Research Article

Expolring how sex difference impacts bone response to high salt diet in adult albino rats

Adel H. Saad*, Hanaa M. Ibrahim*, Wagdy N. Habeeb* and Sara M. Abdel Hafez**
* Department of Physiology , Faculty of Medicine, Minia University, Egypt
** Department of Histology, Faculty of Medicine, Minia University, Egypt

Abstract
Background and aim: High salt intake is related to great risk for developing many diseases such as hypertension, cardiovascular diseases (CVDs), metabolic syndrome and osteoporosis. This work aimed to assess the effects of the high salt diet (HSD) on adult albino rats' bone and exploring the sex disparity of this effect. **Materials and methods:** Forty eight adult albino rats, 24 males and 24 females, were divided into four groups: control males, control females, HSD-fed males, and HSD-fed females. **Results:** The results showed that administration of HSD (8 %) for seven weeks to both male and female rats resulted in increased food intake, decreased body weight gain and BMI, as well as osteoporosis. Osteoporosis was evidenced by increased parathormone hormone and the bone turnover markers; alkaline phosphatase, acid phosphatase, and osteopontin levels. Decreased bone weight, dry weight, fat-free dry weight, ash weight, organic matrix weight, and percentage of non-organic matrix weight were detected. Osteoporosis was more severe in female rats than male rats. **Conclusion:** Accordingly, the results obtained from the present study revealed that the severity of HSD-induced osteoporosis is sex-dependent. The decreased severity of osteoporosis in males may be attributed to high body weight and muscle bulk, inhibition of parathormone secretion, and other different mechanisms of protection exerted by testosterone hormone.
Key words: high salt diet, sex difference, osteoporosis, bone turnover markers, osteopontin and parathormone.

Introduction
Bone remodeling is a continuous coordinated process in which old bone is removed (resorption), and new bone is added (bone formation). The balance between bone resorption and bone formation is determined by the activity of both osteoclasts and osteoblasts. This process is regulated by several hormones as parathormone (PTH), calcitonin, vitamin D3, growth hormone, insulin-like growth factor-1 (IGF-1), cortisol and thyroid hormone, as well as sex hormones; estrogens and androgens, in both males and females[1].

Osteoporosis is a progressive skeletal disease characterized by a decrease in bone mass and a disruption of bone architecture, leading to enhanced bone fragility and consequent increase in fracture risk especially in the elderly. According to WHO criteria, osteoporosis is defined as bone mineral density decreased to ≤ 2.5 standard deviations from the mean bone mineral density in healthy young population[2].

Multiple special habits and lifestyle factors as physical activity, smoking, alcohol consumption and diet are important determinants of human longevity. The common salt (sodium chloride; NaCl) is an important micronutrient added to food. Its daily requirement equals about 10–20 mmol (0.58–1.16 g). However, salt intake till now is generally greater, and can exceed 200mmol (10g) / day in many populations[3].

High salt diet (HSD) is related to high risk of many diseases as hypertension, cardiovascular diseases, diabetes mellitus, metabolic syndrome and renal impairment. So many well developed societies recommended limiting salt intake to 3.75–6 g/day[4]. HSD also increases the urinary calcium excretion, which may increase the risk of osteoporosis and kidney stones[5].

The sex hormones exert potent influences on the size and shape of the skeleton during growth. In addition, these hormones contribute to skeletal homeostasis during adulthood. Therefore, they have an important role in sexual disparity in the response of bone to HSD[6].

The present study was conducted to assess the effect of HSD on bone in adult albino rats, and to evaluate the impact of sex disparity on bone response and exploring the possible underlying mechanisms.

Materials and Methods

Animals:

A total of 48 adult albino rats, 24 males and 24 females, of Sprague-Dawley strain, weighing between 150 and 200g, were obtained from Minia University animal`s house center. Rats were housed at room temperature with natural dark/light cycles in 12 mesh cages (4 rats each). The dimensions of each cage were 30 cm x 30 cm x 20cm that offered an adequate space for free movement and wandering. The rats had free access to water and commercial rat chow (Nile Company, Egypt) for one week before the start of the experiment for acclimatization. The experimental protocol was documented according to the rules of the animal care and use committee, Faculty of Medicine, Minia University.

The rats were divided into the following four groups (12 rats each):

I- Control males (CM): Male rats fed normal salt diets containing 0.3% salt.

II- Control females (CF): Female rats fed normal salt diets containing 0.3% salt.

III- High salt diet fed males (HSD males): Male rats fed high salt diets containing 8% salt.

IV- High salt diet fed females (HSD females): Female rats fed high salt diets containing 8% salt.

Experimental protocol:

The HSD (8%) was prepared by adding additional amount of NaCl to the normal salt powdered chow (7.7% NaCl is added weight/ weight to the normal 0.3% NaCl which is originally present in the standard rat chow). After that, the powder was mixed with a small amount of water, formed into pellets and dried overnight in an oven at 60°C[7].

For all the studied groups, the food intake was calculated daily for 7 weeks which was the experimental duration. Initial and final body weights were measured to assess body weight gain and body mass index (BMI). Rats were weighed using electronic balance (FY 2000), the nasoanal length was measured using strip meter from the nose to the anus, and BMI was calculated according to the formula put by Novelli et al.,[8]: BMI = body weight (g) / length^2 (cm^2)

At the end of the 7th week, all rats were subjected to an overnight fasting. After that, the rats were anaesthetized by light ether anesthesia and were sacrificed by decapitation. The blood samples were immediately collected from the jugular veins in 10ml tubes, allowed to clot, and then centrifuged at 3000 rpm for 20 minutes. The serum samples were separated in 2 ml Eppendorf tubes, and stored at -20°C until used for estimating the level of serum calcium "MG Co., England", phosphorous "MG Co., England", alkaline phosphatase "MG Co., England", acid phosphatase "FAR Co., Italy", and parathormone (PTH) "Shanghai Korain Biotech, China" according to the manufacturer's instructions.

The hind limbs of all rats were also dissected. The left and right femurs of each rat were gently removed and cleaned from adhering muscles and soft tissues. The left femurs were used to assess femur length, weight (W), dry weight (DW), fat-free dry weight (FFDW), ash weight (AW), organic and nonorganic components, % of water content, and bone mineral density (BMD). The right femurs were preserved for histopathological and immunohistochemical studies.

