ORIGINAL ARTICLE

Effect of Zinc on Salt Induced Impaired Remodeling in Long Bones of Rats

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ABSTRACT

Objective: To determine the effect of zinc on salt induced bone damage in rats.

Study Design: Laboratory based randomized control trial.

Place and Duration of Study: The Anatomy department of Islamic International Medical College, Rawalpindi, hosted the conduction of research with the cooperation of National Institute of Health, Islamabad. The study commenced on 17th September 2015 and completed on 17th March 2016.

Materials and Methods: Forty five female Sprague Dawley rats, 10-12 weeks old were used in the study. The animals were randomly divided into 3 groups. The rats in experimental group A fed on high salt diet (8%NaCl) whereas animals in experimental group B were given high salt diet supplemented with zinc (50mg/kg/day) for eight weeks however, the diet of control group was not tempered with. Blood samples were drawn at the start of intervention through tail vein and at the end of the experiment by intracardiac puncture for hormonal assay. All rats were dissected, left humeri and femora were removed, decalcified and five micrometer (µm) sections were obtained after tissue processing. Tissues were stained with Haematoxylin and eosin (H&E) for histological parameters. The quantitative data was analyzed by using Statistical Package for Social Sciences (SPSS) version 21 and was expressed as Mean + S.D.

One Way Analysis of Variance (ANOVA) followed by Post hoc tukey test was applied for inter group comparison of parameters. T-test was applied for intragroup comparison of values. Result having p-value <0.05 was considered statistically significant.

Results: Marked histological changes were identified in the experimental groups. These changes were of greater severity in high salt diet group as compared to the zinc supplemented group in which reverse beneficial effects were observed. Fall in serum calcium and alkaline phosphatase levels were deemed substantial in group A with respect to group B.

Conclusion: Zinc has a Protective role against High salt exposed diet induced damage on the histomorphology of bone tissue.

Key Words: *Cortical Bone, Hypercalciuria, Osteoblast, Salt, Zinc.*

Introduction

The world is under continuous threat of increase diet-related non-communicable ailments.[1] Unbalanced and excessive salt intake is often closely associated with development of hypertension and other cardiovascular diseases.[2] However, awareness regarding relationship of zinc to sodium induced osteoporosis is still in a gray area. Despite of previous researches, precise associations of the trace elements with bone health are not clear as yet. Inverse of negative balance between bone formation and resorption has been evaluated with the help of trace elements.[3]

Low bone mass is a silent epidemic of the 21st century and figures are set to increase worldwide. Considering the elements which affect bone metabolism is of utmost importance for the prevention of osteoporosis. Although nutrition is an important determinant of bone health, but the effects of the micronutrients is little understood.[4]

Bone is a systematized tissue which acclimates and changes according to certain factors and its organization varies due to diverse functional requirements.[5] The net result of unaltered healthy bone mass is sustained by a balanced bone formation and resorption activity.[6] Imbalance results in a progressive metabolic ailment called osteoporosis,[7] becoming a public health problem,[8]

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upsetting 200 million people worldwide.[9] Characterized by lessened structural integrity and proneness to fractures, it is more prevailing than myocardial infarct, breast cancer and stroke[10] It is imperative to explore and develop nutritional strategies for osteoporosis prevention as the life threatening outcomes and increase in annual cost associated with disease morbidity requires a quick fix.[11]

Salt being most ubiquitous of food flavorings[12] and a known risk factor for osteoporosis,[13] imposes hazards on human wellbeing. High urinary excretion of calcium with increase salt intake leads to impaired bone health.[14]

Human population has exceeded the daily limit of 2000 mg of Na /day as recommended by WHO.[15] Different communities have different intakes (Western 2300-4300 mg Na/day , Asian 5300mg-6000mg of Na/ day)[16] Sodium in this range is adversely affecting people including osteoporosis, hypertension , increase urinary tract stones and stroke.[17]

It took 75 years to realize that zinc is a crucial trace element[18] although it has been used therapeutically in Ayurveda but its nutritional significance in public health was recognized recently.[19] As it is a vital element[20] and human body contains only 2-3 grams. even a small deficiency is a disaster.[21] Zinc can be a hidden link for the prevention of osteoporosis due to its regulatory role in bone metabolism.[22] It has the ability to stimulate the differentiation and proliferation of osteoblasts and inhibiting osteoclast like cells formation from bone marrow[22] Zinc ,by stimulating apoptotic cell death of mature osteoclasts can inhibit bone resorption and have direct positive effect on bone metabolism.[23] Other than bones which act as a zinc sink zinc is stored in muscles and skin.[24] So free available quantity is negligible and only food source can be utilized when required[25] to prevent conditions like bone loss, gastric ulcers[26] night blindness.[27]

Therefore, this experimental study will highlight the potential benefits of Zn supplementation in reducing bone loss more accurately and eventually will give desired awareness to masses regarding positive link between zinc and bone health.

