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IODINE AND SELENIUM DEPLETION AFFECT DIFFERENTLY GROWTH, BIOMARKERS OF BONE METABOLISM AND OXIDATIVE STATUS OF MALES AND FEMALES GROWING RATS

Fanta TOURE [1*], Ousmane SANGARE [1], Cheick Abdoul Gadiri KABA [2] et Barbara STOECKER [3]

[1] Département des Sciences Alimentaires et Nutritionnelles, Ecole Supérieure du Tourisme et de l'Hotellerie de Guinée, Conakry, Guinée
[2] Service de Tromatologie de l'Hopital Ignass Deen, Conakry, Guinée
[3] Department of Nutritional Sciences, Oklahoma State University, Stillwater, Oklahoma, USA

* Correspondance, e-mail : *fantatoure30@yahoo.fr*

ABSTRACT

This study investigated the effects of experimental Iodine (I) and/or selenium (Se) depletion on biomarkers of bonne metabolism and oxidant status of growing rats. Dams were fed experimental diets beginning at week 1 of lactation. Pups were weaned at 3 weeks of age and a sub-sample of males and females was fed the experimental diet of their mother for an additional 7 weeks. I and Se status and growth in the animals were assessed. Biomarkers of bone metabolism and oxidant status of rats were also investigated. Weight gain was decreased by I depletion in all animals and by Se depletion in males. Males had higher weight gain than females. Iodine and selenium depletion did not significantly affect serum alkaline phosphatase. However, the values were higher in males than females ($P < 0.03$). Serum osteocalcin levels were decreased by selenium depletion ($P < 0.04$) and there were significantly higher values in females than males ($P < 0.02$). Serum FRAP concentrations were decreased by selenium depletion ($P < 0.04$) in all the rats and there was significantly higher serum FRAP in females than males when iodine was deficient ($P < 0.02$). Serum FRAP was also increased by iodine depletion in females ($P < 0.01$). Hepatic TBARS as expressed in MDA levels in liver extract were increased by selenium depletion ($P < 0.0001$), and iodine depletion ($P < 0.03$); and males had higher levels of TBARS than females ($P < 0.0001$). Iodine and/or selenium depletion differently affected biomarkers of bone metabolism and oxidative status of males and females growing rats. There was higher growth, bone turnover and lipid oxidation, and lower bone ash weight in male compared to female rats.

Keywords : *Iodine, selenium, biomarkers of bone metabolism, oxidative status, young rats.*

RÉSUMÉ

La carence en iode et en sélénium affecte différemment la croissance, les biomarqueurs du métabolisme de l'os et le statut oxydatif des rats et rates en état de croissance

Cette étude investigua l'effet de la carence expérimentale de l'iode (I) et du sélénium (Se) sur la croissance, les marqueurs biochimiques du métabolisme de l'os et sur le statut oxydatif des jeunes rats. Des dames rates ont été alimentées de diètes expérimentales à partir de la semaine 1 de la lactation. Les ratons ont été sevrés à 3 semaines d'âge et un sous-échantillons de mâles et de femelles a été mis sur la diète de leurs mères respectives pour 7 semaines. Les statuts de l'I et du Se, la croissance, les biomarqueurs du métabolisme de l'os ainsi que le statut oxydatif des jeunes rats ont été déterminés. La croissance exprimée en gain pondéral a été réduite par la déplétion de l'I et du Se chez tous les animaux et par celle du Se chez les mâles. Les mâles avaient de gains pondéraux plus grands que ceux des femelles. La carence en I et du Se n'a pas affecté significativement l'alcaline phosphatase sérique, mais les valeurs étaient plus élevées chez les males ($P < 0,03$). Les taux d'ostéocalcine sérique ont été diminués par la carence en Se ($P < 0,04$) et ces taux étaient plus élevés chez les femelles ($P < 0,02$). Les concentrations du FRAP ont été diminuées par la carence en Se chez tous les rats ($P < 0,04$) et les taux étaient plus élevés chez les femelles quand l'iode était déficient ($P < 0,02$). Le FRAP sérique a été augmenté par la carence en iode chez les femelles ($P < 0,01$). Les taux de TBARS dans l'extrait hépatique ont été augmentés par la déficience du Se ($P < 0,0001$) et par celle de l'iode ($P < 0,03$); et ces taux étaient plus élevés chez les males ($P < 0,0001$). La carence en iode et en sélénium a diffréremment affecté les biomarqueurs du métabolisme de l'os ainsi que le statut oxydatif des rats et rates en état de croissance. Il y a eu une plus grande croissance, un taux plus élevé de remodelage de l'os, une plus grande oxidation des lipids et un plus faible poids des cendres chez les mâles que chez les femelles.

