Title	カルシウム欠乏食摂食により誘発する骨粗鬆症の特徴
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Characteristics of Osteoporosis Due to Calcium Deficient Food in Rats

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Abstract: Seven-week old female Wister rats fed calcium deficient food with estrogen, vitamin D_3 and K_2, and 2% oxalic acid food had osteoporosis with significantly low values of bone breaking energy and bone mineral density, and high level of femoral X-ray image density.
Rats fed spinach food containing dry powdered spinach in stead of 5% fiber avoided development of osteoporosis. Osteoporosis due to calcium deficient food intake was similar to osteoporosis due to 2% oxalic acid food intake in body weight, food intake, uterus weight, estradiol level and concentration of dexypyridinoline and calcium in urine, but perfectly different from osteoporosis due to restricted food intake and due to protein deficient food in body weight, food intake, uterus weight, estradiol level, calcium concentration in urine and inhibition by estrogen, vitamins D_3 and K_2 Increase of parathyroid hormone(PTH) in serum was observed in rats fed calcium deficient food, but not in rats fed 2% oxalic acid food. Extremely low concentration of urinary calcium is supposed to be great sign for the osteoporosis and to be useful for judgment of it. Osteoporosis due to a lack of various nutrients is similar in a significant fall of bone breaking energy and bone mineral density, but the characteristics of the osteoporosis and its treatment diet are expected to be different. Therefore, alkaline phosphatase activity and concentrations of deoxypyridinoline and calcium in urine are analyzed in decision of osteoporosis and its treatment should be done according to the results.

Key words: osteoporosis clcium deficient food, bone fracture energy, bone mineral density, deoxypyrisinoline

Intoroduction

Osteoporosis is divided in type I and type II[1]. The former contains postmenopausal osteoporosis and is also called as bone absorption type. Extreme decrease of serum estradiol[2] and increase of urinary deoxypirdinoline[3)4)] as a marker are main signs of osteoporosis. Female hormone as estrogen[2] and bisphosphonates[5] have been used as treatment for osteoporosis. On the other hand, later includes involutional osteoporosis and is slso called as bone formation type. Rise of alkaline phosphatase as a marker is characteristic of osteoporosis[6]. In cure of the osteoporosis, active vitamin D_3 and K_2 have been utilized.

Rats fed a restricted diet containing a 50% less carbohydrate and oil, but normal levels of protein, mineral and vitamins had osteoporosis[8)10)] with significant reductions of bone mineral density and bone mineral density. Estrogen[9] and exercise[10] are reported to inhibit the osteoporosis. Rise of desoxypyridinoline in feces[11] and decreases of serum estradiol level, uterus weight and body weight are main features of the osteoporosis[12]. The osteoporosis is similar to type I with respects to increase of urinary Dpd and inhibitions by estrogen and exercise. Type I appears to induce by loss of appetite and decrease of food intake are due to menopausal symptoms after menopause.

Rats fed protein deficient food are reported to have osteoporosis[13-15] with significant decreases of fracture energy and bone mineral density. Increase of urinary alkaline phosphatase activity as a marker of bone formation type and decreases of serum estradiol concentration, womb weight, and body weight are main signs of the osteoporosis. The osteoporosis is similar to type II in increase of urinary alkaline phosphatase activity and effectiveness of vitamins D_3 and K_2 as an inhibitor. Many old men 70 to 80 year old are supposed to have type II osteoporosis, which is induced by decrease of food intake and especially reduced protein intake due to

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Table 1. Dietary composition and amount of exercise loaded in a day.

	Group 1	Group 2	Group 3	Group 4	Group 5
(g/100g diet)					
Casein	20.0	20.0	20.0	20.0	20.0
β-Cornstarch	39.7	39.7	39.7	39.7	39.7
α-Cornstarch	13.2	13.2	13.2	13.2	13.2
Sucrose	10.0	10.0	10.0	10.0	10.0
Soybean oil	7.0	7.0	7.0	7.0	7.0
Celluose	5.0	5.0	5.0	3.0	-
Mineral mix	3.5	-	-	3.5	3.5
Vitamin mix	1.0	1.0	1.0	1.0	1.0
L-Cystine	0.3	0.3	0.3	0.3	0.3
Choline bitartrate	0.3	0.3	0.3	0.3	0.3
t-Butylhydroquinone	0.0014	0.0014	0.0014	0.0014	0.0014
Calcium-free mineral	-	3.5	3.5	-	-
Oxalic acid				2.0	
Spinach					5.0
Energy (kcal/100g)	394.8	394.8	394.8	394.8	394.8
Vitamin D_3 (μg/100g)			0.0242		
Vitamin K_2 (μg/100g)			1089.9		
Estrogen (μg/100g)			15.137		

