EFFECT OF GENDER, GENOTYPE, AND DIETARY SODIUM CHLORIDE ON URINARY CALCIUM AND BONE MINERAL CONTENT

Honors Thesis

Presented in Partial Fulfillment of the Requirements for Graduation with Distinction

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INTRODUCTION AND BACKGROUND

Calcium has always been associated with the terms “bone loss” and “osteoporosis”. Has it ever occurred that along with calcium, another mineral such as sodium could be involved? Sodium is known to be linked with urinary calcium excretion and bone loss (1-2). Over time, this bone loss may have an impact on bringing about osteoporosis. In a study done on young females, aged 8-13, it was discovered that urinary sodium was one of the biggest determinants of urinary calcium output (3). In the early years of puberty, ample bone mass and calcium balance are essential for development and growth. In order for a child to increase skeletal mass and enhance development, it is necessary to be in positive calcium equilibrium. The results of the study indicate that urinary calcium excretion is inversely related to the bone mass of young females. Consequently, as more calcium is found in the urine, a decline in bone mass is detected (3). For adults, calcium is mainly needed in bones in order to prevent breakage and degradation (3).

There have been many studies done in humans and animals regarding bone mass, urinary calcium excretion, and dietary sodium chloride intake (1,3-6). The studies that involve human subjects have many limitations such as failure in following guidelines, lack of 24-hour supervision, and inadequacy of reporting accurate results. Due to these conditions, many subjects are eliminated from the study. On the other hand, studies done on rats are more controlled since the rat diet is completely determined by the investigator. The researcher knows the exact activity and consumption of the rat. Consequently, studies done in rats tend to be more defined and accurate.
Dietary sodium chloride increasing urinary calcium levels is observed in a majority of experiments. However, in each example, there are variations in the amounts of sodium chloride required, calcium excreted, and bone mass retained (4-5). These differences occur because each individual has a distinct sensitivity to the ions, sodium and calcium. Factors such as age, sex, genetics, diet, and environment help explain these variations (1). For example, postmenopausal women tend to lose more calcium from bone than premenopausal women (1). In another study, elderly women were either placed on a low salt diet (~70 mmol/day) or were given salt supplements (~170 mmol/day) for ten days. Any additional consumption of salt was avoided. A twenty-four-hour urine sample collected on the tenth day of the high salt diet showed a 30% increase in the excretion of hydroxyproline in the urine. This indicates increased resorption of bone due to increased parathyroid activity (4). Likewise, urinary calcium excretion rose about 25% in the females that were on the high salt diet. Therefore, variations in sodium chloride intake do influence the resorption and formation mechanisms of bone (4).

The main mechanism behind the sodium-calcium-bone connection involves the role of parathyroid hormone (PTH). PTH, along with 1,25-dihydroxy vitamin D and calcitonin, regulates the plasma concentration of calcium (7). Serum concentration of calcium is tightly regulated. As soon as it decreases, PTH is released and calcium is moved into the extracellular fluid, thus bringing the calcium concentration back to a homeostatic level (8). This is the role of PTH in the bone.

In the kidney, PTH functions to increase calcium retention (8). Sodium and calcium interact in the proximal and distal tubules of the kidney. Although sodium is filtered at the glomerulus, 99% of it is reabsorbed back into the body. Therefore, about
1% of the filtered sodium is excreted into the urine. This holds true for a healthy individual who does not have any renal complication (9). Calcium is also reabsorbed in the kidney and therefore, affected by sodium (5,10). The calcium reabsorbed in the proximal tubule of the kidney is closely linked to sodium, and independent of PTH. PTH only influences the calcium that is reabsorbed in the ascending limb of Henle and the distal convoluted tubule. Together these account for only 15% of the calcium that is reabsorbed. Sixty-five percent of calcium reabsorption is paralleled with sodium. Thus, an increased dietary source of sodium chloride will cause more urinary sodium, which in turn will pull more calcium into the urine (8-9).