Mots-clés : *Iode, sélénium, biomarqueurs du métabolisme de l'os, statut oxydatif, jeunes rats.*

I - INTRODUCTION

Osteoporosis is a serious public health problem in the world. Currently it is estimated that over 200 million people worldwide suffer from this disease (1). By 2050 the incidence of hip fracture in men is projected to increase by 240 % and 310 % in women (2). While we experience this alarming situation, several factors have been implicated in the etiology of bone and articular diseases. Bone quality is aspects of bone composition and structure that contribute to

bone strength independently of bone mineral density (3). The factors that may influence bone quality include gender (4), age, family history, ethnicity, hormone levels, nutrition (5), the use of some drugs, and some chronic diseases, bone turnover (3). Nutritional and dietary factors that have been shown to influence bone health include calcium (6), phosphorus, vitamin C (7), magnesium, zinc (8), iron (9), copper (10), manganese (11), vitamin K (12, 13), vitamin D, and vitamin E (14). Iodine and selenium deficiencies have been associated with osteoarthritis (15, 16,) and osteoarthritis may lead to bone loss (17, 18, 19). The mechanism whereby iodine and selenium may affect bones and joints is not clear. However, thyroid hormone (T_3) is believed to have an important role in the development and maintenance of both endochondral and intramembranous bone (20). Selenium is required for thyroid hormone metabolism. In addition, selenium may protect bone and cartilage cells against oxidative damage (21). Biochemical markers of bone metabolism are byproducts that are released into the blood stream and urine during the process of bone remodeling, which involves bone resorption and bone formation (22). They measure bone cell activities (23). Bone density determination is valuable for evaluation of patients at risk for osteoporosis, but it does not give any information about the rate of bone turnover, therefore, supplementing bone density information with measurement of markers of bone turnover may enhance the prediction of fracture risk.

Serum and urine tests can detect these markers and provide information about the rate of bone resorption and formation. Bone formation can be evaluated using serum non-specific alkaline phosphatase (ALP), bone-specific alkaline phosphatase (B-ALP), osteocalcin, carboxyterminal propeptide of type I collagen (PICP), and aminoterminal propeptide of type I collagen (PINP) (22). Indicators of bone resorption such as cross-linked C-telopeptide of type I collagen, tartrate resistant acid phosphatase (TRAP), N-telopeptide of collagen cross-links (NTx), and C-telopeptide of collagen cross-links (CTx) can be determined in serum. Other bone resorption markers such as hydroxyproline, free and total pyridinoline, free and total deoxipyridinoline as well as NTx and CTx can be assessed in urine (22). Bone specific alkaline phosphatase is an osteoblast product that is believed to be an essential enzyme for bone mineralization (22). Both bone specific and tissue non-specific alkaline phosphatase can promote mineralization by hydrolyzing a variety of phosphate compounds to make inorganic phosphate available for bone mineralization (24). It has also been suggested that alkaline phosphatase may destroy inhibitors of mineral crystal growth and behave like a calcium binding protein (25). Osteocalcin (bone gla-protein) is a peptide synthesized and secreted by osteoblasts during bone formation. It is mostly incorporated into bone matrix with some escaping into the blood; therefore, osteocalcin is accepted as a

marker of bone formation. However, osteocalcin is also released from bone to the circulation during bone resorption. Therefore osteocalcin is more a marker of bone turnover than of bone formation (22). Amino-teminal and carboxyterminal propeptide of type I collagen direct the assembly of the collagen triple helix and are separated from the newly formed collagen molecules and released into the circulation. Therefore, their concentration in serum may be an index of bone formation. However these byproducts of collagen synthesis are also produced by other type I collagen-containing tissues such as the skin. Serum N-terminal and C-terminal propeptide of type I collage are less useful than ALP and OC as indicators of bone formation (26). Tartrate resistant acid phosphatase (TRAP), also known as type-5 acid phosphatase) is an iron-containing protein produced in different tissues with acid phosphatase activity and is one of the most abundant enzymes in osteoclasts (27). Serum TRAP is used as a biochemical marker of osteoclastic activity and bone resorption. However, it lacks specificity because other cells that are not related to bone such as erythrocytes and platelets also release TRAP into serum (28). NTx and CTx are degradation products of type I collagen, mainly produced by cathepsin K. Pyridinoline, deoxypyridinoline, and cross-linked C-telopeptide of type I collagen (ICTP) are also degradation products produced by matrix metalloproteases (29).

Pyridinoline and deoxypyridinoline are the two cross-links present in the mature form of type I collagen. Urine levels of pyridinoline and deoxypyridinoline correlate with the breakdown of collagen released from bone matrix by the osteoclasts (30). This cross-linking structure, which is unique to collagen and elastin molecules, creates bonds between polypeptide chains in collagen fibrils to enhance stability. Pyridinoline and deoxypyridinoline cross-links can be excreted free or still bound to the peptide chains and either form can be measured. Deoxypyridinoline is the more abundant cross-link in bone collagen and is generally the one measured (30). Despite the studies showing effects of iodine and selenium on bone, there is limited information on the effects of these trace elements on bone quality through biochemical markers of bonne metabolism and on the oxidative status. There is also insufficient data on gender difference in bone response to the elements with respect to the above-mentioned characteristics. *The objectives of this study were : 1.* To investigate the effects of iodine and/or selenium depletion on bone quality in growing male and female rats by assessing indicators of selected biochemical markers of bone metabolism and oxidant status of growing rats. 2. To investigate gender differences in bone response to iodine and/or selenium depletion with respect to the biomarkers of bone metabolism and oxidative status of growing rats.