The basal composition of experimental diets was dased on AIN-93G (American Institute of Nutirition) formulation.

kidney disease, diabetes or rheumatism.

Moreover, osteroporosis is known to occure due to calcium deficient or short food intake[16]. Signs of the osteoporosis and connection with types I and type II are unknown.

Furthermore, osteoporosis is reported to induce due to inhibition of calcium absorption by oxalic acid.[17] It is expecting to be similar with osteoporosis due to calcium deficient food intake. As spinach contains oxalic acid[17], spinach food containing a lot of it is supposed to cause osteoporosis.

Therefore, the characteristics of osteoporosis due to calcium deficient food intake were investigated to compare with signs of osteoporosis due to restricted food intake and protein free food intake, inhibition of osteoporosis due to calcium deficient food intake by estrogen, vitamin D_3 and K_2 was studied to know connection with osteoporosis due to restricted food intake and protein deficiency food intake, and studies were undertaken to determine if osteoporosis due to calcium deficient food intake is similar with osteoporosis due to oxalic acid food intake, and spinach food including oxalic acid much cause osteoporosis.

Materials and Methods

1. *Preparation of foods used*

Standard food according to the AIN-93G[18)19)] formula, calcium deficient food used calcium deficient mineral mixture made by special order (Oriental Yeast Co.) in stead of the mineral mixture, calcium deficient food with 2.50 μg of conjugated estrogen (calculated by converting 0.625mg of estrogen (amount of it using at hormone replacement) per unit weight of a 50kg women to an equivalent amount of estrogen per unit body weight of (100g) of a rat and doubled to increase its effect), 0.04 μg of active D_3 (calculated in the way as estrogen) and 217.98 μg of K_2 (calculated in the same way as estrogen), oxalic acid food with 2% instead of fiber, and dry powdered spinach food used 5% dry powdered spinach in return for fiber were prepared. Composition of the foods was showed in Table 1.

2. *Experimental animals.*

Female rats (6 weeks old) of the Wistar strain were kept on standard feed of the AIN-93G for 1 week before start of the experiment. The animals were then separated into five groups with 5 rats each. Group 1 was fed *ad libitum* the standard AIN-93G feed. Groups 2 was fed *ad libitum* calcium deficient food, Group 3 was fed *ad libitum* calcium deficient food with estrogen, vitamins D_3 and K_2, Group 4 was fed *ad libitum* 2% oxalic acid food and Group 5 was fed *ad libitum* dry powder spinach food for 2 weeks. The body weight and food intake of each animal were measured every day. Urine was collected for 3 hours between six to nine o'clock in the morning before the day killed the animals. The animals were killed and blood was drawn. The serum was collected by centrifuging the blood at 12,000 rpm for 20 min. The liver, kidney, adrenal gland and uterus were then re-

Table 2. Body weight and food intake.

	Body weight			Food intake
Groups	Initial(g)	Final(g)	Gain(g)	Totial(g)
Group 1	125.7±2.9[a]	150.2±7.3[a]	24.5±5.3[a]	139.3±10.9[a]
Group 2	125.7±7.0[a]	147.3±8.7[a]	21.7±4.1[a]	140.3±14.6[a]
Group 3	125.6±5.6[a]	144.6±4.7[a]	19.0±5.1[a]	139.0±10.8[a]
Group 4	125.2±4.0[a]	134.4±7.0[b]	9.2±4.6[b]	116.1±11.1[a]
Group 5	125.2±4.2[a]	148.0±6.6[a]	22.2±7.7[a]	130.8±19.9[a]

Means±SD. Values not sharing common superscript letter (a and b) are significantly different ($p<0.05$).

moved, and their total weights were measured. The right and left femurs were removed and stored at −60℃ to estimate fracture force, BMD, and density of X-ray image.

