61- and 4.21-folds of mRNA and protein expressions in control), and weakest NF-κB (0.35- and 0.32-fold mRNA and protein expressions in control) expressions (Fig. 1). The periodontitis induced (control group) reduce the IκB-α, TGF-β1, IL-10 mRNA and protein expressions and raise NF-κB expressions. Liensinine raised the IκB-α, TGF-β1, IL-10 expressions and reduced NF-κB expressions compared to the control mice, and the 200 mg/kg liensinine increased the IκB-α (1.28 and 4.70-fold of mRNA and protein expressions in control), TGF-β1 (1.83 and 2.11-fold of mRNA and protein expressions in control), IL-10 (11.01 and 1.65-fold of mRNA and protein expressions in control) expressions and reduced the NF-κB (0.49 and 0.57-fold of mRNA and protein expressions in control) expressions than 100 mg/kg liensinine; these

Table 5 Serum CAT, GSH-Px, NO, MDA, and ET levels of liensinine-treated periodontitis mice at 10 weeks

Group	CAT (U/ mL)	GSH-Px (U/ mL)	NO (μmol/L)	MDA (nmol/L)	ET (pg/mL)
Normal	6.34 ± 0.22^{a}	30.52 ± 2.21^{A}	33.17 ± 2.69^{d}	13.25 ± 0.74^{E}	12.08 ± 0.42$^{\mathbf{d}}$
Control	2.38 ± 0.17^{d}	11.62 ± 0.52^{E}	64.52 ± 5.14^{a}	37.68 ± 1.28^{A}	35.17 ± 2.23$^{\mathbf{a}}$
Liensinine (mg/kg)					
100	3.75 ± 0.29^{c}	16.28 ± 1.71^{D}	52.02 ± 3.89^{b}	30.52 ± 1.44^{B}	27.85 ± 1.33$^{\mathbf{b}}$
200	5.05 ± 0.48^{b}	22.68 ± 2.06^{C}	42.36 ± 4.46^{c}	22.53 ± 1.28^{C}	18.91 ± 1.75$^{\mathbf{c}}$
Metronidazole (200 mg/kg)	5.25 ± 0.53^{b}	27.21 ± 1.856^{B}	40.25 ± 3.96^{c}	19.30 ± 0.72^{D}	17.63 ± 2.06$^{\mathbf{c}}$

a–d, A–E, *a–d*, *A–E*, **a–d** Mean values with different letters in the column are significantly different ($p < 0.05$) according to Duncan's multiple range test

Table 6 Cytokine IL-4, IL-6, TNF-α, and IFN-γ levels of liensinine-treated periodontitis mice at 10 weeks

Group	IL-4 (pg/mL)	IL-6 (pg/mL)	TNF-α (pg/mL)	IFN-γ (pg/mL)
Normal	26.31 ± 1.78^{a}	42.33 ± 3.49^{E}	44.65 ± 3.65^{e}	43.20 ± 2.41^{D}
Control	8.52 ± 0.38^{d}	117.25 ± 11.30^{A}	125.61 ± 12.01^{a}	95.62 ± 6.23^{A}
Liensinine (mg/kg)				
100	13.65 ± 0.72^{c}	87.69 ± 8.52^{B}	102.31 ± 9.15^{b}	74.30 ± 3.68^{B}
200	18.82 ± 1.13^{b}	65.44 ± 5.52^{C}	75.21 ± 6.42^{c}	58.75 ± 4.26^{C}
Metronidazole (200 mg/kg)	19.31 ± 1.32^{b}	51.25 ± 4.26^{D}	62.36 ± 5.18^{d}	55.37 ± 4.82^{C}

a–d, A–E, *a–e*, *A–D* Mean values with different letters in the column are significantly different ($p < 0.05$) according to Duncan's multiple range test

expressions were similar to those of metronidazole-treated mice.

mRNA and protein expressions of Mn-SOD, Gu/Zn-SOD, and CAT in periodontal tissue of mice

The mRNA and protein expressions of Mn-SOD, Gu/Zn-SOD, and CAT of normal mice were highest in all mice (Fig. 2), and liensinine- and metronidazole-treated mice also showed higher Mn-SOD, Gu/Zn-SOD, and CAT expressions compared to control mice. The levels of Mn-SOD (1.37- and 1.40- fold of mRNA and protein expressions in control), Gu/Zn-SOD (1.34- and 1.39-fold of mRNA and protein expressions in control), and CAT (1.64- and 1.50-fold of mRNA and protein expressions in control) when treated with the 200 mg/kg liensinine were similar to those when treated with metronidazole; they could make the Mn-SOD, Gu/Zn-SOD, and CAT expressions close to the normal mice.