II - MATERIAL AND METHODS

The methodology section of this paper consists of presentation of an experimental design involving the dietary manipulation of male and female rats as well as the determination of the effect on iodine and/or selenium status. It also consists the assessment of growth, biochemical markers of bone metabolism, and lipid peroxidation status of the rats. A statistical analysis of main outcome measures ends the section.

II-1. Animal experiment and study design

- *Animal Feeding and Handling.*

Fourteen Sprague Dawley pregnant rats (120 g to 186 g) were ordered from Harlan Teklad, (Indianapolis, IN) and fed an adaptation diet (low iodine, low selenium) for the last 5 to 7 days of pregnancy and a week of lactation. Then the lactating dams were randomly assigned to four experimental diets: adequate selenium, adequate iodine (+Se+I); adequate selenium low iodine (+Se-I); low selenium, adequate iodine (-Se+I); low selenium, low iodine (-I -S) as best illustrated elsewhere (31). Dams were assigned to the experimental diets one week after delivery. At three weeks of age, nine male and nine female pups per dietary treatment were randomly selected and continued on the diets of their respective mothers for seven more weeks. The young rats were fed *ad libitum* with free access to reverse osmosis water. During the feeding experiment, two young rats (one male and one female) died from the group consuming (low iodine, adequate selenium) diet. The male died of an unknown cause at the vivarium during the 6^{th} week and the female died in our laboratory during the anesthesia during the 7^{th} week at the end of the experiment. Thus the total number of pups remaining for the experiment was 70. The use of rats in this study was approved by the Institutional Animal Care and Use Committee (IACUC) of Oklahoma State University.

- *Experimental diets*

The preparation of the experimental diets was done following the recommendations of the American Institute of Nutrition (AIN) for growing rodents (32), with some modifications as reported elsewhere (31) and resumed in ***Table 1***.

Table 1 : *Composition of the experimental diets (g/kg diet)*

Ingredients	**+I +Se**	**+I -Se**	**-I +Se**	**-I -Se**
Torula yeast	340	340	340	340
Dextrose	389.49	389.49	389.49	389.49
Sucrose	100	100	100	100
Soybean oil	70	70	70	70
Fiber (celufil)	50	50	50	50
Mineral mix +I+Se	35	-	-	-
Mineral mix +I-Se	-	35	-	-
Mineral mix -I +Se	-	-	35	-
Mineral mix -I-Se	-	-	-	35
Vitamin mix	10	10	10	10
L-cystine	3	3	3	3
Choline	2.5	2.5	2.5	2.5

I = Iodine. Se = Selenium. (+I+Se) = Adequate iodine, adequate selenium. (+I-Se) = Adequate iodine, low selenium. (-I+Se) = Low iodine, adequate selenium. (-I-Se) = Low iodine, low selenium.

II-2. Necropsy of the pups

The necropsy of the rats was done as described elsewhere (31). Portions of liver were taken and stored at -70 °C for TBARS analyses. The thyroid glands were weighed and discarded. The left leg (left femur and the left tibia together) were excised and kept in -20°C for later bone ash weight assessment.

- *Determination of growth through weight gain*

Weight gain was determined by subtracting the weight of the rat recorded on the day of the start of the experimental diet from the weight recorded on the day of necropsy according to the following formula : *Weight gain = B-A*, where B = Weight of the rats on the day of necropsy and A = weight of the rats at the start of the experimental diet.

II-3. Biochemical analyses

To ascertain the iodine status of the rats, thyroid weight, serum thyroxin (T_4) and serum triiodothyronine (T_3) were assessed. Serum T_4 and T_3 were assessed in the pups using radioimmunoassay (RIA) kits (Diagnostic Products Corp., Inc, Los Angeles, CA) following the manufacture's instruction as reported elsewhere (31). The selenium status of the rats was determined by measuring liver glutathione peroxidase activity by a spectrophotometric method described by Lawrence and Burk (33). Liver thiobarbituric acid reactive substances (TBARS), mostly composed of malondialdehyde (MDA) were assessed as an