It has also been shown that sodium alone does not cause calcium excretion. In fact, the combination of both Na and Cl is the major cause of calcium excretion. The chloride ion does play a role because studies done with sodium bicarbonate and sodium citrate indicate no increase in urinary calcium excretion. The chloride ion contributes to increased plasma volume and acid production (11-12). In addition, experiments have been done with KCl to test the importance of the sodium ion (13). It was found that KCl also has no effect on urinary calcium. Therefore, it is the combination of sodium and chloride that plays a role in urinary calcium excretion (1).

High/low protein diets have also been tested to see if they influence calcium and bone mass. A high protein diet does decrease the reabsorption of calcium, but not to the extent that a high sodium chloride diet does. Goulding and associates (6) actually showed a high protein diet to increase the phosphate in bone. This is beneficial to the bone since phosphorous is the second most abundant mineral in bone.
PROBLEM IDENTIFICATION AND JUSTIFICATION

The rats used in this study are all lean SHHF/ Mcc-fa^{GP} (abbreviated SHHF) rats. This model is selectively bred for spontaneous hypertension and heart failure (14). The Mcc stands for McCune, and the fa^{GP} indicates that many members of the family carry the cp gene. This strain of rats are at risk for hypertension and all of them develop congestive heart failure (CHF) (15-16). Gender and genotype are the two main factors that determine the age of onset of CHF (15). The SHHF homozygote lean (+/+ ) do not carry a defect in the leptin-receptor whereas the SHHF heterozygote lean (+/fa^{GP} ) have one gene for the leptin-receptor defect (16). Leptin is a hormone that is known to increase bone growth (17). It is a product of the "obesity gene" and is secreted from adipose tissue. Since the heterozygotes have a deficiency in the leptin-receptor, they may have a decreased size and density of bone as compared to the homozygotes.

An earlier study indicated that an 8.0% salt diet brings heart failure earlier in lean male SHHF rats (15). The high salt group has a shorter life span than the low salt group (14.8 mo vs. 21.8 mo). From this study, there is also some indication that the high salt seems to have a greater effect on the life span of the homozygote lean as compared to the heterozygote lean (McCune unpublished results). Therefore, part of the reason for this second experiment involving a high salt diet is to see if this difference can be repeated again in the lean SHHF males and to see if SHHF females show a similar response.

High doses of NaCl have been observed to have a destructive effect on the bone mass of rats (1,6). Studies have shown that the bone loss is due to an increase in bone resorption, not due to a decrease in bone formation. The reduction in bone mass is due to the loss of calcium, the most abundant mineral in bones (6). Goulding et al (6) showed
that salt supplements do increase urinary calcium although the rats were taking a sufficient amount of dietary calcium (0.60%). The rats used in our experiments are also taking an adequate amount of calcium (0.93%). Therefore, there must be a link between sodium and calcium.

The above relationship is very important to understand because it has a strong influence on our lives. For example, we may be able to fight osteoporosis not only by increasing calcium, but also by decreasing sodium chloride. What is the point of taking calcium supplements if we are consuming a high sodium chloride diet? Most of the calcium will simply exit our bodies through urine. Aside from bone density, calcium also plays a role in many other events: muscular contraction, neurotransmission, membrane transport, enzyme reactions, hormone secretion, and blood coagulation (9). These other roles are all very important for proper functioning of the body.

In order to carefully assess urinary calcium excretion, precise values need to be measured. It is not certain whether frozen urine samples will yield valid results. Studies done in humans suggested that a urine sample should be acidified to a pH of 1.5-2.5 upon collection (18). Since Ca\(^{++}\) is a positively charged ion, it will attract and bind to negative charges. If a solution is basic (-), then Ca\(^{++}\) will be bound to the negative charges. If a solution is acidic (+), then Ca\(^{++}\) will most likely be unbound and free floating. This is the mechanism behind acidifying a solution before taking a calcium measurement (8, 18-20). Ng and associates (18) also recommended heating and centrifuging the urine samples before assessing calcium. Since we had previously frozen (at -20°C) urine samples, we needed to observe the effects of acidifying, heating, and freezing on the level of urinary calcium measured.
From the information mentioned above, it is evident that a relationship between sodium, calcium, and bone mass exists, but the goal of this study was to explore how it affects lean male and female SHHF rats. Another extension of this project was to accurately measure urinary calcium excretion, and then look at how it was related to dietary salt and bone mineral content (BMC).