Materials and Methods

The study was a laboratory based randomized control trial carried out in the Anatomy department of Islamic International Medical College Rawalpindi. It was initiated after the approval of the Ethical Review Committee. The research was carried out with the collaboration of National Institute of Health (NIH) Islamabad and Army Medical College. It took six months to complete this study. 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.

Forty five rats grouped by using random number table method, selected by non-probability convenient sampling, were randomly divided in to three groups (15 animals in each group) and were allowed to adjust in well aired new environment in a temperature range of 20-26°C. The rats in group A (N=15) were given diet having 8% NaCl[28] for eight weeks. Rats in group B (N=15) were given high salt diet supplemented with zinc at a dose of 50mg/kg body weight.[29] The rats of group C (N=15) served as controls, they were given standard laboratory diet. Water was provided ad libitum. The dose of NaCl and Zinc was set based on previous studies.

Dissection was done after eight weeks. Blood was drawn through intracardiac puncture for assessing serum calcium and alkaline phosphatase (ALP) level at the end of intervention. The left humeri and femora of rats were removed and immediately fixed in 10% neutral buffered formaldehyde for 2 days. Decalcification was performed using aqueous solution of 5-10% nitric acid for 24-48 hours. Transverse sections from the mid diaphysis were obtained, processed and embedded in paraffin wax to form blocks. Five µm[30] thick sections were obtained by mounting blocks on rotary microtome. Haematoxylin and eosin was used for histological study of specimen.

Cortical bone thickness of diaphysis of humeri and femora was measured with the help of ocular micrometer. The thickness of cortical bone was measured by counting the number of divisions of eye piece of linear ocular micrometer, placed perpendicularly from underneath the periosteum to endosteum. Cortical bone width of opposite side was measured in a same manner per section under 4X objective and results were averaged.

Parametric data was analyzed by using Statistical

Package for Social Sciences (SPSS) version 21. Quantitative data was expressed as Mean + S.D. One Way Analysis of Variance (ANOVA) followed by Post hoc tukey test was applied for inter group comparison of parameters.t-test was applied for intra group comparison of values. Result having p-value <0.05 was considered statistically significant.

Results

Mean thickness of the humeral cortical bone was 53.766±9.066 µm in control group C, 53.666±7.596µm in experimental group B and lowest of all, 41.8000±15.254 µm in experimental group A. The results were significant (p<0.05) amongst different groups (Table I) (Fig 2, 3).

The difference between group C and A was 11.966 µm, being highly significant (p=0.014).The result between group C and B was insignificant (p=1.000) with difference of 0.100µm. The mean cortical thickness of group B was greater than group A with difference of -11.866µm (p<0.05) (Table II) (Fig 1).

Mean thickness of the femoral cortical bone was 44.600±8.437µm in control group C, 39.366±10.677µm in experimental group B and lowest of all, 30.433±9.350µm in experimental group A. The results of difference in cortical bone thickness were significant between groups (p<0.05).

The difference between group C and A was 14.166 µm, the result was highly significant (p=0.001).The insignificant difference of 5.233µm (p=0.300) was recorded between group C and B. The mean of thickness was greater in group B than group A difference being -8.933µm (p=0.036).

Mean random initial and final serum calcium was 8.680±0.90333 mg/dl and 8.5333±0.9559mg/dl in control group C, 7.826±0.6123 mg/dl and 7.153±1.364mg/dl in experimental group A and 8.666±0.952 mg/dl and 8.816±0.9635mg/dl in experimental group B Initial calcium levels revealed p-value of 0.010 whereas the final levels were different in all groups (p=0.004).The mean difference between initial and final value in Control group C was 1.3800, 0.3466 in experimental group A and -1.0333 in experimental group B. Decrease in calcium level was highly significant between experimental group C and A

(p=0.004), insignificant (p=0.672) between group C and B and there is significant result between group A and group B (p=0.038) (Table IV).

Initial and final mean serum alkaline phosphatase level was 487.800±51.669 U/L and 478.066±53.620 U/L in control group C, 466.200±45.874U/L and 349.9333±56.0484 U/L in experimental group A and 486.066±47.373 U/L and 416.666±62.009 U/L in experimental group B (Table III) (Fig 5).

Initial Serum alkaline phosphatase showed inconsequential value in all the groups (p=0.405) whereas the final levels were significant (p=0.000) The mean of difference between initial and final value in Control group C was 9.7333 U/L,116.2666 U/L in experimental group A and 69.4000 U/L in experimental group B.