The Ryukyu University Guidelines for the Care and Use of Laboratory Animals was followed in this study.

3. *Analysis of bone mechanical properties.*

Mechanical properties of the left femur used in the foregoing experiment[11] were analyzed with an experimental compressor (TM-3, Toyo Boldwin Co.) and an automatic balance record meter. The three-point bending form of the method of Peng *et al*[20] was used in this study. The left femur was turned down part of the femur front, and both ends of the femur were lightly fixed by a thread on the stand. Width of the stand was 40kg weight, 10mm/min speed, and 100mm/min chart speed. Strength, ductility, and stiffness of the left form of the left femur used in the foregoing experiment[11] were measured based on the recorded stress/strain curve.[20] Toughness of the femur was measured by integrating the covered area of stress/strain curve by use of image analysis software (NIH image).

4. *Measurement of bone mineral density.*

Dual-energy X-ray absorptionmetry (DXA) was used to measure mineral density.[11] Calcium images absorbed X-rays in the left femur were taken by scanning at 1 mm pitch by a DCS-600 (Aloka Co.). Mineral density of the femur was analyzed by processing scanned graphic images on computer.

5. *Femur photograph of X-ray picture.*

Femur photograph of X-ray[11] was taken by the Montography system (Senograph 600 T, Yokogawa Medical System) with Kodak film (Ektasan B/RA) was used for the X-ray. The density of a negative image was examined from the X-ray picture by an X-ray film equipment (Konishiroku Picture Co.)

6. *Measurement of bone metabolic marker.*

Alkaline phosphatase activity in the urine was determined by an Alkaline Phospha K test kit (Wako Co.) and urinary deoxypyridinoline was analyzed by METRA Dpd EIA kit (Sumitomo Co.).

7. *Analysis of estradiol, calcium, magnesium, phosphorus, parathyroid hormone (PTH), albumin, and creatine.*

The estradiol concentration in the serum was measured by an ELA kit (Funakoshi Co.). The levels of calcium and albumin in serum, and calcium, and creatinine in urine was determined by Wako Kits. The parathyroid hormone in the serum was investigated by an ELISA kit (Amerdham Phamacia Biotech Co.).

8. *Statistical Analysis.*

Data were tested by one-way analysis of variance followed by inspection of differences between means by Duncan's new multiple range test throughout these experiments. The different superscript letters in the figures shows statistically significant differences at $p < 0.05$.

Results

1. *Body weight, food intake and tissues weight.*

The body weight of all Groups was about 125g at beginning of the experiment. The rats of Groups 1, 2, 3, and 5 except Group 4 rat gradually grew to reach 150g, an increase of 25g in 2 weeks. Body weight of Group 4 rat was lower with only 9g gain, compared with those of other groups as shown in Table 2. On the other hand, food intake in 2 weeks was 131-140g in all groups and appeared to be almost same among the Groups. In various tissues, the weights of heart in Group 4, and spleen in Group 4 were significantly high, but those of liver in Groups 4 and 5 was low (Table 3).

2. *Length, thickness and weight of a femur*4.

Length (cm) of the bone was 2.84, 2.86, 2.86, and 2.88 for Groups 1, 2, 3, 4, and 5 respectively. Thickness (cm) of the thigh bone was 0.28, 0.28, 0.28, 0.28, and 0.28 for Groups 1, 2, 3, 4, and 5 respectively. Weight

Table 3. Oregan weights per 100g of body weight.

	Heart(g)	Liver(g)	Kidney(g)	Adrenal(g)	Spleen(g)	Pancreas(g)
Group 1	0.28±0.01[a]	2.30±0.07[a]	0.60±0.04[a]	0.028±0.005[a]	0.20±0.01[a]	0.44±0.06[a]
Group 2	0.29±0.02[a]	2.44±0.30[b]	0.61±0.03[a]	0.023±0.005[a]	0.20±0.01[a]	0.42±0.08[a]
Group 3	0.29±0.02[a]	2.33±0.09[a]	0.63±0.02[a]	0.025±0.002[a]	0.20±0.02[a]	0.38±0.06[a]
Group 4	0.30±0.03[b]	2.16±0.07[c]	0.63±0.03[a]	0.028±0.004[a]	0.21±0.02[a]	0.43±0.04[a]
Group 5	0.29±0.02[a]	2.22±0.15[c]	0.65±0.07[a]	0.028±0.006[a]	0.19±0.02[a]	0.38±0.04[a]

Means±SD (n=5). Values not sharing common superscript letter (a, b and c) are significantly different ($p<0.05$).