**OBJECTIVES**

The object of the preliminary experiment is to see how the following will affect the urinary calcium measurement:

1. Various treatments of urine, such as acid and/or heat.
2. Different storage techniques, such as fresh and frozen samples.

The preliminary experiment hypotheses to be tested are:

1. Adding acid to the urine will increase the urinary calcium value.
2. Heating will not have a significant effect.
3. Fresh and frozen (at -20°C) urine samples will give similar results.

The object of the experiment is to see how different doses of NaCl will affect SHHF rats. The following criteria will be observed as a function of the high salt/low salt diet:

1. Determine the differences in urinary calcium excretion.
2. Determine the changes in bone mineral content, with different levels of dietary salt.
3. Determine how gender plays a role in the sodium-calcium relationship.
4. Determine if genotype has any role in sensitivity to sodium.
The hypotheses to be tested are:

1. High salt rats will excrete more calcium in their urine than the low salt rats.
2. Male SHHF rats will have a higher bone mineral content than female SHHF rats.
3. The low salt rats will have a higher bone mineral content than the high salt rats in each male/female category.
4. Heterozygote lean male SHHF rats will excrete more calcium into the urine than homozygote lean male SHHF rats.

**METHODS AND PROCEDURES**

*Animals*

SHHF lean male and female rats were used in this study. Group A rats (8 males) were about 1.5 months older than Group B rats (10 males and 16 females). The zygosity was known for the Group A rats. Hence, a comparison between urinary calcium and zygosity could be observed. All rats were about 2.5 months old at the beginning of the study. The rats were housed and bred in Dr. McCune’s colony at The Ohio State University. The animals were caged in pairs in a temperature-controlled environment (23° C) with a half light-half dark 24 hour cycle. At the age of 2.5 months, Group A and Group B rats were either placed on a high salt or low sat diet. Beginning with Group A, half the males were put on a high salt diet and the other half on a low salt diet. Out of Group B, 6 males and 8 females were placed on a high salt diet and 4 males and 8 females received the low salt diet. The high salt diet (TD 92012) had 8.0% NaCl. The low salt diet (TD 7034) contained 0.21% NaCl. A normal salt diet in rats would be
around 1.0% NaCl. The other nutrients such as protein, fat, and calcium were the same for the two diets. The rats had access to chow and water ad libitum.

Collection of data

Animals were put in individual Nalgene metabolic cages for collection of 24-hour urine samples. The rats were allowed water ad libitum but no food was permitted in the metabolic cage (except for the one experiment that was done to see the differences in fasted vs. fed calcium excretion). The urine sample was immediately stored at -20°C until it was used for an assay. Twenty-four-hour urine samples were collected from all of the rats in the study at 3, 11 and 13 months of age. In Group B, an additional urine sample was taken at 8 months of age.

Bone mass measurements

A bone mass measurement for each rat was performed initially at 3 months and then at 11 months of age. All measurements were done by using dual energy X-ray absorptiometry (DXA) with a Lunar DPX-L machine (Dr. John Landoll, Department of Physical Medicine, performed the procedure and provided a print out of the whole body scan). DXA was used because it was non-invasive so sacrifice of the rat was not required for the calculation of the bone mineral density (BMD). The whole-body of the rat was scanned in a standard position (See Appendix). In order to prevent movement of the rat, intraperitoneal (~50mg ketamine and 5 mg xylazime/ kg body wt.) was injected about 30 minutes prior to the bone scan. Bone mineral content (BMC) was expressed in grams and
divided by the measured area of the bone to determine the BMD in g/cm² (3). In this experiment, the machine used assumed that 38% of the bone was calcium.

Assay Procedures

Calcium assays were performed on the urine samples. To determine total urinary calcium, a Sigma Calcium Kit was used (20). This kit utilized a calcium-binding dye, o-cresolphthalein complexone (CPC) which changed color when it selectively binded calcium (8-9). In a basic solution, CPC formed a red chromophore with calcium, which was measured at 575 nm on a spectrophotometer (8-9,20).