Comparison among groups demonstrated the highest decrease in alkaline phosphatase level between group C and A being 109.2000 U/L with significant value (p=0.000).The mean of decrease between group C and group B was 42.4666 U/L (p=0.021) which was less group A and B -66.7333 U/L (p=0.038

Table I: Multiple comparison of cortical bone thickness among all groups of Humerus and Femur by Post Hoc Tukey test

	Humerus			Femur		
Groups	C	A	B	C	A	B
Mean value	53.766	41.800	53.666	44.600	30.433	39.366
Std. Deviation	9.066	15.254	7.596	8.437	9.350	10.677
SEM	2.341	3.938	1.961	2.178	2.414	2.757
p-value	0.006*			0.001*		

Table II: Mean Cortical Bone thickness in Humerus and femur (µm) of all groups

	Humerus			Femur		
Groups	C vs. A	C vs. B	A vs. B	C vs. A	C vs. B	A vs. B
Mean Difference	11.966	0.100	-11.866	14.166	5.233	-8.933
p-value	0.014*	1.000	0.015*	0.001*	0.300	0.036*

*p<0.05

Discussion

Bone acclimates and changes under the influence of certain elements and its organization varies due to diverse functional requirements.[5] The healthy bone mass is sustained by a balanced between bone

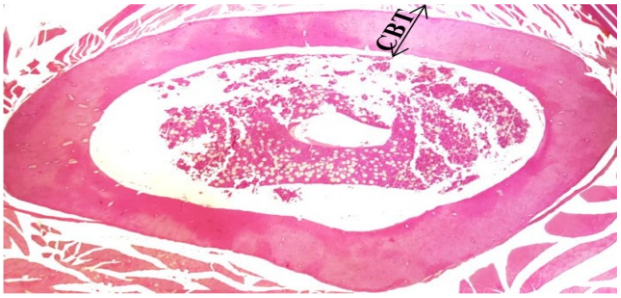

Fig 1 : Cross-section of Humerus diaphysis of A13 showing decreased cortical bone thickness (CBT). H&E, X4.

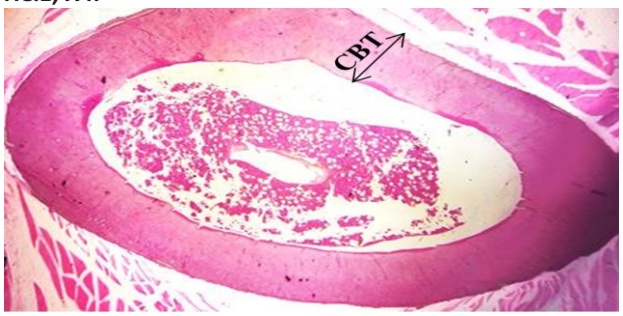

Fig 2: Cross-section of Humerus diaphysis of B7 showing increased cortical bone thickness (CBT). H&E, X4.

Table III: Initial-final serum calcium (mg/dl) and Alkaline Phosphatase (U/L) level of all groups

Parameter	Group	Initial level	Final level	Std. Deviation	*p*-value
	Group A	7.8267± .61233	7.1533± 1.3642	0.9346	.000*
	Group B	8.6667± .95219	8.1867± .96353	1.6249	.l02
	Group C	8.680± 0.903	8.533± 0.9553	0.2587	0.000*
	p-value	0.010*	0.004*	-	-
	Group A	466.20000± 45.8743	349.93333± 56.04847	74.5419	.833
	Group B	486.0667± 47.3730	416.6667± 62.0099	58.11190	.083
	Group C	487.800± 51.6695	478.0667± 53.6209	22.6604	0.000*
	p-value	0.405	0.000*	-	-

*p < 0.05

Table IV: Multiple comparison of final calcium (mg/dl) and Alkaline Phosphatase (U/L) level

Parameter	Groups	Mean Difference	*p*-value
Serum Calcium (mg/dl)	Group C vs. Group A	1.3800	.004*
	Group C vs. Group B	0.3466	.672
	Group A vs. Group B	-1.0333	.038*
Serum Alkaline phosphatase (U/L)	Group C vs. Group A	109.20000	.000*
	Group C vs. Group B	42.46667	0.021*
	Group A vs. Group B	-66.73333	.038*

*p < 0.05

formation and resorption activity.[6] Life style, genetic and dietary factors have impact on its prevalence. Although dietary factors have limited influence but are nonetheless crucial because they modulate the achievement of maximum peak bone mass and subsequent better bone health. By developing nutritional strategies for osteoporosis prevention, the annual cost and debilitation associated with its morbidity can be lessened.

