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Experimental Research

Anatomy Section

The Effects of Dietary Supplements of Calcium, Vitamin D and Estrogen Hormone on Serum Levels of OPG and RANKL Cytokines and their Relationship with Increased Bone Density in Rats

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ABSTRACT

Introduction: Osteoprotegerin (OPG)-Receptor activator of nuclear factor kappa-B ligand (RANKL) pathway is one of the contributing factors in the regulation of osteogenesis and bone resorption routes.

Aim: The purpose of this study was to evaluate the effects of various dietary supplements on this pathway.

Materials and Methods: The samples for this study (24 newborn rats) were divided in three groups according to the experiment applied for each group. Rats were given special diet according to their group plan for six weeks. Blood samples were collected to measure their serum levels of OPG and RANKL and all organs of rats were used to measure their bone density too. The results were analysed using appropriate statistical analysing tests.

Results: Levels of whole-body bone mineral density in calcium plus vitamin D plus Estrogen (Ca + D + E) group and calcium plus vitamin D (Ca + D) group were significantly increased compared to control group. Mineral density was highest in calcium plus vitamin D plus Estrogen group and was about 0.1357 g/cm^2. RANKL had a significant decrease in calcium plus vitamin D plus Estrogen group compared to control and calcium plus vitamin D groups. There was a significant increase in the mean calcium and OPG in both experimental groups rather than control. Also, significant increase in estrogen was observed in Ca + D group than the control group.

Conclusion: The results showed that intake of calcium and vitamin D and estrogen at determined dose led to an increase in OPG and RANKL cytokines reduction which ultimately led to an increase in bone mineral density. But Ca, D and E synergies were more effective in increasing bone mineral density compared to only the use of Ca and D.

Keywords: Estradiol, Osteoblast, Osteoclast

INTRODUCTION

Bone is considered to be one of the hardest living tissues which has an extensive blood supply and is constantly undergoing change [1]. Osteoblasts account for the organic components of bone matrix. Bone surface growth is promoted by deposition of calcium salts in a newly formed matrix [2]. Alkaline phosphatase secreting Osteoblasts, generate matrix vesicles in which crystals of hydroxyapatite (calcium and phosphate deposits) are shaped [3].

Osteoclasts secrete collagenase and other enzymes into the sub-cellular space to make a local acidic environment which dissolves hydroxyapatite and accelerates localized absorption of collagen. Therefore, both cells (osteoblasts, osteoclasts) are coordinated and essential for bone remodeling [4].

The bone remodeling process happens with a rate of close to a 100% in the first year of life and 10% in the adult population [5]. The most important micronutrients in maintaining bone health which contribute most to bone mineral density at all ages are calcium, phosphorus, vitamin D [6]. Other studies showed that taking supplements such as calcium and vitamin D promotes bone absorption and resorption [7]. Studies on laboratory animals have also shown that calcium deficiency leads to inhibition of bone formation [8]. Vicky Tai et al., studied the special effects of a diet rich in calcium on bone metabolism in rats. Results indicated that Bone Mineral Density (BMD) of the femur in groups which had consumed calcium rich nutrients were notably higher than that of the control group [9]. During the process of bone formation, fluctuations in estrogen affect bone formation [10].

Receptor activator of nuclear factor kappa-B ligand (RANKL), a member of the Tumour Necrosis Factor (TNF) superfamily, is an effective stimulator of both, osteoclast formation and their bone-resorbing activity [11]. Upon binding to its receptor, RANK located on osteoclasts, RANKL signaling increases differentiation and activation of the osteoclasts resulting in expression of osteoclast specific molecules. During normal bone remodeling, marrow stromal cells and osteoblasts produce RANKL, which binds to the transmembrane receptor RANK on osteoclast precursors and induces differentiation and activation [12]. This occurs through the transcription factor, nuclear-factor kappa B(NFkB), which is responsible not only for activating osteoclastogenesis but also the body's inflammatory response. Although it has been proposed for a long time that the main source of RANKL are stromal and osteoblastic cells, a very recent study examiners proved that the main RANKL production site resides within osteocytes [13].

