PROTECTIVE EFFECTS OF ZINC ON HISTOMORPHOMETRY OF FEMUR IN SALT LOADED FEMALE SPRAGUE DAWLEY RATS

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ABSTRACT

Objective: To evaluate the protective effect of zinc on histomorphometry of femur of Sprague Dawley rats under high salt diet.

Study Design: Analytical randomized control trial.

Place and Duration of Study: The studied was carried out at Islamic International Medical College, Rawalpindi, with the collaboration of National Institute of Health; Islamabad. The study spanned over a period of six months, from Sep 2015 to Mar 2016.

Material and Methods: Forty five female Sprague Dawley rats, 10-12 weeks old were used in the study. The rats were randomly divided into 3 groups. Group-A rats were fed high salt diet (8% NaCl) and group B rats were given high salt diet augmented with zinc (50mg / kg / day) for eight weeks, however, the diet of control group was not modified. Rats were dissected and left femora were removed. Decalcification was performed. Tissue from proximal femur was obtained to study the trabecular structure and collagen staining while midshaft of femur was transversely sectioned to measure the medullary cavity diameter. Processing was done to obtain five micrometer (µm) thick sections. Tissues were stained with haematoxylin and eosin (H&E) and Masson's Trichrome for histological parameters. Comparison was done amongst all groups.

Results: Obvious histological variations were observed in experimental groups. These changes were of more severity in rats of group-A who took high salt diet as compared to group B who received zinc supplementation in addition to salt.

Conclusion: High salt diet exerted harmful effects on bones due to increased sodium chloride induced hypercalciuria leading to bone loss. Zinc is valuable in ameliorating the detrimental effects of salt on bones by enhancing osteoblast activity and inhibiting bone resorbing cells.

Keywords: Hypercalciuria, Osteoblast, Salt, Zinc.

INTRODUCTION

Bone is a highly vascular tissue which adapts to external as well as internal factors and its organization varies in different anatomical locations even in same bone[1]. Balance interaction between bone forming and bone resorbing cells is mandatory for healthy bone mass. Negative inclination in this balance results in death-defying disease, osteoporosis,[2] affecting nearly 200 million people worldwide[3]. Decrease in mechanical integrity and increase vulnerability to fracture is the hallmark of this disease. Its prevalence is high than myocardial infarction, breast cancer and stroke[4]. It is imperious to discover and improve nutritional strategies for osteoporosis deterrence due to life threatening outcomes and substantial annual cost related to its morbidity. Salt being omnipresent and a known threat for osteoporosis, inflicts health risks. It interacts with calcium absorption and excretion leading to hypercalciuria[5].

WHO has recommended daily intake of 2000 mg sodium[6] but the human population has exceeded the limits. Western inhabitants has daily intake of 2300-4300 mg sodium per day whereas asian residents has much more, 5300mg-6000mg of sodium per day[7] and this much quantity is adversely influencing the health

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resulting in grave diseases like osteoporosis, hypertension, increase urinary tract stones and stroke[8].

Zinc is a crucial trace element[9]. Although its role as therapeutic agent has been observed in Ayurveda many years back but its nutritional benefits have been recognized recently. Being vital element, human body contains only 2-3 grams, even a small deficiency is a disaster[10]. Due to its role in bone metabolism, zinc can be a veiled connection for the prevention of osteoporosis. It has the ability to stimulate the differentiation and proliferation of osteoblasts and inhibiting osteoclast formation from bone marrow[11].

Table-I: Histological scoring system for trabecular architecture.

Grades	Structure of trabecular bone	Quantity of trabecular bone (% of area)
0	Normal	90-100
1	Partially reduced	60-90
2	Markedly reduced	30-60
3	Absent	0-30

Zinc, by stimulating apoptotic cell death of mature osteoclasts can inhibit bone resorption and have direct positive effect on bone metabolism[12]. Other than bones which act as a zinc sink[13], zinc is stored in muscles and skin. So free available quantity is negligible and only food source can be utilized when required[14] to prevent conditions like bone loss, gastric ulcers[15] and night blindness[16]. The present study was carried to determine the valuable effects of zinc on high salt diet induced morphometric changes in femur of female rats.

MATERIAL AND METHODS

The study was conducted at Islamic International Medical College, Anatomy department by the approval of Ethical Review Committee. National Institute of Health (NIH) and Army Medical College contributed in the accomplishment of this research. Forty five three month's old female Sprague Dawley rats, weighing 250-300 grams were assembled in to three groups by using random number table method, selected by non-probability convenient sampling (15 animals in each group). They were acclimatized in new situation having temperature range of 20-26°C. The desired temperature was maintained by using electric blower. Inclusion criteria were forty five, 12 weeks old, adult female Sprague Dawley rats weighing 250-300g. Pregnancy, male rats and any evident pathology were also considered as exclusion factors. Group C received standard laboratory diet and served as controls. Diet having 8% NaCl[12] was given to group A for eight weeks. Rats in group B were given zinc at a dose of 50mg/kg body weight[13] along with high salt diet. Water was provided ad libitum.

