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Original Article

Effects of GW1929 on uterus, ovary and bone metabolism function in perimenopause rats

Xiaoli Wang, Kehui Xu, Ying Xiong, Qiao Li, Xia Zhao

Department of Gynecology and Obstetrics, Key Laboratory of Birth Defects and Related Diseases of Women and Children, Ministry of Education, West China Second Hospital, Sichuan University, Chengdu 610041, P. R. China

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Abstract: This study was aimed to investigate the effect of GW1929, a novel peroxisome proliferator-activated receptors gamma (PPARγ) agonist, in perimenopause rats. Female Sprague-Dawley (SD) rats were treated with 4-vinylcyclohexene diepoxide (VCD) to induce perimenopause rat model. Then they were given GW1929 in low, middle and high dosage. Histopathology observation of uterus and ovary tissues was measured by hematoxylin and eosin staining. The levels of serum hormones, oxidative stress related factors, bone formation and bone metabolism associated factors in serum were detected by kits. Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling (TUNEL) was employed to evaluate cell apoptosis. Furthermore, the expression of PPARγ and apoptosis associated proteins were measured by western blotting. The results revealed that there was no thickening of endometrium and no mature follicular development in ovaries of model group rats. GW1929 treatment recovered endometrial function with a tendency of thickening and there were mature follicle in the ovary. In addition, GW1929 increased the expression of PPARγ in both uterus and ovary tissues. The contents of estrogen (E2) were increased, whereas follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were decreased after being intervened with GW1929 in perimenopause rats. Concurrently, GW1929 reduced the levels of oxidative stress in a dose-dependent manner. Following treatment with GW1929, cell apoptosis in uterus and ovary tissues were attenuated, accompanied by a downregulation of Bax expression and an upregulation of Bcl-2 and cleaved caspase-3 expression. Moreover, In the GW1929-treated perimenopause rats, the levels of alkaline phosphatase (ALP), osteocalcin (OCN), osteopontin (OPN), bone alkaline phosphatase (BALP) and bone mineral density (BMD) were enhanced, while tartrate-resistant acid phosphatase (TRAP) was reduced. Taken together, we conclude that GW1929 could improve uterus, ovary and bone metabolism function in perimenopause rats, which is of great significance for the treatment of perimenopause.

Keywords: Perimenopause, uterus, ovary, peroxisome proliferator-activated receptors gamma, bone metabolism

Introduction

In middle age, women develop from the reproductive (premenopause) to non-reproductive (postmenopause) life through a transitional period named perimenopause, which is the time around menopause, from the appearance of a menopausal profile (serum hormone concentrations) to 1 year after the last menstrual period [1]. This transition period is marked by the irregular menstrual cycles, endocrine and metabolism and increased vulnerability to anxiety [2]. These symptoms occur along with the onset of cyclical irregularity and changes in ovarian hormone secretion, including reduced production of estrogen (E2) and enhanced production of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which is closely associated with a plenty of physiological changes that sometimes impair the quality of life.

Peroxisome proliferator-activated receptors gamma (PPARγ) is closely implicated in modulating reproductive cycles, which contributes to the significant role of PPARγ in gynecologic endocrine diseases [3, 4]. A current study has demonstrated that there is an association between the expression of PPARγ in bovine endometrium and the stage of oestrous cycle [5]. And deletion of PPARγ can impair glucose metabolism and estrous cycling in female mice [6]. In addition, it has been well documented that activation of PPARγ could protect against ovarian ischemia reperfusion injury of female

rats [7]. As a novel selective PPARγ agonist, GW1929 could restore the inhibition of FSH-induced follicle development and steroidogenesis [8]. However, the effect of GW1929 in perimenopause remains to be further elucidated, which attracts our research interests.

In this current study, we examined whether GW1929 could protect against perimenopause in rats. And we investigated the effect of GW1929 on uterus, ovarian and bony metabolic function in perimenopause rats, which will be of critical significance to the clinical treatment of perimenopause.

Materials and methods

Chemicals

The 4-vinylcyclohexene diepoxide (VCD, ≥96% purity)) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and stock solutions were diluted with corn oil (Sigma-Aldrich). Progynova was the product of Bayer Xianling Pharmaceutical Co., Ltd. (Guangzhou, China).