Preparation of urine sample

Twenty-four hour urine samples were tested for calcium in the various combinations listed in the table below.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frozen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Acid</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Heat</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Urine samples are tested within 4 hours of collection.

b Samples are frozen at -20°C within 2 hours of collection.
c 10 μL of 6N HCl was added to a 1-mL aliquot of urine (18-20).
d A 1-mL aliquot was heated for 10 minutes at 56°C (18).
**Statistics**

SPSS was the computer program we used to analyze the results. ANOVA (Analysis of Variance) was used in performing multiple comparisons. The paired t-test and also general linear model repeated measures were also used in analyzing the data. All data are presented as mean ± SEM (Standard Error of Mean). Differences are considered statistically significant at p< 0.05.
RESULTS AND DISCUSSION

Preliminary experiment

Table 1 shows the results of heat vs. no heat. There was no significant difference between heating and not heating the sample. The averages of the samples were very close in value.

Table 1 (mg Ca\textsuperscript{++}/ 24 hrs.)
The effect of heat vs. no heat on urinary calcium measurements

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Heat</th>
<th>No-Heat</th>
</tr>
</thead>
<tbody>
<tr>
<td>6481</td>
<td>2.76</td>
<td>2.57</td>
</tr>
<tr>
<td>6498</td>
<td>1.03</td>
<td>1.03</td>
</tr>
<tr>
<td>6500</td>
<td>1.24</td>
<td>1.29</td>
</tr>
<tr>
<td>6511</td>
<td>1.87</td>
<td>2.08</td>
</tr>
<tr>
<td>6522</td>
<td>1.72</td>
<td>1.70</td>
</tr>
<tr>
<td>6523</td>
<td>1.46</td>
<td>1.50</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>1.68 ± 0.25</td>
<td>1.70 ± 0.23</td>
</tr>
</tbody>
</table>

Table 2 shows the outcome of acidifying frozen and fresh samples of urine for ten rats. For the ten urine samples, there were no significant differences among the various sample treatments. The means of all four methods yielded comparable results. No consistent pattern was seen.
Repeat tests to determine accuracy of acidifying urine

<table>
<thead>
<tr>
<th></th>
<th>Assayed fresh</th>
<th>Assayed frozen</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 1.5</td>
<td>Untreated</td>
<td>pH 1.5</td>
</tr>
<tr>
<td>2.26</td>
<td>2.42</td>
<td>2.34</td>
</tr>
<tr>
<td>2.95</td>
<td>3.35</td>
<td>2.61</td>
</tr>
<tr>
<td>1.62</td>
<td>1.67</td>
<td>1.59</td>
</tr>
<tr>
<td>1.37</td>
<td>1.44</td>
<td>1.50</td>
</tr>
<tr>
<td>1.90</td>
<td>2.03</td>
<td>1.82</td>
</tr>
<tr>
<td>1.18</td>
<td>1.18</td>
<td>1.01</td>
</tr>
<tr>
<td>1.70</td>
<td>1.80</td>
<td>1.68</td>
</tr>
<tr>
<td>0.76</td>
<td>0.84</td>
<td>0.89</td>
</tr>
<tr>
<td>2.02</td>
<td>2.09</td>
<td>1.79</td>
</tr>
<tr>
<td>1.64</td>
<td>1.69</td>
<td>1.51</td>
</tr>
<tr>
<td>Mean</td>
<td>1.74</td>
<td>1.67</td>
</tr>
<tr>
<td>SEM</td>
<td>0.19</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Ng and associates (18) also found no significant difference for urine samples that were acid-treated to pH 1.5, pH 9, and untreated. However, there was a noticeable discrepancy between the numbers as the concentrations of calcium increased. Yet at low concentrations, the values were similar (18). Since we were measuring low concentrations of calcium, this may be the reason why no discrepancy was seen. If the samples were not assayed fresh, they were frozen (at -20°C), not just refrigerated (at 4°C). Ng and associates (18) observed that calcium was lost when the samples were untreated and stored at 4°C. Although our samples were also untreated, they were frozen. This may also account for the consistency of the results and the fact that acid/no-acid or heat/no-heat do not make a difference. Therefore, in this experiment the hypothesis of acid increasing urinary calcium values was proved false.
Fasted vs. Fed

At 8 months of age, urine was collected for Group B rats in both the fasted and fed state. Figure 1 shows the differences in urinary calcium excretion among the HSM (high salt males, n=6), LSM (low salt males, n=4), HSF (high salt females, n=8), and LSF (low salt females, n=8) with respect to the fasted vs. fed state.