BMD was calculated according to **"Archimedes' principle"**;
$Bone\ density = \frac{bone\ weight\ in\ air}{bone\ weight\ in\ air - bone\ weight\ in\ water} \times density\ of\ distilled\ water$[9].

DW was assessed by drying the femur to a constant weight at 100 °C for 24 h and weighing them again. The water content was assessed according to the following formula;
$\%\ of\ water\ content = \frac{bone\ weight\ in\ air - \ dry\ weight}{bone\ weight\ in\ air} \times 100$ [10].

FFDW was obtained by submerging the femurs in 90% petroleum ether for 48 h, then drying them in a forced-air oven at 90 °C until constant weight was obtained, and then reweighing them[11].
AW, which contains only the minerals, was obtained after heating at 100°C for 48h[12]. Concentrated nitric acid (0.1ml) and 30% hydrogen peroxide (0.05ml) were added to each sample, and then placed in a sand bath heated over 100 °C. This treatment was repeated until a whitish residue was obtained. On the basis of the obtained weights (W, DW, and AW), the following calculations were made[10]:

Percentage of non-organic components:
$Weight\ of\ non-organic\ component = Ash\ weight$
$\%\ of\ non-organic\ component = \frac{weight\ of\ nonorganic\ component}{W} \times 100$

Percentage of organic components:
$Weight\ of\ organic\ component = DW - AW$
$\%\ of\ organic\ component = \frac{weight\ of\ organic\ component}{W} \times 100$

The excised parts of the right femurs from each rat were cut transversely at their upper ends (to study the cancellous bone morphology) and their shafts (to study the compact bone morphology). Multiple small specimens were rapidly fixed in 10% neutral formal saline for about 48 hours, and then decalcified in daily exchanges using the chelating agent formalin-EDTA for 4 weeks. An ample volume of decalcifying solution, 50 times the bone volume, was used. The decalcified specimens were dehydrated in ascending grades of alcohol, cleared in xylene, and impregnated in paraplast carefully for 3 h in an oven at 58°C. Serial sections of the bone tissue were cut at a thickness of 7µm and stained with:
a. Hematoxylin and Eosin (H&E) stain for studying the general histological structure[13].
b. Masson's Trichrome stain for demonstration of mineralized and unmineralized bone matrix[14].
c. Von Kossa's stain: for demonstration of Calcium deposition.
d. Additional slides were used for an immune-histochemical study using osteopontin monoclonal rabbit antibodies (Lab Vision Laboratories, USA) according to the manufacturer's protocol.
Slides were photographed using an Olympus digital camera connected to an Olympus, made in China (U.TV0.5XC-3) light microscope at the Histology and Cell Biology Department, Faculty of Medicine, Minia University. Images were processed using Adobe Photoshop 7.

Parameters were measured in five fields from three serial sections of each rat; cortical bone thickness, trabecular bone thickness, mean diameter of Haversian canals eroded surface percentage, and mean surface area fraction for ostopontin immunoreactive cells. Measurements were taken using Image J program analysis software (version 1.5) at magnification × 400[15].

Statistical analysis:
Data were expressed as means ± standard errors of the mean (SEM). Statistical analysis was performed using Graph pad Prism 5 software and significant difference between groups was done by one-way ANOVA test followed by Tukey's Multiple Comparison Test with a P value of <0.05 considered statistically significant.

Results

A- Evaluation of the food intake, body weight gain, and BMI:

The data presented in table (1) show that HSD significantly increased the food intake compared to the control groups. This increase in food intake was significantly higher in HSD male more than in HSD female groups. On the other hand, the body weight gain and BMI were significantly lower in HSD compared to the control groups. However, they were still significantly higher in HSD male than in HSD female groups.

Table (1): Effect of HSD on food intake, body weight gain and BMI

Groups (*n* = 12) / Parameters	Control Male	Control Female	HSD Male	HSD Female
Food intake (g/day)	16.8 ± 0.72	12.5 ± 0.5	21.8 ± 1.16 [a,d]	17.3 ± 1.16 [b,c]
Body weight gain (g)	103.8 ± 2.64	83.5 ± 2.477	51 ± 9.14 [a,d]	25.6 ± 1.39 [b,c]
% of weight gain	+55.66%	+46.29%	+28.38% [a,d]	+14.93% [b,c]
BMI (g/cm^2)	0.607 ± 0.17	0.584 ± 0.18	0.577 ± 0.205 [a,d]	0.519 ± 0.012 [b,c]

Data represent mean ± S.E. ***n*:** number of rats in each group. **HSD:** high salt diet. **BMI:** body mass index. **[a]:** significant difference from the control male group. **[b]:** significant difference from the control female group. **[c]:** significant difference from the HSD male group, $P < 0.05$. **[d]:** significant difference from the HSD female group, $P < 0.05$.

B- Evaluation of the serum parameters:

The data presented in table (2) showed that HSD did not significantly affect both serum calcium and phosphorous levels compared to the control groups. Regarding the bone turnover markers; alkaline phosphatase and acid phosphatase, HSD significantly increased these parameters compared to the control groups. However, there was no significant difference between HSD male and HSD female groups. As regards PTH, HSD significantly increased its level compared to the control groups. This increase was higher in HSD female than in HSD male groups.

Table (2): Effect of HSD on the serum parameters

Groups (*n* = 12) / Parameters	Control Male	Control Female	HSD Male	HSD Female
Calcium (mg %)	8.31± 0.056	8.19 ± 0.05	8.3 ± 0.07	8.16 ± 0.27
Phosphorous (mg %)	5.34 ± 0.039	5.32 ± 0.03	5.28 ± 0.02	5.26 ± 0.02
Alkaline phosphatase (U/L)	119.1 ± 2.01	117.1 ± 1.9	206.2 ± 4.4 [a]	206.2 ± 3.4 [b]
Acid phosphatase (U/L)	26.96 ± 0.69	26.41 ± 0.53	77.55 ± 2.2 [a]	81.39 ± 1.1 [b]
PTH (pg/ml)	28.33 ± 1.66	40.19 ± 2.7	57.76 ± 1.5 [a,d]	72.94 ± 1.93 [b,c]

Data represent mean ± S.E. ***n*:** number of rats in each group. **HSD:** high salt diet. **PTH:** parathormone. **[a]:** significant difference from the control male group. **[b]:** significant difference from the control female group. **[c]:** significant difference from the HSD male group, **[d]:** significant difference from the HSD female group, $P < 0.05$.