Animals

Female Sprague-Dawley (SD) rats (Eight-weeks-old, body weight 220-250 g) were obtained from Shanghai SLAC Laboratory Animal Company Limited (Shanghai, China). Animals were given water and food *ad libitum* and kept on 12/12 h light/dark cycles. They were allowed to acclimate to the environment for at least two weeks before the experiment. This study was conducted in strict accordance with the guidelines for the Care and Use of Laboratory Animals approved by the Ministry of Science and Technology of China. All the study protocols were approved by the Ethics Committee on Animal Experiments of Sichuan University.

Treatments

Before the experiment started, time estrous cyclicity was monitored daily by vaginal lavage. Only rats which possessed regular 4-5 day cycles were used for the investigation. The animals were divided into 1 of 6 groups randomly (n=10 in each group): control, model (VCD), positive (VCD+0.09 mg/kg progynova), low (VCD+1.5 mg/kg GW1929), middle (VCD+3.0 mg/kg GW1929) and high (VCD+6.0 mg/kg GW1929). VCD was employed to establish perimenopause rat model as described in the previous study [9]. In brief, rats were administrated with 40 mg/kg VCD daily by intraperitoneal injection for 15 d. Stock solutions were diluted with corn oil. Rats in the control group were injected with the same volume of corn oil. Then, progynova or different doses of GW1929 were used to treat rats with gavage for 21 d, and animals in the control group were administrated with equal volume of distilled water. The day after the last dose, the rats were euthanized. Blood, uterus and ovaries were harvested immediately for further analysis.

Histopathology observation

Appropriate weight uterus tissues or ovary tissues were conventionally fixed in 10% formalin over night at 4°C. Subsequently, these tissues were routinely included in paraffin and cut into sections (4 μm thick). Strips of tissue were stained with hematoxylin and eosin (HE) for morphological evaluation. Then, all the sections were dehydrated with graded ethanol and xylene. The slides (n=8) were examined by an experienced pathologist blind to the treatments, under a light microscope (Olympus Corp., Tokyo, Japan) using 200× magnification.

Measurement of the levels of serum hormones

Rat blood samples were collected into Eppendorf tubes and centrifuged at 3500 rpm for 10 min to obtain the serum. The levels of E2, FSH and LH in serum were detected by Enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions, which were all purchased from Shanghai Xitang Biotechnology Co., Ltd. (Shanghai, China).

Measurement of the levels of oxidative stress related factors

The levels of oxidative stress related factors including reactive oxygen (ROS), superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) in serum were measured by the assay kits using the manufacturer's instructions, respectively. Above kits were the products of Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Measurement of bone formation and bone metabolism associated factors

The levels of bone formation associated factors including alkaline phosphatase (ALP), osteocal-

