RANKL EXPRESSION AND METABOLIC CHANGES IN OVARIECTOMIZED RATS AND THE POSSIBLE PROTECTION BY VEGETABLE FORMULA

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INTRODUCTION
Osteoporosis is a major public health issue leads to pain, functional limitation, higher probability of fracture and death especially in postmenopausal women [1]. Lack of estrogen, hyperthyroidism, immobilization, deficiency of vitamins and minerals, elevation of cytokines due to chronic inflammatory disease and oxidative stress play an important role in the pathogenesis of osteoporosis [2]. Osteoprotegerin (OPG) and RANKL are bone-specific genes. RANKL is a 317 amino acid peptide [3]. Its major role in the bone is the stimulation of osteoclasts differentiation [4], activity and inhibition of osteoclasts apoptosis [5]. The RANKL/RANK signaling pathway has an effect on cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor-α and that is why cytokines play a role in the regulation of bone resorption [6]. Studying mRNA transcription of one or both of these genes is helpful to follow-up the process of osteogenesis. It was found that OPG mRNA decreased and RANKL mRNA increased in ovariectomized rats. Estrogen supplementation in such rats elevated the expression of OPG mRNA and suppressed the RANKL mRNA, thus minimizing osteoclastic activity [7]. The common manifestation of osteoporosis is the fragility fracture which affects the quality of life and add more financial burden [8]. Thus, it is important to define the factors contributing to the development of osteoporosis and try to provide the patient with the supplement that enables him to withstand the condition with minimal side effects or complications and in the same time help in osteogenesis.

There is increasing evidence suggesting a positive association between intake of vegetables and bone health as they are major source of antioxidant and anti-inflammatory compounds so that, they regulate the cytokines and control ROS elevation [9]. In the same time, phytoestrogens (isoflavones) is a popular natural compound used as an alternative to estrogen replacement therapy for prevention and treatment of osteoporosis [10]. Cauliflower, turnip, Allium cepa, parsley, spinach, and wild leek have high nutritive values such as Vitamin A, thiamine, riboflavin, niacin, Vitamin C and minerals such as calcium, iron, phosphorous, and traces such as zinc and selenium [11]. Furthermore, they contain polyphenols, flavonoids, kaempferol, quercetin, and their glycosides [12,13].

Soya bean phytochemicals showed positive linear correlation to antioxidant activity [14]. Isoflavones found in soybeans have a positive effect on estrogen receptors. It can be helpful for treatment of osteoporosis, and menopausal symptoms [15].

Objective
The aim was first, to study the effect of ovariectomy on RANKL gene expression, bone mineral density, bone formation marker and other associated biochemical changes in minerals, blood sugar level, oxidative status, and lipid profile. The second objective was to formulate a novel vegetable and soybean mixture to fulfill the requirement for healthy bone and control the deterioration of bone mass in the ovariectomized rats.
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METHODS

Diet formulation

Vegetables (cauliflower, turnip, A. cepa, parsley, spinach, and wild leek) were purchased from the local market, washed, sliced into small pieces and dried in air oven at 60°C till complete dryness. Weighed and ground into fine powder. White skinned unsalted cheese (karish) was dried in air oven at 60°C till complete dryness then mixed with ground soya bean and added to the previously mentioned vegetable mixture. All ingredients of this formula were mixed in equal weights and refrigerated until use. It was added to rat basic diet (Table 1) as an additive (20% on the expense of starch).

Chemical analysis of the vegetable mixture

The formula was analyzed for total isoflavonoids as catechin [11], total polyphenols as tannic acid [17], and total antioxidant (TAO) capacity (TAC) (1,1-diphenyl-2-picrylhydrazyl radical [DPPH]) [18]. The phenolic compounds of the mixture were extracted by methanol, and some of these active compounds were identified using high-performance liquid chromatography (HPLC).

Experiment

Thirty female Sprague Dawley rats (200–250 g body weight) were obtained from the animal house of the National Research Center. Animals were treated according to the known ethical approval for laboratory animals. 20 animals were surgically bilaterally ovariectomized according to Khajuriam et al. [19] while the skin of other 10 animals has been surgically incised without ovariectomy (Sham operation). All animals were acclimatized at room temperature and given control diet for 2 weeks till complete healing of the surgical wound. Animals were divided into three groups (10 each). The first group, which had the Sham operation, was considered as control (non ovariectomized control group [NOVXC]). The second group is considered as ovariectomized control rats (OVXC). Both control groups were fed on control diet (Table 1). The last group (OVXT) was fed on a diet similar to control one, but 20% of the starch was replaced by the vegetables mixture for 8 successive weeks. At the end of the experiment period, animals were fasted overnight and sacrificed. Blood samples were collected into three different tubes. The first was collected on EDTA and kept in −80°C till be used for RNA extraction and preparation of cDNA which was kept in −20°C till further mRNA transcription of RANKL gene assay. The second blood sample was collected on sodium citrate, for rapid analysis of blood sugar. The last blood sample was collected on EDTA, for separation of plasma and determination of Ca, ph, Mg, Zn, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride, malondialdehyde (MDA), TAO, and bone formation marker (human procollagen 1 N terminal peptide [PINP]). The rats' carcasses were frozen in a certain position to evaluate the bone mass density (BMD) by DEXA scan.