C- Evaluation of the bone parameters (measured from the left femurs):

The data presented in table (3) showed that HSD did not significantly affect both femur length and % of water content compared to the control groups. Regarding bone weight, dry weight, fat-free dry weight, ash weight, organic matrix weight, % of non-organic components,

and BMD; HSD significantly decreased these parameters compared to the control groups. However, they were still significantly higher in HSD male than in HSD female groups. On the other hand, HSD did not significantly affect both % of organic components and ratio of non-organic to organic components. The % of organic components was significantly higher in HSD male than in HSD female groups, while the ratio of non-organic to organic components was significantly lower in HSD male than in HSD female groups.

Table (3): Effect of HSD on the bone parameters measured from the left femurs

Groups (*n* = 12) / Parameters	Control Male	Control Female	HSD Male	HSD Female
Femur length (cm)	3.42 ± 0.041	3.19 ± 0.055	3.33 ± 0.058	3.17 ± 0.049
Bone Weight (g)	0.6012±0.027	0.468 ± 0.0072	0.509 ± 0.014 [a,d]	0.401 ± 0.001 [b,c]
Dry weight (g)	0.494± 0.0017	0.359 ± 0.0078	0.3086 ± 0.039 [a,d]	0.302 ± 0.008 [b,c]
FFDW (g)	0.485± 0.0018	0.351 ± 0.0076	0.393 ± 0.01 [a,d]	0.295 ± 0.001 [b,c]
Ash weight (g)	0.261 ± 0.164	0.234 ± 0.02	0.187 ± 0.002 [a,d]	0.158 ± 0.001 [b,c]
Organic matrix weight (g)	0.233 ± 0.015	0.125 ± 0.021	0.215 ± 0.012 [a,d]	0.144 ± 0.001 [b,c]
Ratio of non-organic to organic	1.207 ± 0.12	1.43 ± 0.13	0.912 ± 0.10 [d]	1.1 ± 0.10 [c]
% of non-organic Components	43.63 ± 2.63	50.102 ± 4.19	36.88 ± 0.512 [a]	39.47 ± 0.319 [b,c]
% of organic components	38.82 ± 2.64	26.61 ± 4.45	42.11 ± 2.18 [d]	35.88 ± 0.2 [c]
% of water content	17.80 ± 0.215	23.28 ± 0.84	21.01 ± 2.07	24.66 ± 0.26
BMD (g/cm^3)	3.847 ± 0.017	3.55 ± 0.041	2.99 ± 0.0048 [a,d]	2.38 ± 0.016 [b,c]
%changes of BMD from the of her sex	8.8%	-8.8%	20.6% [a,d]	-20.6% [b,c]
%changes of BMD from the corresponding control group	23.3%	33.3%	-23.3% [a,d]	-33.3% [b,c]

Data represent mean ± S.E. ***n*:** number of rats in each group. **HSD:** high salt diet. **FFDW:** fat-free dry weight. **BMD:** bone mineral density. **[a]:** significant difference from the control male group. **[b]:** significant difference from the control female group. **[c]:** significant difference from the HSD male group, **[d]:** significant difference from the HSD female group, $P < 0.05$.

D- Histopathological and immunohistochemical study:

***1*- Hematoxylin and Eosin (H&E) stain results:**

Transverse sections of rat femoral diaphysis of compact bone of both control male and female groups revealed the same morphological structure. They were formed of regularly arranged multiple bone lamellae arranged in a regular concentric manner. The covering periosteum and the lining endosteum were noticed. The bone matrix appears homogenously eosinophilic. The Haversian system contains blood vessels and multiple distinct cement lines. The osteocytes were noticed settle inside their lacunae. Whoever, HSD male group showed distorted bone structure as some areas were noticed completely devoid of osteocytes. Faintly stained bone matrix was frequently seen. On the other hand, HSD female group exhibited massive distortion than HSD male group. Some areas were seen completely devoid of both osteocytes and bone matrix.

Additionally, transverse sections of rat's femoral metaphysis of spongy bone of both control male and female groups showed the same morphological structure. They were consisting of cancellous bone with multiple network of thick branching and anastomosing bone trabeculae enclosing red bone marrow cavities of variable sizes. Bone matrix appeared homogenously eosinophilic. The HSD male group showed distorted bone trabeculae with widening of bone marrow cavities containing more numerous fat cells. Focal eroded surfaces were also noticed. Prominent ossification was

clearly noticed. Meanwhile, HSD female group displayed massive distortion of bone trabeculae. Apparent thinning of the bone trabeculae was also noticed. More widening of bone marrow cavities was clearly observed than in the HSD male group. Examining wide fields in this group revealed more extensive eroded surface and less prominent ossification center compared to HSD male group **(figure 1).**

