The preventive and therapeutic roles of phytoestrogen α-Zearalanol on osteoporotic rats due to ovariectomy

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ABSTRACT

Objective(s): The aim of this study was to observe the influence of phytoestrogen α-Zearalanol on ovariectomy-induced postmenopausal osteoporosis in rats.

Materials and Methods: 40 SD female rats were randomly divided into four groups: Sham group, OVX group (ovariectomized and fed estrogen), α-Zearalanol group (ovariectomized and fed α-Zearalanol), and untreated group (ovariectomized). Three weeks later after surgery, α-Zearalanol and estradiol valerate were administered by oral gavage for 12 weeks to the α-Zearalanol group and the OVX group, respectively. In contrast, the sham and untreated controls were treated with distilled water in a daily basis. After the treatments, uterus histomorphometry, bone mechanical strength, bone histomorphometry, bone mineral density (BMD) of femur, and serum biochemical indicators, such as serum E2, CT and PTH, as well as the levels of TNF and IL-1 were examined.

Results: The BMD was overall declined rigorously in the OVX rats, and that could be mitigated through feeding on either estrogen or α-Zearalanol. Estrogen or α-Zearalanol was found to decrease the levels of serum ALP and BGP in OVX rats, while α-Zearalanol was found to increase the levels of serum E2 and CT, the thickness of the endometrium, and decrease the levels of PTH, TNF and IL-1 in serum in OVX rats. Feeding the OVX rats on α-Zearalanol improved the bone histomorphometric parameters impaired due to estrogen deficiency and enhanced the bone mechanical properties in the ovariectomized rats.

Conclusion: α-Zearalanol treated rats reduced the resorption of bone, and showed a preventive and therapeutic effect of α-Zearalanol on postmenopausal osteoporosis.

Introduction

Osteoporosis is often characterized by reduced bone mass and structural deterioration, and increased bone fragility and susceptibility of bone fracture, which is believed to be increasing as the extension of the average life expectancy in the worldwide, and which thereby turns to be a main cause of increased morbidity and mortality in human beings (1).

The common treatment for postmenopausal osteoporosis is hormone replacement therapy (HRT), which reduces the rate of postmenopausal bone loss effectively (2), while risking the occurrence of endometrial or breast cancer as side effects. It is therefore needed for finding new substitutes owning estrogenic effects in osteoporosis treatment but showing less undesirable side effects on endometrium or breast.

Recently, plant-derived phytoestrogen have been reported to be capable of reducing the side effects of estrogen on the endometrium or breast, while retaining the benefits in osteoporosis prevention (3). α-Zearalanol (α-ZAL) is a reductive product of the Gibberella zeae metabolite zaenalone, which owns estrogenic property, and can potentially be useful in the treatments of estrogen-related human disorders like postmenopausal osteoporosis (4).

Several investigations have demonstrated that α-ZAL retards the development of atherosclerosis with fewer side effects on the growth of mammary gland and uterine (5). However, the mechanism underlined has not been fully elucidated. In this work, we have investigated the effects of α-ZAL on the estrogen-deficiency caused bone loss in the ovariectomized (OVX) rats, and compared the similarities and differences with the effects of estrogen physiologically and biochemically.

Materials and Methods

Experimental animals and preparations of experimental materials

All animal experiments were undertaken with the approval of Animal Ethics Committee of Hebei
University. Forty adult virgin female (6-month-old) Sprague-Dawley rats were obtained from Laboratory Animal Center of Hebei Province. The animal house is maintained at temperature 22±2 °C with relative humidity 50±15% and 12 hr dark/light cycle throughout the study. Rats had free access to food and water ad libitum.

