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PROTECTIVE EFFECT OF ZINC;

AGAINST SALT INDUCED MACROSCOPIC CHANGES IN HUMERUS AND FEMUR OF RATS

Dr. Kaukab Anjum[1], Dr Asma Ali[2], Dr. Uzma Shahid[3]

1. M.B.B.S, M.Phil.
   Assistant Professor, Anatomy.
   Wah Medical College, Wah Cantt.
2. Demonstrator, Anatomy
   Islamic International Medical College.
3. Associate Professor, Anatomy
   Wah Medical College, Wah Cantt.

Correspondence Address:
Dr. Kaukab Anjum
Assistant Professor, Anatomy
Wah Medical College, Wah Cantt.
kanjumq@gmail.com

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ABSTRACT… Background: Nutrition is an important determinant of bone health**.** Micronutrients, other than calcium has been paid less attention to-date in the prevention and treatment of bone diseases. **Objective:** To evaluate the protective effect of zinc on high salt induced gross changes in humerus and femur of rats. **Study design:** Analytical control randomized trial. **Place and duration of study:** Islamic International Medical College, Rawalpindi, hosted the research with the cooperation of National Institute of Health; Islamabad. The study was approved by Ethical review committee of Riphah international university before its initiation. It took six months to complete the research (Sep 2015-March 2016). **Material and methods:** Forty five adult female Sprague Dawley, 10-12 weeks old rats were used in the study. Three groups were made, each having fifteen rats. Control group C (N=15) received laboratory diet without any alteration. Experimental group A (N=15) were served with high salt diet (8%NaCl) whereas experimental group B (N=15) animals were given high salt diet augmented with zinc (50mg/kg/day).All groups were given the diet for eight weeks. Animals were weighed at the start and end of study after which they were sacrificed. Left humeri and femora of all rats were obtained. Weight and mid shaft diameters of bones were recorded. The results were compiled after comparison amongst all the groups. **Results:** Marked gross changes were witnessed in 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 noticed. After zinc administration, there was substantial increase in the weight of animals and bones with concurrent increase in mid shaft diameters. **Conclusion:** Zinc has a Protective role against high salt induced damage on the gross parameters of bones.

Key words: Gross parameters, mid shaft diameters, Salt, Zinc.

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INTRODUCTON

The world is under continuous threat of increase diet-related non-communicable ailments. 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]

Bone metabolism is affected by endocrine, mechanical, and nutritional factors with extensive interactions between them. Imbalance results in a progressive metabolic and degenerative disease of the bones characterized by micro architectural defects, bone mass reduction and decrease resistance to mechanical injuries.[4] Osteoporosis is a silent epidemic of the 21st century,[5] Being most frequent ailment of human beings, eventually leads to more vulnerability to fractures. Over 2000 million people,[6] one in every tenth person in the world is its victim.[7,8] It reflects a disparity between bone formation and bone resorption hence increasing skeletal turnover and bone fragility.[9,10] Osteoblasts, mesenchymal in origin, flourish and discriminate prior to bone formation and are present on the forming surfaces of growing or remodeling bone. They are accountable for the production and mineralization

of the matrix. Multinucleated osteoclasts are derived from circulating precursors of the monocyte-macrophage cell line[11] and are the only bone resorbing cells which require RANKL and Macrophage colony stimulating factor for their differentiation.[12] Healthy bone depends on the balanced activities of bone cells. Shift of balance results in cluster of abnormalities in which bones have low mass and altered microstructure leading to increase fracture risk.[13]

One of the most significant variable factor in the development and maintenance of bone mass is nutrition which in balance form can play a major role in the prevention of osteoporosis.[14]

Salt being oldest and most ubiquitous of food flavorings, within recommended levels, is necessary for lives of organisms. International recommendations suggest that average population intake should be less than 5-6 g whereas most adult populations have exceeded the nutritional recommendations of salt intake average being 6-12g.[2,15]

Increase salt in diet can disrupt the equilibrium of formation and resorption of bone ultimately resulting in increased excretion of sodium in urine along with calcium which in turn stimulates bone resorption activities.[16,17] NaCl intake is one factor that influences calcium requirements, bone resorption, and the pathogenesis of osteoporosis.[18] Increase sodium intake is a risk factor for osteoporosis.[19,20] due to change in bone mineral density and detrimental effect on calcium homeostasis.[21,22]

Zinc is a component of more than 200 enzymes[14,23] and 23 most abundant element in the earth's crust with enzymatic function reduces osteoclast resorption activities and increase markers of osteoblast differentiation, matrix maturation and mineralization.[24] Zinc may increase bone formation through stimulating cell proliferation, alkaline phosphatase activity and collagen synthesis.[25] Zinc inhibits bone loss by bone protein synthesis, and exerting beneficial effect on IGF-I and TGF-β1 production in the bone tissues.[26,27]

