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**ORIGINAL ARTICLE**

# The Protective Effect of Zinc on Salt Induced Histological Changes in Femur of Sprague Dawley Rats-Data Driven Approach

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## ABSTRACT

**Background:** Osteoporosis, a degenerative skeletal illness, is a widespread medical issue that can affect a person's way of life. **Aim:** To evaluate how salt played a protective role in the Sprague Dawley rats' femur's microscopic alterations.
**Study Design:** Random control trial
**Methodology:** The study used 30 female, Sprague Dawley rats aged between 0 and 12 weeks. Three groups of the animals were randomly chosen. For eight weeks, group B rodents received a high salt diet supplemented with zinc (50 mg/kg/day), while group A rats were administered a high salt diet (8% NaCl). The control group's diet was left unaltered. The left femora of the rats were excised during dissection. There was a decalcification process. To quantify the cortical bone, tissue from the mid-shaft of the femur was taken. To acquire five micrometer (m) sections, processing was done. Hematoxylin and Eosin (H&E) was used to stain tissues as a histological parameter. Each group was compared to the others. **Statistical analysis:** MATLAB was used to conduct the data analysis. Mean+ S.D. was used to express quantitative data. For group comparisons, a 2-sample t-test was used. Statistical significance was defined as p 0.05.
**Results:** Group A of the experimental study had a considerably smaller area of cortical bone from the mid shaft of the femur. When compared to group A, the experimental zinc-administered group B showed statistically significant improvements.
**Practical Implication:** Osteoporosis is a multifactorial disease and numerous micro/macro nutrients and dietary components can influence bone health including high salt intake. Zinc plays a pivotal role in maintenance and growth of skeletal system and its deficiency results in growth failure, epidermal, gastrointestinal, central nervous, immune, skeletal, and reproductive systems disorders so present study was planned. **Conclusion:** A diet high in salt caused bone loss due to a reduction in the cortex's surface area. By promoting osteoblast activity and preventing bone-resorbing cells, zinc is useful in reducing the negative effects of salt on bones.
**Keywords**: Cortex, Femur, Osteaoprosis, Salt and Zinc.

## INTRODUCTION

Osteoporosis, a degenerative skeletal illness, is a widespread medical issue that can affect a person's way of life. Due to the decrease in bone mineral density, it is characterised by bone fragility. This weakens bone density and makes bones more prone to fractures, with the likelihood of its occurrence rising with age. Alcohol consumption, tobacco usage, gonadal hormone deficiency, immobility, and insufficient calcium or vitamin D intake are a few of the many risk factors for osteoporosis[1]. Moreover, osteoporosis is not only improperly diagnosed but also improperly treated, which lowers quality of life. As a result, osteoporosis is seen as a major global public health concern, particularly for women and the elderly. 200 million individuals are thought to be affected by the worldwide harmful effects of osteoporosis[2]. It is a disease that goes undetected until there are fractures, after which it can be fatal or create serious secondary health issues[3]. The body contains almost all cell types, and zinc is a cofactor for 200 enzymes. It is an essential trace element. It is essential for the growth, development, and maintenance of healthy bones because it regulates bone homeostasis. It influences the development of hard tissues by preventing osteoclast differentiation and enhancing osteoblast activity. A significant amount of the body's total zinc content, concentrated in the layer of osteoid just before calcification, is found in the skeleton (30% of the body's entire zinc content is kept in bone mass). The range of Zn concentrations required for the best therapeutic outcome localised in the osteoid layer just before calcification. A Zn delivery mechanism must be used in order to improve bone density because the range of Zn concentrations necessary for an ideal treatment response is relatively constrained[4,5]. Zinc is a structural component of transcription factors and enzymes involved in cellular signalling pathways, which promote osteoblastic cell proliferation, differentiation, and mineralization. Zinc supplementation may therefore play a crucial role in osteoporosis prevention[6].

Furthermore, because a vitamin D deficit has a detrimental impact on PTH, it is necessary for the physiological action of vitamin D on calcium metabolism. Nutritional zinc insufficiency, however, is a problem for global health[7]. Humans have two families of zinc transporters, the Slc30a (ZnT) and Slc39a (Zip), which are in charge of importing and removing zinc, respectively[8].