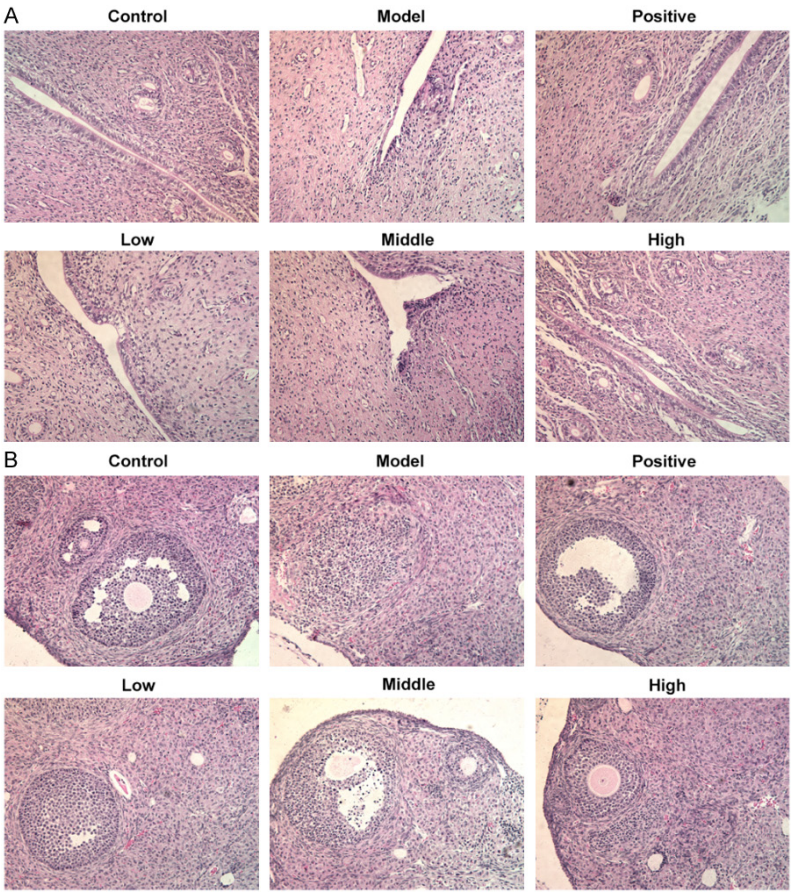

Figure 1. GW1929 improved the pathological changes of uterus tissues and ovarian tissues in perimenopause rats induced by VCD. The histopathology changes of (A) uterus tissues and (B) ovarian tissues were measured by H&E staining. Magnification ×200. VCD, 4-vinylcyclohexene diepoxide.

cin (OCN) and osteopontin (OPN) as well as bone metabolism associated factors containing tartrate-resistant acid phosphatase (TRAP) and bone alkaline phosphatase (BALP) in serum were detected using ELISA kits according to the manufacturer's instructions. These kits were obtained from Shanghai Xitang Biotechnology Co., Ltd. (Shanghai, China).

Bone mineral density (BMD) assay

To investigate the effect of GW1929 on BMD, the limbs of rats were placed on the platform of dual-energy X-ray absorptiometry.

Terminal-deoxynucleoitidyl Transferase mediated nick end labeling (TUNEL)

TUNEL fluorescein kit (Promega, Madison, WI, USA) was applied to examine the apoptosis in paraffin-embedded uterus tissues or ovary tissues. Integrated optical density (IOD) analysis was used to determine the indirect reaction of apoptosis. Specimens were mounted and examined using routine light microscopy.

Western blotting analysis

Tissues were homogenized on ice in RIPA Lysis Buffer (Beyotime, Shanghai, China). The protein concentration of the supernatant was detected using a BCA Protein Quantitation kit (Beyotime, Shanghai, China). Protein was isolated by SDS-polyacrylamide gels (PAGE), and subsequently electrophoretically transferred onto polyvinylidene fluoride membranes. After being blocked by 5% skimmed milk, membranes were exposed to primary antibodies overnight at 4°C. Then these membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Abcam, Cambridge, UK) for 2 h. An enhanced chemiluminescence (ECL) reagent was used for visualization and ImageJ software was employed to analyze the results. Anti-PPARγ, anti-Bcl-2, anti-Bax, anti-cleaved caspase-3, anti-caspase-3 and anti-GAPDH antibodies were all purchased from Cell Signaling Technology (Boston, MA, USA). The protein expression was normalized to GAPDH levels.

Statistical analysis

All results were confirmed in at least three independent experiments and all statistical analyses were conducted using SPSS 14.0 software (Chicago, IL). All experimental results were expressed as mean ± SD. Statistical comparisons were made by two-tailed Student's t test or one-way analysis of variance (ANOVA). A significance level of $P<0.05$ was adopted for all analyses.