Osteoprotegerin (OPG) is a secreted by TNF receptor super family member acting as a decoy receptor molecule for RANKL, thereby counteracting its osteoclastogenic activity. It is produced by a variety of cells, including stromal cells, B lymphocytes and dendritic cells [14].

Estrogen, the major hormone regulating bone remodeling, is essential for both men and women. Estrogen's role in maintaining bone health is far reaching from maintaining calcium homeostasis by stimulating the release of calcitonin and activation of vitamin D (1,25(OH)2D) receptors in the gut, enabling its function on an osteo-endocrine-immunological basis. Estrogen limits bone loss by affecting osteoblasts and osteoclasts. Estrogen-receptor

activation of the osteoblasts stimulates release of growth factors TGF and IGF-1, and OPG. This in turn, limits M-CSF and RANKL, which reduces osteoclastogenesis and increases osteoclast apoptosis. Decline in estrogen levels leads to increased osteoclastic activity, promoted by reduced hormonal control over the proinflammatory cytokines, IL-1, IL-6, and TNF [15]. It has been shown in a study that estrogen has an inhibitory effect on the bone resorption process and stimulates osteoclast apoptosis [16].

In this study, we sought to evaluate the fluctuations of intervening cytokines and finally the bone density by providing a diet containing various factors such as estrogen and calcium.

MATERIALS AND METHODS

In this experimental study 24 newborn Wistar rats, 15-20-day-old, were randomly placed into three groups. Rats in all groups were kept in the same suitable light and temperature conditions during six weeks.

It should also be noted that the rats had freely access to food and water during the study. Injections and dietary supplement feeding at different periods were performed by the person responsible for the animal home.

Control group received only natural food and did not get any dietary supplement during this trial.

Calcium plus vitamin D group (Ca + D group) received supplements of calcium and vitamin D in addition to natural food.

The calcium plus Vitamin D plus estrogen group (Ca + D + E group) received 30μg/kg of 17 beta-estradiol hormone subcutaneously five days a week.

The subjects of this group received 35mg of soluble calcium per kilogram of body weight (according to the weekly weighing) by daily oral gavage for five days per week for a duration of six weeks. Calcium used in this study is known as osteocare liquid, containing 300mg of calcium, 150mg of magnesium, 6mg of zinc and 3.8 micrograms (150 IU) of vitamin D per 200ml of serving [17] 30 micrograms of estradiol hormone was injected subcutaneously on a daily basis, 5 times a day, for six weeks [18]. After six weeks under the influence of listed supplements, whole-body bone mineral density was measured using Norland densitometer (made in America) at a speed of 60 mm per second and a resolution of 1.0 x 1.0 mm at nuclear medicine center of Kurdistan, Iran. Finally, after the measurement of bone mineral density, the rats in different groups were killed considering the ethical issues by inhalation of ether and chloroform and immediately blood samples were taken from the heart through direct method. After taking blood samples, serum separation was carried out using a centrifuge at a speed of 2500 rpm for 12-15 minutes. Serum levels of estrogen, calcium and cytokines OPG and RANKL were measured using special kits of East Biopharm Company made in America by ELISA as previously mentioned [19-22].

Statistical methods: Data averages were analysed using one-way analysis of variance (ANOVA) and Dantte test in SPSS 22 software. Significant level for all groups were considered as ($p<0.05$).

Also, since live animals were used in this research, before the beginning of the work tracking code of ethics within study at a number of EC/93/A/113 was received from certain organizations and all phases of the research were conducted under the supervision of University of Medical Sciences of Ilam, Iran.

