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Zinc inhibits ovariectomy induced microarchitectural changes in the bone tissue

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Purpose: The purpose of the current study was to examine the effect of zinc supplementation as a nutritional supplement in case of osteopenia induced microarchitectural changes in rat model.

Methods: Forty eight animals in two batches of twenty four animals each were assigned to four groups: Control, Zinc, Ovariectomized (OVX) and OVX + Zinc. The treatment period was continued for eight weeks. Histoarchitecture analysis was performed on both the bones i.e. femur and tibia using light as well as electron microscopy. Also, the bone calcium content was estimated using atomic absorption spectrophotometer.

Results: The body weight of the animals in the OVX group was significantly higher in comparison to the control animals. The body weight was found to increase significantly upon zinc supplementation to OVX animals till the 4th week and then was almost comparable till the termination of treatment period. Calcium content in both femur and tibia were found to be significantly reduced in the ovariectomized group. The connectivity of trabeculae was lost following ovariectomy. Zinc administration restored bone calcium content as well bone tissue morphology including trabecular thickness.

Conclusion: These findings suggest that changes in the trabecular bone attributed to estrogen deficiency are arrested by zinc supplementation.

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1. Introduction

Osteoporosis is a skeletal disease characterised by low bone mass and structural weakening of the bone material that leads to reduced bone strength [1]. Postmenopausal osteoporosis is by far the commonest form of age-related bone loss. In women, bone loss is exacerbated after menopause due to a decrease in estrogen production. It is estimated that 10 million individuals have osteoporosis while another 34 million suffer from low bone density. Upon survey, it has been found that 61 million individuals will develop osteoporosis or low bone density by 2020 [2]. Beyond medical costs, there is the physical burden of living with osteoporosis and its impact on the daily life style, including restrictions in daily activities, loss of confidence (due to fear of falling and fracture) and loss of independence [3].

The development of osteoporosis is thought to be primarily related to ageing, genetic factors. Some modifiable factors, such as smoking, excess alcohol consumption, life style and deficiency or excess of some of the components of diet, are also associated with osteoporosis [4]. Therapies for osteoporosis fall into two categories: antiresorptive drugs, which slow bone resorption, and anabolic drugs, which stimulate bone formation. However, the adverse effects of these medications include malignant tumour formation with hormone therapy and gastrointestinal tolerance problems with bisphosphonates, which may exclude their long-term use [5,6]. Thus, there is a need for some alternative that can improve bone health without inducing adverse effects. Growing evidence of the benefits of natural foods for bone health presents alternatives for the prevention or treatment of osteoporosis [7,8]. Trace elements play a major role in the growth and development of skeleton, of which zinc is of particular interest to us.

The fact that the organic component in the bone is mainly composed of protein and that most of the bone mineral portion is calcium implies that the essential nutrients required for bone health are protein and calcium [9]. In addition this, certain minerals, vitamins and trace metals are also required for the

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maintenance of bone composition and microarchitecture [10]. Zinc is an essential trace element, is a component of 200 enzymes and is also well known to be necessary for normal mineralization and collagen synthesis in bone. Yamaguchi et al. studied the direct effects of zinc on the proliferative activity of bone cells and found that it has a stimulatory effect on osteoblastic bone formation and an inhibitory effect on osteoclastic bone resorption. Zinc produced remarkable increases in alkaline phosphatase activity and protein concentration in osteoblasts [11,12]. The role of zinc has been studied previous in terms of zinc deficiency or adequate zinc diet. Zn-deficient diets have impairment in growth plate chondrocyte proliferation, body length gain [13,14] and bone mass [15] in animals.

However, to the best of our knowledge, little research on the association between zinc supplementation and bone micro architecture in the osteopenic condition has been conducted. To address this issue, the present study investigated the effect of zinc supplementation on bone calcium content and microarcitecture in osteopenic rat model.

2. Material and methods

To carry out the present study, forty eight female Wistar rats (12 weeks old, 100–150g) in a batch of two were procured from the Central Animal House of Panjab University, Chandigarh. The animals were housed in polypropylene cages bedded with rice husk. They were given clean drinking tap water and standard animal pellet diet (Ashirwad Industries, Kharar, Punjab, India), throughout the experiment *ad libitum*. The animals were randomly divided into four groups comprising twelve animals in each group. They were acclimatized for one week to laboratory conditions prior to the start of the experimental procedure. Group 1 (Control) was given standard diet as well as drinking water. Group 2 (Zinc) was given zinc supplement as zinc sulphate in drinking water at a dose level of 227 mg of $ZnSO_4.7H_2O$ per litre [16]. Group 3 (OVX) animals were bilaterally ovariectomized and were kept under normal feeding conditions without any zinc supplementation. Group 4 (OVX + Zinc) animals were bilaterally ovariectomized and given zinc in drinking water (227 mg of $ZnSO_4.7H_2O$ per litre). The treatment period was continued for eight weeks. The experimental design and procedures were approved by the Ethical Committee on Animal Experiments of the Central Animal House, Panjab University, Chandigarh, India.