The rats were randomly divided into four groups, each group comprised of ten rats. After 1 week acclimation, three groups were ovarioctomized bilaterally and one group underwent a sham operation as described before (6). After the surgery, the rats were housed individually for one week, and then housed in a group of five each for subsequent treatments: Drug administrations were performed three weeks later after the surgery. Both sham group and an untreated OVX group were used as controls, and were administered orally with appropriate amount of distilled water. The other groups were treated orally with either estradiol valerate (Bayer Inc., Germany) or α-ZAL (Sigma Inc., Germany) by 10 mg/kg BW per day.

After 12 weeks treatments, blood samples were collected from abdominal aorta, and blood serum was prepared and preserved at -20 °C till further analysis. Femur and tibia of all rats were excised and their soft tissues were removed. The femurs were parceled with saline gauze and preserved at -20 °C till further analysis. And the tibias were preserved in 70% ethanol till further analysis. Uterus were isolated, and the absolute weight of uterine tissue was recorded and normalized with body weight (relative weight of uterus, i.e., weight of uterus per 1 kg of BW) of animals, then fixed in 10% phosphate buffered formalin for further analysis.

Histomorphometry of uterus
The uterus samples fixed by using 10% phosphate buffered formalin were further processed with paraffin-embedding and sectioned by 5 µm. The biopsies were stained with hematoxylin and eosin for histological examination. The stained sections were observed under a microscope and subjected to morphometric analysis using the eye piece scale and the stage micrometer.

Biochemical analysis on bone markers
Serum alkaline phosphatase (ALP), serum calcium (Ca) and phosphorus (P) were quantified by using an automatic biochemical analyzer (OLYMPUS, Japan).

Serum estradiol, calcitonin (CT), bone glaprotein (BGP), TNFα and IL-1
Radioimmunoassay (RIA) was carried out by using a kit (Huaying biotechnology institute, Beijing, China) for estimating the circulating levels of estradiol, CT, BGP, TNFα and IL-1.

Measurements of the bone density
Bone density of the left femurs was measured using dual-energy X-ray absortiometry (Lunar, USA). During which, the femurs were carefully brought to the room temperature in a saline bath before measurements.

Measurements of the bone mechanical strength
Mechanical strength of right femurs was measured by three point bending test (TPBT) (7) using mechanical testing machine. Before the testing, the femurs were taken out from freezer and thawed at room temperature for 12 hrs. During the TPBT testing, the femurs were positioned on 2 supports 15 mm apart. Load was applied to midshaft, with a loading speed of 15 mm/min. The bone was compressed in the middle of the femur shaft until fracture occurred. During testing, data on displacement and loading were displayed and recorded by using a computer connecting to the interface of the machine. Indexes of structural mechanics of bone, including maximal load, maximal deflection, elastic load, and elastic deformation were observed on the computer. The inner and outer diameters of cross section were measured with slide gauge, and the indexes of mechanics of materials (maximal stress and maximal strain) of bone were calculated.

The histomorphometry of bone
The proximal tibias of the left limb were fixed with 10% formaldehyde for 18 hrs, followed by decalcification with 15% EDTA, and then dehydrated in 95% (v/v) ethanol and embedded in paraffin. 7 µm-thick sections were cut from the proximal tibias and stained with Haematoxylin and eosin. The sections were then examined under light microscopy, and analyzed by using an image analyzing system (NIS-Elements D 3.1 image analyzing system, Nikon corp, Japan). The following indexes were recorded (8): 1) Total trabecular bone volume (BV/TV; %) of mineralized and unmineralized, which are presented as a percentage of the total medullar volume. 2) Mean trabecular plate thickness (MTPS; µM), the mean thickness of trabecular plates in ten visual fields. 3) Mean trabecular plate separation (MTPS; µM), an index of the mean distance between trabecular plates in ten visual fields. 4) Mean trabecular plate density (MTPD; /mm), the mean number of trabecular plates in ten visual fields.

Statistical analysis of the data
All data obtained in this study were presented as mean±SD. The means between groups were compared using analysis of variance (ANOVA). Two group means were tested using Fisher’s protected least-significant difference (PLSD) post hoc tests. For all tests, differences were considered significant when P<0.05.