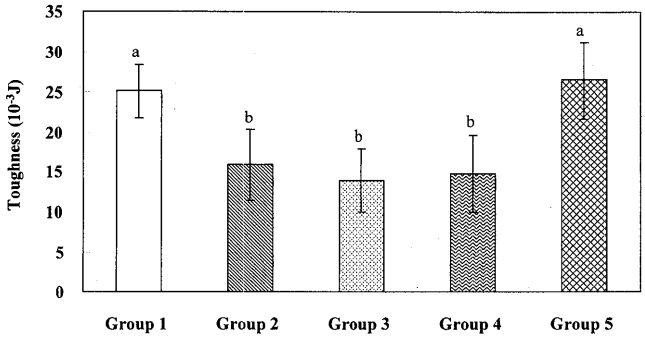

Fig. 1. Breaking energy of the left femur of rats fed standard food (Group 1), calcium deficient food (Group 2), calcium deficient food with estrogen, active vitamin D_3 and vitamin K_2 (Group 3), 2% oxalic acid food (Group 4) and dry powdered spinach food (Group 5).
Mean±SD (n=5). Values not sharing common superscript letters (a and b) are significantly different ($p<0.05$).

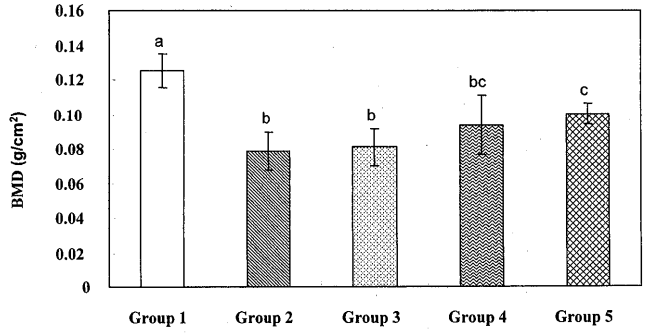

Fig. 2. Bone mineral density (BMD) of the femur by Dual energy X-ray absorptionmetry (DXA) method.
Mean±SD (n=5, Group 4:n=6). Values not sharing common superscript letters (a and b) are significantly different ($p<0.05$).

(g) of the bone was 0.42, 0.40, 0.37, 0.40, and 0.41 for Groups 1, 2, 3, 4, and 5 respectively.

3. *Bone breaking energy, bone mineral density and X-ray image density of the femur.*

The values for the breaking energy (Toughness, 10^{-3} J) of the femur for Groups 1, 2, 3, 4, and 5 were 25.06, 15.93, 13.96, 14.80, and 26.48 respectively. As can be seen in Fig.1, the Groups 2, 3, and 4 rats fed calcium deficient food, calcium deficient food with estrogen, active vitamin D_3 and vitamin K_2, and oxalic acid food had low breaking energy levels, compared with those of Groups 1 and 5 rats fed standard food and dry powdered spinach food. Similar results were observed in the bone mineral density, the values for the Groups 2, 3, 4, and 5 rats had low than those of Groups 1 and 5 rats. The bone mineral density levels of Groups 1, 2, 3, 4, and 4 were 0.125 (g/cm^2), 0.078 (g/cm^2), 0.080 (g/cm^2), 0.093 (g/cm^2), and 0.100 (g/cm^2) respectively. (Fig.2) In density of X- ray image, bone rich in calcium is white because it absorbs X-rays. The values for density of the bone X-ray image for groups 1, 2, 3, 4, and 5 were 0.64, 0.78, 0.78, 0.73 and 0.58 (Fig. 3). The densities of Groups 2, 3, and 4 were remarkably higher than those of Groups 1 and 5.