One of the main dietary influences on urinary calcium excretion is salt consumption. Calcium excretion from the urine is decreased when salt consumption is decreased. While there is a negative calcium balance when salt intake is increased due to the stimulation of processes that increase intestinal calcium absorption as well as calcium mobilisation from bone[9]. One of the risk factors for bone loss in humans has been identified as nutritional deficit, particularly a calcium (Ca) shortage. The renal proximal tubules contain a common transport system for sodium and calcium. Increased sodium chloride (NaCl) consumption decreases bone mineral content and increases the amount of calcium lost through urine, which affects the body's ability to maintain a healthy level of calcium.

The process of bone resorption is speeding up as a result of increased salt consumption, which also causes a rise in hydroxyproline excretion in the urine[10]. Although though lowering salt intake is one of the simplest, most effective and economical strategies to manage public health and avoid issues like hypertension and osteoporosis, people routinely go beyond the recommended salt intake in their diets, leading to significant public health issues[11]. It is beyond a shadow of a doubt that salt addiction causes millions of fatalities every year due to the aggravation of osteoporosis and hypertension[12], with food processing emerging as the most practical source of human dietary salt intake, which has increased up to tenfold[13].

In summary, zinc deficiency is a global health issue that can cause growth failure, epidermal, gastrointestinal, central nervous, immune, skeletal, and reproductive system disorders. High salt

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intake is one of many micro/macro nutrients and dietary factors that can negatively affect bone health. Osteoporosis is a multifactorial disease. A condition with anaemia, hypogonadism, and dwarfism has also been linked to zinc deficiency, according to reports[14].

## MATERIAL AND METHODS

The laboratory-based randomized control trial, which lasted for six months and was approved by the ethical review committee, conducted in the anatomy department of the Islamic International Medical College in Rawalpindi. The success of this research was made possible by the National Institute of Health (NIH) in Islamabad and the Army Medical College in Rawalpindi. It was planned to use 30 female Sprague Dawley rats that were three months old and weighed between 250 and 300 grammes. The animals were divided into three groups of ten each, where they became used to the new environment's temperature range of 20–26°C. Group C served as the controls and was fed a typical laboratory meal.

Group A had a diet containing 8% NaCl[15] for eight weeks. Zinc was administered to the rats in group B at a dosage of 50 mg/kg body weight[16] along with a high salt diet. Water was available at all times. Based on earlier research, the zinc and sodium chloride dosages were chosen.

After eight weeks, animals were sacrificed. Right femora were extracted and put in 10% neutral buffered formaldehyde for two days right away. An aqueous solution of 5–10% nitric acid was used to decalcify the material for 24–48 hours. The midshaft's transverse sections were taken, prepared, and then formed into blocks by being immersed in paraffin wax. By putting blocks on a rotating microtome, 5 m thick sections were produced. Typical histological studies were conducted using hematoxylin and eosin.

The images of the cross-sectional slides of the samples were examined using the image processing toolbox provided in MATLAB. For this particular analysis, MATLAB 2019a was used. Each 2988 by 5312 image was individually loaded and segmented using the image segmentation tool. None of the automatic segmentation methods produced satisfactory results as they failed to isolate the cortex of the samples under examination; hence, the graph-cut semi-automatic segmentation method was used to obtain the desired area of the cross-section.

Certain preprocessing steps were performed prior to the analysis to facilitate segmentation, and to filter out any unnecessary information that may not be needed in the analysis. Many raw images obtained from under the microscope after being converted from color to grayscale, might not have optimal contrast values. The images, therefore, after being loaded into MATLAB, were contrast adjusted using the native 'imadjust' function and fed into the image segmentation toolbox. After obtaining the segmented image, a binary mask of the segmented cortex was exported to allow for the calculation of the area.