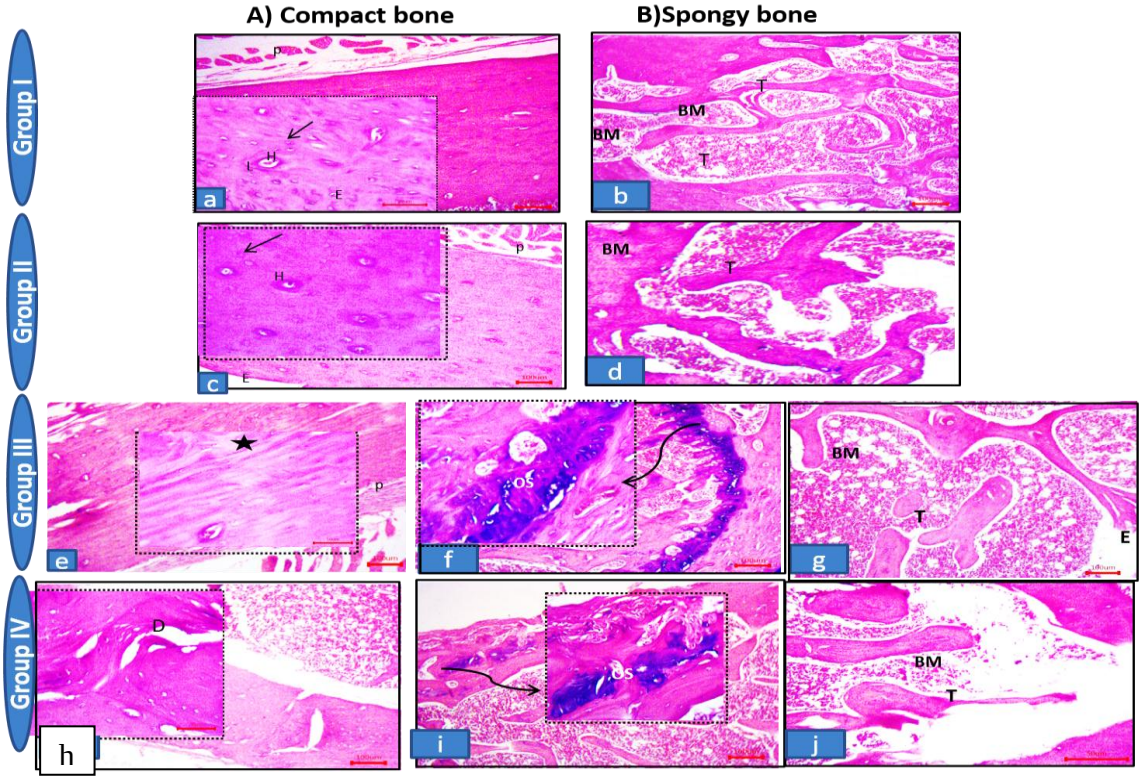

Figure (1): Representative photomicrographs of transverse sections of rat femoral diaphysis of compact bone:

Compact bone: (a & c); control male and female groups respectively. periosteum (p) and lining endosteum (e). The bone matrix appears homogenously eosinophilic. Haversian system (H) cement lines (L). The osteocytes (black arrows) settle inside their lacunae.

(e) Male HSD group showing distorted bone structure. Devoicing osteocyte cells (black arrow). Faintly stained bone matrix (star).(h) Female HSD group showing massive distortion (D). devoid of osteocytes and bone matrix (L).

Spongy bone: (b & d) control male and female groups respectively. Bone matrix appears homogenously eosinophilic. (f) Male HSD group showing prominent ossification center (OS). Distorted bone trabeculae (T) with widening of bone marrow cavities (BM) containing more numerous fat cells (f). Notice the eroded surface (E). (i) Female HSD group showing less prominent ossification center (OS). Massive distortion of bone trabeculae. Apparent thinning of the bone trabeculae is also noticed (T). More widening of bone marrow cavities (BM) is clearly observed. Notice the more eroded surface (E). **H&E-stained× 100, insets × 400.**

2- Masson's trichrome stain results:

Transverse sections of rat femoral diaphysis of compact bone and metaphysis of spongy bone of both control male and female groups showed the bony trabeculae formed mainly of mineralized bone appearing red in staining and few areas of unmineralized bone appearing blue in staining. HSD male group displayed bony trabeculae formed of both mineralized bone matrix with more areas of unmineralized bone matrix. Group IV (HSD female showed bony trabeculae formed mostly of unmineralized bone matrix and few areas of mineralized bone matrix **(figure 2).**

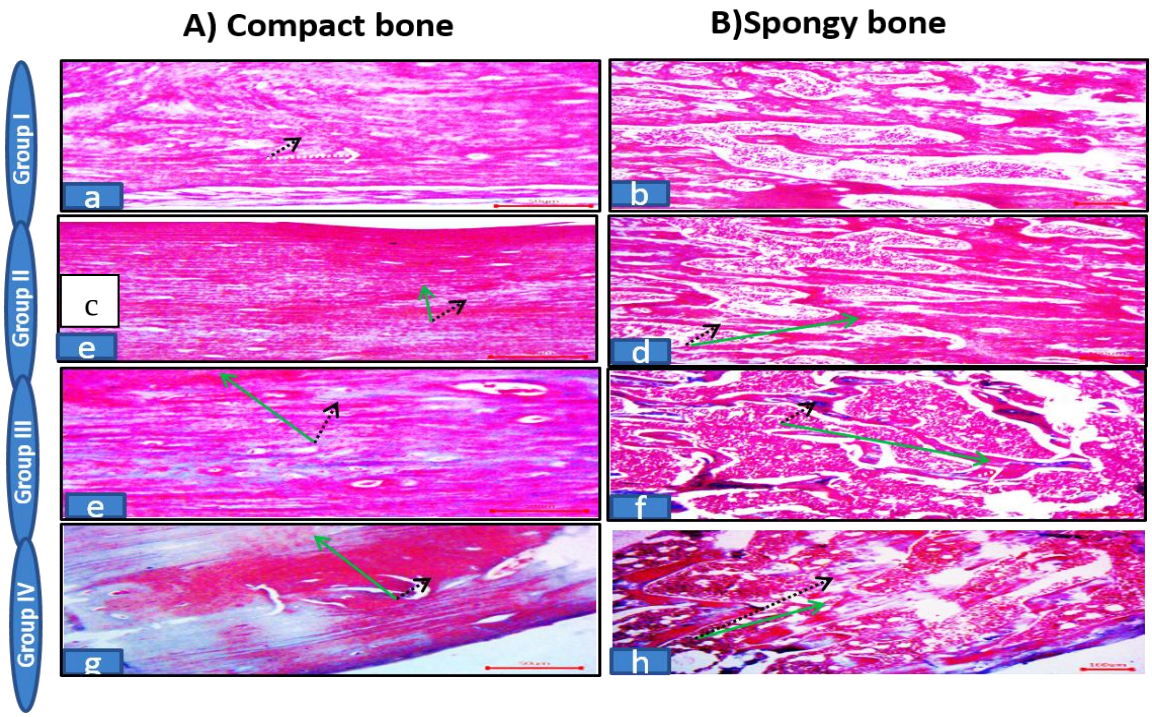

Figure (2): Representative photomicrographs of transverse sections of rat femoral diaphysis of compact bone:

Compact bone: (a & c) control male and female groups respectively. Unmineralized bone appearing blue in staining (black arrow). (e) Male HSD group showing both mineralized bone matrix (green arrow) with more areas of unmineralized bone matrix (black arrow).(g) Female HSD group formed mostly of unmineralized bone matrix (black arrow), few areas of mineralized bone matrix (green arrow).