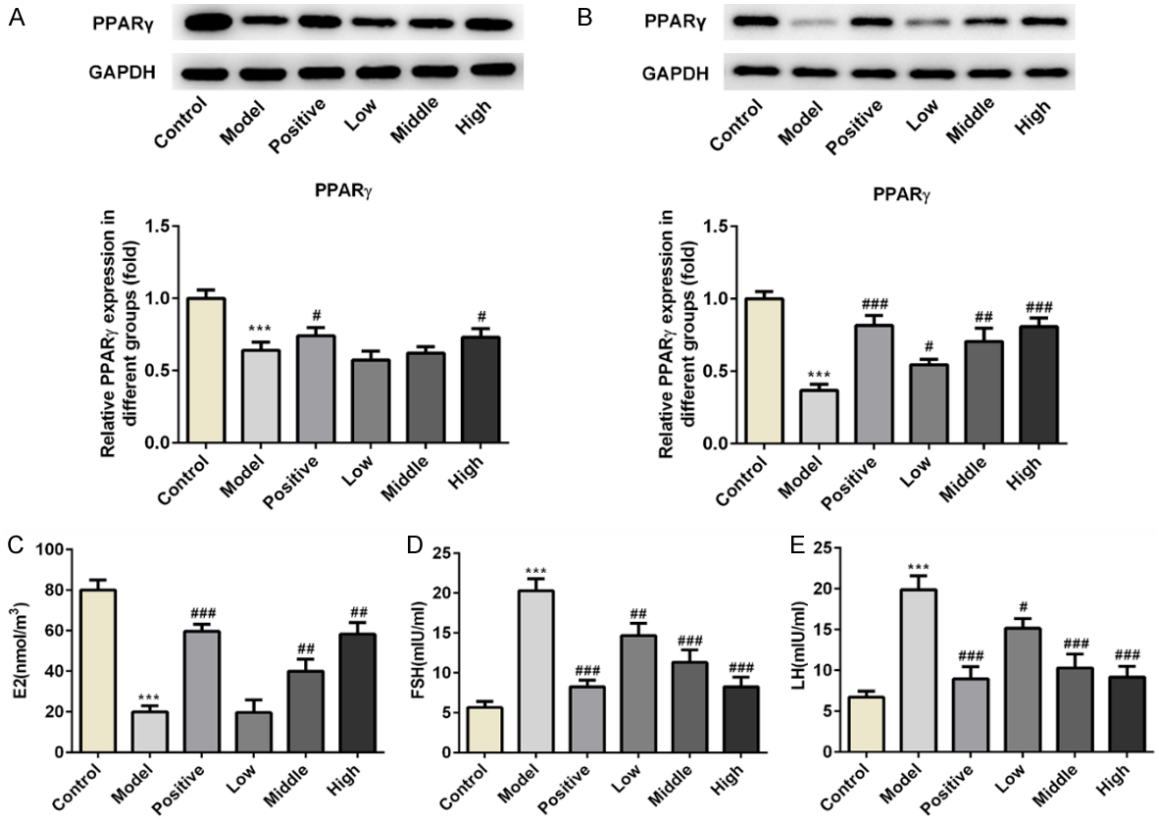

Figure 2. GW1929 upregulated the expression of PPARγ and affected the levels of serum hormones in perimenopause rat induced by VCD. The expression of PPARγ in (A) uterus tissues and (B) ovarian tissues were evaluated by Western blotting. The levels of (C) E2, (D) FSH and (E) LH in serum were detected by ELISA kits. Data were presented as mean ± SD and represented three separate experiments. ***P<0.001 *vs.* control; #P<0.05, ##P<0.01, ###P<0.001 *vs.* model. VCD, 4-vinylcyclohexene diepoxide; PPARγ, peroxisome proliferator-activated receptors gamma; E2, estrogen; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

Results

GW1929 improved the pathological changes of uterus tissues and ovarian tissues in perimenopause rats induced by VCD

Hematoxylin and eosin (H&E) staining was employed to investigate the pathological changes of uterus tissues and ovarian tissues extracted from rats in the present study. As presented in **Figure 1A**, the endometrium was thickened and shed in the control group, but there was no thickening of endometrium in model group. GW1929 treatment recovered endometrial function with a tendency of thickening and shedding in a dose-dependent manner. In addition, normal ovarian structure and follicular development or ovulation was observed in rats of control group. After induction with VCD, there was no mature follicular development in ovaries (**Figure 1B**). And there were mature follicle in the ovary following treatment with GW1929. Above results indicated that the model of perimenopause rats induced by VCD was established successfully, and GW1929 treatment notably improved the pathological changes of uterus tissues and ovarian tissues.

GW1929 affected the levels of serum hormones in perimenopause rat

To the best of our knowledge, GW1929 serves as a novel selective PPARγ agonist. As shown in **Figure 2A** and **2B**, the expression of PPARγ in uterus tissues and ovarian tissues were obviously downregulated in model group. Following treatment with GW1929, the expression of PPARγ was upregulated in a dose-dependent manner. Then, we measured the levels of serum hormones in rats of each group. It was found that the contents of E2 were reduced significantly in model group with an augment of FSH and LH (**Figure 2C-E**). Speaking of the effect of GW1929 treatment, the level of E2 in the