indicator of lipid peroxidation. Liver MDA was assessed using the method described by Phelps and Harris (34) with modification pertaining to the preparation of the liver extract. The supernatant of liver homogenate from liver samples stored at -70 C was prepared in the following way : liver sample was homogenized in 50mM phosphate buffer (PBS with 1 mM EDTA, 1 mM phenymethylsulfonylfluoride, and 1 µM pepstain A and trypsin inhibitor at 80 mg/L buffer PH = 7.4) using a homogenizer with cold teflon-glass and overhead stirrer (Wheaton Science Product Cat # 903475). The homogenate was centrifuged at 900 g at 4 ºC for 15 min. The supernatant was decanted, and recentrifuged at 12,000 g (11,400 rpm) at 4 ºC for an additional 15 min. The latter supernatant was aliquoted and stored at -80 ºC for TBARS analysis. As an indicator of antioxidant status, serum ferric reducing ability of plasma (FRAP) was determined. Serum FRAP was assessed colorimetrically with a commercially available kit from Roche Diagnostics (Somerville, NJ) using a Cobas-Fara II Clinical Analyzer (Montclair, NJ). As indicators of biochemical markers of bone metabolism we assessed serum osteocalcin, serum alkaline phosphatase (ALP), serum tartrate resistant acid phosphatase (TRAP), urinary deoxypyridinoline (DPD), urinary calcium, urinary magnesium, and urinary phosphorus. Serum osteocalcin was analyzed to assess bone formation using a rat osteocalcin immunoradiometric assay (IRMA) kit (Immunotopics, Inc, San Clemente, CA) following the manufacturer's instructions.

Two different antibodies to rat osteocalcin are used in the assay. An affinity purified polyclonal goat antibody recognizing the C-terminal portion of the molecule was immobilized onto plastic beads for capture and another affinity purified polyclonal goat antibody recognizing the amino terminal portion of the molecule was radiolabeled for detection. A sample containing rat osteoclacin was incubated simultaneously with an antibody coated bead and the ^{125}I labeled antibody. Osteocalcin contained in the sample is immunologically bound by the immobilized antibody and the radiolabeled antibody to form a "sandwich" complex : Bead/Anti-Rat Osteocalcin-Rat Osteocalcin-^{125}I Anti-Rat Osteocalcin. At the end of the incubation period, the bead is washed to remove any unbound labeled antibody and other components. The radioactivity bound to the beads is then measured in a gamma counter. The radioactivity of the antibody complex bound to the bead is directly proportional to the amount of rat osteocalcin in the sample. Serum alkaline phosphatase as a non-specific indicator of bone formation was assessed using a colorimetric kit from Roche Diagnostics (Somerville, NJ). The test was performed on a Cobas-Fara II Clinical Analyzer (Montclair, NJ). The Roche reagent for alkaline phosphatase uses 4-notophenylphosphate as the orthophosphate monoester and 2-amino-2-methyl-1, 3-propanediol as the phosphate receptor and buffer. The 4-nitrophenylphosphate is colorless, but the resultant 4-nitrophenoxide ion has a strong absorbance at 405 nm. The rate

of increased absorbance at 405 nm is proportional to the enzyme (alkaline phosphatase) activity. Serum TRAP was assessed as an indicator of bone resorption using a colorimetric kit from Roche Diagnostics System Inc (Nutley, NJ) and the Cobas-Fara II Clinical Analyzer (Roche, Montclair, NJ). Urinary DPD was analyzed as an indicator of bone resorption using the Metra DPD EIA kit (Quidel Corporation, San Diego, CA) following the manufacturer's instruction and using a plate reader. The assay is a competitive enzyme immunoassay in a microtiter strip-well format using a monoclonal anti-DPD antibody coated on the strip to capture DPD. DPD in the sample competes with conjugated DPD-alkaline phosphatase for the antibody and the reaction is detected with a p-Nitrophenyl phosphate substrate using a plate reader at 405 nm. Metra DPD results were expressed based on urinary concentrations of creatinine. Urinary creatinine was assessed colorimetrically using a kit from Roche Diagnostics Inc. (Nutley, NJ) and the Cobas-Fara II clinical analyzer (Roche, Montclair, NJ). Urinary Ca, Mg, and P were determined to know the amounts of the minerals lost in the urine. Urinary calcium and magnesium were assessed using flame atomic absorption spectrometry (Perkin Elmer, 5100PC). Urinary phosphorus was assessed using a colorimetric kit from Roche Diagnostics (Nutley, NJ) and the Cobas-Fara II clinical analyzer (Roche, Montclair, NJ). During the test, phosphorus reacts in acid medium with ammonium molybdate to form a phosphomolybdate complex with a yellow color. The intensity of this color, measured at 340 nm, is proportional to the concentration of inorganic phosphorus in the sample.