Spongy bone: (b & d) control male and female groups respectively showing mineralized bone appearing red in staining (green arrow),d few areas of unmineralized bone appearing blue in staining (black arrow).(f) Male HSD group showing bony trabeculae formed of both mineralized bone matrix (green arrow) with more areas of unmineralized bone matrix (black arrow).(h) Female HSD group showing bony trabeculae formed mostly of unmineralized bone matrix (black arrow) and few areas of mineralized bone matrix (green arrow). **Masson's trichrome-stained X400**

3- Von Kossa's stain results:

Both male and female control groups showed calcium deposition in bone tissue that appeared black in color. Group III (HSD male) showed an apparent decrease in this color if compared to the previously mentioned groups. Additionally, group IV (HSD female) showed less calcium deposition than HSD male group **(figure 3A).**

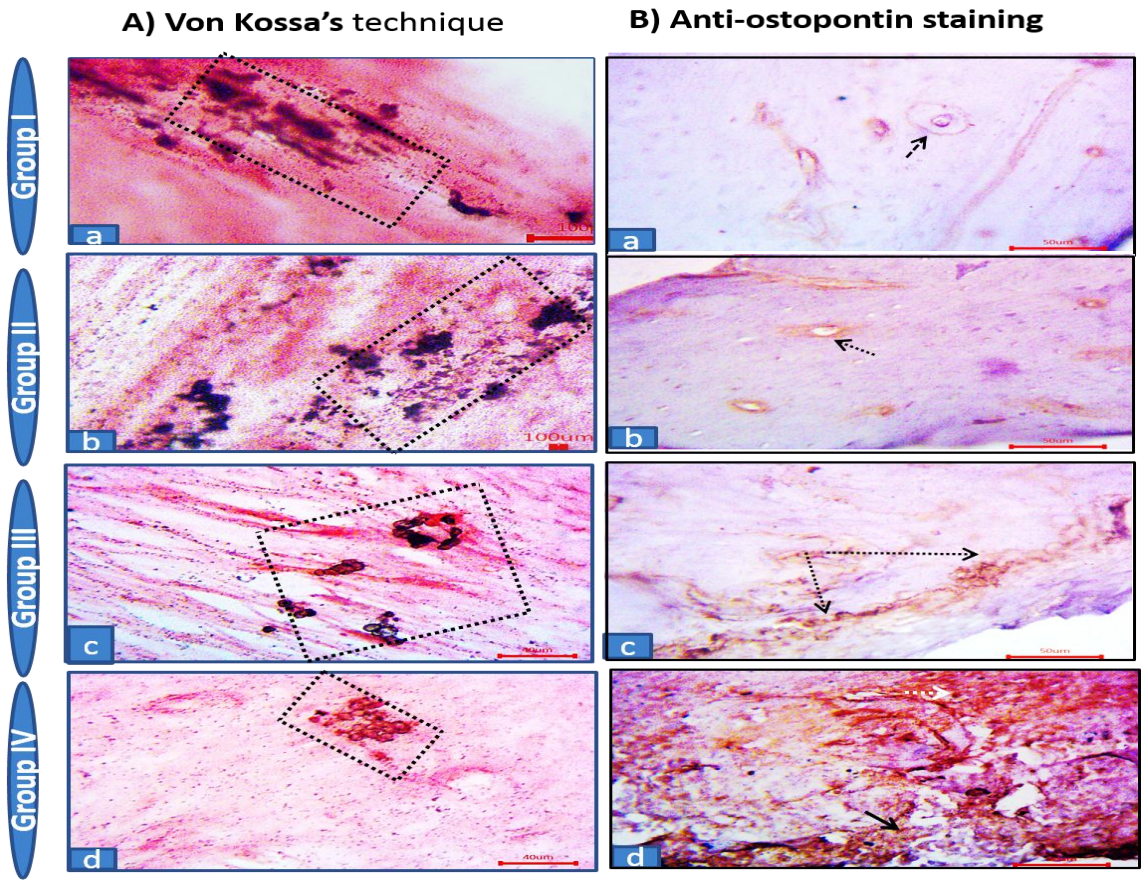

Figure (3): Representative photomicrographs of bone tissue:
A) Von Kossa`s stain: (a & b) control male and female groups respectively showing calcium deposition in bone tissue that appeared black in color. (c) Male HSD group showing apparent decrease in this color if compared to previously mentioned groups.(d) Female HSD group appears to contain less calcium deposition than group III. **Von Kossa's staining technique× 400)**
B) Immunohistochemistry for anti-osteopontin: (a & b) control male and female groups respectively showing faint cytoplasmic immunoreactivity, mainly in the Haversian system (arrow).
(c) Male HSD group showing more (arrows) positive cytoplasmic expression in the bone matrix.
(d) Female HSD group showing more intense and extensive (arrows) positive cytoplasmic expression in the bone matrix. **anti-osteopontin counterstain with Hematoxylin**

4- Immunohistochemical results for anti-osteopontin:
Both male and female control groups showed cytoplasmic immunoreactivity mainly in the Haversian system. Group III (HSD male) showed more positive cytoplasmic expression in the bone matrix. Furthermore, group IV (HSD female) displayed more intense and extensive positive cytoplasmic expression in the bone matrix than observed in HSD male **(Figure 3B).**

5- Morphometrical and statistical results:
The data presented in table (4) showed that HSD significantly decreased both the cortical and trabecular bone thickness, and significantly increased the Haversian canal diameter, eroded surface, and surface area of ostopontin-positive cells compared to the control groups. All these changes were significantly greater in HSD female than HSD male groups.