If 100 grams of diet contain 8 grams of NaCl then 18000 grams of diet will contain NaCl=1440 grams (18000 x 8/100).

If the dose of Zinc is 50 mg/Kg body weight then for 4.5 Kg, the estimated dose of Zinc =50x4.5= 225mg/4.5 Kg.

Table-II: Naito's score for the histological grading of collagen staining.

Grade	Staining
0	Normal staining
1	Slight reduction
2	Moderate reduction
3	Severe reduction
4	No dye no

Approximate dose of Zinc per rat per day= 225mg/15 rats = 15mg.

The dose of Zinc for 60 days=225x60=13500 mg = 13.5 grams.

Animals were sacrificed after eight weeks. The left femora were removed and fixed in 10% neutral buffered formaldehyde for 2 days. Decalcification was performed using aqueous

solution of 10% nitric acid for 24-48 hours. Transverse sections from the midshaft and longitudinal sections from proximal femur just below the greater trochanter were obtained, processed and embedded in paraffin wax to form blocks. Sections having thickness of 5µm were obtained by mounting blocks on rotary microtome. Haematoxylin and eosin (H&E) was used for routine histological study and Masson's Trichrome was employed for the demonstration of collagen fibers.

The architecture of trabecular bone was observed per unit area in four different fields per slide and then averaged.

Grading of trabecular bone was done according to the scoring criteria of Bitto et al[17] It was observed under H & E stain (table-I).

Intensity of collagen fiber staining was graded according to the scoring criteria of Naito's[18] (table-II). The slides were stained with Masson's Trichrome.

Diameter of medullary cavity of left femora was taken by using linear micrometer. Scale of the eyepiece was overlaid on the medullary cavity. Number of division of the eye piece from one side endosteum to opposite endosteum was counted and then averaged. This procedure was performed in perpendicular and transverse direction.

Table-III: Trabecular architecture and collagen staining scores in femur of all groups.

	Femur								
	Trabecular Architecture				Collagen Staining				
Groups	Grade 0	Grade 1	Grade 2	Grade 3	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Group C	10	0	0	0	10	0	0	0	0
Group A	1	3	6	0	1	2	5	2	0
Group B	2	7	1	0	1	6	2	1	0

Table-IV: Mean score, standard deviation and comparison of medullary cavity diameter in femur of all groups.

	Femur			
Groups	**C**	**A**	**B**	***p* value**
Mean score	378.400	426.333	394.267	0.002*
Std. deviation	44.969	37.238	17.919	
SEM	11.611	9.614	4.626	
Post-Hoc comparison	C vs. A	C vs. B	A vs. B	
Mean difference	-47.933	-15.866	32.066	
p value	0.002*	0.441	0.043*	

Table-V: Naito's score for the Histological grading of collagen staining.

	Femur		
Groups	**C vs. A**	**C vs. B**	**A vs. B**
Mean difference	-1.467	-0.800	0.667
p value	0.000*	0.001*	0.004*

Data analysis were done by using Statistical Package for Social Sciences (SPSS) version 21. Quantitative data were expressed as Mean ± S.D. One Way Analysis of Variance (ANOVA) and Post hoc tukey test was applied for inter group comparison. T-test was applied for intra group comparison of values. Pearson Chi Square test was employed for analysis of qualitative data.

A *p*-value <0.05 was considered statistically significant.

RESULTS

Total 45 spraguedawley rats were selected (15 in each group).

Scoring of Trabecular Bone Architecture

In group C; 100% animals had grade 0 trabecular bone architecture. When stained

sections of femur in group A were looked upon for intensity scoring, 10% had grade 0, 30% had grade 1, 60% had grade 2, none fell in grade 3. In experimental group B, 20% had grade 0, 70% had grade 1, 10% had grade 2 and none had grade 3 trabecular bone structure (table-IV).

The comparison of trabecular structure showed that it was more significant ($p<0.001$) in femur of group A and after administration of zinc, reversal of pathological changes were observed in group B ($p<0.05$). After Intergroup comparison, difference in scores between groups C and A and group A and B in both bones were highly significant ($p<0.001$) while significant results were observed between group A and B ($p<0.05$) (table-III). Histological sections of group A slides exhibited moderate to severe trabecular change whereas mild changes were observed in group B (fig-1).