Discussion

In this study, the standard of liensinine was researched in periodontitis mice, and metronidazole was used as drug control. Gingival index can show preliminary conditions to detect the change of gum color and dentine, as well as bleeding tendency, which can be clinically used as an evidence of periodontitis. Gingival index of mice can also check the degree of periodontitis in mice. Two weeks later, because of the induced periodontitis, gingival index of the control group without treatment increases with time (Pan et al. 2011). Metronidazole (200 mg/kg) and 200 mg/kg liensinine can significantly reduce gingival index, which comes close to normal after 10 weeks, while 100 mg/kg liensinine can reduce gingival index to a certain extent and alleviate periodontitis. As a result, liensinine has great effect on prevention and treatment for periodontitis, even recovering the periodontal health under a certain concentration.

Studies have shown that periodontal tissue damage is primarily caused by the host immune and inflammation response to infection, and periodontal pathogens and their toxic products lead to inflammation of periodontal tissues and periodontitis (Jin et al. 2015). Th1 and Th2 cells are mutual regulatory cells, restraining each other through the secretion of different cell factors, whose balance maintains the normal function of the body's immune system. During periodontitis, the body's immune response shifts to Th1, and Th1 polarization in periodontitis promotes inflammation (Hienz et al. 2015). Th2 cell reaction can reduce the destruction of periodontal tissues and Th2 immune response can protect from periodontal infection. The production of antibodies is regulated by Th1 and Th2