Analysis

Plasma calcium [20], phosphorus [21], magnesium [22], zinc [23], glucose and cholesterol [24], LDL [25], HDL [26], triglycerides [27], MDA [28], and TAC [29] were estimated using colorimetric method commercial kits from Biodiagnostic Egypt. Corn starch 64.957, Choline chloride 0.025, L-cystine 0.018, Vitamin mixture 1, Slat mixture 4, Cellulose 10, Casein 10, and Vitamin mixture 1 were purchased from local market. L-lysine 1, Choline chloride 0.025, Corn starch 64.957 were washed, sliced into small pieces and dried in air oven at 60°C till complete dryness. Weighed and ground into fine powder. White skinned unsalted cheese (karish) was dried in air oven at 60°C till complete dryness then mixed with ground soya bean and added to the previously mentioned vegetable mixture. All ingredients of this formula were mixed in equal weights and refrigerated until use. It was added to rat basic diet (Table 1) as an additive (20% on the expense of starch).

Gene mRNA transcription quantification

Rats RANKL and GAPDH mRNAs were semi-quantified by RT-PCR kits. Blood total RNA was extracted using Qi-easy TM plus blood RNA extraction mini kit [31]. Taq DNA polymerase was supplied by Invitrogen® (Carlsbad, CA, USA). ReverTraAce® Random primers [32], forward and reverse primers and the product size of the genes were as follows: RANKL, 5’-AGC GTC GCC CTG TTC TTC TAT TT-3’/5’-ACT TGG GAT TTT GAT GCT GGT TTT-3’; GAPDH, 5’-TCC ACT CAC GGC AAA TTC AAC G/TAG ACT CCA GGA CAT ACT CAG C-3’, 145 bp. All chemicals were of the highest available purity from commercial suppliers.

DNA isolation was then performed using ReverTrNAc® and then cDNA was amplified under the conditions recommended by the supplier (Invitrogen®). Real-time analysis was performed using qPCR Green Master (Jena Bioscience) using the fluorescent DNA stain Eva Green®.

Statistical analysis

Results were analyzed by one-way analysis of variance followed by least significant difference post hoc test (SPSS ver. 17.0) p≤0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Evaluation of the RANKL/GAPDH mRNA transcription ratio showed a marked increase in ovariectomized rats (OVXC) versus the control (NOVXC) group (1.00 vs. 0.199, respectively). Supplementing rats with the formula in OVXT group lowered the mRNA expression of RANKL and controlled bone deterioration and consequently led to controlled osteoporosis on a molecular level (0.51).

The receptor activator of RANKL/RANK/OPG system regulates bone cell function [33]. It was shown that RANKL/RANK signaling regulates the formation of multinucleated osteoclasts, activating and keep it survived. The role of OPG is critical in the protection of the skeleton from excessive bone resorption, and this is accomplished by binding to RANKL and preventing it from binding to its receptor, RANK. Unless RANKL is blocked by OPG in the preosteoblastic/stromal cells, RANKL will bind its receptor RANK on osteoclasts lineage cells, and the differentiation of osteoclasts precursor into mature osteoclasts is accelerated [34].

Results showed a marked drop in BMD values of OVXC compared to NOVXC group (0.157 vs. 0.25 mg/cm², respectively). Rats given the vegetable formula with the diet (OVXT) showed a relatively high BMD value (0.183 mg/cm²) compared with those not given the formula (OVXC).

In the same time, serum level of PINP revealed a significant drop in OVXT group compared to NOVXC (27.9±2.8 and 34±2.4 µ/L, respectively). Supplemeting the plant formula in OVXT showed a marked increase in ovariectomized rats (OVXC) versus the control (NOVXC) group (1.00 vs. 0.199, respectively). Supplementing rats with the formula in OVXT group lowered the mRNA expression of RANKL and controlled bone deterioration and consequently led to controlled osteoporosis on a molecular level (0.51). This can be explained in the light of the absence of estrogen due to ovariectomy and the elevated RANKL mRNA expression that promotes osteoclastic activity in bone [7]. The formula supplemented diet showed a high content of isoflavonoids as catechin [100 mg/100 g
of dry weight) and polyphenols as tannic acid (1000 mg/100 g dry weight). Furthermore, the TAO was high (estimated by DPPH method which showed inhibition percentage of 91.81%).

The HPLC results of the current formulation (Table 2) revealed the presence of important polyphenols necessary for bone health. These polyphenols and the high antioxidant power of the formula are considered an essential factor is contributing to its effective role in counteracting the drop in BMD due to ovariectomy. It has been reported that the presence of compounds having antioxidant power in food can ameliorate the damaging effects of free radicals and in turn oxidative stress thereby prevent the risk of osteoporosis in postmenopausal women [36].

Results showed (Table 3) that the level of calcium, phosphorus, magnesium, and zinc of OVXC rats was all lower than corresponding levels in NOVXC ones. Addition of the formula to the diet of the OVXT rats relatively improved the level of some of these minerals if compared to OVXC group.