Figure-1: Overview of the workflow followed to segment the desired structure:

**Area Calculation:** The area of the structures present in the image could not be calculated in physical units because of the unavailability of the mapping required to convert pixels into their physical measurements. Therefore, the pixels present in the binary map were counted and the area was represented as a normalized measure of the pixels representing the cortex of the cross-section with respect to the pixels of the whole image. At a constant magnification factor, this normalized area can be used to accurately gauge the atrophy of the structure. The normalized area of the structure is given by,

$$Normalized\ Area = \frac{Area\ of\ the\ cortex\ in\ pixels}{The\ total\ pixels\ in\ the\ image}$$

The Normalized area has no units because of the division of two similar quantities.

**Statistical Analysis:** MATLAB was used to conduct the data analysis. Mean+S.D. was used to express quantitative data. For group comparisons, a 2-sample t-test was used. Statistical significance was defined as p 0.05.

## RESULTS

We compare the results for the cortical bones of groups C, A, and B using a two-sample t-test. Three combinations are used for the t-test: groups C and A, groups C and B, and groups A and B. The following is a list of the null hypothesis for each of these scenarios:

- Groups C and A: There exists no significant difference between the areas of the cortical bones of the control group and the salt loaded experimental group A.
- Groups C and B: There exists no significant difference between the areas of the cortical bones of the control group and the zinc protected, salt loaded experimental group B.
- Groups A and B: There exists no significant difference between the areas of the cortical bones of the salt loaded experimental group A and the zinc protected salt loaded experimental group B.

A 2-sample t-test between control group C and group A demonstrates a p-value of 0.000011. This suggests that the effect of salt significantly affects the area of the cortical bone of rats. A similar analysis between the control group C, and the zinc-protected, salt-loaded group B, fails to reject the null hypothesis. This suggests that there exists no significant difference between the areas of the cortical bones in groups C and A. Additionally, 2-sample t-test of groups A and B also exhibits a statistically significant difference, thus rejecting the null hypothesis; suggesting that there is a significant difference between the cortical bone areas of the two groups.

Area of cortical bone from mid shaft of femur was calculated. The mean value of normalized area in the femur for group C was 0.1728, and the area was decreased to 0.1212 in group A, while a beneficial effect was seen in group B, 0.1556. The difference in normalized area among different groups was significant ($p<0.05$) (Table 1).

Table-1: Showing Mean Normalized Area in Femur of All Groups

| Groups | C | A | B |
|---|---|---|---|
| Mean Score | 0.1728 | 0.1212 | 0.1556 |
| St. Deviation | 0.0151 | 0..227 | 0.0296 |
| Sem | 0.0048 | 0.0072 | 0.0094 |
| *p* value | 0.00012* | | |

*Statistically Significant

Multiple comparisons revealed a substantial difference of 0.0516 between group C and A (p=0.00001175). Between group C and B, a value of 0.0172 was obtained (p=0.1196) and the difference in apoptotic osteocytes number between group A and B was -0.0344 (p=0.009239) (Table 2).

Table-2: Multiple Comparison of Mean Normalized Area in Femur among All Groups.

| Groups | C vs A | C vs B | A vs B |
|---|---|---|---|
| Mean differences | 0.0516 | 0.0172 | -0.0344 |
| *p* value | 0.0000117 | 0.1196 | 0.009239* |
| Null Hypothesis | Rejected | Failed to reject | Rejected |

Results clearly indicated the decrease in mean of normalized area in cortex of group A as compared to group C and increase in normalized area due to zinc in group B (Fig-2).