Osteoclasts are exquisitely sensitive to zinc which is a highly effective inhibitor of bone resorption.[28] It has been shown that zinc can increase the production of osteocalcin[29] and stimulate the proliferation and function of osteoblastic cells in bone tissues.[30] Therefore, zinc can influence the skeletal growth by stimulation of osteogenesis, escorted by a parallel inhibition of osteoclastogenesis.[31] It can also induce matrix formation, mineralization in bone[32] and increase in osteoid area by augmenting collagen production.[25] Besides its important role in immune system functioning, zinc has a protective role in diarrhea, wound healing, maintaining sense of taste and smell, insulin storage, and dark vision adaptation.[33]

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, Riphah University. 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[34] 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.[35]

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.

Calculation of salt dose

Dose of NaCl is 8% i.e. 8 grams of NaCl per 100 grams of diet.
Diet intake of one rat is 15-20 grams per day.
Diet of 15 rats = 20x15 = 300 grams per day.
Diet of 15 rats for 60 days = 18000 grams.

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

Calculation of zinc dose

Dose of Zn is 50mg per kilogram per day.
Weight of one rat is approximately 300 grams.
Weight of 15 rats =15x300 =4500 grams =4.5 Kg.

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.

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 weighed before the dietary intervention and before sacrifice. All rats were dissected after eight weeks. The left femora and humeri were removed and cleaned off the soft tissue.

Mid shaft diameter of humerus and femur

The mid shaft diameters[36-38] of all left humeri and left femora were recorded with the help of digital vernier caliper.[6,39]

Weight of humerus and femur

Left humeri and left femora were weighed by digital weighing scale.[39] By using the following formula, relative tissue body weight index (RTBWI)[21] was calculated:
RTBWI= weight of organ (g)/ body weight (g) x100.

Statistical Package for Social Sciences (SPSS) version21 was used for analysis of quantitative data and was expressed as Mean + S.D. Intra-group comparison was done with t-test. One Way Analysis of Variance (ANOVA) and Post hoc tukey test was applied for inter group comparison. p-value <0.05 was considered statistically significant.

RESULTS

Body weight of animals

In control group C, mean body weight at the start and end of the study was 249.466 ±7.3762g and 303.000 ±7.8923g respectively. Mean body weight of animals in group A were 248.8000 ±5.1713g and 270.8667 ±6.3904g whereas the mean body weight of animals at beginning and after completion of specified time in group B was 250.866 ±5.7179g and 295.7333 ±10.2083g (Table-I). Initial weights showed insignificant values whereas significant values were noted in the final weights of groups (p<0.001). The weight gain was highest in group C (p=0.000) followed by group B (p=0.950) and least increase in weight was observed in group A (p=0.004).

Intergroup comparison revealed noteworthy alteration between the final body weight of group A and C (p=0.000). Insignificant result was found (p=0.054) between C and B which demonstrates that the animals in group B had gained nearly equivalent weight as control group. Variation between the weights of group A and B was significant (p=0.000) showing protective effect of zinc against bone damage (Table-II).

Weight of humerus and femur

The mean humerus weight was highest in group C (0.3013±0.0266g). In group A, it was 0.2560±0.0264g and in group B, the mean weight of 0.2867±0.0271g was observed. The mean femur weight was highest in group C, 0.5947±0.0650g. In group A, the mean was 0.5107±0.0365g and in group B the mean

recorded was 0.5580±0.0344g (Table-III).

Multiple comparison showed that weight of humerus and femur in group C was significantly different from that of group A (p=0.000). Weight of humerus and femur in group B remained almost the same as that of the control group C revealed by significant p value 0.0301 and 0.099 respectively. Significant p value showing protective effect of zinc was observed between group A and B in both bones having p□0.05 (Table-IV).

Mid shaft diameter of humerus and femur

The mid shaft diameter of humerus was 2.4127±0.1851mm in group C being highest. In group A, diameter was 2.2613±0.1525mm and in group B the mean of mid shaft diameter was 2.3500±0.2045mm.

Femur of group C was found to have highest diameter of 3.2093±0.2221mm. In group A, the mean of mid shaft diameter was 3.0607±0.1720mm and in group B the mean was 3.1367±0.2108mm (Table-V).

The difference of mean in humerus and femur diameter between group C and A was 0.15mm and 0.14mm respectively. Difference of 0.06mm in humerus and 0.08mm in femur was observed between group C and B whereas between group A and B, the difference of -0.08 mm and –0.07mm in humerus and femur was observed respectively (Table-VI).