II-4. Bone ash weight and mineral content using atomic absorption spectrometry

The left femur was ashed to determine the amounts of individual minerals in it using atomic absorption spectrometry. The concentrations of Ca, Mg, Zn, and Fe were determined using flame atomic absorption spectrometry (Perkin Elmer, 5100PC). The femurs were weighed, dried at 105 ºC for 24 hours, re-weighed, and placed in individual acid-washed crucibles. An acid digestion with concentrated nitric acid and hydrogen peroxide at 85 ºC was followed by a dry ashing in a muffle furnace. Bone was ashed in the muffle furnace at 375 ºC for 24 hours three times with intermittent acid digestions and dryings until white ash was obtained (35). After cooling, bone ash was weighed and dissolved in 12.2 ml of 5.2 % nitric acid solution (11.6 mL water + 600 µL concentrate nitric acid). Then appropriate dilutions of the stock solution were done for Ca, Mg and Zn for the analysis, while the Fe was directly assessed in the stock solution. For Ca analysis the stock solution was diluted (1 : 2805) using 0.05 % nitric acid containing 0.1 % lanthanum. For Mg and Zn the stock solution was diluted (1:14) in 0.05 % nitric acid solution.

II-5. Statistical Analyses

Data were analyzed using SAS (Statistical Analysis System) version 8 (SASInstitute Inc., Cary, NC). A split plot arrangement in a completely randomized design with sub sampling of the subunits (9 male and 9 female young rats) and considering the main units (dams) as random factors, along with Proc Mixed and Least Square Means were used to determine the main and interaction effects of the independent variable (iodine, selenium and sex) on the dependent variables (weight gain, I and Se status, biomarkers of bone metabolism, and oxidative status of the pups). Whenever an interaction effect was significant, slice analysis in Proc Mixed was used to determine the significance of differences between different levels of an independent variable at a given level of the other interacting independent variable with respect to the outcome variable. An effect was considered to be significant at $P < 0.05$.

III - RESULTS

III-1. Weight gain, iodine and selenium status

Serum thyroxin (T_4) was significantly decreased by iodine deficiency ($P < 0.0001$) and selenium depletion tended to increase T_4 ($P < 0.08$) ***(Table 2)***. For serum triiodothyronine (T_3), there were no significant differences between males and females when selenium was adequate. But when selenium was deficient, females had higher serum T_3 than males. In males as well as in females, there were no significant differences between adequate selenium and deficient selenium with respect to serum T_3 ***(Figure 1)***. Selenium depletion markedly decreased hepatic glutathione peroxidase activity ($P < 0.0001$) regardless of sex and iodine status of the rats ***(Table 2)***.

III-2. Biochemical markers of bone metabolism and oxidative status

The measured bone formation markers were serum alkaline phosphatase and serum osteocalcin. Iodine and selenium depletion did not significantly affect serum alkaline phosphatase. However, the values were higher in males than females ($P < 0.03$) ***(Table 2)***. Serum osteocalcin levels were decreased by selenium depletion ($P < 0.04$) and there were significantly higher values of serum osteocalcin in females than males ($P < 0.02$) ***(Table 3)***.

Table 2 : *Effects of sex and diet on weight gain, thyroid weight, serum thyroxin, serum triiodothyronine, and hepatic glutathione peroxidase activity*

	Weight gain (g)	Thyroid weight (mg/100g BW)	Serum T_4 (µg/dl).	Serum T_3 (ng/dl).	Liver GSHPx Activity (mmole of NADPH oxidized/min/g)
Sex					
Males	191 ± 4	33 ± 3	2.2 ± 0.2	54.0 ± 2.3	8.22 ± 0.57
Females	134 ± 4	39 ± 3	2.4 ± 0.2	67.1 ± 2.2	7.43 ± 0.57
Diet					
+Se	175 ± 4	36 ± 3	2.0 ± 0.2	61.0 ± 2.60	14.20 ± 0.58
-Se	151 ± 4	37 ± 3	2.5 ± 0.2	60.0 ± 2.5	1.45 ± 0.56
+I	172 ± 4	7 ±3	3.9 ± 0.2	62.0 ± 2.60	7.79 ± 0.56
-I	153 ± 4	65 ±3	0.6 ± 0.2	59.1 ± 2.5	7.86 ± 0.58
Source of Variation	*P-values*	*P-values*	*P-values*	*P-values*	*P-values*
I	<0.008	<0.0001	<0.0001	0.45	0.93
Se	<0.002	0.80	<0.08	0.78	<0.0001
I*Se	0.43	0.91	0.18	0.30	0.49
Sex	<0.0001	0.15	0.53	<0.0001	0.33
I*Sex	0.87	0.30	0.18	0.11	0.76
Se*sex	<0.004	0.47	0.54	<0.05	0.56
I*Se*Sex	0.99	0.40	0.96	0.85	0.71

Values are least square means ± SEM. Effect is significant at P<0.05. +Se = Adequate selenium, –Se = Low selenium. +I = Adequate iodine. -I = Low iodine. BW = Body weight. T_4 = Thyroxin. T_3 = Triiodothyronine. GSHPx = Glutathione peroxidase.