Table (4): Effect of HSD on the morphometric bone parameters

Groups (n = 12) / Parameters	Control Male	Control Female	HSD Male	HSD Female
Cortical bone thickness (µm)	518.5 ± 7.6	541.5 ± 10.5	380.7 ± 20.1 [a,d]	291.8 ± 3.7 [b,c]
Trabecular bone thickness (µm)	126.2 ± 1.4	121.8 ± 0.9	84.5 ± 1.4 [a,d]	79 ± 0.9 [b,c]
Haversian canal diameter (µm)	13.2 ± 0.5	13.3 ± 0.6	24.2 ± 1.1 [a,d]	30.5 ± 2.3 [b,c]
Eroded surface (%)	1.2 ± 0.1	1.3 ± 0.2	4.3 ± 0.6 [a,d]	6.3 ± 0.6 [b,c]
Surface area of ostopontin-positive cells (%)	6.8 ± 0.3	7 ± 0.6	27.3 ± 2.5 [a,d]	34.5 ± 2 [b,c]

Data represent mean ± S.E. ***n*:** number of rats in each group. **HSD:** high salt diet. **[a]:** significant difference from the control male group. **[b]:** significant difference from the control female group. **[c]:** significant difference from the HSD male group, **[d]:** significant difference from the HSD female group $P < 0.05$.

Discussion

The results obtained in the present study showed that there is a decrease in weight gain and BMI in spite of increased food intake in the HSD groups when compared to the control groups. These results are in line with Oloyo et al.,[7], who found that salt intake stimulates appetite, food intake and body metabolism, with subsequent increase in energy expenditure that leads to a decrease in body weight. This decrease in weight gain may also be related to the increase in body water loss through excessive urination observed during the experiment.

The results obtained in the present study showed also that food intake, weight gain & BMI were higher in HSD male than in HSD female groups. These results are in line with Alrabadi et al.,[16], who reported that testosterone increases the appetite and hence the food intake *via* increasing neuropeptide Y and ghrelin levels. The higher weight gain & BMI can be also attributed to the anabolic effects of testosterone which affects mainly the muscle tissue.

On studying the direct effects of HSD on the bones, the present study demonstrated that HSD induced osteoporosis as evidenced by: (a) Increased levels of PTH as well as bone turnover markers; alkaline phosphatase (a marker of bone formation) and acid phosphatase (a marker of bone resorption). (b) Decreased bone weight, dry weight, fat-free dry weight, ash weight, organic matrix weight, % of non-organic components, and BMD. (c) The histopathological results which confirmed all the previously changed parameters. They showed osteoporotic changes in the compact and spongy bone tissues in HSD male and HSD female groups in the form of distorted bone structure, widening of bone marrow cavities, eroded surface, less calcium deposition, and apparent thinning of bony trabeculae which included some areas of unmineralized bone matrix within the normal mineralized bone matrix. (d) The immunohistochemical results also showed increased expression of osteopontin (a marker of bone resorption) in the bone matrix.

The HSD-induced osteoporosis comes in line with Fatahi et al.,[17], who reported a positive association between dietary sodium intake and the risk of increased loss of BMD which, in turn, results in osteoporosis. Ahmed and Abd EL Samad[18], also suggested that HSD altered bone integrity, denoted by enhanced bone resorption, decreased bone formation, and decreased BMD. As well, Fodor et al.,[19]

reported that osteopontin plays some role in the pathophysiology of osteoporosis.

The HSD-induced osteoporosis can be explained by the increased PTH level. When salt intake is increased, calcium excretion in urine increases. This should be compensated by increasing calcium mobilization from bone and absorption from intestine *via* increasing serum PTH and calcitirol respectively[20].

The effect of high serum Na^+ level on bones is not only through increasing the urinary calcium excretion, but also due to a direct stimulatory cell-mediated effect of increased Na^+ concentration on osteoclasts[21]. HSD also induces CD-4 T helper (Th17) cells and impairs the function of regulatory T (Treg) cells. Th17 cells are one of the major cells responsible for enhanced osteoclastogenesis and bone loss by producing higher levels of IL-17, receptor activator of nuclear factor kappa-B ligand (RANKL) and TNF-α, and lower levels of IFN-γ. Treg cells suppress the effector functions of Th17 cells through their production of IL-10 and TGF-β1[22]. Tregs can also lead to suppression of bone loss by inhibiting osteoclast differentiation and functions[23].

The results obtained in the present study showed that HSD did not significantly affect both serum calcium and phosphorous levels compared to the control groups. The insignificant changes of serum calcium level in spite of increased urinary calcium excretion is explained by the consequent increase in PTH and calcitirol levels that resulted in increased bone resorption and intestinal calcium absorption, respectively[2]. The normal serum phosphorous level in HSD groups can also be explained by the balance between increasing its intestinal absorption secondary to increased Na^+ level, and decreasing its renal reabsorption secondary to increased PTH level[24].

On studying the effect of sex difference on the bone response to HSD, the present study demonstrated that HSD-induced osteoporosis was more severe in the female group than in the male group as evidenced by: (a) higher levels of PTH in HSD female group. (b) lesser bone weight, dry weight, fat-free dry weight, ash weight, organic matrix weight, % of non-organic components, and BMD in HSD group. (c) the histopathological results showed more severe osteoporotic changes in the compact and spongy bone tissues in HSD female group in the form of massive distortion of bone structure, more widening of bone marrow cavities, more extensive eroded surface, lesser calcium deposition, and greater thinning of bony trabeculae which were formed mostly of unmineralized bone matrix with few areas of mineralized bone matrix. (d) The immunohistochemical results also showed more intense and extensive expression of osteopontin in the bone matrix in HSD female group when compared to HSD male group.

The more severe HSD-induced osteoporosis in female group comes in line with Wentz et al.,[25], who demonstrated a higher bone fracture rate in female than male. As well, Zborowskiet al.,[26] reported that women with polycystic ovary syndrome who developed variable hyperandrogenism at puberty showed an increased peak bone density compared to age-matched controls.

The lower severity of osteoporosis in HSD male group can be explained by the results of the current study as the body weight gain and BMI are less affected, and PTH level is less elevated in HSD male group compared to HSD female group. The body weight has been positively correlated with BMD, and the increased body weight is thought to exert protective effects on bone through increasing the mechanical loading[27]. Testosterone also inhibits PTH and PTH-induced cAMP production, thus decreasing the osteoclast activity[28].