The present study focused on determining the beneficial effects of zinc on high salt diet induced bone damage in long bones of rats by observing microscopic quantitative and biochemical parameters. The results suggested that zinc supplementation can prevent the high salt induced deleterious effects on bones.

ALP is a marker enzyme of osteoblast activity[31] reevaluated by Ahmed[32] who documented the decrease in calcium, ALP levels and subsequent impaired bone integrity after salt loaded diet. Decrease in the osteoblast activity due to salt overload can be the reason of low ALP levels. Furthermore, decline in the ALP activity has been demonstrated in animal models of experimental induced osteoporosis.[33] As > 99% of Na and 95% of the calcium are reabsorbed in the kidneys, it is speculated that impaired renal function may be responsible for Na induced calciuria and temporarily depress calcium levels.[14] Substandard kidney function also causes hypophosphatemia and fall in 1, 25 (OH) 2 D3. All these events lead to less intestinal absorption of calcium as well as decrease availability to bones.[16] Reduction in the biomarkers of bone formation (ALP) and significant increase in the biomarkers of bone resorption has been observed due to high PTH secretion secondary to low calcium levels and consequently increase in bone remodeling.[16] In line with other publications, Creedon[34] also observed the decrease in calcium levels due to sodium induced increase urinary excretion of calcium. As a compensatory mechanism, the PTH secretion increases which causes calcium mobilization from bones at the expense of bone loss.[15]

As Zn is a cofactor of ALP[22] which is an enzyme expressed by osteoblasts close to the blood vessels and is a valuable index for bone tissue development. Administration of zinc results in increase of enzyme

activity indicating enhance osteoblastic activity.[35] Increase in levels of Calcium and ALP with significant difference ($p<0.05$) in the present study is also validated by Otsuka[36] who observed increase in levels after measured zinc discharge on bone mineral density from injectable Zn-containing B-Tricalcium Phosphate. It could be attributed to intensified differentiation of osteoblastic cells to raise ALP activity.[37] Zinc plays an important role in preventing osteoporosis by stimulating bone formation, reported by Ma[31] by demonstrating increase in calcium and ALP in the femoral-diaphyseal and metaphyseal tissues. Decrease in calcium content by bone resorbing factors can be prevented by zinc supplementation.[19] Our outcome is in agreement with above results, further firming up my research.

Cross section of long bones reveals four different bone types: periosteum, cortical bone, endosteum and cancellous bone. Femur diaphysis is mainly composed of compact bone[38] and cancellous bone forms a very thin layer on the inner aspect of diaphysis of long bones.[5] The cortical bone thickness is an important parameter to evaluate bone quality and strength[39] so in the present study the bone damage is assessed by measuring the cortical bone thickness in cross sections of mid diaphysis. It is revealed that humerus and femur of control group has maximum thickness of 50.7um and 44.6um respectively followed by experimental group B who took salt and zinc supplementation whereas the lowest dimensions are found in experimental group A fed on high salt diet.

My results are in harmony with the work of Ahmed[32] who observed decline in the thickness of cortical bone of rats. He anticipated that high salt intake can be related with increased plasma levels of creatinine, urea, phosphate and potassium due to deranged kidney function which finally led to bone changes. Furthermore increased serum phosphate inhibits 1αhydroxylase and produced fall in 1, 25(OH) 2 D3. As a result intestinal absorption of calcium is decreased with subsequent increase in PTH secretion leading to increase osteoclastic activity. Degenerative changes in osteoblasts, osteocytes and hyperactivity of osteoclasts results in inaccurate bone remodeling with decrease in cortical bone thickness.[40] Changes in bone remodeling which is mediated by bone cells, increased osteoclastic activity and multiple resorption cavities can be the reason of decrease in the thickness of cortical bone.[41] My result is in conformity with the results of all above periodicals sharing a common point that salt intake results in osteoporosis with decrease in cortical bone thickness.

Increase in cortical bone thickness after zinc supplementation in experimental group B is documented in the present study. As many published studies has confirmed that zinc has positive role in improving bone health , it is further strengthened by Brzoska[22] who reported the shielding effect of zinc diet on bone homeostasis. He postulated that increase in the bone alkaline phosphatase activity may be due to zinc adequacy. Increase in the osteocalcin level produced by osteoblasts after zinc supplemented diet might have resulted in increase in cortical bone thickness.[42] Zinc is required for growth of osteoblasts and zinc showed decreased was bone resorption.[43]

Conclusion

This research indicates that zinc supplementation can be considered an appropriate dietary strategy to reduce risk of osteoporosis. Cortical bone thickness, alkaline phosphatase activity and calcium levels were considerably increased after zinc administration showing that zinc has protective role against high salt induced impaired remodeling in long bones of rats.

Recommendations

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.

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