Figure-2: Comparison of Mean of Normalized Area in Cortex among Groups

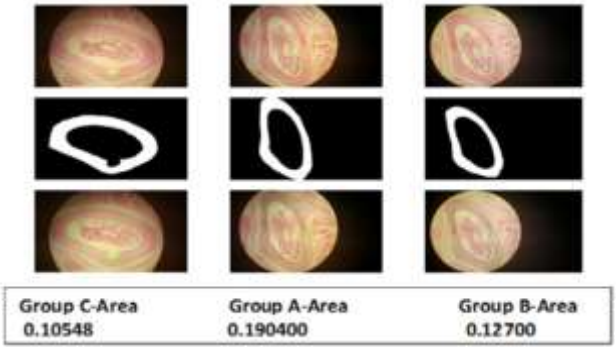
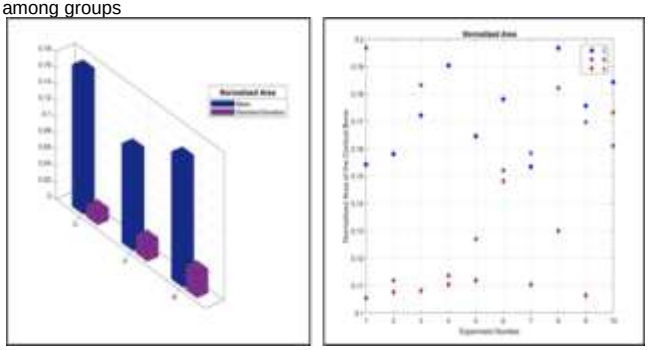



Figure-3: Both bar chart (a) and scatter plot (b) showing the normalized area among groups



## DISCUSSION

The beneficial effects of zinc on salt-loaded rats were statistically examined as part of the study design for this article. By sustaining and controlling a high salt diet for rats, which led to the weakening of long bones, the required case scenarios were realized. By utilizing a microscope to observe quantitative factors like bone thickness, diameter, etc., the impairments were confirmed. The findings imply that zinc supplementation may be able to mitigate, occasionally even fully prevent, the negative effects of a diet high in salt on bones.

Rat long bones can be divided into four main bone types: periosteum, cortex, endosteum, and cancellous. The long bone's shaft, the femur diaphysis, is primarily made of compact bone. In the inside of the shaft of long bones, cancellous bone, a meshwork of spongy tissue, forms a very thin layer. Quantitative measures that may be compared and statistically evaluated for significance are needed for the evaluation of bone quality and health. The hard outer layer of bones, known as the cortical bone, serves as a protective covering for the internal bone structure. An important criterion to consider when assessing the quality and state of bones is their thickness, which may be measured quantitatively. In this work, this parameter is measured and examined. The cortical bone's dimensions serve as a qualitative indicator of the quality of the bone; the larger the dimensions, the thicker the protective covering over the inner bone structure and, consequently, the healthier the bone.[5] The damage to the bones is quantified in order to determine the negative effects of salt overload. By measuring the cortical bone area in mid-diaphysis cross sections, the damage is determined. The statistical analysis for this study uses this numerical measurement. Three groups of rats—the control group (group C), the experimental groups A and B—were used in the investigation. The plan of the experiment examined two effects[6]:

1. How a high-salt diet affects bone health

2. How zinc supplementation affects bone health

The experimental groups A and B were both given high salt diets in order to design the best experiment possible. Rats' bones were negatively impacted as a result of this. The control group, which was not given a high-salt diet, provided the appropriate baseline for evaluating the impact of salt induction on the bones of rats By contrasting the diets of the experimental groups A and B, the impact of zinc supplementation was seen. To compare the beneficial effects of zinc on bone health with the benchmarked control group and the salt-loaded experimental group A, group B was given a zinc supplement. The results of the hypothesis tests show that the experimental group B's femur has the largest cortical area, followed by the control group's femur. Group B received zinc and salt supplements, resulting in less harm than group A, which consumed a high-salt diet[7].

The findings of this study are consistent with those of Ahmed et al., who found that rats fed a high-sodium diet had thinner cortical bones[17]. According to the authors, excessive salt consumption may result in elevated plasma levels of potassium, phosphate, creatinine, and urea because of abnormal kidney function, which then affects bone structure. Moreover, a rise in serum phosphate inhibits 1-hydroxylase and causes a decrease in 1,25(OH)2 D, the physiologically active form of vitamin D. This decrease in intestinal calcium absorption causes an increase in PTH release, which in turn causes an increase in osteoclastic activity. Inaccurate bone remodelling is caused by degenerative alterations in osteoblasts, osteocytes, and the hyperactivity of osteoclasts, which leads to a reduction in cortical bone thickness[18].The entire area in the research is reduced as a result of a diet-induced salt excess in rats, according to the findings. The reduction in cortical bone thickness may be caused by changes in bone remodeling, which are mediated by changes in bone cells, increased osteoclastic activity, and numerous resorption cavities[19-21]. The findings reported in this article are consistent with the findings of the aforementioned journals, and they all point to the same conclusion: a high salt intake causes osteoporosis and a reduction in cortical bone thickness.