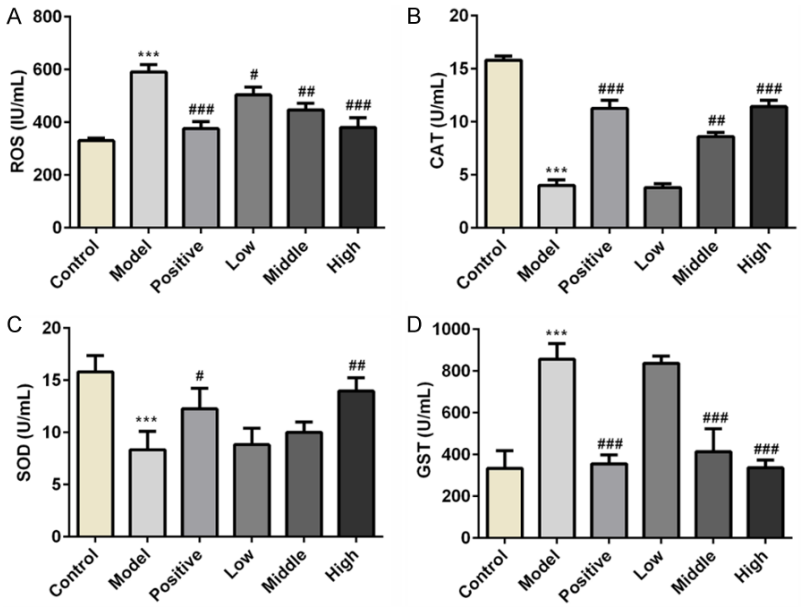

Figure 3. GW1929 alleviated the oxidative stress in perimenopause rat induced by VCD. The levels of (A) ROS, (B) CAT, (C) SOD and (D) GST were measured by kits. Data were presented as mean ± SD and represented three separate experiments. ***P<0.001 *vs.* control; #P<0.05, ##P<0.01, ###P<0.001 *vs.* model. VCD, 4-vinylcyclohexene diepoxide; ROS, reactive oxygen; CAT, catalase; SOD, superoxide dismutase; GST, glutathione S-transferase.

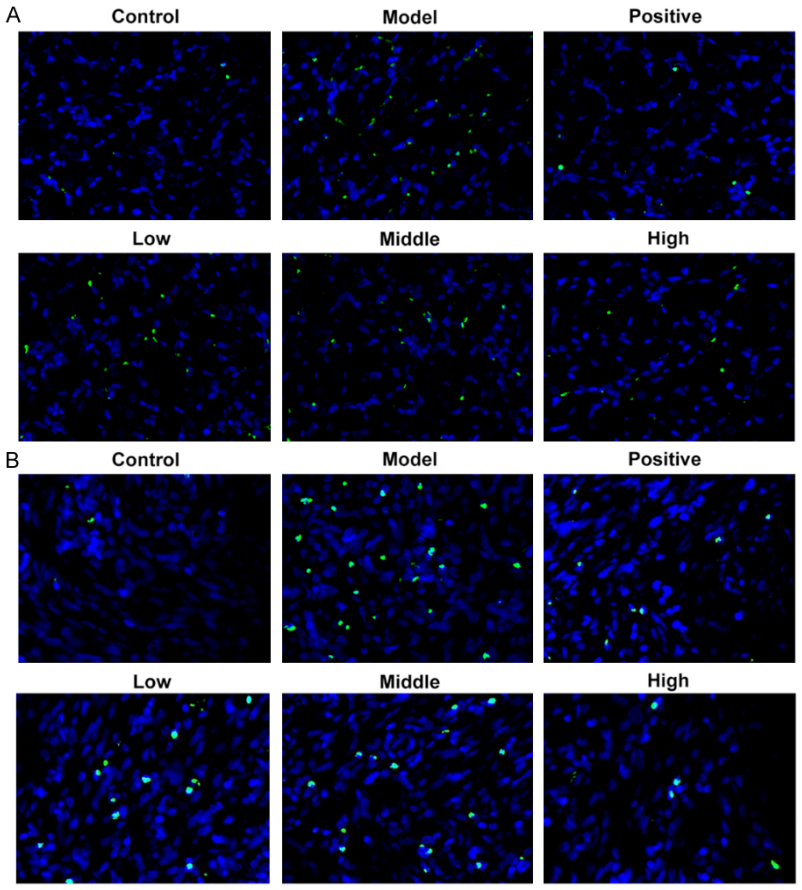

Figure 4. GW1929 inhibited apoptosis of uterus and ovarian cells in perimenopause rat induced by VCD. Photomicrographs of representative images of (A) uterus cell apoptosis and (B) ovarian cell apoptosis were assessed by TUNEL assay. Magnification ×100. VCD, 4-vinylcyclohexene diepoxide; TUNEL, Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling.