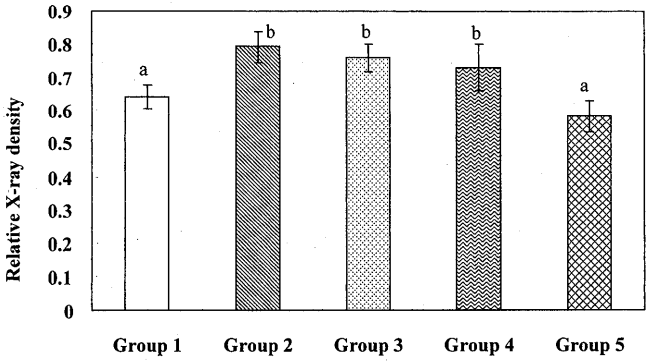

Fig. 3. X-ray film images of the femur.
Mean±SD (n=5, Group 4:n=6). Values not sharing common superscript letters (a, b, and c) are significantly different ($p<0.05$).

4. *Concentration of estradiol in serum and weight of a womb.*

The levels of estradiol in serum were 38.55 pg/mL, 22.59 pg/mL, 25.25 pg/mL, 40.37 pg/mL, and 37.77 pg/mL for Groups 1-5 respectively. The values of a womb weight (g/100g of body weight) were 0.27, 0.25, 0.25, 0.25, and 0.27 for Groups 1-5 respectively. No difference was found for uterine weight and serum estradiol concentrations for Groups 1-5. The results were shown in Fig. 4 and 5.

5. *Contents of bone metabolism markers.*

The alkaline phosphatase activities in urine were 4.99K-A, 4.99 K-A, 4.91 K-A, 4.96 K-A, and 8.01 K-A for Groups 1, 2, 3, 4, and 5 respectively. The values of

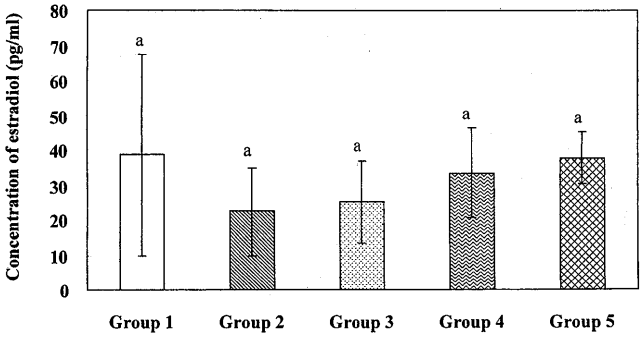

Fig. 4. Concentration of estradiol in serum.
Mean±SD (n =5, Group 4:n=6). Values not sharing common superscript letters (a and b) are significantly different ($p<0.05$).

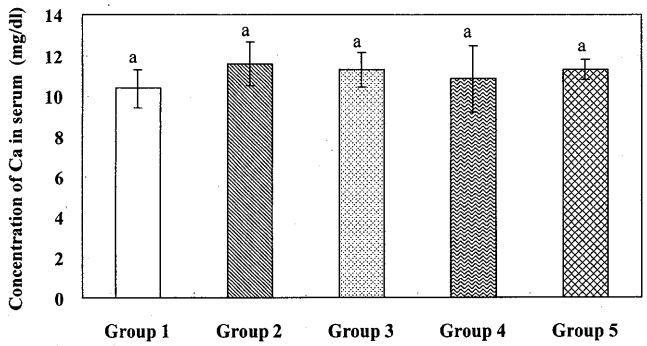

Fig. 7. Content of calcium in the serum.
Mean±SD (n =5, Group 4:n=6). Values not sharing common superscript letters (a and b) are significantly different ($p<0.05$).

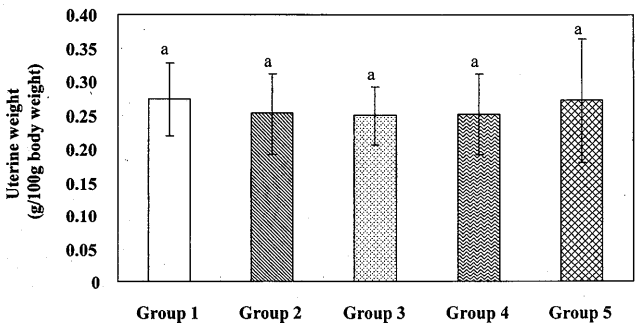

Fig. 5. Uterus weight per 100g of body weight.
Mean±SD (n =5, Group 4:n=6). Values not sharing common superscript letters (a and b) are significantly different ($p<0.05$).