Figure 1. Urine calcium excretion for HSM (high salt males, n=6), LSM (low salt males, n=4), HSF (high salt females, n=8), and LSF (low salt females, n=8) in the fasted and fed state. Error bar represents SEM. Significant differences are described in Table 3.
Table 3  Urine Calcium Excretion (Fasted vs. Fed)

<table>
<thead>
<tr>
<th></th>
<th>Fasted</th>
<th>Fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSM</td>
<td>1.18 ± 0.20*&lt;sub&gt;a&lt;/sub&gt;</td>
<td>2.65 ± 0.43*&lt;sub&gt;c&lt;/sub&gt;</td>
</tr>
<tr>
<td>LSM</td>
<td>0.73 ± 0.15&lt;sub&gt;a&lt;/sub&gt;</td>
<td>0.39 ± 0.04&lt;sub&gt;d&lt;/sub&gt;</td>
</tr>
<tr>
<td>HSF</td>
<td>2.65 ± 0.26*&lt;sub&gt;b&lt;/sub&gt;</td>
<td>5.24 ± 0.51*&lt;sub&gt;e&lt;/sub&gt;</td>
</tr>
<tr>
<td>LSF</td>
<td>2.13 ± 0.15*&lt;sub&gt;b&lt;/sub&gt;</td>
<td>0.97 ± 0.06*&lt;sub&gt;d&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

* Significant differences between fasted and fed within a group
** Different letters of the same column indicate significant difference due to diet and/or gender.

From the fasted to the fed state, HSM and HSF significantly increased urinary calcium, whereas LSF showed a significant decrease in urinary calcium. LSM probably did not show a notable difference because the sample size was small, consisting of only 4 rats. These observations can not be explained thoroughly but it is thought that an increase in dietary sodium for the HS groups will cause more calcium to be excreted in the urine. The LS groups are probably deficient in sodium, so in the fed state when they do acquire some sodium, they tend to reabsorb it instead of excreting it into the urine (8-9). Another explanation could be related to the mechanism of the PTH. In a normal or low sodium diet, NaCl would decrease serum calcium and therefore trigger the release of PTH. PTH would then stimulate calcium absorption by releasing 1,25-dihydroxy vitamin D (1). This expected mechanism does not work under high sodium intake because urinary calcium is being excreted at high levels so it is not being absorbed (6). Possible reasons could be that excessive salt inhibits the PTH reaction, or that high salt may depress the calcium intestinal absorption response (6).

The purpose of doing this experiment between the fasted vs. fed was to show that there was an important distinction between the two conditions. Therefore, one must be consistent in comparing results over time, either the fasted state or the fed state should be
used. In this experiment, urine was collected in the fasted state at 3, 8, 11, and 13 months of age.

**Bone Mineral Content and Bone Mineral Density**

Figure 2 displays the results of female and male BMC. Figure 3 represents the BMD of females and males. BMD is calculated from BMC by dividing the rat’s measured area of bone. Therefore, there is a high correlation between BMC and BMD.

![Graph showing BMC and BMD](image)

**Figure 2.** Bone mineral content (BMC) of male and female SHHF rats at 3 and 11 months of age. Error bar represents SEM. Significant differences among the various groups are shown in Table 4. HSF (high salt females, n=8), LSF (low salt females, n=8), HSM (high salt males, n=10), LSM (low salt males, n=8)
For BMC, at 3 and 11 months of age, a significant sex difference was observed. The BMC of the females was considerably lower than that of the males. This was expected because males have more bone and muscle mass than females (21). At 3 months, there was no difference between the HSM and the LSM, as well as the HSF and the LSF. This was anticipated because at this point the rats were not on altered diets. At 11 months, there was a significant increase in BMC for both the males and females as compared to the 3 month age group. This was also predicted because the rats were growing and developing skeletal mass. The increase in the males was almost double that of the females. There was also a significant difference between the diets in the males at 11 months. The low salt group had a higher BMC than the high salt group. This observation followed with the hypothesis that the low salt rats retain more calcium in the urine than the high salt rats. The dietary sodium had a greater effect on males since they have more bone mass than females (3,21).