Although on gross evaluation the midshaft diameters amongst all groups showed variation but statistically the diameters in humerus and femur exhibit p-values of 0.085 and 0.146 showing insignificant results.

Group A showed decrease in gross parameters as compared to group C whereas group B had protective effect against salt induced damage, results being confirmed statistically.

Groups	Mean Initial Weight(g)	Mean Final Weight(g)	Std. Deviation	*p* value
Group C	249.466±7.3762	303.000±7.8923	3.9617	.000*
Group A	248.800±5.1713	270.8667±6.3904	4.6516	.004*
Group B	250.866±5.7179	295.7333±10.2083	11.7890	.950
p value	0.647	0.000*		

Table-I. Initial and final mean Body Weight (g) of all groups
***p* < 0.05**

Groups	Mean Difference (g)	p value
Group A vs. Group C	32.13333	.000*
Group B vs. Group C	7.26667	.054
Group A vs. Group B	-24.86667	.000*

Table-II. Showing multiple comparison of mean body weight gain (g) by post hoc tukey test.
***p < 0.05**

	Humerus			Femur		
Groups	**C**	**A**	**B**	**C**	**A**	**B**
Mean(g)	0.3013	0.2560	0.2867	0.5947	0.5107	0.5580
Std. Deviation	0.0266	0.0264	0.0271	0.0650	0.3654	0.3448
SEM	0.0068	0.0068	0.0070	0.1679	0.0094	0.0089
p value	0.000*			0.000*		

Table-III. Mean weight (g) of humerus and femur of all groups.
***p < 0.05**

	Humerus			Femur		
Groups	C vs. A	C vs. B	A vs. B	C vs. A	C vs. B	A vs. B
Mean Difference	0.0453	0.01467	-0.0306	0.08400	0.03667	-0.0473
p-value	0.000*	0.030*	0.009*	0.000*	0.099	0.024*

Table-IV. Showing multiple comparison of mean weight (g) of humerus and femur.
***p < 0.05**

	Humerus			Femur		
Groups	C	A	B	C	A	B
Mean(mm)	2.4127	2.2613	2.3500	3.2093	3.0607	3.1367
Std. Deviation	0.1851	0.1525	0.2045	0.2221	0.1720	0.2108
SEM	0.0478	0.3940	0.5262	0.5737	0.0444	0.05444
p value	0.085			0.146		

Table-V. Showing mean midshaft diameter of humerus and femur (mm) in all groups
***p < 0.05**

	Humerus			Femur		
Groups	C vs. A	C vs. B	A vs. B	C vs. A	C vs. B	A vs. B
Mean Difference (mm)	0.15	0.06	-0.08	0.14	0.08	-0.07
p value	0.085			0.146		

Table-VI. Mean difference of midshaft diameter (mm) of humerus and femur in all groups.
***p < 0.05**

DISCUSSION

Bone acclimates and changes under the influence of certain elements and its organization varies due to diverse functional requirements.[40] The healthy bone mass is sustained by a balanced between bone formation and resorption activity.[41] 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.

Several studies on animals have highlighted that high salt diet decreases weight gain.[21,34,35] Although, increase in the total body weight (RTBW) of all the groups during eight weeks was observed, rats in group C had highest weight gain of 54g followed by weight gain of 37g in experimental group B. Increase in weight was observed in both experimental groups but the least weight gain of 22g was observed in group A fed on high salt diet. Our result was in accordance with previous work done by Adeniyi[35] who reported significantly less weight gain of 49.8g in salt group as compared to control group. He inferred that rats in salt loaded group excreted more urine and consumed more water than control group C and subsequent more weight loss at the expense of food intake. This observation is also supported by Gibbs who reported that supercedance of hunger center by the thirst center during salt loading[42] can lead to increase in water intake with resultant net weight loss.[43]

Same effects were seen by Yatabe[34] who demonstrated decrease in weight gain with significant result p□0.05 after feeding high salt diet (0.8% NaCl) to a group of rats for eight weeks. This could be due to increased calcium excretion with subsequent volume expansion and competition between Na and Ca ions in proximal

convoluted tubules. Furthermore, Creedon[21] while explaining effects of more salt ingestion observed less body weight gain in salt loaded group. This could be due to sequential bone resorption due to increased urinary calcium excretion and PTH secretion hence strengthening our research. This fact has been confirmed by other investigators as well who testified decreased weight gain in salt-treated rats.[21,44]

The factual increase in the weight of rats after zinc supplementation is provided by Ovesen research[45] who worked with different concentrations of zinc on animals. Animals fed on zinc concentrated diet (60mg/kg) had significantly higher weight gain than those who received standard dietary zinc having 47mg/kg. He proposed that the positive influence of zinc on periosteum and bone tissue area, mimicking growth hormone or insulin like growth factor effects, results in bone formation causing the increase in weight.[45] This observation has been seen by Adeniyi[35] and Cho[46] who witnessed increase in weight gain after zinc diet. As increase in the body weight is a parameter of bone formation[28] therefore it is concluded that increase in weight after zinc diet might be due to enhanced bone formation, hence our conclusion is being reinforced by the above researcher's findings.