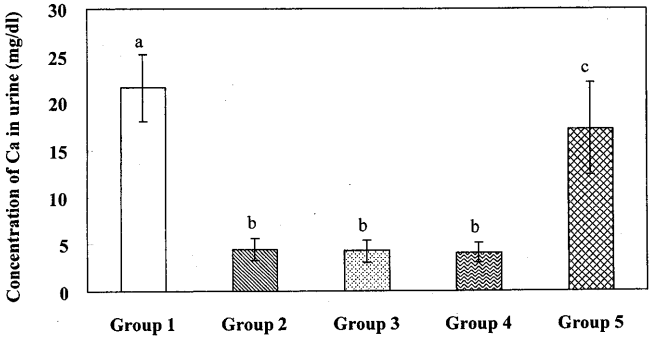

Fig. 8. Density of calcium in the urine.
Mean±SD (n =5, Group 4:n=6). Values not sharing common superscript letters (a and b) are significantly different ($p<0.05$).

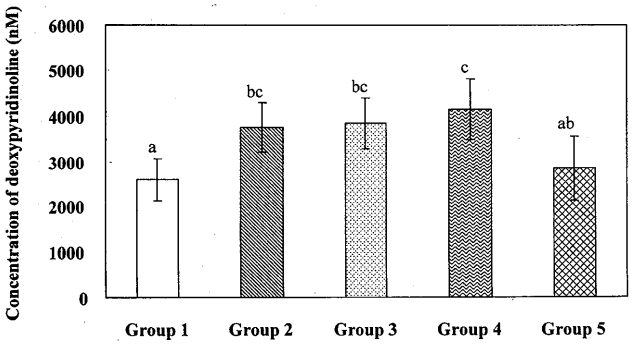

Fig. 6. Concentration of deoxypyridinoline in urine.
Mean±SD (n =5, Group 4:n=6). Values not sharing common superscript letters (a and b) are significantly different ($p<0.05$).

Groups 1-4 were almost same but that of Group 5 was extremely high. Concentration of deoxypyridinoline in urine was significantly increased for Groups 2-4, compared with those of Groups 1 and 5. Actual average deoxpridinoline values were 2591.87(nM), 3747.79(nM), 3840.67 (nM), 4139.91 (nM), and 2838.21 (nM) for Groups 1, 2, 3, 4, and 5 respectively (Fig. 6).

6. *Contens of minerals, parathyroid hormone, creatine, and albumin in serum and urine.*

Concentrations of serum calcium for Groups 1-5 were 10.38 (mg/dL), 11.59 (mg/dL), 11.31 (mg/dL), 10.82 (mg/dL), and 11.31 (mg/dL) respectively.

No significant difference was found among the Groups (Fig.7). Content of urinary calcium was extremely low for Groups 2-4 rats induced osteoporosis, compared with those of Groups 1 and 5 (Fig.8). Average numbers for Groups 1-5 were 21.64 (mg/dL), 4.46 (mg/dL), 4.25 (mg/dL), 4.08 (mg/dL), and 17.24 (mg/dL) respectively. No change was found in magnesium and phosphorus of urine among the Groups. The values of PTH in serum were 37.26 pg/mL, 52.96 pg/mL, 54.72 pg/mL, 41.30 pg/mL, and 39.83 pg/mL for Groups 1, 2, 3, 4, and 5. Concentrations of urinary creatinine were 6.05 mg/dL, 7.09 mg/dL, 6.30 mg/dL, 8.93 mg/dL, and 7.67 mg/dL for Groups 1, 2, 3, 4, and 5 respectively. Albumin levels in serum were 3.84g/dL, 4.34g/dL, 3.53g/dL, 3.50g/dL and 3.40g/dL for Groups 1, 2, 3, 4, and 5 respectively.