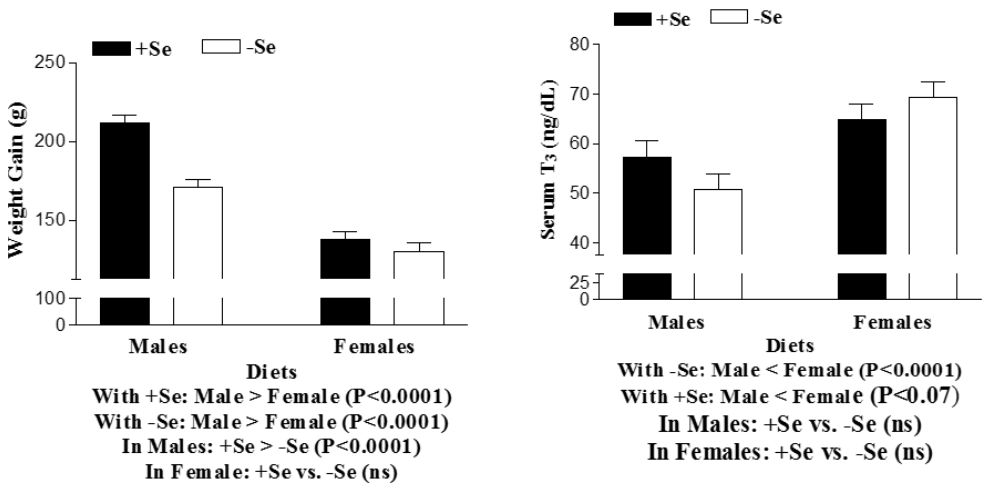

Figure 1 : *Interaction effects of selenium and sex on weight gain and serum triiodothyronine (T_3)*

There were no significant effects of either diet or sex on urinary DPD and urinary Ca. However, urinary Mg (P < 0.007), urinary P (P < 0.04) ***(Table 4)*** were higher in males than females. As markers of oxidative status we determined liver glutathione peroxidase (GSH-Px) activity, which was also an index of selenium status, serum FRAP, and liver TBARS as measured by malondialdehyde. Hepatic GSH-Px activity was decreased by selenium depletion (P<0.0001), but was not affected by iodine depletion ***(Table 2)***. Serum FRAP concentrations were decreased by selenium depletion (P<0.04) ***(Table 4)*** and there was significantly higher serum FRAP in females than males when iodine was deficient (P < 0.02) ***(Figure 2)***. Serum FRAP was also increased by iodine depletion in females (P < 0.01).

Table 3 : *Effects of sex and diet on serum alkaline phosphatase, osteocalcin, tartrate resistant acid phosphatase, and urinary deoxypyridinoline*

	Serum ALP (µmole/L)	**Serum osteocalcin (ng/mL)**	**Serum TRAP (µmole/L)**	**Urinary DPD (nmole/mmole creatinine)**
Sex				
Males	91.3 ± 7.3	31.3 ± 2.8	6.62 ± 0.37	168.0 ± 40.4
Females	71.8 ± 7.3	37.2 ± 2.8	5.86 ± 0.37	246.9 ± 40.9
Diet				
+Se	86.1 ± 9.2	41.0 ± 3.8	6.39 ± 0.47	198.0 ± 42.2
-Se	76.9 ± 9.0	27.5 ± 3.7	6.10 ± 0.46	216.9 ± 39.0
+I	88.6 ± 9.6	30.7 ± 4.0	6.38 ± 0.48	231.4 ± 39.6
-I	74.4 ± 8.6	37.8 ± 3.5	6.11 ± 0.44	183.5 ± 41.7
Source of variation	*P-values*	*P-values*	*P-values*	*P-values*
I	0.30	0.21	0.69	0.41
Se	0.49	<0.04	0.67	0.74
I*Se	0.31	0.17	0.82	0.82
Sex	<0.03	<0.02	<0.04	0.18
I*Sex	0.27	0.21	<0.06	0.70
Se*sex	0.32	0.96	0.46	0.13
I*Se*Sex	0.21	0.23	0.59	0.49

Values are least square means ± SEM. Effect is significant at P<0.05. +Se = Adequate selenium. -Se = Low selenium. +I = adequate iodine. –I= Low Iodine. ALP = Alkaline phosphatase. TRAP = Tartrate resistant acid phosphatase. DPD = deoxypyridinoline.

Hepatic TBARS as expressed in MDA levels in liver extract were increased by selenium depletion (P < 0.0001), and iodine depletion (P < 0.03); and males had higher levels of TBARS than females (P < 0.0001) ***(Table 4)***. As bone resorption markers, we assessed serum TRAP, urinary DPD, urinary Ca, Mg,

and P ***(Tables 3 and 4)***. When iodine was adequate, serum TRAP was higher in males than females (P < 0.004), but when iodine was deficient there were no significant differences between males and females in serum TRAP ***(Figure 2)***

Table 4 : *Effects of sex and diet on urinary Ca, urinary Mg, urinary P, serum FRAP and Liver thiobarbituric acid reactive substances*

