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Regulatory effect of Traditional Chinese Medicinal Formula Zuo-Gui-Wan on the Th17/Treg paradigm in mice with bone loss induced by estrogen deficiency

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ABSTRACT

Ethnopharmacological relevance: Bone loss is a common pathological condition induced by estrogen deficiency. The Th17/Treg paradigm, which can be skewed by estrogen, plays an important role in regulating bone metabolism

Aim of the study: The purpose of this study was to determine the role of the Th17/Treg shift in estrogen deficiency-induced bone loss in mouse models and to elucidate the immunopharmacologic mechanism of Zuo-Gui-Wan (ZGW) in preventing bone loss in this process by regulating Th17/Treg paradigm.

Materials and methods: Splenocytes of ovariectomized (Ovx) mice and naturally aged mice were isolated and Flow cytometry was used to detect the Th17/Treg subsets. In addition, serum estrodiol (E_2) and serum C-terminal telopeptides of type I collagen (CTx) were detected by ELISA assay. Bone mineral density (BMD) of the left tibiae was measured by dual-energy X-ray absorptiometry. Moreover, Ovx mice were administrated with different doses of ZGW for 12 weeks, and BMD and Th17/Treg subsets were determined. Bone histomorphometry was observed by Hematoxylin and eosin (H&E) staining and serum protein levels of IL-6 were analyzed by ELISA assay. In addition, the mRNA and protein expression of RORγt and Foxp3 were detected by RT-PCR and Western blot respectively.

Result: The Th17/Treg paradigm shifted to Th17 in estrogen-deficient mice both in the Ovx mice and the naturally aged mice. BMD and E_2 levels negatively correlated with the Th17/Treg ratio. After ZGW administration, the BMD was enhanced markedly in the Ovx mice as well as in the naturally aged mice.

Abbreviations: ANOVA, Analysis of variance; BMD, Bone mineral density; CIA, Collagen-induced arthritis; CTLA-4, Cytotoxic T lymphocyte antigen 4; CTx, C-terminal telopeptides of typeI collagen; DXA, Dual-energy X-ray; E_2, Estradiol; Foxp3, Forkhead box P3; GAPHD, Glyceraldehyde-3-phosphate dehydrogenase; HE, Hematoxylin and eosin; IgG, Immunoglobulin G; MACS, Magnetic affinity cell sorting; OB, Osteoblast; OC, Osteoclast; Ovx, Ovariectomized mice; PBS, Phosphate buffered saline; RA, Rheumatoid arthritis; ROR, Retinoic acid-related orphan receptor; SDS, Sodium dodecyl sulfate; Sham, Sham-ovariectomized mice; T.Ar, The totle tissue area; Tb.Ar, The trabecular area; TBST, Tris-buffered saline with Tween 20 buffer; TCM, Traditional Chinese Medicine; TGF-β, Transforming growth factor-beta; Th, T-helper cell; ZGW, Zuo-Gui-Wan

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Both the mRNA and protein expressions of IL-6 and RORγt were decreased, whereas those of Foxp3 were increased significantly after ZGW administration.
Conclusion: Th17/Treg shift involved in the bone loss induced by estrogen deficiency. ZGW prevented bone loss efficiently and skewed Th17/Treg paradigm towards Treg without enhancing E_2.

1. Introduction

Estrogen-deficient bone loss is a result of an increase in osteoclast recruitment and accelerated bone reabsorption, which results in an increased risk of adverse health outcomes including decreased quality of life, disability, recurrent hospitalizations, and death (Campbell and Buchner, 1997; Bortz, 1993; Kelchtermans et al., 2009). Although estrogen replacement therapy has been shown to have a considerable bone protecting effect, the adverse reaction of enhanced serum estrogen levels have been reported to be associated with headache and increased cancer risk such as breast cancer, endometrial cancer, and ovarian cancer (Loder et al., 2005; Ingle, 2013; Le Ray et al., 2012). As the world's population continues to age, bone loss preventing medication with minimal adverse reactions is urgently needed (Shin et al., 2010). However, up to now, the pathogenesis of estrogen-deficient bone loss still remains unclear. Understanding the mechanism by which estrogen deficiency promotes bone loss is critically important.