Goulding and associates (6, 22) have studied the effects of dietary sodium on bone content. In adult female oophorectomized rats, high NaCl did promote bone loss of the rat (22). In addition, male albino rats, which consumed salt supplements, also showed a decrease in mineral ash, calcium, and phosphate from bone as compared to the controls, which did not receive salt (6). Direct skeletal effects of sodium have been seen in many rat models, but only one human study has shown that sodium intake directly influences

<table>
<thead>
<tr>
<th></th>
<th>HSF</th>
<th>LSF</th>
<th>HSM</th>
<th>LSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mo</td>
<td>4.11 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.12 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.76 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.69 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>11 mo</td>
<td>5.96 ± 0.18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.67 ± 0.13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.92 ± 0.34&lt;sup&gt;*d&lt;/sup&gt;</td>
<td>12.02 ± 0.37&lt;sup&gt;*d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Significant effect of diet within the same gender
<sup>a-d</sup> Different letters of the same row indicate significant differences due to diet and/or gender.

"Table 4  BMC With Age"
BMD (23). It may be harder to produce such results in humans because human subjects tend to be more difficult to observe in long-term studies. Therefore, it is more feasible to perform prolonged studies on rats than on humans.

**Figure 3.** Bone mineral density (BMD) of male and female SHHF rats at 3 and 11 months of age. Error bar represents SEM. Table 5 summarizes the significant differences among various groups. HSF (high salt females, n=8), LSF (low salt females, n=8), HSM (high salt males, n=10), LSM (low salt males, n=8)

**Table 5 BMD With Age**

<table>
<thead>
<tr>
<th></th>
<th>HSF</th>
<th>LSF</th>
<th>HSM</th>
<th>LSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mo</td>
<td>0.28 ± 1.7×10⁻³&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.28 ± 1.1×10⁻³&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.28 ± 1.5×10⁻³&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.28 ± 1.5×10⁻³&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>11 mo</td>
<td>0.29 ± 1.6×10⁻³&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.30 ± 1.1×10⁻³&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.32 ± 1.6×10⁻³&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.33 ± 2.2×10⁻³&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Significant effect of diet within same gender
** Different letters of the same row indicate significant differences due to diet and/or gender.
A similar trend was seen with BMD (Figure 3). Since BMD took into account the projected area of the bones, the deviation among the rats was very low. The SEM was in the range of $1 \times 10^{-3}$ to $3 \times 10^{-3}$, thus, at 11 months each group was significantly different from the other groups. Although the numbers were very close, the low salt rats in each group were still higher than the high salt rats.

**Urinary Calcium Excretion**

Figure 4 summarizes urinary calcium excretion over time for females and males.

![Graph showing urinary calcium excretion over time for different groups.](image)

**Figure 4.** Urine calcium excretion for HSM (high salt males, n=6), LSM (low salt males, n=4), HSF (high salt females, n=8), and LSF (low salt females, n=8) for Group B SHHF rats at 3, 8, 11, and 13 months of age. Error bar represents SEM.
At each time period, with the exception of 3 months, the high salt rats were excreting more calcium in the urine than the low salt rats of the same gender. The only significant difference noted with diet was in the males at 13 months. The deviation of the 13 month high salt male rats was elevated because the two rats that died of CHF excreted large amounts of calcium in the urine (≈2.60 mg/24 h for each rat). The statistical analysis, not including the two expired rats, also indicated a significant difference in diet of the males at 13 months of age. The mean was 1.97 ± 0.21 for the four living rats, whereas the mean for all the rats was 2.74 ± 0.57. Hence, an increase from the 11 month interval was still observed.

There was a significant sex difference at 3 and 8 months of age. At 11 months, there was only a significant difference of calcium excretion between the LSM and HSF. After 8 months of age, a decline in the female urine calcium was noticed; however, there were no significant differences. The results of Figure 4 supported the hypothesis that the high salt rats excreted more calcium in the urine than low salt rats of the same gender. This was a result of the increased dietary sodium chloride consumed by the high salt rat (1).