RESULTS

By analysing data and using one-way ANOVA test in the evaluation of Bone Mineral Density (BMD) in all three groups of rats studied, after six weeks the effect of supplements diet and estradiol hormone were determined in which the average lowest BMD was observed in control group (0.1105 g/cm^2) and the average highest bone mineral density was found in Ca + D + E (0.1357 g/cm^2) [Table/Fig-1,2]. Also, using Dantte test on this variable, a statistically significant correlation was observed between two groups of Ca + D and Ca + D + E groups with and the control group ($p<0.006$, $p<0.004$). In assessment of calcium levels in the blood serum of rats tested in Ca + D and Ca + D + E groups, there was significant increase compared to the group that did not receive mineral supplement and estradiol (control group), which this increase showed statistically significant relationship between two experimental groups and the control group ($p<0.05$). The least amount of calcium was seen in the control group (8.762 mg/dl) and the highest average was found in Ca + D + E group (11.108 md/dl). The lowest serum estradiol levels in tested rats were seen in the control group (4.811 Ng/l) and the highest level was obtained from Ca + D + E group (27.271 Ng/l) which had significant relationship ($p<0.045$), but the considerable point in examining of this variable was a high level of estradiol in calcium and vitamin D group without injection of estradiol compared to the control, perhaps because the secretion of this hormone in the blood increases through mineral supplement. In the study of OPG and RANKL cytokines that are respectively involved in bone building and resorption, it was found that using statistical analysis, the most cytokine OPG was in the Ca + D and Ca + D + E and the highest RANKL was in the control group. OPG increasing in the group which in addition to mineral supplements also received estrogen represents an increase in osteoblasts cell activity in rats of the groups which the highest bone mineral density compared to the control group and the group that received only mineral supplement without injection of estrogen were observed as well as an increase in RANKL was observed in the control group who received no supplement, as a result, the lowest bone mineral density was in this group.

DISCUSSION

Growth rate and bone regeneration in young children is very active and much faster than adults [23]. Several studies have shown that high bone density acquisition is dependent on physiological factors such as type of nutrition (especially calcium content in diet), endocrine factors (sexual steroids, active vitamin D 3, insulin-dependent growth factor) [24].

Of the most important micronutrients involved in maintaining bone health can be noted to calcium and vitamin D that have beneficial effects on bone mineral density at all ages, especially age of puberty. The main source of calcium in the body is crystals of hydroxyapatite in the bones as well as increasing vitamin D enhances intestinal absorption of calcium; therefore taking supplements of calcium and vitamin D in age of puberty leads to the accumulation of calcium into crystals and increased bone mineral density [25].

In line with the above mentioned issues, Peacock M et al., in a study on bone density in people with decreased serum levels of calcium and under the influence of calcium intake found that the bone mineral density in these patients was more than the control group and also bone fractures rate is decreased on them [26].

Tania Winzenberg et al., found that taking vitamin D supplements in children who had vitamin D deficiency led to an increase in BMD at the lumbar spine [27]. Also in this study, an increase in bone density was dramatically observed in all rats that were under the treatment of calcium and vitamin D supplements for six weeks, it is obvious that there is relationship between the simultaneous intake of calcium and vitamin D on the activity of osteoblasts.

Estrogen which is a sexual steroid hormone is secreted by the ovaries. Increasing osteogenic activity, determining the maximum bone mass and bone mineral homeostasis during development of bone are considered from the physiological functions of estrogen [28]. In a study conducted by Mawi et al., to determine the effect of estradiol on osteoporosis found that postmenopausal women at a risk of osteoporosis had shown a reduction in bone mineral