GW1929 treated groups was increased notably, whereas the levels of both FSH and LH were decreased in a concentration-dependent manner. These findings suggested that GW1929 could regulate the levels of serum hormones in perimenopause rat.

GW1929 alleviated the oxidative stress in perimenopause rat

To explore the effect of GW1929 on oxidative stress in perimenopause rat induced by VCD, the levels of ROS, CAT, SOD and GST in serum were assessed by kits and concentrations of different groups are presented in **Figure 3A-D**. The levels of ROS were enhanced significantly in the model group, but CAT, SOD and GST were reduced. Treatment with GW1929 attenuated VCD-induced increase in ROS levels and decrease in CAT, SOD and GST in comparison with the model group. These observations revealed that GW1929 was able to relieve the oxidative stress in perimenopause rat.

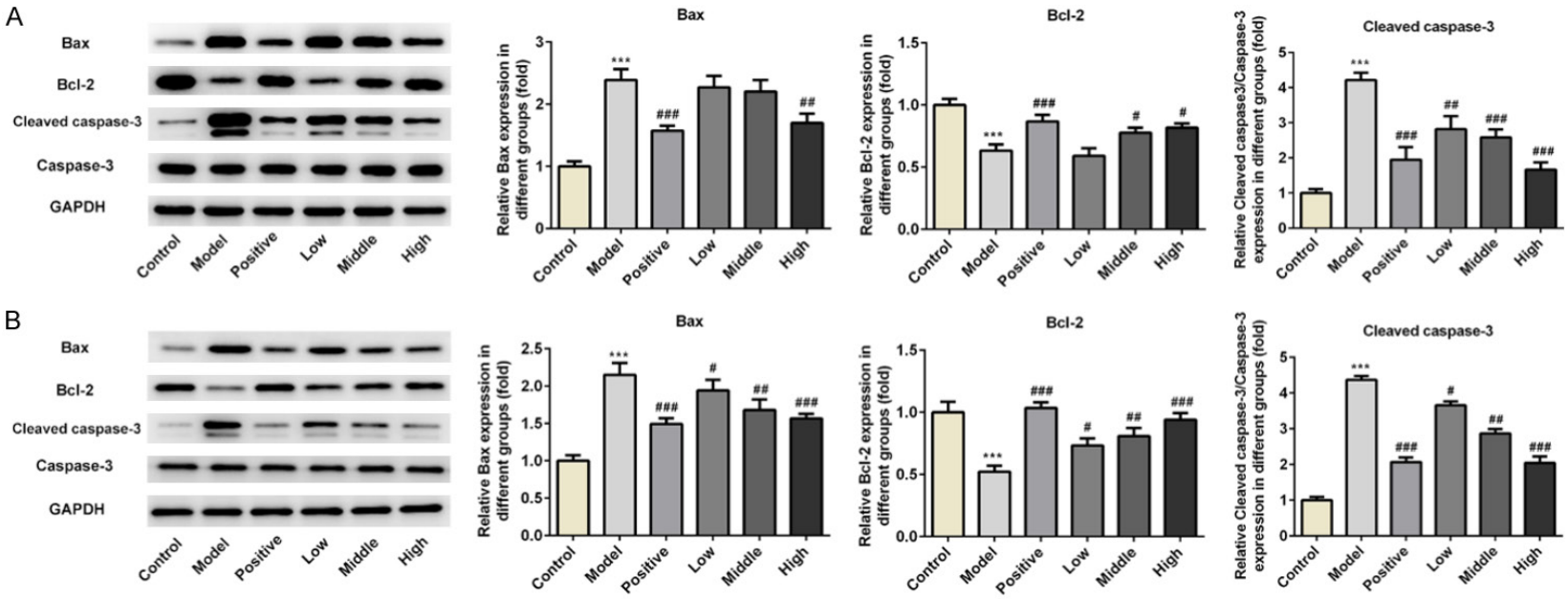

Figure 5. GW1929 reduced the expression of apoptosis-associated proteins in uterus tissues and ovarian tissues. Western blotting shows the expression levels of Bax, Bcl-2 and cleaved caspase-3 in (A) uterus tissues and (B) ovarian tissues. Data were presented as mean ± SD and represented three separate experiments. ***P<0.001 *vs.* control; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 *vs.* model.