**Heterozygote/ Homozygote Effect**

Figure 5 summarizes the percentage change in BMD, BMC, and BW (body weight) of HSHO (high salt homozygote, n=3), HSHE (high salt heterozygote, n=3), LSHO (low salt homozygote, n=3), and LSHE (low salt heterozygote, n=3) SHHF rats. These statistics were based on the zygosity that we knew for 12 SHHF male rats.
Figure 5. Percentage change of BMD, BMC, and BW (body weight) from 3 months of age to 11 months of age. Percentage change measured in 12 male SHHF rats. Error bar shows SEM. Significant differences are described in Table 6. HSHO (high salt homozygote, n=3), HSHE (high salt heterozygote, n=3), LSHO (low salt homozygote, n=3), LSHE (low salt heterozygote, n=3)

Table 6  Percentage change of BMD, BMC, and BW over a period of 8 months

<table>
<thead>
<tr>
<th></th>
<th>HSHO</th>
<th>HSHE</th>
<th>LSHO</th>
<th>LSHE</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD</td>
<td>111 ± 1.39&lt;sub&gt;ab&lt;/sub&gt;</td>
<td>109 ± 0.14&lt;sub&gt;a&lt;/sub&gt;</td>
<td>117 ± 1.62&lt;sub&gt;b&lt;/sub&gt;</td>
<td>116 ± 0.68&lt;sub&gt;b&lt;/sub&gt;</td>
</tr>
<tr>
<td>BMC</td>
<td>187 ± 4.05&lt;sub&gt;cd&lt;/sub&gt;</td>
<td>173 ± 6.22&lt;sub&gt;c&lt;/sub&gt;</td>
<td>212 ± 7.60&lt;sub&gt;c&lt;/sub&gt;</td>
<td>208 ± 1.40&lt;sub&gt;de&lt;/sub&gt;</td>
</tr>
<tr>
<td>BW</td>
<td>136 ± 4.28&lt;sub&gt;fg&lt;/sub&gt;</td>
<td>128 ± 1.51&lt;sub&gt;f&lt;/sub&gt;</td>
<td>148 ± 2.39&lt;sub&gt;g&lt;/sub&gt;</td>
<td>142 ± 2.06&lt;sub&gt;fg&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

<sup>a-g</sup> Different letters of the same row indicate significant differences due to zygosity and/or diet.

A very high correlation (>0.921) was detected between BW gain and BMD and BMC. LS rats were heavier, whereas HS rats were lighter.
In each case, the HSHE had the smallest percentage increase, whereas the LSHO had the largest percentage increase in the 8 month period. The other 2 groups, HSHO and LSHE fell in the middle range of percentage increase. This trend went along with the hypothesis: In relation to CHF, HE showed more sensitivity to salt (15, McCune unpublished results). Therefore, they may have excreted more calcium, which in turn decreased the amount of calcium in bone. The increased sensitivity to salt may be a result of the leptin receptor defect carried by the HE (16). The HSHE would be the most susceptible to salt because it has two conditions working against the retention of calcium; thus, the percentage increases of BMD, BMC, and BW would be the least.

To date, four rats have suffered CHF; they were all male HSHE rats. This outcome supports the relationships discussed in the study. High sodium chloride intake has a negative influence on bone mass of SHHF rats. High urinary calcium loss reduces skeletal calcium retention, which decreases the growth of the SHHF rat (3). Gender and genotype function as the main determinants of bringing on the age of CHF (15).

Although the results show indications of a zygosity effect, the statistics are a product of only 12 SHHF male rats. A larger group of SHHF rats would supply more insight and knowledge of the HE/HO differences.
SUMMARY AND CONCLUSIONS

The results of the study expand the knowledge accumulated in previous investigations of the relation between sodium intake, urinary calcium excretion, and bone mineral content of SHHF rats. The mechanisms of the human are similar to that of the rat. Nutritional factors influence bone mineral content and bone mineral density. Therefore, as in the SHHF rat, it has been shown that dietary sodium chloride increases urinary calcium excretion which in turn decreases bone mass in humans as well (1, 3-6). This deficiency of calcium and loss of bone mass may result in osteoporosis, one of the major diseases of old age (21). This study has definitely shown that a relationship exists between sodium, calcium, and bone content. However, further investigations are needed in order to deduce the exact mechanisms behind the relationship in lean SHHF male and female rats.
REFERENCES