Discussion

Rats fed calcium deficient food, calcium deficient food containing estrogen, vitamins D_3 and K_2 and 2% oxalic acid food succumbed to osteoporosis with significant decreases of fracture energy and bone mineral density and increase of bone X-ray density. Calcium deficient food intake is well known to cause osteoporosis due to shortage of calcium[15]. On the other hand,

oxalic acid is reported to induce osteoporosis due to inhibit absorbance of calcium.[16]

Spinach food intake used dry powdered spinach containing a large number of oxalic acid did not cause osteoporosis. Spinach contains oxalic acid in rate of 400-600mg/100g. Dry powdered spinach food used include 0.4-0.6% of oxalic acid. Amount of oxalic acid for spinach food corresponded to 1/3 of oxalic acid content for 2% oxalic acid food, so spinach food intake was considered to void development of osteoporosis due to less content of oxalic acid, compared with the 2% oxalic acid food. Daily food intake is not supposed to cause osteoporosis, even taking a large quantity of spinach with much oxalic acid. However, people who restrict food intake for health reason fail to consider the deficiency of nutrients and seem likely to suffer from osteoporosis due to calcium deficient food intake.

Osteoporosis due to calcium deficient food intake was similar to osteoporosis due to 2% oxalic acid food intake with respects to body weight, food intake, uterus weight, estradiol level and concentrations of deoxpyridinoline and calcium in urine but totally deferent from osteoporosis due to restricted food intake, and osteoporosis due to protein deficient food in body weight, food intake, uterus weight, estradiol level, concentration of calcium in urine and inhibition by estradiol, vitamins of D_3 and K_2.

Concentration of urinary calcium was extremely low for Groups 2, 3, and 4 rats induced the osteoporosis, compared with those of Groups 1 and 5.

Extremely low concentration of urinary calcium like this is supposed to be great sign for the osteoporosis and to be useful for judgment of it.

Osteoporosis due to a lack of various nutrient is similar in significant fall of bone breaking energy and bone mineral density, but the characteristics of the osteoporosis and its treatment by diet are expected to be different.

Therefore, alkaline phosphatase activity and concentrations of deoxypyridinoline and calcium in urine are analyzed in decision of osteoporosis and its treatment should be done according to the results.

Rats fed calcium deficient food had high concentration of PTH, but rats fed 2% oxalic acid food did not have high level of it. Calcium deficient food intake is known to cause increase of PTH. Calcium concentration in the intestine is estimated to decrease for calcium deficient food intake, but keep normal for 2% oxalic acid food intake. A rise of PTH is supposed to be induced due to low level of intestinal calcium. Osteoporosis due to calcium deficient food intake is similar to that due to 2% oxalic acid food intake, so a rise of PTH seems to have no influence to the osteoporosis.

An increase of urinary deoxypyridinoline was observed for rats fed calcium deficient food intake and 2%oxalic acid food intake, so the osteoporosis appears to cause due to increase of osteoclast surface.

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カルシウム欠乏食摂食により誘発する骨粗鬆症の特徴

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要　約

7週齢のWistar系雌ラットにカルシウム欠乏食，エストロゲン，ビタミンD_3とK_2を含むカルシウム欠乏食，2％蓚酸食を2週間投与すると骨の破断エネルギーと骨密度が減少し，X線写真の透過度が上昇し，骨粗鬆症になった．5％乾燥粉末ほうれん草食は骨粗鬆症を誘発しなかった．カルシウム欠乏食による骨粗鬆症では，体重，摂食量，子宮重量，血中エストロゲン量が変わらぬ点と，尿中デオキシピリジノン量と尿中カルシウムが減少の点で，2％蓚酸食による骨粗鬆症とよく類似した．食餌制限による骨粗鬆症とたんぱく質欠乏食による骨粗鬆症は，体重，摂食量，子宮重量，血中エストロゲン量が減少し，尿中カルシウム量がかわらない，そのため異なった．血中の副甲状腺ホルモンは，カルシウム欠乏食では，上昇したが，2％蓚酸食では上昇しなかった．尿中のカルシウムが極端に減少するのはカルシウム欠乏食による骨粗鬆症の大きな特徴で，骨粗鬆症の判定に有用であると思われた．栄養素の不足欠乏による骨粗鬆症は，骨の破断エネルギーと骨密度の減少の点では類似するが，その特徴や対応は異なる．そのために，骨粗鬆症の判定では，尿中のデオキシピリジノリン濃度，アルカリホスファターゼ活性，カルシウム濃度を測定し，その結果に即した対応が必要である．