Results
The effects of α-ZAL on uterus index and histomorphometry
The final body weights of the rats in the treated groups and the control groups were unable to find differences of significance (Table 1). However, the final uterine indexes were found to be decreased in all the rats in the O VX group compared to the rats in the sham group. Through treatments of estradiol valerate and α-ZAL, the O VX rats showed elevated uterine indexes,
Table 1. The effect of estradiol and α-ZAL on uterus index and endometrial thickness (ET) in ovariectomized rats

| Group     | Uterus index | Body weight (g) | ET (μM) |
|-----------|--------------|-----------------|---------|
| Sham      | 2.54±0.919 a | 308.4±38.52     | 5.38±1.22 a |
| OVX       | 0.35±0.061 b | 312.3±37.46     | 235.9±4.74 b |
| E2        | 0.84±0.029 ab| 301.0±17.34     | 444.5±70.40 ab |
| α-ZAL     | 0.61±0.305 abc| 312.7±32.36     | 351.0±28.63 abc |

Values are means±SD and values with different letters are significantly different (P<0.05). Sham, sham-operated group; OVX, ovariectomied group; E2, ovariectomied fed estradiol valerate; α-ZAL, ovariectomied fed α-ZAL; ET, Endometrial thickness.

However, the uterine indexes of the α-ZAL treated rats were found to be lower than those rats treated by using E2 (Table 1).

The endometrial thickness (ET) were measured at the time of death, and the efficacy of OVX, E2 and α-ZAL replacement were assessed and characterized to see the uterine response to these interventions in the groups. Compared with sham group, endometrial atrophy was observed in OVX rats, but those rats treated with estradiol valerate and α-ZAL experienced increases in endometrial thickness. Similar to these, the degree of endometrium in the α-ZAL treated rats were smaller than those rats treated with E2 (Table 1).

The effects of α-ZAL on bone biochemical markers

After the treatments for 12 weeks by feeding α-ZAL, the levels of Ca and P in the serum of the rats were measured, however no significant differences between groups were found (Table 2), while the levels of serum ALP and BGP were found to be higher significantly in the OVX rats compared to the rats in the sham group (Table 2), indicating the treatments of estradiol valerate to the OVX rats elevated the levels of serum ALP and BGP, respectively. Interestingly, a similar effect of α-ZAL and estrogen on the levels of serum ALP and BGP in the E2 group and α-ZAL group was observed (Table 2).

The effects of α-ZAL on serum E2, CT and PTH

As expected that OVX rats did show a significant reduction in serum E2 compared to the rats in the sham group, we then observed that E2 could be significantly enhanced through feeding the OVX rats on either E2 or α-ZAL in a similar way. Further, the serum level of CT was also low in the OVX rats compared to the sham rats, but it could be enhanced by feeding on either E2 or α-ZAL. In contrast, the level of PTH in serum were found to be higher in OVX rats than it was in the rats in the sham group, while it can be reduced by feeding on E2 or α-ZAL (Table 3).

The effects of α-ZAL on bone density

The bone of OVX rats was found less condensed than those rats in the sham group, while it could be increased through the treatments of estradiol valerate and α-ZAL (Table 5), indicating that both estradiol valerate and α-ZAL increased the bone densities significantly.

Table 2. The effects of ovariectomy, E2 and α-ZAL treatments on serum Ca, P, ALP and BGP levels in rats

| Group     | Ca (mg/dl) | P (mg/dl) | ALP (IU/L) | BGP (ng/ml) |
|-----------|------------|-----------|------------|-------------|
| Sham      | 2.45±0.326 | 1.889±0.346 | 45.92±4.83 | 3.23±0.543   |
| OVX       | 2.427±0.563 | 1.721±0.414 | 91.5±11.7 | 4.74±0.37   |
| E2        | 2.46±1.624 | 1.855±0.260 | 68.59±6.97 | 3.84±0.22   |
| α-ZAL     | 2.405±0.568 | 1.863±0.262 | 66.15±1.1 | 3.64±0.379  |

Values are means±SD and values with different letters are significantly different (P<0.05). Sham, sham-operated group; OVX, ovariectomied group; E2, ovariectomied fed estradiol valerate; α-ZAL, ovariectomied fed α-ZAL; ALP, alkaline phosphatase; BGP, Osteocalcin.