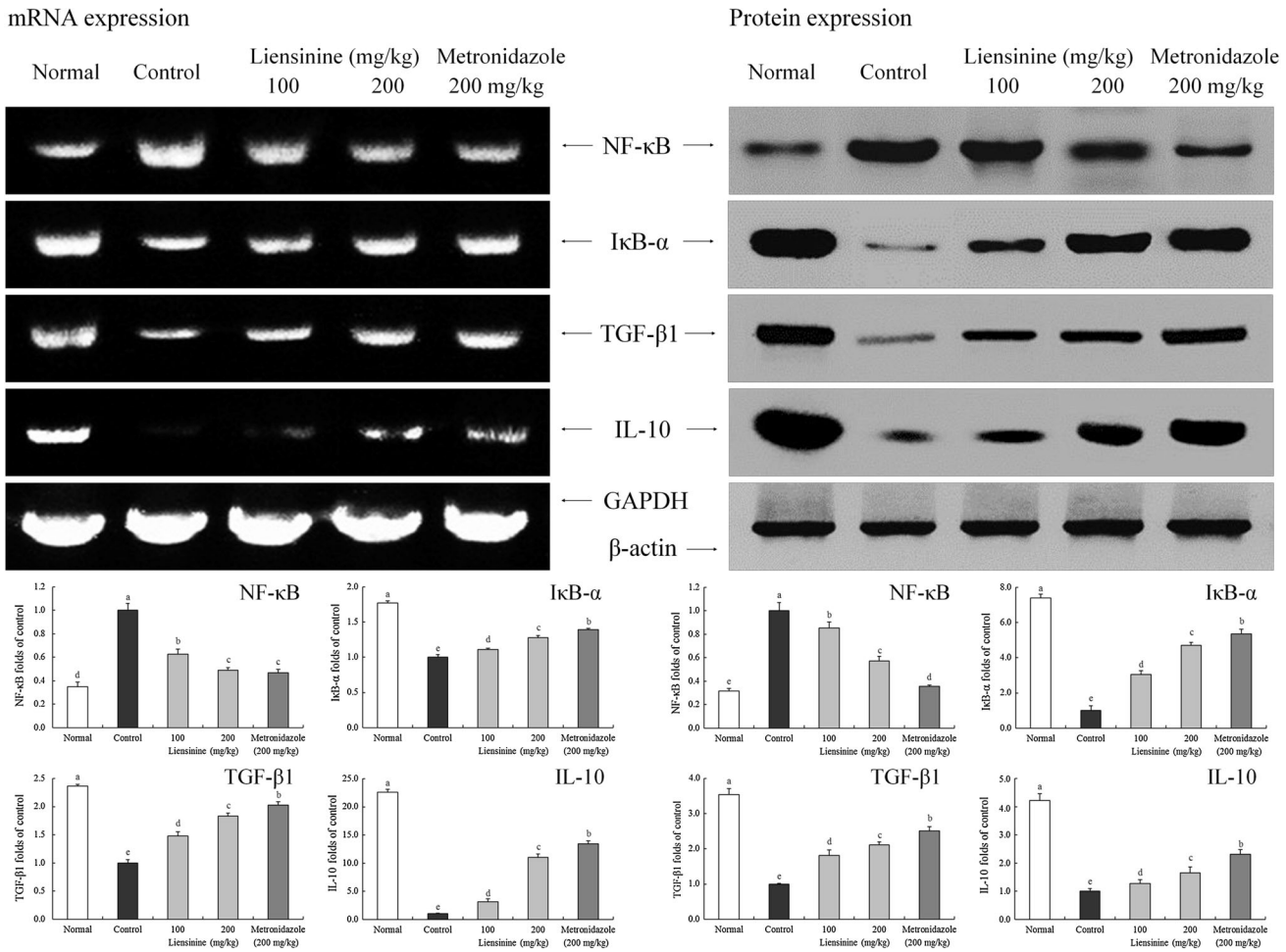

Fig. 1 Effect of liensinine on the mRNA and protein expressions of NF-κB, IκB-α, TGF-β, and IL-10 in periodontitis mice. Fold ratio: gene expression/GAPDH (β-actin) × control numerical value (control fold ratio: 1). **a–e** Mean values with *different letters* over the bars are significantly different ($p < 0.05$) according to Duncan's multiple range test

cytokines, which is also associated with the expression level of specific antibody IgG subcategories (Nouri et al. 2015). Th1 cytokine stimulates the production of IgG_{2a}, while Th2 cytokine promotes the synthesis of IgG_1. During periodontitis, IgG_1 level goes down, while IgG_{2a} level rises (Verma et al. 2010). That is to say, Th2 cell reaction is inhibited, while Th1 cell reaction is active. Immune response of periodontitis mice shifts to Th1, so periodontal inflammation worsens. Liensinine can obviously improve IgG_1 level of periodontitis mouse and can lower its IgG_{2a} level, showing its effect on prevention and treatment of periodontitis.

The imbalance of oxidative reaction and antioxidant defense in periodontitis tissues has direct relationship with disorder of the body's immune response (Balaji et al. 2015). Because the immune cell membranes contain high-concentration unsaturated fatty acids which are highly sensitive to peroxidation, immune cells are very sensitive to oxidative reaction, so immune cells are stimulated by excessive free radicals and its immune function declines, which affect the function of the immune system (Tkachev et al. 2015). The immune ability to pathogenic bacteria declines, causing inflammation and worsening periodontitis (Hajishengallis 2015). When human body lesions occur, Th1/Th2 cytokines and the level of free radicals in the body will change accordingly. So testing oxidation-related index and corresponding cytokine levels in mice serum can directly observe the development trend of periodontitis (Chen et al. 2015a, b). As an important active substance in body, SOD can destroy pathogens and exclusively scavenge harmful free radicals and harmful substances produced by pathogenic bacteria and inflammation, which is beneficial for the prevention of inflammation and the recovery of the body (Abbès et al. 2015).

Three types of enzymes sorted (Cu/Zn-SOD, Mn-SOD, Fe-SOD) by different metal ions in SOD have similar effect (Huseynova et al. 2014). Studies have confirmed that NO has direct influence to maintain the balance of normal

