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The Anti-inflammatory Effect of Z-Ligustilide in Experimental Ovariectomized Osteopenic Rats

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ABSTRACT—The purpose of the present study was to investigate the anti-inflammatory activity of Z-Ligustilide (LIG) in experimental ovariectomized (OVX) osteopenic rats. The anti-inflammatory potential of LIG in the regulation of nuclear factor kappa B (NF-κB), maleic dialdehyde (MDA), polymorphonuclear cells (PMN), interleukin-1β (IL-1β), inducible nitric oxide synthase (iNOS) and tumor necrosis factor-α (TNF-α), adhesion molecule (ICAM-1) and cyclooxygenase-2 (COX-2) was determined by ELISA. LIG significantly inhibited OVX-induced up-regulation of NF-κB activation and the production of IL-1β, TNF-α, iNOS, ICAM-1 and COX-2. Moreover, LIG suppressed MDA and infiltration of PMN. The results of the present study clearly demonstrate that there may be an inflammatory component in the etiology of osteoporosis. It revealed a significant anti-inflammatory effect of Z-Ligustilide in experimental OVX osteopenic rats.

KEY WORDS: Z-Ligustilide; inflammation; osteoporosis; ELISA.

INTRODUCTION

Several inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, celiac disease, cystic fibrosis and chronic obstructive pulmonary disease have been associated to bone resorption [1]. Clinical observations reveal coincidence of systemic osteoporosis with period of systemic inflammation as well as co-localization of regional osteoporosis with areas of regional inflammation [2]. Recently, growing understanding of the bone remodelling process suggests that factors involved in inflammation are linked with those critical for bone physiology and remodelling, supporting the theory that inflammation significantly contributes to the etiopathogenesis of osteoporosis [3, 4]. Many studies report an increase in the risk of developing osteoporosis in various inflammatory conditions [5, 6].

Popularity of herbal drugs is increasing all over the world because of lesser side effects as compared to synthetic drugs. Herbal or medicinal plant products in various forms have been available for many hundreds of years for treatment of diseases in both Eastern and Western cultures [7]. The roots of *Angelica sinensis* (Oliv.) Diels (Dang Gui; Apiaceae) have a long history in traditional Chinese medicine as a remedy for women's disorders [8–12]. It has been reported that extracts isolated from *A. sinensis* exert antiproliferative effects on tumor cells, reduce oxidative stress and protect cardiomyocytes against oxidant injury by increasing cellular GSH [13–15]. Z-Ligustilide (LIG), isolated and purified from the essential oil of *A. sinensis*, facilitates blood circulation and attenuates inflammatory pain behaviour in mice [16]. However, the anti-inflammatory effect of LIG has not been examined. In the current study, we investigated the anti-inflammatory effect of LIG in experimental ovariectomized osteopenic rats.

MATERIAL AND METHODS

Plant Material

Z-Ligustilide was obtained from Shanghai Yixin bio-technology Co. Ltd., China.

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Animals

Healthy female adult Wistar rats (2 months old and weighing 225±25 g) were used in the study. All animal experiments followed the guidelines published by the Ministry of Science and Technology of the People's Republic of China. Care was taken to minimize discomfort, distress and pain to the animals.

Experimental Design

The rats were randomly divided into five groups of animals: four ovariectomized (OVX) and another given a sham operation (control). Then groups 1 (n=10, sham) and 2 (n=10, OVX) were treated with vehicle (PBS). Group 3 (n=10) was treated with 20 mg LIG (OVX + LIG at 20 mg/kg/day). Group 4 (n=10) was treated with 40 mg LIG (OVX + LIG at 40 mg/kg/day), and group 5 (n=10) was treated with 80 mg LIG (OVX + LIG at 80 mg/kg/day) for 8 weeks, respectively.

Eight weeks later, the rats were sacrificed; the blood was collected, and serum was stored immediately at −20 °C to estimate inflammatory cells and inflammatory mediators.

Measurement of Maleic Dialdehyde (MDA)

MDA was determined with thiobarbituric acid (TBA) using the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute). Total protein content of the samples was analyzed using Coomassie blue assay (Nanjing Jiancheng Bioengineering Institute).

Measurement of Infiltration of PMN

Meloperoxidase (MPO) activity was measured to assess the extent of PMN infiltration. The method of assaying MPO activity was according to the guide of the assay kit (Nanjing Jiancheng Bioengineering Co Ltd, China).

Measurement of IL-1β and TNF-α Level

The concentration of IL-1β and TNF-α was determined using a commercial ELISA kit (Shanghai Jinma Biological Technology, Inc., China) following the manufacturer's instruction.