Table 3. The effects of ovariectomy, E2 and α-ZAL treatments on serum E2, CT, PTH in rats

| Group     | E2 (ng/ml) | CT (μg/L) | PTH (μg/ml) |
|-----------|------------|-----------|-------------|
| Sham      | 76.9±12.03 | 312.3±29.99 | 56.3±8.22 |
| OVX       | 43.77±6.95 | 229.6±32.78 | 68.0±6.14 |
| E2        | 78.26±10.14 | 320.92±42.80 | 55.6±6.56 |
| α-ZAL     | 72.55±10.13 | 311.07±44.48 | 54.2±1.16 |

Values are means±SD and values with different letters are significantly different (P<0.05). Sham, sham-operated group; OVX, ovariectomied group; E2, ovariectomied fed estradiol valerate; α-ZAL, ovariectomied fed α-ZAL; E2, Estradiol; CT, calcitonin; PTH, parathyroid hormone.
Table 4. The effects of ovariectomy, E2 and α-ZAL treatments on serum IL-1 and TNFa in rats

| Group          | IL-1     | TNFa    |
|----------------|----------|---------|
| Sham           | 0.194±0.04 9* | 0.308±0.0549* |
| OVX            | 0.30l±0.030 6* | 0.47±0.043 6* |
| E2            | 0.203±0.030 a  | 0.36±0.054*  |
| α-ZAL         | 0.210±0.035  b | 0.348±0.087*  |

Values are means±SD and values with different letters are significantly different (P<0.05). Sham, sham-operated group; OVX, ovariectomized group; E2, ovariectomized fed estradiol valerate; α-ZAL, ovariectomized fed α-ZAL.

Table 5. The effects of ovariectomy, E2 and α-ZAL treatments on left femur density in rats

| Group          | BMD [g/cm²] |
|----------------|-------------|
| Sham           | 0.188±0.020 9* |
| OVX            | 0.110±0.030  b |
| E2            | 0.176±0.010 a  |
| α-ZAL         | 0.164±0.037 a  |

Values are means±SD and values with different letters are significantly different (P<0.05). Sham, sham-operated group; OVX, ovariectomized group; E2, ovariectomized fed estradiol valerate; α-ZAL, ovariectomized fed α-ZAL.

**The effects of α-ZAL on bone mechanical strength**

The mean maximal load, maximal deflection, elastic load, and elastic deformation were found to be reduced significantly in the OVX rats (Table 6). Treatments with either estradiol valerate or α-ZAL were found to be capable of improving the maximal load, maximal deflection, elastic load, and elastic deformation of femurs without big differences (Table 6). Similarly, the maximal stress and maximal strain in the OVX rats were found to be elevated significantly by the treatments of estradiol valerate and α-ZAL, compared to those of rats in the sham group (Table 6).

**The effects of α-ZAL on bone histomorphometry parameters**

Total trabecular bone volume was found to be significantly decreased in the OVX rats compare to those in the sham group, indicating that estradiol valerate and α-ZAL could improve the total volume of trabecular bone. Similarly, the mean thickness of the trabecular plate (MTPT) was decreased significantly in OVX rats, while it could be increased by the treatments of estradiol valerate and α-ZAL. The mean trabecular plate separation (MTPS) was found to be enlarged in OVX rats, but it became narrowed in the rats of E2 group and α-ZAL group by the treatments of estradiol valerate and α-ZAL. The mean trabecular plate density (MTPD) was seen to be decreased in OVX rats, but it was enhanced in the rats of E2 and α-ZAL groups by the treatments of estradiol valerate and α-ZAL, respectively (Table 7 and Figure 1).