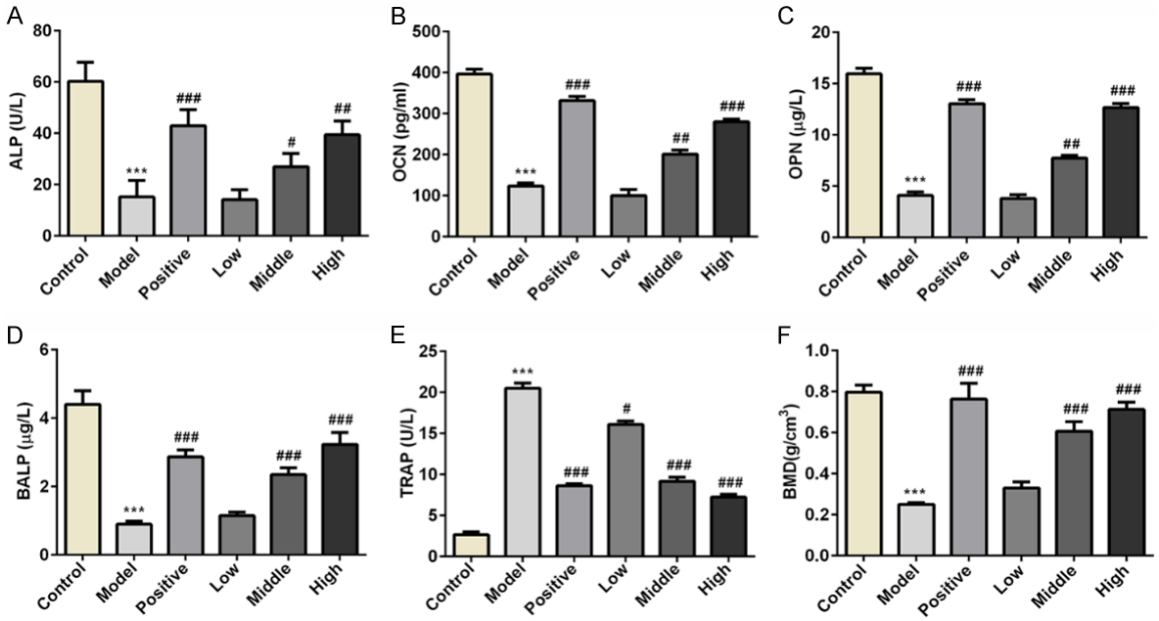

Figure 6. GW1929 regulated bone metabolic function in perimenopause rats induced by VCD. The levels of (A) ALP, (B) OCN, (C) OPN, (D) TALP and (E) TRAP in serum were measured by kits. (F) The levels of BMD were evaluated by dual-energy X-ray absorptiometry. Data were presented as mean ± SD and represented three separate experiments. ***P<0.001 *vs.* control; #P<0.05, ##P<0.01, ###P<0.001 *vs.* model. VCD, 4-vinylcyclohexene diepoxide; ALP, alkaline phosphatase; OCN, osteocalcin; OPN, osteopontin.

GW1929 suppressed cell apoptosis of uterus and ovarian tissues in perimenopause rat

In this study, the TUNEL assay was used to detect the degree of apoptosis of uterus and ovary. As exhibited in **Figure 4A** and **4B**, animals in the perimenopause model group showed high levels of apoptosis. Following treatment with GW1929, the apoptosis index of uterus and ovarian cells were significantly decreased in compassion with the model group. Concurrently, the expression levels of apoptosis-associated proteins were evaluated by Western blot in the present study. As shown in **Figure 5A** and **5B**, the expression of anti-apoptosis protein Bcl-2 was downregulated markedly coupled with upregulation of the expression of pro-apoptotic proteins Bax and cleaved caspase-3 in the perimenopause rat compared with the control group. Following treatment with GW1929, the expression level of Bcl-2 was increased, while Bax and cleaved caspase-3 were decreased in a concentration-dependent manner. Overall, these data suggested that GW1929 inhibited cell apoptosis of uterus and ovarian tissues in perimenopause rat.