Measurement of NF-κB Activation

The activation of NF-κB was determined using an ELISA kit (Shanghai Jinma Biological Technology, Inc., China), following the manufacturer's instruction. This kit specifically detects the p50 member of NF-κB.

Measurement of COX-2, iNOS and ICAM-1 Level

The procedures were processed according to the protocols recommended for the COX-2, iNOS and ICAM-1 immunohistochemistry kit (Hengdabaisheng Biotechnology, Beijing, China).

Statistical Analysis

The data were expressed as mean ± S.E.M., and results were analyzed by ANOVA followed by Dunnett's t test. $P<0.05$ was considered significant.

RESULTS

The MDA in the sham group was found to be 0.963±0.006 nmol. A significant decrease in the MDA level was observed in the OVX group, as compared to the sham rats ($P<0.01$). The levels of MDA decreased after administration of 80 mg LIG (1.225±0.036 nmol, $P<0.01$) and 40 mg LIG (1.553±0.066 nmol, $P<0.05$). However, the same results did not occur in the OVX-20-treated group (2.558±0.088 nmol) (Fig. 1).

The activity of MPO was determined as an indicator of PMNs migration. In this study, the MPO activity was 0.90 U/g in sham rats and significantly increased in the OVX group. Treatment with 80 mg LIG significantly reduced MPO activity (1.30 U/g, $P<0.05$). Treatment with 20 mg LIG and 40 mg LIG reduced MPO activity also. However, it is not significant (Fig. 2).

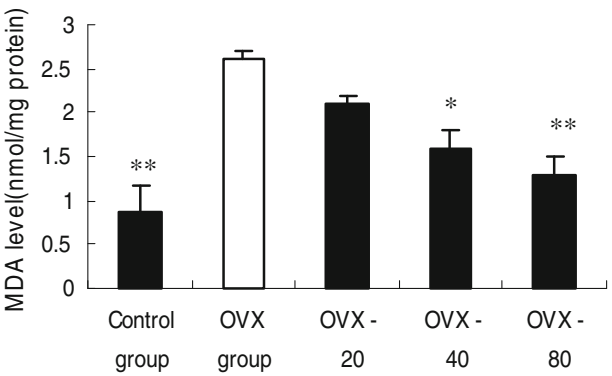

Fig. 1. Effect of Z-Ligustilide on MDA level. The levels of MDA decreased after administration of 80 mg LIG ($P<0.01$) and 40 mg LIG ($P<0.05$). However, the same results did not occur in the OVX-20-treated group. Values are shown as means ± SEM. *$P<0.05$ vs. OVX group, **$P<0.01$ vs. OVX group.

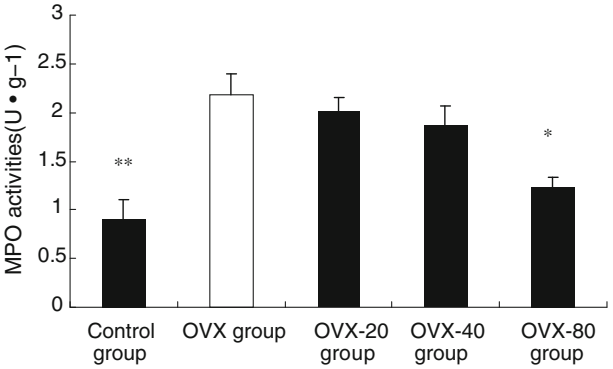

Fig. 2. Effect of Z-Ligustilide MPO activities. Treatment with 80 mg LIG significantly reduced MPO activity ($P<0.05$). Treatment with 20 mg LIG and 40 mg LIG reduced MPO activity also. However, it is not significant (Fig. 2). Values are shown as means ± SEM. *$P<0.05$ vs. OVX group, **$P<0.01$ vs. OVX group.

Figure 3 shows that OVX significantly increased protein concentration of IL-1β in the blood. Treatment with 80 mg LIG decreased the level of IL-1β (13.93 pg/mg) as compared to the OVX group, respectively ($P<0.05$). As shown in Fig. 4, the levels of TNF-α elevated significantly after OVX; 80 mg LIG suppressed this response (0.16 ng/μg, $P<0.05$). As shown in Fig. 5, OVX significantly induced activated NF-κB above control levels, and 80 mg LIG significantly suppressed this response (0.290, $P<0.05$).

Rats subjected to OVX showed typical markers of inflammation up-regulation of adhesion molecule and induction of prooxidative enzymes (COX-2 and iNOS).