Table 6. The effects of ovariectomy, E2 and α-ZAL treatments on mechanical properties of left femur

| Parameters               | Sham          | OVX           | E2            | α-ZAL          |
|--------------------------|---------------|---------------|---------------|---------------|
| maximal load             | 8.44±7.59 a  | 6.27±6.60 b  | 8.33±4.48 a  | 8.27±6.55 a   |
| maximal deflection       | 1.04±0.13 a  | 0.71±0.12 b  | 1.04±0.10 a  | 0.95±0.11 a   |
| elastic load             | 63.10±5.84 a | 44.92±6.91 b | 64.28±4.88 a | 58.42±7.62 a  |
| elastic deflection       | 0.9±0.12 a   | 0.39±0.21 b  | 0.90±0.11 a  | 0.74±0.11 a   |
| Maximal stress           | 1339.26±2.386 a | 817.07±2.134 b | 1213.04±3.139 a | 1338.32±4.7950 a |
| maximal strain           | 0.19±0.02 a  | 0.15±0.01 b  | 0.18±0.01 a  | 0.16±0.01 a   |

Values are means±SD and values with different letters are significantly different (P<0.05). Sham, sham-operated group; OVX, ovariectomized group; E2, ovariectomized fed estradiol valerate; α-ZAL, ovariectomized fed α-ZAL.

Table 7. The effects of ovariectomy, E2 and α-ZAL treatments on mechanical properties of left femur

| Group         | TBV/TTV (%) | MTPT [μm] | MTPD [mm²] | MTPS [μm] |
|---------------|-------------|-----------|------------|------------|
| Sham          | 44.13±6.61 a | 81.31±9.65 a | 2.35±0.56 a | 54.63±10.23 a |
| OVX           | 27.09±6.98 b | 58.37±16.09 b | 1.02±0.33 b | 965.52±3.534 a |
| E2            | 40.13±4.62 a | 76.65±11.20 a | 1.96±0.97 a | 873.49±28.54 a |
| α-ZAL         | 35.43±4.35 a | 66.27±15.81 a | 1.67±0.18 a | 719.37±4.66 a |

Values are means±SD and values with different letters are significantly different (P<0.05). Sham, sham-operated group; OVX, ovariectomized group; E2, ovariectomized fed estradiol valerate; α-ZAL, ovariectomized fed α-zearalanol; TBV/TTV: Total trabecular bone volume; MTPT: Mean trabecular plate thickness; MTPD: Mean trabecular plate density; MTPS: Mean trabecular plate separation.

Figure 1. Histomorphologies of bone trabeculae by ovariectomy and by treatments with estrogen and α-ZAL (Haematoxylin-Eosin Staining, 100×). In sham group, the cancellous bone showed intervening trabecular bone with connectivity of the trabecular elements. While in ovariectomized rats, there was significant thinning and disconnection of trabeculae. When the rats were fed estrogen and α-ZAL for three months, the trabeculation in the cancellous bone was significantly higher than that of ovariectomized rats.
Discussion

Deficiency in estrogen due to post-menopause is responsible for postmenopausal osteoporosis, for which hormone replacement therapy has been designed and utilized as alternative treatments (7, 8). However, the therapy shows serious side effects on the uterus and also risk breast cancer occurrence when performed for a long-term.

In this study, we have investigated the possible roles of α-zearalanol, a novel phytoestrogen, in the prevention of bone loss in the experimentally established rat models. Our results showed that α-zearalanol was capable of improving the bone density, the bone biomechanics properties and the bone histomorphometry parameters with fewer side effects to uterus, while giving less effect on uterus. By contrast, treatments using estradiol increased uterus index and endometrium thickness significantly.