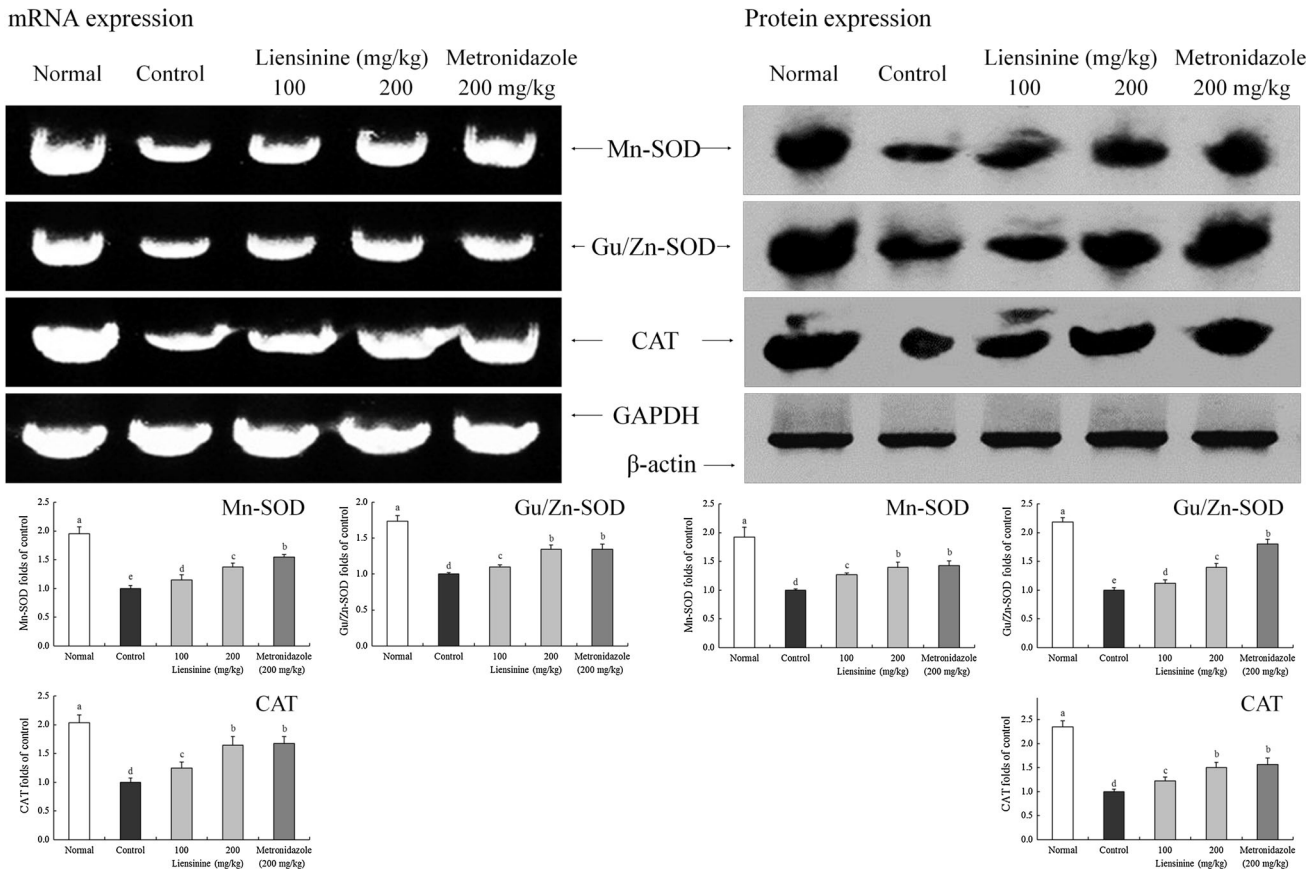

Fig. 2 Effect of liensinine on the mRNA and protein expressions of Mn-SOD, Gu/Zn-SOD, and CAT in periodontitis mice. Fold ratio: gene expression/GAPDH (β-actin) × control numerical value (control fold ratio: 1). **a–e** Mean values with *different letters* over the bars are significantly different ($p < 0.05$) according to Duncan's multiple range test

flora in oral cavity and anti-microbial infection. During periodontitis, with the increase of ET in body, the level of NO also increases to fight against the strong contraction of small blood vessels and capillaries in periodontal tissues caused by ET, but excessive NO produced in the process of defense reaction causes destruction of periodontal tissues and worsens periodontitis, so clinical tests suggest that patients with periodontitis show high level of NO in the body (Chen et al. 2000; Ring and Stremmel 2000).