In the present study certain gross parameters were also considered to assess the alteration in the bone organization. The weight of bones was recorded and 0.28% decrease in weight was observed in group A whereas group C had bone weight comprising 0.30% of the total body weight. There was reversal of adverse effects caused by high salt and was revealed by increase up to 0.29% of the total body weight in group B fed on zinc supplemented diet.

The difference in weight was significant between group C and A (p□ 0.000). This result correlates with the study of Saric[43] who reported that high salt diet slightly but not significantly decreased the weight of bones. He speculated that the observed results might be due to increase water intake and high urinary output in rats which led to dehydration and concurrently did not disturb the development of bones. However other researchers have recorded less bone weight and calcium content in salt treated rats.[21,44] and support my study. The divergence of my results from other similar animal studies may be due to the less duration of my study. This is inferred from the research of another scholar Chan[44] who studied the weight of bones during different time periods of research. He observed significantly lower bone weight after extended period of intervention whereas the rats that experienced short duration of research had no significant difference. Significant histological findings with less effect on femoral weight were observed by Martiniakova.[38]

When the weight of bones was compared per total body weight of rats, there was subtle variation. This finding is supported by work of Creedon[21] who found that although the total femoral mass was reduced in rats after experimental osteoporosis but relative to total body weight, it was trivial.

High salt induces detrimental effects on Ca homeostasis, increase bone turnover by increasing resorption and subsequent reducing bone mass.[20,21] The reason for disturbance in Ca homeostasis may be due to the fact that decrease serum calcium stimulates PTH secretion leading to increased bone resorption and decrease bone weight. Hence, it can be concluded from my and other studies that may be if the research is carried out for prolonged period, there could be more significant results regarding bone weight.

In present study, after administration of zinc to experimental group B, there was considerable increase in the weight of bones. Ma[30] observed the effect of zinc and vitamin k2 on bone components in rats. He was of the view that increased bone weight after zinc and vitamin K2 is due to bone forming effect of zinc although zinc alone is not responsible for increasing the bone. An increase in the rate of radius bone mineral density (BMD) has been demonstrated with high intake of zinc.[47]

One of the critical indicator of bone strength is external diameter and it predicts up to 55% of

the variation in bone strength.[48,49] The present study agrees with this statement as the midshaft diameters of humerus and femur decreased in both interventional groups however protective effect was obvious in rats fed on zinc invigorated diet. Although on gross evaluation there was decrease in the midshaft diameters of bones but statistically, results were not significant. This result is in agreement with previous work of Yong Zhong[39] in which no significant decrease in midshaft diameter was recorded after removal of ovaries to induce bone damage in rats. Increase in the length and cross sectional area of mid diaphysis of femur has been proved by Ovesen.[45] Further research is required to assess the diet induced noticeable variation in the midshaft diameters of bones after excessive salt diet.

The available literature shows that high sodium salt is a risk factor for osteoporosis by inducing hypercalciuria[50] characterized by decrease quality of bone and increased risk of fragility fracture.[51] The fact is based on the maintenance of balance activities between osteoclasts and osteoblasts which are the main target for explaining the cell-based mechanisms responsible for this metabolic disorder.[52]

CONCLUSION

This research indicates that zinc supplementation can be considered an appropriate dietary strategy to reduce risk of osteoporosis. It was observed in present study that zinc supplementation exerted protective effects on the gross parameters of the rat osseous tissue. High salt diet caused significant changes in the weight of animals and bones and their mid shaft diameters with decrease in total body weight, weight of bones and diameters whereas zinc supplementation resulted in considerable reversal of changes.

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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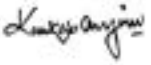
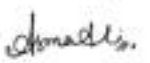
AUTHORSHIP AND CONTRIBUTION DECLARATION

Sr. #	Author-s Full Name	Contribution to the paper	Author=s Signature
1	Dr. Kaukab Anjum	Research, Thesis writing, formatting and editing,	
2	Dr Asma Ali	results compilation and statistical analysis Literature writing, Formatting	
3	Dr. Uzma Shahid	Thesis formatting, Results compilation