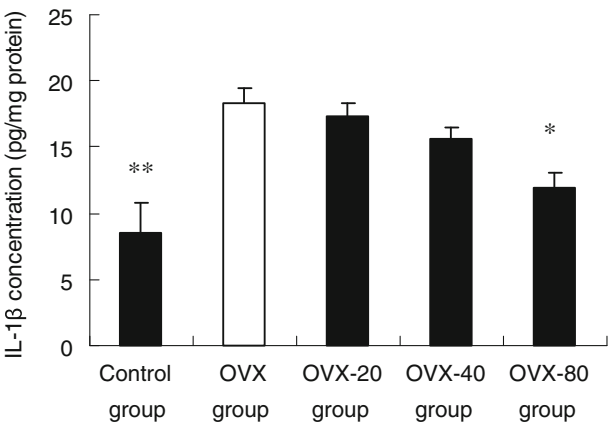

Fig. 3. Effect of Z-Ligustilide on IL-1β concentration. Treatment with 80 mg LIG decreased the level of IL-1β as compared to the OVX group, respectively ($P<0.05$). However, the same results did not occur in the OVX-20-treated group and OVX-40-treated group. Values are shown as means ± SEM. *$P<0.05$ vs. OVX group, **$P<0.01$ vs. OVX group.

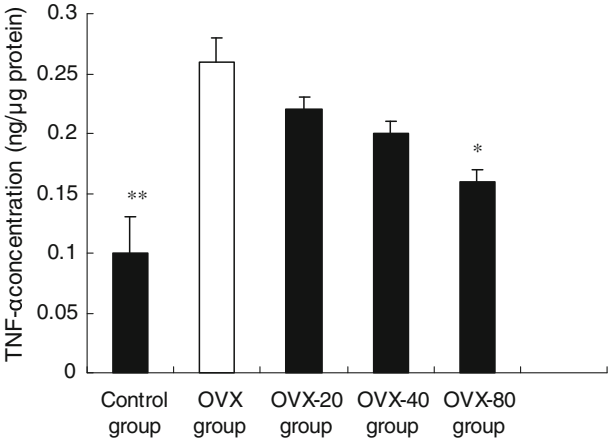

Fig. 4. Effect of Z-Ligustilide on TNF-α concentration. The levels of TNF-α elevated significantly in OVX group. Treatment with 80 mg LIG suppressed this response ($P<0.05$). However, the same results did not occur in the OVX-20-treated group and OVX-40-treated group. Values are shown as means ± SEM. *$P<0.05$ vs. OVX group, **$P<0.01$ vs. OVX group.

The protein expressions of COX-2 decreased in 80 mg LIG-treated groups (58.33/mm^2, $P<0.05$). In this study, 80 mg LIG suppressed OVX-induced iNOS expression (50.30/mm^2, $P<0.05$). Treatment with 20 mg LIG and 40 mg LIG reduced iNOS expression also. However, it is not significant (Table 1).

The protein expressions of ICAM-1 in the OVX group significantly increased (20.33/mm^2) compared with those in the control group (140.22/mm^2). Treatment with 80 mg LIG (121.36/mm^2) decreased

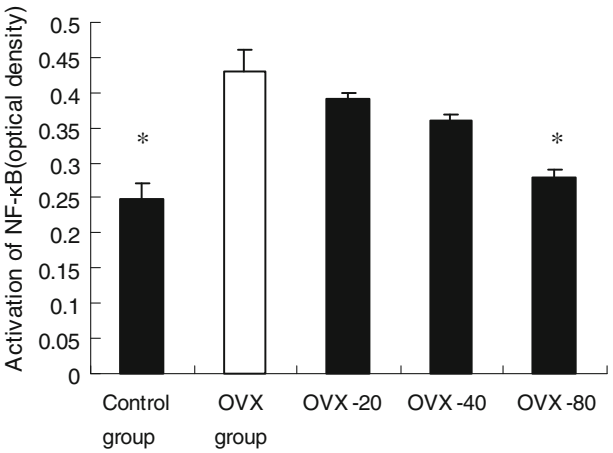

Fig. 5. Effect of Z-Ligustilide on activation of NF-κB. OVX significantly induced activated NF-κB above control levels, and 80 mg LIG significantly suppressed this response ($P<0.05$). However, the same results did not occur in the OVX-20-treated group and OVX-40-treated group. Values are shown as means ± SEM. *$P<0.05$ vs. OVX group.