Beside our previously mentioned results, the protective effect of androgens can be attributed to other several mechanisms. Testosterone may affect osteoblast expression of alkaline phosphatase, osteocalcin, type 1 collagen, and mineralization of extracellular bone matrix, by its stimulatory effects on osteoblast differentiation. It also decreases the osteoblast and osteocyte apoptosis indirectly by regulation of cytokines and growth factors present in bone, upregulation of transforming growth factor (TGF)-β and insulin-like growth factors (IGFs),

which stimulate bone formation[29], and downregulation of IL-6 which stimulates the osteoclastogenesis[30]. Dihydrotestosterone (DHT) reduces osteoprotegerin (OPG) level which is considered a powerful stimulator of osteoclast activity [31]. Androgens also appear to exert their bone protective effects, in part, indirectly through the osteoblasts. Orchiectomy normally results in increased osteoblast precursor cells that indirectly stimulate osteoclast proliferation and activation via RANKL expression, leading to bone resorption and loss[32].

Conclusion:

Administration of HSD (8%) for seven weeks to both male and female rats resulted in osteoporosis. HSD may produce this effect through increasing calcium loss in urine, which stimulates excessive secretion of parathormone hormone with its vigorous resorbing effects on bone. Also HSD may produce induction of Th17 cells along with impairment of Treg cells. Osteoporosis is more severe in female rats than male rats as evidenced by laboratory measurements and histopathological examination. This significant decrease in the severity of osteoporosis in male rats may be due to higher body weight and muscle bulk, inhibition of parathormone secretion, and the different mechanisms of protection exerted by testosterone hormone.

Recommendations:

- The ability of calcium absorption is decreased in postmenopausal women due to the attenuation of vitamin D synthesis. So the compensatory increase in case of loss of calcium due to HSD is impaired. So postmenopausal women must take care of their diet salt content more than younger women
- Hypernatremia is a dangerous situation. Dehydration can lead to increased NaCl concentration in the body. Old people are known to lose the appropriate thirst to balance their water intake so they are liable to dehydration and consequently to all hazards of HSD. So the amount of water and salt intake must be taken seriously in old people.
- For renal patients, they must observe their diet and reduce table salt for prevention of more electrolyte imbalance and kidney injury.
- Further studies can be done to evaluate the protective effects of sex hormones by doing orchiectomy for male rats and ovariectomy to female rats.

References

1. Lavoie, B., Lian, J. B., and Mawe, G. M. (2017). Regulation of bone metabolism by serotonin. In "Understanding the Gut-Bone Signaling Axis", pp. 35-46.
2. Vafa, M., Soltani, S., Zayeri, F., Niroomand, M., and Najarzadeh, A. (2016). The relationship between sodium intake and some bone minerals and osteoporosis risk assessment instrument in postmenopausal women. Medical journal of the Islamic Republic of Iran 30, 377.
3. Brown, I. J., Tzoulaki, I., Candeias, V., and Elliott, P. (2009). Salt intakes around the world: implications for public health. International journal of epidemiology 38, 791-813.
4. Hoffmann, I. S., and Cubeddu, L. X. (2009). Salt and the metabolic syndrome. Nutrition, Metabolism and Cardiovascular Diseases 19, 123-128.
5. Massey, L. K. (2005). Effect of dietary salt intake on circadian calcium metabolism, bone turnover, and calcium oxalate kidney stone risk in postmenopausal women. Nutrition Research 25, 891-903.
6. Dempster, D. W., and Raisz, L. G. (2015). Bone physiology: Bone cells, modeling, and remodeling. In "Nutrition and Bone Health", pp. 37-56.
7. Oloyo, A. K., Imaga, N. O., Fatope, Y., and Sofola, O. A. (2019). Sex differences in cardiac and renal responses to a high salt diet in Sprague-Dawley rats. Heliyon 5, e01665.
8. Novelli, E., Diniz, Y., Galhardi, C., Ebaid, G., Rodrigues, H., Mani, F., Fernandes, A. A. H., Cicogna, A. C., and Novelli Filho, J. (2007). Anthropometrical parameters and markers of obesity in rats. Laboratory animals 41, 111-119.
9. Keenan, M. J., Hegsted, M., Jones, K. L., Delany, J. P., Kime, J. C., Melancon, L. E., Tulley, R. T., and Hong, K. D. (1997). Comparison of bone density measurement techniques: DXA and Archimedes' principle. Journal of bone and mineral research 12, 1903-1907.
10. Brzoska, M., Moniuszko-Jakoniuk, J., Jurczuk, M., Gałażyn-Sidorczuk, M., and