Dependent Variable	(I) group[a]	(J) group[a]	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Bone mineral density	CA+D	Control	0.011475*	0.003930	0.031	0.00120	0.02175
	CA+D+E	Control	0.024550*	0.003930	0.000	0.01428	0.03482
Calcium	CA+D	Control	2.965000*	0.383045	0.000	1.96374	3.96626
	CA+D+E	Control	2.785000*	0.383045	0.000	1.78374	3.78626
Estrogen	CA+D	Control	7.815250*	2.766175	0.036	0.58460	15.04590
	CA+D+E	Control	15.538250*	2.766175	0.001	8.30760	22.76890
Osteoprotegrin	CA+D	Control	0.670500	0.284690	0.0015	-.07367	1.41467
	CA+D+E	Control	0.999750*	0.284690	0.012	0.25558	1.74392
Receptor activator of nuclear factor kappa-B ligand	CA+D	Control	-30.961000	19.481641	0.244	-81.88507	19.96307
	CA+D+E	Control	-65.005500*	19.481641	0.016	-115.92957	-14.08143

[Table/Fig-1]: Multiple Comparisons using Dunnett t (2-sided)[a]for different variables in trial groups compared to control group.
*The mean difference is significant at the 0.05 level.
[a]Dunnett t-tests treat one group as a control, and compare all other groups against it.

Variabl/group	BMD g/cm^2	Calcium mg/dl	Estrogen ng/l	OPG ng/ml	RANKL pg/ml
Cal+D	0.1327	11.062	12.154	1.774	73.134
Ca+VitD+Est.	0.1357	11.108	27.271	1.793	64.755
Control	0.1105	8.762	4.811	0.499	87.956

[Table/Fig-2]: Mean values of different variables amongst three groups

density due to low levels of estradiol. In this study, 49.5% of postmenopausal women aged 47 to 60 years had estradiol levels less than 5 picograms. As a result, the researchers found that there was a significant correlation between estradiol and BMD at the femoral neck in postmenopausal women who received more than 5 picograms estradiol [29]. Mawi et al., reported that bone weight loss was prevented in ovariectomized rats that received estrogen compared to a group of rats that without estrogen their bone activity was decreased. They concluded that there was a significant correlation between estradiol and bone mineral density [30].

In the present study, it was found that the bone mineral density in the group of rats that were under the influence of estrogen and mineral supplements (group 3) showed a significant increase compared to mineral supplements (group 2) and control (group1) groups, which it is evidence of the truth of past studies and significant direct connection of estradiol with the activity of osteogenesis. Several studies have examined the effect of calcium deficiency or lack of estrogen; their results showed that the combination of both of these factors have further effects on increased bone mass [31].

OPG and RANKL cytokines are factors affecting the activity of osteoblast cells and osteoclastogenesis process. OPG cytokine by binding to RANKL prevents from RANKL binding to RANK receptor on osteoclast cells and inhibits from bone resorption and reduce the process of osteoclastogenesis [32].

In a study carried out by Anna Stern et al., to assess the level of OPG, RANKL and sexual steroids and associated with BMD found that with declining estrogen levels, RANKL is increased and thus BMD is reduced and the result is osteoporosis [33]. According to the results of the present study, OPG levels and bone mineral density were greater in the group receiving calcium, vitamin D and estrogen compared to the other groups. There has been also a significant relationship between Bone Mineral Density (BMD) and the mean calcium and estrogen ($p \leq 0.007$). The highest RANKL cytokine and also the lowest BMD were observed in the control group.

LIMITATION

Working with rats in a long period trial is a difficult job. There was some difficulty providing the experimental materials such as kits for the study and also very hard for the measurements of bone density as the facilities were in another state.

CONCLUSION

These results reflect the impact of calcium and estradiol on increased production and activity of osteoblast cells. With the increase in OPG produced by osteoblast cells the process of bone resorption or osteoclastogenesis were reduced and bone mineral density was increased in this group. Also, increased selective mineral supplement (Ca+ D) and estradiol at specific doses led to an increase in the production of OPG cytokine and decrease in production of RANKL cytokine which ultimately led to an increase in bone mineral density. However, synergistic mineral supplement and estradiol (Ca+D+ E) had greater impact on increased bone mineral density compared to only intake of minerals (Ca+ D).

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