It was not possible to carry the measurements in a single batch. Therefore, measurements of all the parameters were done in two batches. These batches were segregated in different time over a period of one year. The first batch includes the ash content analysis and the second batch includes the histological analysis using light and electron microscopy.

2.1. Surgical procedure for bilateral ovariectomy

Ovariectomy was performed in female Wistar rats by the method as described in our previous paper [17]. The ovariectomized Wistar rat model is the FDA recommended model and has been widely used for the rapid development of osteoporosis for purposes of investigating aspects of pathogenesis and treatment of postmenopausal bone loss [18].

At the completion of termination period, the animals were sacrificed by decapitation under anesthesia (using diethyl ether) and both the femora and tibiae were extracted. Bones of all the animals were cleared off from the muscle and cartilage and were further processed.

2.2. Elemental analysis

The method of Szpunar et al. [19] was partially modified for bone digestion. Bone specimens were digested in concentrated nitric acid, and trace element analysis for the bone tissue was performed using an atomic absorption spectrophotometer (model 3100; PerkinElmer). Calcium hollow cathode lamp was operated at a slit width of 0.7 nm, which was selected to isolate 422.7 nm lines. Calcium standard (1000 ppm) was used for calibrating the system.

2.3. Micro architectural analysis

Microscopy was done to analyze the microstructure of bone that includes arrangement and geometry of the trabecular region. Also, the adipocytes present in the bone marrow were analyzed using light microscopy.

2.3.1. Light microscopy

2.3.1.1. Bone sample preparation

2.3.1.1.1. Bone tissue preparation (fixation and embedding) for Hematoxylin-eosin staining. After excision, the trabecular regions (sectioned from the metaphyseal region along the growth plate) of the femora and tibia of all the groups were sectioned and fixed immediately in 4% formaldehyde prepared in 0.1M phosphate buffer (pH 7.2) overnight. After this, all the samples were decalcified in 10% Ethylene Diamine Tetra Acetic acid (EDTA) prepared in 0.05M Tris–HCl buffer, pH 7.2. All the samples were removed from demineralising solution and washed in phosphate buffer saline. Then they were treated in five different gradients of alcohol (starting from 30% ethanol to absolute alcohol) for 1hr. After this they were dipped in absolute alcohol and benzene (1:1) for half an hour, only benzene for half an hour and then in wax for 6 h. Samples were then embedded in the paraffin wax (melting temperature 60 °C) using plastic cassettes. Plastic cassettes were then inserted into the microtome so that the wax block faces the blade. The thin bone tissue sections (of about 4–10 μm thin) were then sliced and further processed for different staining protocols. HE staining was done to visualize the diaphyseal cortical bone thickness of both the femora and the tibia upon ovariectomy and zinc supplementation. After deparaffinising in xylene for about 15 min, the sections were immersed in the graded alcohol solutions (90% ethanol to 30% ethanol). After this the slides were dipped in Hematoxylin for 1–2 min. The slides were rinsed under running tap water in staining box until the water is no longer coloured (~5 min). Sections were again immersed in eosin stain for 1–2 min and rinsed until water became clear. Then again the slides were kept in gradient of ethanol and were shifted to xylene till they were cover slipped. Then the slides were viewed and analyzed under the light microscope (Leica).

2.3.1.2. Bone marrow preparation. Adipocytes were analyzed by extracting bone marrow cells from the femur bone. The marrow cells were then stained with h and e stain as according to the standard protocol. The stained sections were then analyzed under light microscope (Leica).

2.3.2. Scanning electron microscopy

2.3.2.1. Bone sample preparation. The bone samples were processed for the assessment of microarchitecture as per the method followed by Chang et al. [20]. Longitudinal section of all the bone samples (trabecular region sectioned from the metaphyseal growth plate) were immersed in a tissue fixative solution (4% formaldehyde in phosphate buffer saline) for at least one day and then rinsed with the Phosphate buffer saline (PBS) before characterization. For SEM, the bone was polished and etched in a hydrogen peroxide

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