In clinic, changes in bone density is often seen as one of the main indicators of osteoporosis, decreased bone density increases the incidence of bone fracture (9). Therefore, roles of increasing bone density by α-zearalanol in OVX rats was meaningful to demonstrate its potential in bone protection, which in this regard, was found to be similar to the roles of estradiol valerate. These can further be supported by the bone-sparing effects of α-ZAL as indicated by the improvements of bone mechanical strength test and bone histomorphometry parameters.

Biomechanics is a straight forward test for evaluating the risk of bone fractures due to osteoporosis (10). We found the biomechanics properties were indeed decreased in OVX rats when compare with the rats in the sham group. Interestingly the bone losses can be prevented by treating the rats with estradiol valerate or α-zearalanol. Consistence with this, the improvements on bone histomorphometry parameters also indicated the bone protective effects of α-zearalanol on OVX rats.

The positive effect of α-zearalanol on bone may be associated with its role of inhibiting the bone turnover, which behaves somehow similar to estrogen. Indeed, the osteoporotic rats when treated with α-zearalanol are clearly demonstrating an increase in bone turnover as associated with elevated rates of bone formation and bone resorption. It has been reported that estrogen blunts the bone turnover of osteoporosis and decreases the markers of bone formation and resorption (11). Similarly α-zearalanol regulates the bone metabolic processes like estrogen (12). We found that, as indicated by the variations of serum biochemical markers, α-zearalanol decreased the alkaline phosphatase and osteocalcin in serum, which served as markers of bone formation, but didn’t affect the blood calcium and phosphorus, suggesting that α-zearalanol behaves similarly to the estrogen that inhibits the bone formation and reduces the level of bone turnover. It was known that hormones, such as PTH (13-15), CT (16, 17), are also involved in the regulations of bone formation and bone resorption. PTH and CT up-regulate the activity of the bone-resorbing cells by regulating the blood calcium. Interestingly, in this study, we found that estradiol valerate showed an up-regulating effect on CT and a down-regulating effect on PTH, while α-zearalanol can only up-regulate the level of serum CT, but didn’t affect the level of serum PTH, suggesting that α-zearalanol may regulate bone turnover through regulation to endocrinium.

It has been argued that bone-protective effects of estrogen are due partially to its capacities of suppressing the productions of osteoclastogenic cytokines from osteoblasts, bone marrow stromal cells and T-cells (18), which otherwise increase the number of osteoclasts. The decrease of the level of estrogen at menopause is often associated with increases of interleukins, such as IL-1, IL-6, IL-7 and tumor necrosis factor α (TNFα) (19-22). Estrogen replacement therapy, predominantly exert bone-protective effects through regulating the expression of cytokine such as IL-1, IL-6, TNFα. Consistence with this, in this research, our data showed that serum IL-1 and TNFα levels were significantly elevated in OVX rats and all the pharmacological interventions including both estradiol valerate and α-zearalanol treatments significantly decreased the concentration of TNFα in serum, while α-zearalanol can only increase the level of serum TNFα, but cannot increase the IL-1, suggesting that regulations on the expression of cytokine by α-zearalanol may indeed be involved in its bone-protective effects.

Conclusion

Our study clearly showed that α-zearalanol can effectively abate bone loss due to ovariectomy in a manner similar to that of estrogen. However, α-zearalanol shows anti-atherosclerotic activity that presumably through binding to estrogen receptors (23), and an antioxidant ion effect through inhibiting the homocysteine-induced endothelin-1 expression and oxidative stress (24, 25). Based upon this idea, we conclude that α-zearalanol can be a new substitute of estrogen in preventing postmenopausal osteoporosis. However, further investigation on any possible adverse actions, and more detailed mechanism of α-zearalanol actions should also be addressed.

Acknowledgment

This work was jointly supported by grants from Medical Commission of Hebei Province, China (20130048), Population and Family Planning Committee of Hebei Province, China (2010-A03), Advance Research Foundation of Hebei University (2007)Y04 and Youth Foundation of Hebei University (2010Q37).

Conflict of interest

All the authors state that they have no conflicts of interest.
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