	Urinary Ca (mg/12 hours)	**Urinary Mg (mg/12 hours)**	**Urinary P (mg/12 hours)**	**Serum FRAP (µmole/L)**	**Liver TBARS (nmole/mg protein)**
Sex					
Males	0.85 ± 0.41	2.64 ± 0.21	7.74 ± 0.63	528.4 ± 53.5	0.291 ± 0.003
Females	1.25 ± 0.42	1.89 ± 0.21	5.82 ± 0.64	714.1 ± 53.6	0.267 ± 0.003
Diet					
+Se	1.28 ± 0.43	2.46 ± 0.24	7.52 ± 0.66	730.6 ± 61.0	0.267 ± 0.003
-Se	0.82 ± 0.41	2.07 ± 0.23	6.04 ± 0.61	511.9 ± 59.8	0.290 ± 0.003
+I	1.31 ± 0.43	2.59 ± 0.24	7.54 ± 0.62	550.6 ± 63.3	0.273 ± 0.003
-I	0.79 ± 0.42	1.94 ± 0.23	6.01 ± 0.65	691.9 ± 57.4	0.284 ± 0.003
Source of variation	*P-values*	*P-values*	*P-values*	*P-values*	*P-values*
I	0.40	0.09	0.09	0.13	<0.03
Se	0.46	0.28	0.11	<0.04	<0.0001
I*Se	0.40	0.27	<0.07	0.18	0.20
Sex	0.51	<0.007	<0.04	<0.02	<0.0001
I*Sex	0.29	0.35	0.78	<0.03	0.20
Se*sex	0.30	0.18	0.43	0.55	0.62
I*Se*Sex	0.37	0.69	0.92	0.67	0.06

Values are least square means ± SEM. Effect is significant at P < 0.05. +Se = Adequate selenium. –Se = Low selenium. +I = Adequate iodine. -I = low iodine. Ca = Calcium. Mg = Magnesium. P = Phosphorus. FRAP = Ferric reducing ability of plasma. TBARS = Thiobarbituric acid reactive substances.

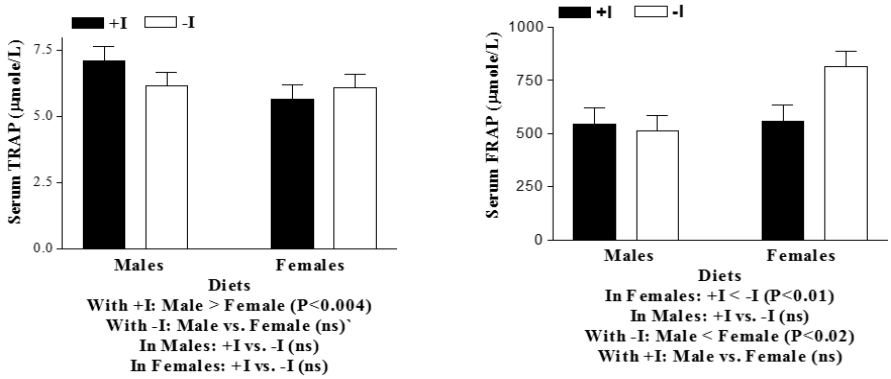

Figure 2 : *Interaction effect of iodine and sex on serum tartrate resistant acid phosphatase (TRAP) and serum ferric reducing ability of plasma (FRAP)*

III-3. Bone ash weight and mineral content

There were no significant effects of iodine and/or selenium on femur wet weight, dry weight, and ash weight. However, femur wet weight and dry weight were both higher in males than females ($P < 0.02$), but femur ash weight as percent of either wet or dry weights was higher in females than males ($P < 0.002$ and $P < 0.03$ respectively). Femur concentrations of calcium, magnesium, zinc and iron as measured by flame atomic absorption spectrometry were not affected by iodine and/or sex of the animals. Selenium depletion increased femur Zn ($P < 0.01$) and decreased femur Fe ($P < 0.05$) without significantly affecting the bone concentrations of Ca and Mg.

IV - DISCUSSION

The discussion on iodine and /or selenium status of the rats in our study has been reported elsewhere (31). Our data indicate that there is a significantly higher bone turnover in male than female rats. This was evidenced by higher serum ALP and TRAP activities in males than females. In addition, there were higher urinary excretions of P and Mg in males than females. Serum osteocalcin was higher in females and was decreased by selenium depletion. The mechanism by which selenium depletion can decrease serum osteocalcin levels is not yet established. Moreno-Reyes and colleagues (16) also found decreased serum osteocalcin in selenium deficient male rats. There are 18 different selenoproteins identified in the mammalian system (36), and the functions of many of them have not yet been identified. The mechanism whereby selenium may affect serum osteocalcin levels and the reason for higher serum osteocalcin in females are not known and further investigation in this area is needed. It has been suggested that estrogen increases the rate of bone mineralization in female rats at puberty compared to male rats (37). The mechanism of this high mineralization of female bone at this time of high circulating estrogen levels is not known. Because females had higher osteocalcin in this study and better bone status, it would be interesting to investigate whether this action of estrogen is mediated by osteocalcin. An increase in liver MDA and a decrease in serum FRAP due to selenium-depletion may indicate a higher propensity of lipid peroxidation in selenium-deficient rats. MDA is a secondary product of lipid peroxidation, and its levels have been shown to be high in plasma of patients with rheumatoid arthritis including children with juvenile rheumatoid arthritis (38). The TBARS values obtained for our control rats are similar to the values found in seven-week old male (control) rats by Poon et al. (39) using a fluorescence method. Liver MDA was higher and serum FRAP was lower in males than females. Lipid peroxidation might have contributed to the poorer bone quality observed in