The study that is being presented shows that experimental group B experienced an increase in cortical bone area following zinc supplementation. The work of Brzoska et al., which demonstrated the protective impact of zinc diet on bone homeostasis, further strengthens the positive and beneficial effects of zinc supplementation in bone health, as documented in this article[22]. He proposed that adequate zinc levels may be the cause of an increase in bone alkaline phosphatase activity. Following a zinc supplementation, osteoblasts manufacture more osteocalcin, which causes cortical bone thickness to rise[23]. Also necessary for osteoblast proliferation and associated with a reduction in bone resorption is zinc[24].

Increased urine calcium excretion and parathyroid hormone (PTH) secretion are linked to high NaCl intake. As a result, there is more bone resorption since the release of calcium from the bone is stimulated. Further investigation of bone marrow cells that contain bone-resorbing substances (such PTH) showed an increase in osteoclasts. When zinc was added to bone marrow that also included bone-resorbing factors, there was a reduction in the number of osteoclasts. It is known that this effect is brought on by the apoptotic death of osteoclasts when zinc is present[25].

The increase of the medullary cavity on the endocortical side is the first sign of any bone resorption[26]. The increase of the bone marrow cavity is an important sign of bone deterioration since cortical bone loss is relatively slower than changes in other parts of the bone. The diameter of the medullary cavity was measured to determine the extent of bone injury. Subsequent examination of the bone damage also revealed that zinc supplementation had a net beneficial effect on the width of the medullary cavity while high salt intake increased the diameter of the cavity. The increase in cortical bone diameter is indicated by the reduction in the diameter of the medullary cavity. Research of Omara et al[27] on dexamethasone-induced osteoporosis in rats similarly revealed

Haversian and medullary cavity enlargement. According to the authors, secondary hyperparathyroidism may enhance bone resorption as a result of the obstruction of GIT and renal calcium absorption. A considerable bone loss and subsequent reduction in the area of the bone cortex may be caused by an increase in bone resorption[28,29]

The earlier findings of Bhardwaj et al. provide additional evidence for the protective role of zinc, which is described in this article[30]. The authors research the effects of zinc supplementation in an osteoporotic rat model with ovariectomies. They draw the conclusion that zinc supplementation improves the microarchitecture of bones. The exterior diameter is one of the key measures of bone strength. A maximum of 55% of the variation in bone strength can be predicted by the parameter[31,32].

The findings of this study confirm the vital significance of the external diameter because both interventional groups' femur midshaft diameters dropped, resulting in a reduction in the cortex's surface area. Moreover, rats fed a diet enriched in zinc showed the protective effect. Ovesen et al also observed that zinc supplemented rats had increased mid-diaphysis femur cross-sectional area[33].To evaluate the diet-induced significant difference in the midshaft diameters of bones following an excessive salt diet, more research is needed.

**Limitations of study:** This study was conducted only on a small size of population, therefore to generalize the results for larger groups, the study should be performed on a larger scale. Financial constrains and limited resources with no genetic workup and long follow-ups added to limitations.

## CONCLUSION

The effects of salt loading and zinc supplementation on the long bones of rats are examined in this research. Statistics showed that salt loading had a negative impact on bone health, whereas zinc supplementation had a positive impact on bone quality. It has been determined that excessive salt consumption has a negative impact on the microstructure of the bones and that zinc protects against salt-related bone deterioration. The metal of life, zinc is crucial to maintaining good health in people. Even though the zinc-supplemented salt-loaded rats did not show 100% protection, zinc's ameliorative effect was still visible in the form of an increase in the area of the bone cortex compared to the salt-loaded animals.

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