It has been identified that the different systems of the human body are interdependent (Arboleya and Castañeda, 2013). The endocrine system, the bone system and the immune system share many common molecules and cytokines, and the change of one system will inevitably affect the others. Estrodiol (E_2) is one of the most important hormones that play a critical role in reproduction. However, more and more studies have demonstrated that besides the reproductive promoting effect, E_2 also exerts an efficient immunoregulatory effect (D'Amelio et al., 2008). T cells are the most important immunocytes with critical functions in immune activation and maintaining self-tolerance in homeostasis. T-helper (Th) cells have been classified into Th17 and Treg subsets based on their immune function, cell surface molecule expression, and dependence upon transcription regulators (Zhu and Paul, 2010). Th17 cells produce the pro-inflammatory cytokines such as IL-17A, F which play an important role in the induction of inflammation (Bedoya et al., 2013; Crome et al., 2010). In contrast, Treg cells are indispensable in maintaining self-tolerance by secreting cytokines such as transforming growth factor (TGF)-β or by cell–cell contact (Afzali et al., 2007). Cumulative studies reported that IL-17 was involved in elicting OC differentiation and bone reabsorption while TGF-β exert the opposite function in bone mechanism, indicating the potential effect of T17/Treg paradigm in bone remodeling and associated diseases (Shen et al., 2014). Furthermore, it was reported that estrogen deficiency had the inducing effect on the differentiation of IL-17 secreting Th17 cells (Tyagi et al., 2012; Lélu et al., 2011). Therefore, it was hypothesized that Th17/Treg skew induced by estrogen deficiency may be involved in estrogen-deficient bone loss and may be taken as a potential target for clinical therapy.

According to Traditional Chinese Medicine (TCM), dominating the generation and metabolism of bone marrow is one of the critical functions of kidney (Wang et al., 2011). Estrogen deficiency, especially caused by menopause or premature ovarian failure, is considered as a state of kidney deficiency (Wang et al., 2011; Li et al., 2010; Chao et al., 2003). Therefore, kidney nourishing Chinese herbs often been used for treating estrogen deficient bone loss (Min et al., 2010; Wang and Zeng, 2011). Zuo-Gui-Wan (ZGW) – a classical TCM herbal prescription on tonifying kidney yin- is recorded in Jingyue Quanshu (Jingyue's Complete Works) written by Jingyue Zhang in A.D. 1624. ZGW consists of 8 kinds of herbs. The effect of ZGW on attenuate bone loss induced by estrogen deficiency has been proven through research (Li, 2004); however, the underlying therapeutic mechanism is poorly understood (Liu et al., 2011). In our previous study, it was found that ZGW has an inhibitory effect on serum IL-17 protein levels in ovariectomized (Ovx) mice. Therefore, it was hypothesized that the regulatory effect of ZGW on Th17/Treg paradigm contributed to bone loss induced by estrogen deficiency.

In the present study, Ovx mice and natural ageing mice were used to identify the bone loss inducing effect of estrogen deficiency. In addition, Th17 cells ($CD4^+IL\text{-}17A^+$) and Treg cells ($CD4^+CD25^+Foxp3^+$) were accessed by Flow cytometry detection. Bone mineral density (BMD) of the left tibiae of mice was measured by dual-energy X-ray (DXA) to identify the correlation between Th17/Treg shift and bone loss in estrogen deficient state. The Th17/Treg paradigm and bone histomorphometry were analyzed after ZGW administration to evaluate the bone loss preventing effect of ZGW by regulating Th17/Treg paradigm. Furthermore, both the mRNA and protein expressions of Th17- and Treg-specific transcription factors (RORγt, and Foxp3) as well as the signature cytokine for the differentiation of Th17 cells (IL-6) were assayed to elucidate the mechanism underlying the regulatory effect of ZGW on Th17/Treg paradigm in bone loss induced by estrogen deficiency.

2. Materials and methods

2.1. Animals and treatment

Young female BALB/c mice (median body weight, 20 g, 8 weeks old) and natural ageing female BALB/c mice (median body weight, 25 g, 14 months old) were provided by the Institute of Zoology of the Chinese Academy of Sciences (Beijing, China). All animals were caged in a temperatureand light-controlled environment with a 12 h light, 12 h dark cycle, and received pathogen-free water and food for maintenance. All management procedures were approved by the Institutional Animal Care and Use Committee of the Shandong Academy of Medical Science. The registration no. of IACUC was 2012 IACUC-106 approved on 05/06/2012.

There were two sets of experiments in the present study. For the first set of experiments, in order to identify the correlation of Th17/Treg paradigm and bone loss induced by estrogen deficiency, 10 mice per group were taken according to a stochastic averaging principle. The groups were: normal young mice, sham-operated (ovary intact) mice, Ovx, and natural ageing mice. For ovariectomies, mice aged 8 weeks were anesthetized with isofluorane and their ovaries were removed through two small dorsal incisions. Sham operated mice were anesthetized and opened equivalently, but ovaries were not removed. Subsequently, to elucidate the preventing effect of ZGW on bone loss induced by estrogen deficiency, in the second set of experiment 10 mice per group were used according to a stochastic averaging principle. The groups were: sham-operated group, Ovx group, Ovx+vehicle (distilled water) group, Ovx+ZGW low-dose (8 g/kg/d) group, Ovx+ZGW middle-dose (16 g/kg/d) group and Ovx+ZGW high-dose