It is well known that calcium, magnesium, zinc and phosphorus participate in the process of collagen, and other proteins essential for bone formation [37]. These minerals are usually found low in menopausal women, and this occurs due to ovarian hormone deficiency [38]. These minerals participate in the process of bone maintenance through their catalytic action in the synthesis of the bone matrix [39]. It is clear that the body under such condition is in need to replenish these minerals. Addition of the vegetable formula succeeded to partially modulate the mineral concentration in blood plasma and this, in turn, assist the process of bone formation. This proves that the vegetable formula is needed and imposes benefit to the body.

It has been reported that phosphorus and calcium contribute to bone development by inhibiting bone resorption [40]. Zn stimulates osteoblasts and inhibits osteoclasts [41]. Mg is important in matrix and mineral metabolism in bone [42].

The blood sugar level showed a slight increase in OVXC versus NOVXC rats. Alterations in bone and mineral metabolism were reported in some studies as a result of elevated blood sugar [43] which is accompanied with oxidative stress and partly contributing to the development of osteoporosis [44]. Supplementation with the vegetable formula, in OVXT group, showed hypoglycemic effect. Some studies have similarly shown that eating vegetable significantly reduced blood glucose levels [45, 46]. They referred this to its high content of phenolic compounds which act as insulin such as molecules or insulin secretagogues [47]. It is worth to mention that the oxidative stress of OVXC rats was high as evidenced by the high level of serum MDA and the low value of TAO capacity. Such complication was ameliorated when rats (OVXT) were given the vegetable formula which again confirms its high nutritive and antioxidant power mentioned above.

Concerning lipid parameters, ovariectomy caused a sharp increase in serum cholesterol, LDL of OVXC versus NOVXC rats (Table 3). It is known that ovarian cells withdraw plasma lipid to synthesize steroids, in turn, lipids are expected to be saved, and its level in blood is increased [50] therefore, postmenopausal women may be at a higher risk for coronary heart disease [51]. Phytoestrogens present in the given formula produce effects similar to those of estrogen so it may control the level of blood lipids to some extent.

### Table 2: Identification of some phytochemical in the formula using HPLC

<table>
<thead>
<tr>
<th>The compound</th>
<th>mg/100 g dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocatechuic acid</td>
<td>9.12</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>1.62</td>
</tr>
<tr>
<td>Coumarin</td>
<td>1.84</td>
</tr>
<tr>
<td>Rosmarinic acid</td>
<td>11.28</td>
</tr>
<tr>
<td>Cinnamic</td>
<td>1.84</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>1.67</td>
</tr>
<tr>
<td>Chrysin</td>
<td>1.16</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>2.29</td>
</tr>
</tbody>
</table>

HPLC: High-performance liquid chromatography

### Table 3: Chemical analysis of serum of supplemented rats

<table>
<thead>
<tr>
<th>Plasma levels</th>
<th>NOVXC</th>
<th>OVXT</th>
<th>OVXC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mg/dl)</td>
<td>9.0±0.3^a</td>
<td>7.52±0.5^a</td>
<td>7.1±0.3^a</td>
</tr>
<tr>
<td>Phosphorus (mg/dl)</td>
<td>3.5±0.14^a</td>
<td>3.5±0.17^a</td>
<td>3.3±0.14^a</td>
</tr>
<tr>
<td>Magnesium (mg/dl)</td>
<td>1.78±0.05^a</td>
<td>1.70±0.01^a</td>
<td>1.22±0.05^a</td>
</tr>
<tr>
<td>Zinc (µg/dL)</td>
<td>12.0±6.05</td>
<td>11.7±7.1</td>
<td>8.4±6.5</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>65.0±0.5^a</td>
<td>53.06±0.96^a</td>
<td>73.18±0.71^a</td>
</tr>
<tr>
<td>MDA (mMol/L)</td>
<td>1.95±0.11^a</td>
<td>6.26±0.07^a</td>
<td>8.58±0.6^a</td>
</tr>
<tr>
<td>TAC (mMol/L)</td>
<td>6.02±0.11^a</td>
<td>5.52±0.12^a</td>
<td>3.62±0.05^c</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>67.63±2.07^a</td>
<td>76.01±0.77^a</td>
<td>143.0±2.5^c</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>30.00±1.77</td>
<td>75.8±2</td>
<td>106.7±5.6^a</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>22.75±1.05</td>
<td>46.1±3</td>
<td>33.8±3.5^a</td>
</tr>
</tbody>
</table>

MDA: Malondialdehyde, LDL: Low-density lipoprotein, HDL: High-density lipoprotein, TAC: Total antioxidant capacity

CONCLUSION

It could be concluded that ovariectomy in rat model cause deterioration of bone and elevated expression of bone resorption RANK, feeding current vegetable formula opposed this effect and was good for health as antioxidant, hypoglycemic, and hypolipidemic. It should be given for longer time to show a better protective effect from bone deterioration.

### CONFLICT OF INTERESTS

There is no conflict of interests between authors.

### REFERENCES


2. Laughlin GA, Yen SS. Nutritional and endocrine-metabolic aberrations