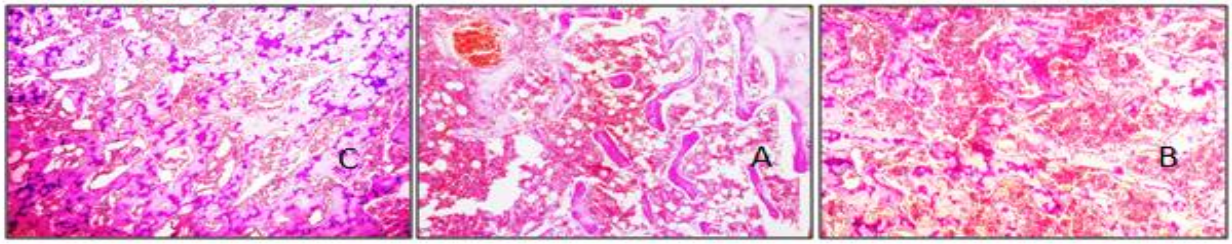

Figure-1: Longitudnal section of femur showing changes in trabecular bone in experimental groups C, A and B.

none had grade 4 staining. In experimental group B, 10% grade 0, 60. 60% had grade 1, 20% had grade 2, 10% had grade 3 and none had grade 4 staining intensity (table-IV).

The staining difference was significant between all the groups in femur ($p<0.001$). Mild alterations of staining in group B while moderate to severe changes in group A staining were noted. The intergroup comparison showed that collagen staining of femur was significantly reduced between groups C and A ($p<0.001$), also significant *p* value was observed between groups C and B ($p<0.05$) and insignificant results were observed between groups A and B (table-I). Histological sections of group A slides exhibited moderate to severe collagen intensity changes whereas mild changes were observed in group B (fig-2).

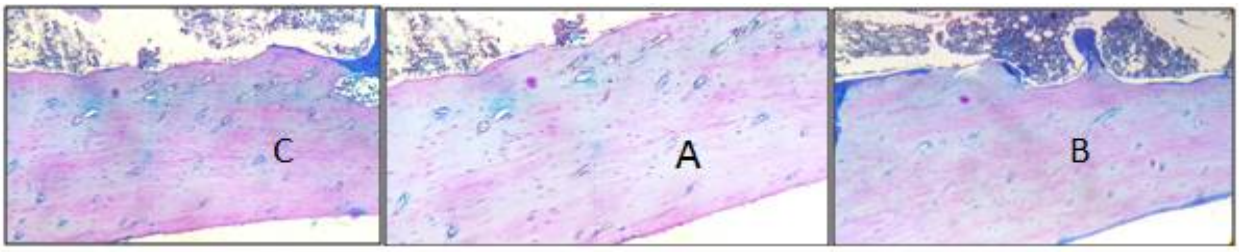

Figure-2: Longitudnal Section of Femur showing changes of collagen staining in experimental groups C, A and B.

Scoring of collagen fibers staining intensity

All (100%) animals had grade 0 (normal) collagen staining in femur of group C.

Histological analysis of femur in group A demonstrated that 10% had grade 0, 20% had grade 1, 50% had grade 2, 20% had grade 3 and

Diameter of Medullary Cavity

Diameter of the medullary cavity of left femur was measured with linear micrometer in all groups. Statistically significant dissimilarity of mean was observed between group C and A and between group A and B ($p< 0.05$) whereas the

distinction between group C and B was insignificant (p=0.078).

Medullary cavity diameter was greatest in group A followed by group B and group C had least diameter. Medullary cavity diameter of femur was 378.400 ± 44.969 μm in control group C, 394.267 ± 17.919 μm in experimental group B and greatest of all 426, 333 ± 37.238 μm in experimental group A (table-V).

DISCUSSION

Different histological parameters were examined in the present study to evaluate the bone damage. Decrease in trabecular architecture has been shown in many models of experimentally induced osteoporosis. When trabecular bone was graded for apparent change in the present research, it was observed that group A had widening of intertrabecular spaces with reduction in trabecular thickness. In salt fed diet, only 13.13% had grade 0 (normal) in femur whereas 60% in femur had grade 2. Adverse effects of high salt on trabecular structure has been observed by Ahmed and Samad[19]. They observed that salt loaded group revealed apparent thinning of the bone trabeculae and widening of bone marrow spaces in between them.