As enzyme remover, there are a lot of CAT in the body, which can remove H_2O_2 in order to protect the cells from the toxic effects of H_2O_2 and maintain periodontal health (Trawczyńska and Wójcik 2015). MDA is a kind of lipid peroxidation product produced in tissues after injury and inflammation. The accumulation of MDA is a sign of worse inflammation and decline immune ability. Lowering MDA level in body can ease the oxidation in body and enhance immune ability, which can alleviate the effect of periodontitis (Kara et al. 2013). GSH-Px is one of the important members of antioxidant enzyme system. It is reported that in periodontal lesion tissues, the activity of GSH-Px decreases, so the increase of GSH-Px in body can help protect the body tissues from oxidation and can protect the periodontal tissues (Tonguç et al. 2011). The experimental results show that liensinine can increase the levels of SOD, CAT, and GSH-Px in periodontitis mice and lower the levels of NO and MDA, thereby showing a strong antioxidant effect in vivo, and thus plays an important role in alleviating periodontitis.

Th1 immune response is the advantageous response in periodontitis, and TNF-α and IFN-γ cytokines change a lot during periodontitis (Chen et al. 2015a, b). Meanwhile, the concentration in serum of Th2 cytokines, such as IL-4 and IL-6, also changes correspondingly. When inflammation happens in body, the level of TNF-α, IFN-γ, and IL-6 significantly increase, while the level of IL-4 level drops (Souto et al. 2014). The same result shows that liensinine can inhibit periodontitis.

Antioxidant system and immune system are inseparable in body, and NF-κB signaling pathway is an important approach to link the two systems (Jayachandran et al. 2015). Oxidative reaction causes autoimmune diseases, in which excessive oxygen-free radicals oxidize serum albumin, induce the synthesis of autoantibody, and cause

disorder of system functions. As oxidative reaction's sensitivity transcription factor, IκB-α is directly involved in inflammation and activate NF-κB that induces the expression of inflammatory cytokines, such as TNF-α and IL-6, causing inflammation (Chen et al. 2015a, b; Kim et al. 2015). IκB-α plays an important role in adjusting the activity of NF-κB. While the degradation of IκB-α, NF-κB phosphorylation and nuclear transcription processes are strengthened, causing severer inflammation. By direct inhibition of the activity of NF-κB, IκB-α can controls the inflammation (Xia et al. 2015). Until now, transcription factor Foxp3 in the cell nucleus has been considered as the most characteristic marker of Treg cells, which can maintain phenotype and inhibition function of Treg cells. Absence or apoptosis of Foxp3 in Treg cells prompt effective T cells and immune response. All damaged tissues of periodontal diseases contain Foxp3, TGF-β, and IL-10 (Bollyky et al. 2009). Among them, IL-10 is an anti-inflammatory cytokine produced by Th2 cells, which can maintain high-level antibody titer and inhibit the synthesis of pro-inflammatory cytokines (Itoga et al. 2015). TGF-β can promote the development of Treg cells and maintain the expression of Foxp3 in cells (Winkler et al. 2015). The simultaneous stimulation of TGF-β and TCR can make Foxp3 negative CD4+ and CD25+ cells express Foxp3, which will transform into immune regulating cells and alleviate inflammation (Amarnath et al. 2007). Therefore, the expressions of TGF-β and IL-10 in patients with periodontitis are significantly lower than that in normal people. In this study, liensinine can significantly increase the expressions of IκB-α, TGF-β, and IL-10 in gingival tissues. Lowering the expression of NF-κB has good treatment effect on periodontitis (Cardoso et al. 2008; Vardar-Sengul et al. 2009), similar to metronidazole. Through further analysis of expressions of mRNA and protein, oxidation-related expression in gingival tissues is influenced by liensinine and the body's antioxidant level improves, which will alleviate the damage of periodontitis to the body.

By simulating the human periodontitis state on mice to establish the animal model, this study tests reveals liensinine inhibition of periodontitis and observes possible liensinine antioxidant mechanism to periodontitis. Liensinine can significantly inhibit periodontitis in mice, and its effect is similar to drug treatment through extracts from harmless food. Meanwhile, the experimental results further proved that liensinine may has periodontitis prevent effects through its antioxidant activities in the mice body. As a result, liensinine is a kind of functional compound from natural food, which can restrain periodontitis through its antioxidant effect and has good development and utilization value.

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