20. Sigma Diagnostics Calcium Kit (Procedure No. 587).


ACKNOWLEDGEMENTS

I would like to extend my sincere thanks to Dr. Sylvia McCune for her help and patience in working with me on this project. I could not have completed it without her continuous advising and support. I would also like to thank my honors committee members for taking the time to evaluate my project.
TOTAL BODY RESULTS
Bone and Mineral Metabolism Lab
The Ohio State University

PATIENT ID: SHHF7501.B15
NAME: SHHF, 6523

SCAN: 1.0e 02/17/00
ANALYSIS: 1.0e 02/17/00

Reference Data Not Available

<table>
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<tr>
<th>REGION</th>
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<th>Young Adult&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Age Matched&lt;sup&gt;3&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>g/cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>% Z</td>
<td>% Z</td>
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<tr>
<td>TOTAL</td>
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<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1 - See appendix E on precision and accuracy. Statistically 68% of repeat scans will fall within 1 SD.
2 - NULL Reference Data Reference Population, Ages 20-45. See Appendices.
3 - Matched for Age, Weight (males 50-100kg; females 35-80kg), Ethnic.

- Standard Analysis.
TOTAL BODY RESULTS
Bone and Mineral Metabolism Lab
The Ohio State University

PATIENT ID: SHHF7501.B15
NAME: SHHF, 6523

SCAN: 1.0e 02/17/00
ANALYSIS: 1.0e 02/17/00

<table>
<thead>
<tr>
<th>Region of Interest</th>
<th>R Value</th>
<th>Tissue % Fat</th>
<th>Region % Fat</th>
<th>Tissue (g)</th>
<th>Fat (g)</th>
<th>Lean (g)</th>
<th>BMC (g)</th>
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<td></td>
<td></td>
<td>10.840</td>
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ANCILLARY TOTAL BODY RESULTS**

- Total Bone Calcium (g): 4.12
- Air Points: 1
- Tissue Points: 58482
- Bone Points: 4823
- Total Points: 58483
- R-Value Points: 32062

**Ancillary results for research purposes, not clinical use.

Standard Analysis.
**TOTAL BODY RESULTS**

Bone and Mineral Metabolism Lab
The Ohio State University

PATIENT ID: SHHF6501.B15
NAME: SHHF, 6483
SCAN: 1.0e 02/24/00
ANALYSIS: 1.0e 02/24/00

---

**ID: SHHF6501.B15 SCAN DATE: 02/24/00**

Reference Data Not Available

**TOTAL BMD (g/cm²)¹**

0.292 ± 0.81

---

<table>
<thead>
<tr>
<th>REGION</th>
<th>BMD¹</th>
<th>Young Adult²</th>
<th>Age Matched³</th>
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<tbody>
<tr>
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<td>g/cm²</td>
<td>% Z</td>
<td>% Z</td>
</tr>
<tr>
<td>TOTAL</td>
<td>0.292</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

---

1 - See appendix E on precision and accuracy. Statistically 68% of repeat scans will fall within 1 SD.
3 - Matched for Age, Weight(males 50-100kg; females 35-80kg). Ethnic.

- Standard Analysis.
TOTAL BODY RESULTS
Bone and Mineral Metabolism Lab
The Ohio State University

PATIENT ID: SHHF6501.B15
NAME: SHHF, 6483

SCAN: 1.0e 02/24/00
ANALYSIS: 1.0e 02/24/00

BODY COMPOSITION**

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<tr>
<th>Region of Interest</th>
<th>R Value</th>
<th>Tissue Value</th>
<th>Region Fat</th>
<th>Region Fat</th>
<th>Tissue Fat</th>
<th>Tissue Lean</th>
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<tr>
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ANCILLARY TOTAL BODY RESULTS**

Total Bone Calcium (g) .. 2.53
Air Points................ 0
Tissue Points............ 53823
Bone Points............... 3162
Total Points............. 53823
R-Value Points.......... 30880

**Ancillary results for research purposes, not clinical use.
Standard Analysis.