GW1929 regulated bone metabolic function in perimenopause rats

To assess the effects of GW1929 on bone function, bone formation associated factors including ALP, OCN, OPN and BALP as well as bone metabolism associated factors TRAP in serum were measured in our study and the results were shown in **Figure 6**. Obvious decrease of ALP, OCN, OPN and BALP levels were observed in the model of perimenopause rat compared with the control group (**Figure 6A-D**). In terms of the effect of GW1929 treatment, the levels of above bone formation associated factors were increased notably in a dose-dependently manner. At the same time, the levels of BMD said the same story with ALP, OCN, OPN and BALP (**Figure 6E**). The levels of TRAP contents presented opposite results (**Figure 6F**). These data revealed that GW1929 could regulate bone metabolic function in perimenopause rats.

Discussion

In the period of perimenopause, a woman might suffer from a variety of symptoms, such as disorders and changes of menstrual cycle,

sleep disorders, dysphoric mood symptoms and body symptoms [10]. It has been well reported that about 90% of women have asked for suggestions on control or relief of the above perimenopause-related symptoms, indicating that the treatment of perimenopause is a crucial focus of clinical practice in the world [11]. In the present study, we found that GW1929, a novel selective PPARγ agonist, could improve uterus, ovarian and bone metabolic function in perimenopause rats induced by VCD, which is of great significance for the treatment of perimenopause.

Many physical and psychological symptoms of perimenopause are resulted from the estrogen depletion and hormone disorders. It has been well reported that ovarian dysfunction is a prognostic marker for perimenopause, which results in the reduced production of E2 and enhanced production of FSH and LH [12]. Consistent with a previous study, VCD treatment led to hormonal disruption of perimenopause rat [13]. It was found that GW1929 treatment increased the level of E2 coupled with decrease of FSH and LH contents, suggesting a protective effect of GW1929 on perimenopause. At this stage of a woman's life, hormonal changes are implicated in enhanced oxidative stress, due to the higher level of ROS generated and reduced antioxidant defenses [14]. Accumulating evidence showed that inhibition of ROS and increase of antioxidant levels could relieve perimenopause [15]. In the present study, the levels of ROS were decreased, whereas antioxidant enzymes including CAT, SOD and GST were increased notably after being intervened with GW1929, in accordance with the previous study [16]. Above results demonstrated that GW1929 is able to alleviate the oxidative stress in perimenopause rat.

Apoptosis is a critical mechanism for cellular homeostasis. A growing body of evidence suggests that apoptosis of ovarian granulosa cells is concomitant with perimenopause-related decline of ovarian function [17]. A previous study reported that moxibustion could decrease ovarian granulosa cell apoptosis associated with perimenopause in a natural aging rat model [18]. Accumulating evidence shows that GW1929 possesses neuroprotective effects on cerebral ischemic-reperfusion injury, which is attributed to its anti-inflammatory and anti-apoptotic potential [19]. In addition, GW1929 was found inhibited Tetrabromobisphenol A-induced apoptosis in mouse primary neuronal cell [20]. In our study, we found that GW1929 treatment reduced cell apoptosis of uterus and ovary, which was in line with downregulation of Bax and cleaved caspase-3 expression as well as upregulation of Bcl-2 expression. These data suggested that GW1929 protect against perimenopause via suppressing cell apoptosis of uterus and ovarian tissues.

In perimenopause, increase of oxidative stress lead to disruption of bone turnover, which ultimately results in bone disease, such as osteoporosis [21]. Bone loss affects most perimenopausal women and reduced life quality of them [22, 23]. Recent researches have provided evidence of Mate tea attenuates bone deterioration in perimenopause [24]. GW1929 enhanced the levels of ALP, OCN, OPN and BALP accompanied by decrease of TRAP content in serum of perimenopause rat, which were all bone markers and the data was consistent with previous researches [25]. And the result of BMD was in accordance with above data. These observations revealed that GW1929 plays a protective role in perimenopause rats through regulating bone metabolic function.

Conclusion

For the first time, our study investigated the effect of GW1929 on perimenopause rats. The present study demonstrated that GW1929 could protect against VCD-induced uterus and ovarian injury via inhibition of oxidative stress and apoptosis. Concurrently, GW1929 can regulate bony metabolic function by affecting the levels of bone formation and bone metabolism associated factors. Our findings demonstrated that MG1929 could be an effective complementary and alternative agent for the therapeutic management of perimenopause.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Xia Zhao, Department of Gynecology and Obstetrics, Key Laboratory of Birth Defects and Related Diseases of Women and Children, Ministry of Education, West China Second Hospital, Sichuan University, No. 20, Section 3, Renmin South Road, Wuhou District, Chengdu 610041, P. R. China. E-mail: xiazhaolight@163.com

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