male rats as male rats also had higher body fat and lower lean mass. Glutathione peroxidase activity and other selenoproteins with antioxidant properties such as thioredoxin reductases and selenoprotein P might have contributed to the value of serum FRAP in selenium adequate rats. Iodine depletion also decreased liver TBARS. The best known role of iodine in the body is for the synthesis of thyroid hormone. However, substantial amounts of the body's iodine are non-hormonal and are concentrated in extrathyroidal tissues where their physiological functions are unknown. Iodine is rich in electrons and can act as an electron donor to hydrogen peroxide and peroxides. It can also bind to the double bonds of polyunsaturated fatty acids of cell membranes making them less sensitive to oxidation. Therefore iodine is believed to have antioxidant properties in tissues where it accumulates (40). For instance, the mammary gland concentrates iodine during pregnancy and lactation, and this concentration is suggested to prevent breast cancer (41). This probable antioxidant property of iodine might have contributed to the decreased liver TBARS values observed in iodine adequate rats. Female rats had also higher femur ash weight than males. There were no significant sex effects on femur bone mineral content as measured by atomic absorption spectrometry. Because there was a higher femur ash weight in females than males, it is possible that some of the minerals we did not assess in the femur are higher in females.

Inorganic phosphates are important components of bone and femur phosphorus concentration was not assessed in this study. Because urinary phosphorus excretion was higher in males than females, it would be interesting to assess the phosphorus concentrations of femur bone solution. The relatively better bone quality in single element-depleted or both iodine and selenium depleted rats compared to control rats and in female rats compared to males may be explained by their relatively slow growth rate. Normal growth requires a balanced bone reaorption and formation. Antiresorptive agents administrated to growing rats impair growth and lead to increased bone mass (42). This indicates that bone resorption is an important part of growth process. In addition, T_3 and estrogen might have had a synergistic or additive effects on females in slowing longitudinal growth by inducing hypertrophy of the chondrocytes and accelerating the closure of the epiphyseal growth plate, which ends longitudinal growth in humans (43). Even though T_3 is believed to induce the differentiation of reserve chondrocytes to proliferative zone chondrocytes either directly (43, 44) or indirectly through GH and IGF-1 (45), its role in inducing hypertrophy in the chondrocytes to stop the clonal expansion of the cells is well recognized (10). We did not assess the estrogen levels of the rats. However, other research indicate that females have higher circulating estrogen than males at puberty (46) and our rats had reached the

state of puberty by the time they were sacrificed. Estrogen (17 β-estradiol) supplementation of both males and females growing rats caused decreased body weight gain, tibial length, and width of growth plate, while the opposite effects were caused in overiectomized rats (47). This tempering effect of growth by estrogen was explained by a decrease in the width of hypertrophic zone of the growth plate chondrocytes and a predominant expression of estrogen receptor α and β in late proliferating and early hypertrophic chondrocytes in estrogen-supplemented rats. Because iodine and selenium deficiencies have been associated with osteoarthritis, we assumed that iodine and/or selenium depletion would cause osteoarthritis and that osteoarthritis would negatively affect bone biomarkers and thus bone quality in growing rats. Yet we did not assess any indices of osteoarthritis. This is an important limitation of this study because it makes it difficult to link our results with Kashin-Beck disease. However Kashin-Beck disease has been suggested to result from oxidative damage (48) and we observed a significant reduction of circulating antioxidant in selenium deficient rats and a significant increase in lipid peroxidation in the liver of Iodine and selenium depleted rats. Based on these observations, we suggest that iodine and selenium depletion studies may contribute to the understanding of the etiology of Kashin-Beck disease.

V - CONCLUSION

These results indicate that the degree of iodine and selenium depletion used in this study impaired iodine and selenium status in the animals, and this might have contributed to following observed effects on growth, biochemical markers of bone metabolism and oxidative status the growing rats. There was a substantial natural sex differences among the rats and iodine and selenium differentially affected the bone of males and female rats. Adequacy of both iodine and selenium caused a rapid growth with relatively impaired bone quality, especially in males, as measured by biomarkers of bone metabolism and bone ash weight. The combined deficiency of the two trace elements reduced growth and bone quality as measured by biomarkers of bone metabolism. Female rats had slower growth rate, better oxidative status, better biomarkers of bone metabolism and higher bone ash weight. Mechanisms whereby iodine and selenium depletion affects bone quality require further investigations.

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