Table 1. Effect of Z-Ligustilide on ICAM-1, iNOS and COX-2 Protein Production (Number of Immunopositive/mm^2)

Different groups	COX-2	iNOS	ICAM-1
Control	9.11±5.00**	11.21±3.00**	20.33±5.20**
OVX	72.20±5.33	70.20±8.30	140.22±18.22
OVX-20	68.11±3.22	66.22±1.22	136.41±21.23
OVX-40	61.23±8.22	59.36±2.33	130.41±33.32
OVX-80	58.33±9.32*	50.30±8.59*	121.36±16.30*

Values are shown as means ± SEM
*P<0.05 vs. OVX group, **P<0.01 vs. OVX group

the level of ICAM-1 as compared to the OVX group (140.22/mm^2), 20 mg LIG group (136.41/mm^2) and 40 mg LIG group (130.41/mm^2) (P<0.05) (Table 1).

DISCUSSION

There is growing evidence that inflammation may be one of the causal factors of osteoporosis. Several cytokines such as IL-1, IL-6, RANKL, OPG and M-CSF were implicated in the pathogenesis of osteoporosis [17, 18]. Z-Ligustilide attenuates inflammatory pain behaviour in mice [16]. It was hypothesized that Z-Ligustilide had the ability to prevent inflammation. The present work was undertaken to evaluate the anti-inflammatory effect of LIG in experimental ovariectomized osteopenic rats.

Peroxidation damage plays an important role in the progression of inflammation. Therefore, the anti-oxidant effects of LIG were investigated by measuring MDA levels. As shown in Fig. 1, the MDA levels in the OVX-40 and OVX-80 groups were significantly lower than those in the saline group (P<0.05 and P<0.01, respectively).

The present study was undertaken to determine whether LIG reduced the number of PMNs in the ovariectomized osteopenic rats. The activity of MPO was determined as an indicator of PMNs migration. In this study, the MPO activity was relatively low in the control group and significantly increased in the OVX group. Treatment with 80 mg LIG/kg wt. resulted in a substantial decrease in the extent of PMN infiltration in the ovariectomized osteoporotic rats.

Increased levels of inflammatory mediators after the menopause have been associated with osteoporosis [19]. Among these inflammatory mediators, IL-1β and TNF-α are of particular importance because they play a major role in coordinating mechanisms that command pro-inflammation. Our results show that 80 mg LIG treatment decreased the level of IL-1β and significantly suppressed the elevated levels of TNF-α.

NF-κB comprises a family of transcription factors that act as regulators of pro-inflammatory mediators [20]. The importance of NF-κB in osteoblasts was revealed by a recent report, which shows that the inhibition of NF-κB in mature osteoblasts by IKK-dominant negative transgenic mice has increased bone mineral density (BMD) and bone volume (BV) due to increased activity of osteoblasts [21]. Therefore, we hypothesized that LIG may potentially show beneficial effects by decreasing the expression of NF-κB. As shown in Fig. 5, 80 mg LIG treatment significantly suppressed OVX-induced NF-κB expression. It is consistent with the results presented in Figs. 3 and 4. We found here that the inhibited activation of the NF-κB p50 suppressed the expression of IL-1β and TNF-α.

The present work was also undertaken to evaluate anti-inflammatory effects of LIG on COX-2 expression, iNOS expression and level of ICAM-1.

Inducible NOS is induced in response to inflammatory-like stimuli and is capable of sustained production of high levels of NO that predominate during inflammation [22]. The excessive or inappropriate production of NO can damage tissue through the superoxide anion (O_2^-) [23]. The protein expressions of iNOS decreased dose dependently in LIG-treated groups. It is consistent with the results of LIG on pro-inflammatory mediators. NO also activates COX enzymes leading to a marked increase in PGE2 production [24]. COX-2 is primarily responsible for increased PGE2 production during inflammation, and PGE2 is generally considered to be a pro-inflammatory agent [25, 26]. In the present work, LIG-80 treatment significantly decreased the expression of COX-2 protein in OVX rats (P<0.05).

Among the immunoglobulin family members, intercellular adhesions molecule-1 (ICAM-1) has been the most extensively investigated in inflammatory process. Patients with acute inflammation had higher soluble ICAM-1 levels compared to patients without disease. In the present work, the treatment with LIG-80 decreased the level of ICAM-1.

CONCLUSION

In the current study Z-Ligustilide treatment showed a significant anti-inflammatory effect by inhibiting up-

regulation of inflammatory cytokines IL-1β and TNF-α, blocking inflammation-related events (MPO and ICAM-1) and expressions of prooxidative enzymes such as COX-2 and iNOS by activation of NF-κB in experimental OVX osteopenic rats.

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