The advantageous effects of zinc on trabecular bone has been seen by Hadley et al[20] in the form of incremental alterations in trabecularization of rats. Furthermore, Maki et al[14] second this finding by observing decrease in trabecular density in rats fed on low zinc diet. He concluded that reduction in phosphatase activity is likely to cause insufficient osseous architecture. Structural changes in the trabecular bone has been presented by Bortolin et al[21]. He added that the positive role of zinc can be due to increased expression of osteocalcin and alkaline phosphatase activity. These results are in agreement with present research in which salt loaded rats have deterioration in trabecular architecture and ameliorative effect by zinc enriched diet is evident by significant p-value <0.05 in group B.

Osteoblast differentiation is considered an essential prerequisite for collagen production. For collagen fiber evaluation, Masson's Trichrome stained sections were studied according to the pre-set criteria of Naito's. The mean score of collagen staining in femur of group A had score of 1.73 and that of group B had 1.33. This can be due to apparently less newly formed osteoid by the osteoblasts in group A. After administering zinc to group B, the staining of collagen has been improved.

The decrease in collagen concentration in the present research has been validated by Mustafa[22] who witnessed changes after inducing osteoporosis with cadmium chloride. Results displayed decrease collagen content as obvious by reduced staining of slides along with alteration in staining pattern in some areas. This can be explained on the basis that decreased number of osteoblasts with degenerative changes and chromatin condensation in nuclei resulted in decreased deposition of extracellular matrix and less collagen content.

Irregular deposition of collagen fibrils could be attributed to disruption of collagen cross-links hence preventing the orderly arrangement of the extracellular matrix. The abnormal deposition of collagen in the extracellular matrix may lead to abnormal cell-matrix interactions[23]. Osteoclasts synthesize MMP-9 to degrade collagen and their activity is greatly increased during bone resorption. Glycosylation of type I collagen due to hyperglycemia could be the most probable cause[24].

In line with previous observations, the decrease in collagen concentration may be due to negative influence of salt on osteoblast activity[21] as excessive salt intake can inhibit the expression of osteogenesis genes which are required for osteoblast differentiation.

Alkaline phosphatase and production of collagen are extensively recognized indicators for osteoblast function[25] and their concentration increases with zinc supplementation. The administration of zinc increased the collagen

content mediated through activation of alkaline phosphatase and subsequent stimulation of collagen synthesis by osteoblasts. Present study is in conformity with above findings as the mean score of staining intensity showed an increase in group B who received zinc as a supplement in their diets.

Earliest sign of any bone resorption is indirectly by the expansion of medullary cavity on the endocortical side since the cortical bone loss is slow to develop. The measurement of medullary cavity diameter was done to evaluate the degree of bone damage. It was observed in the present research that salt increased the diameter while zinc supplementation exerted positive influence. Research of Omara et al[26] on dexamethasone induced osteoporosis in rats showed widening of medullary cavity. Dexamethasone caused marked deformity in the bone tissue in the form of increased endosteal bone resorption with widening of medullary cavity and haversian canals. Both the outer and inner surfaces of bone showed irregularities having multinucleated cells. He stated that inhibition of intestinal and renal absorption of calcium might have resulted in increased bone resorption due to secondary hyperparathyroidism. Increase bone resorption can be the reason of significant bone loss and subsequent enlargement of the medullary cavity[6].

Supplement intake with zinc has stimulatory effects on osteoblastic bone formation and suppressive effects on osteoclastic bone resorption through zinc-finger transcription factor Osterix (OSX). It is present in osteoblastic cells and required for their differentiation. The exact mechanism is poorly understood and further research is required[13]. Stimulatory effect on transforming growth factor (TGF-β) production in maintaining bone homeostasis and by keeping balance between bone forming and bone resorbing cells could be the probable reason of positive reversal of medullary cavity diameter in this research[21].

Reported by Bortolin et al[24] osteoclasts can synthesize matrix metalloproteinase-9 (MMP-9) to degrade bone matrix components and zinc, by reducing the expression of MMP-9 can prevent bone loss. Positive correlation is being established between zinc and bone metabolism by observing its shielding effect against salt induced damage. In nut-shell, zinc is a useful dietary strategy for the prevention and treatment of osteoporosis.

CONCLUSION

High salt diet exerted harmful effects on bones due to increased sodium chloride induced hypercalciuria leading to bone loss. Zinc is valuable in ameliorating the detrimental effects of salt on bones by enhancing osteoblast activity and inhibiting bone resorbing cells.

RECOMMENDATION

Effects of high salt diet can be studied for longer period of time to assess significant gross changes in long bones of rats. Effects of highs salt and zinc can be observed on the osteocytes apoptosis to evaluate their role in development and prevention of osteoporosis. Comparison of high salt diet induced effects can be studied between male and female rats to assess the difference in the degree of damage.

CONFLICT OF INTEREST

This study has no conflict of interest to declare by any author.

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