Rogalska, J. (2001). The effect of zinc supply on cadmium-induced changes in the tibia of rats. Food and chemical toxicology 39, 729-737.
11. WATKINS, K. L., and Southern, L. L. (1992). Effect of dietary sodium zeolite A and graded levels of calcium and phosphorus on growth, plasma, and tibia characteristics of chicks. Poultry science 71, 1048-1058.
12. Zhang, B., and Coon, C. N. (1997). The relationship of various tibia bone measurements in hens. Poultry Science76,1698-1701.
13. Bancroft, J. D., and Layton, C. (2013). The hematoxylins and eosin. Bancroft's Theory and Practice of Histological Techniques, 173-186.
14. Chong, W. C., Wu, R., and Tu, A. (2012). A study on tissue processing. International Journal of Innovative Interdisciplinary Research 1, 37-43.
15. Ali, D., Abdelzaher, W., and Abdel-Hafez, S. (2018). Evaluation of the rivastigmine role against botulinum toxin-A-induced osteoporosis in albino rats: A biochemical, histological, and immunohistochemical study. Human & experimental toxicology 37, 1323-1335.
16. Alrabadi, N., Al-Rabadi, G. J., Maraqa, R., Sarayrah, H., Alzoubi, K. H., Alqudah, M., and Al-U'datt D, G. (2020). Androgen effect on body weight and behaviour of male and female rats: novel insight on the clinical value. Andrologia, e13730.
17. Fatahi, S., Namazi, N., Larijani, B., and Azadbakht, L. (2018). The Association of Dietary and Urinary Sodium With Bone Mineral Density and Risk of Osteoporosis: A Systematic Review and Meta-Analysis. J Am Coll Nutr 37, 522-532.
18. Ahmed, M. A., and Abd El Samad, A. A. (2013a). Benefits of omega-3 fatty acid against bone changes in salt-loaded rats: possible role of kidney. Physiol Rep 1, e00106.
19. Fodor, D., Bondor, C., Albu, A., Simon, S.-p., Craciun, A., and Muntean, L. (2013). The value of osteopontin in the assessment of bone mineral density status in postmenopausal women. Journal of Investigative Medicine 61, 15-21.
20. Robinson, A. T., Edwards, D. G., and Farquhar, W. B. (2019). The Influence of Dietary Salt Beyond Blood Pressure. Curr Hypertens Rep 21, 42.
21. Wu, L., Luthringer, B., Feyerabend, F., Zhang, Z., Machens, H., Maeda, M., Taipaleenmäki, H., Hesse, E., Willumeit-Römer, R., and Schilling, A. (2017). Increased levels of sodium chloride directly increase osteoclastic differentiation and resorption in mice and men. Osteoporosis International 28, 3215-3228.
22. Dar, H., Singh, A., Shukla, P., Anupam, R., Mondal, R., Mishra, P., and Srivastava, R. (2018a). High dietary salt intake correlates with modulated Th17-Treg cell balance resulting in enhanced bone loss and impaired bone-microarchitecture in male mice. Scientific Reports 8.
23. Yuan, F. L. et al., Regulatory T cells as a potent target for controlling bone loss. Biochem. Bio phys Res Commun 402, 173–176 (2010).
24. Liu, Z., Qi, H., Liu, B., Liu, K., Wu, J., Cao, H., Zhang, J., Yan, Y., He, Y., and Zhang, L. (2017). Genetic susceptibility to salt-sensitive hypertension in a Han Chinese population: a validation study of candidate genes. Hypertension Research 40, 876-884.
25. Wentz, L., Liu, P.-Y., Haymes, E., and Ilich, J. Z. (2011). Females have a greater incidence of stress fractures than males in both military and athletic populations: a systemic review. Military medicine 176, 420-430.
26. Zborowski, J. V., Cauley, J. A., Talbott, E. O., Guzick, D. S., and Winters, S. J. (2000). Bone mineral density, androgens, and the polycystic ovary: the complex and controversial issue of androgenic influence in female bone. The Journal of Clinical Endocrinology & Metabolism 85, 3496-3506.
27. Crepaldi, G., Romanato, G., Tonin, P., and Maggi, S. (2007). Osteoporosis and body composition. Journal of endocrinological investigation 30, 42-47.
28. Pilbeam, C. C., and Raisz, L. G. (1990). Effects of androgens on parathyroid hormone and interleukin-1-stimulated prostaglandin production in cultured neonatal mouse calvariae. Journal of Bone and Mineral Research 5, 1183-1188.
29. Kasperk, C. H., Wakley, G. K., Hierl, T., and Ziegler, R. (1997). Gonadal and adrenal androgens are potent regulators of human

bone cell metabolism in vitro. Journal of Bone and Mineral Research 12, 464-471.
30. Callewaert, F., Boonen, S., and Vanderschueren, D. (2010). Sex steroids and the male skeleton: a tale of two hormones. Trends in Endocrinology & Metabolism 21, 89-95.
31. Cheng, B.-H., Chu, T.-M. G., Chang, C., Kang, H.-Y., and Huang, K.-E. (2013a). Testosterone delivered with a scaffold is as effective as bone morphologic protein-2 in promoting the repair of critical- size segmental defect of femoral bone in mice. PLoS One 8 e70234.
32. Clarke B L, M.D. and Khosla S, M.D.: Androgens and Bone. Steroids. (2009) Mar; 74(3): 296–305.

استكشاف كيف يؤثر الاختلاف بين الجنسين على استجابة العظام لنظام غذائي عالي الملح في الجرذان البالغة البيضاء

عادل حسين سعد[١]، هناء محمد ابراهيم[١]، وجدى نشأت حبيب[١] وسارة محمد عبد الحفيظ[٢]
قسم الفسيولوجى[١] والهستولوجى[٢]، كلية الطب، جامعة المنيا

الخلفية والهدف من البحث: يرتبط تناول كميات كبيرة من الملح بارتفاع مخاطر الإصابة بالعديد من الأمراض مثل ارتفاع ضغط الدم وأمراض القلب والأوعية الدموية ومتلازمة التمثيل الغذائي وهشاشة العظام. يهدف هذا العمل إلى تقييم آثار النظام الغذائي عالي الملح على عظام الجرذان البالغة البيضاء واستكشاف التباين الجنسي لهذا التأثير وأسبابه. **المواد والطرق المستخدمه:** تم تقسيم ٤٨ جرذًا بالغًا بيضاء ٢٤ ذكورًا و ٢٤ إناثًا ، إلى أربع مجموعات:مجموعة الذكور الضابطة ، ومجموعة الإناث الضابطة ، ومجموعة الذكور الذين تغذوا على نظام غذائي عالى الملح ، ومجموعة الإناث التي تغذت على نظام غذائي عالى الملح. **النتائج:** أظهرت النتائج أن إعطاء (8٪) نظام غذائي عالى الملح لمدة سبعة أسابيع لكل من ذكور وإناث الجرذان أدى إلى زيادة تناول الطعام ، وانخفاض في زيادة وزن الجسم ومؤشر كتلة الجسم ، وكذلك هشاشة العظام. تم إثبات هشاشة العظام من خلال زيادة هرمون الباراثيرمون وعلامات تحلل العظام ؛ الفوسفاتيز القلوي ، الفوسفاتاز الحمضي ، ومستويات osteopontin. تم الكشف عن انخفاض وزن العظم ، الوزن الجاف ، الوزن الجاف الخالي من الدهون ، وزن الرماد ، وزن المادة الأساس العضوية ، ونسبة الوزن غير العضوي. كانت هشاشة العظام أكثر حدة في إناث الجرذان من ذكور الجرذان. **الخلاصة:** وفقًا لذلك ، كشفت النتائج التي تم الحصول عليها من هذه الدراسة أن شدة هشاشة العظام التي يسببها النظام غذائى عالى الملح تعتمد على الجنس. قد يُعزى انخفاض شدة هشاشة العظام عند الذكور عن الاناث إلى ارتفاع وزن الجسم وكتلة العضلات ، والتأثير المثبط لهرمون التستوستيرون على الباراثورمون ، وآليات أخرى مختلفة للحماية التي قد يسببها هرمون التستوستيرون.