APPENDIX - 4

RAT TESTING RESULT

Effects of nanocalcium supplemented milk on bone mineral metabolism in ovariectomized rats

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This study was conducted to investigate the effects of calcium supplementation in milk on bone loss in ovariectomized rats. Thirty Sprague-Dawley rats of 8 week-old were divided into 4 groups, overiectomized, and fed the diet and 1 mL milk containing:

1) OVX1, the commercial milk by intubation,
2) 2) OVX2, the calcium carbonate supplemented milk,
3) 3) OVX3, ionized calcium supplemented milk, and
4) 4) OVX4, nano-calcium supplemented milk.

After 18 wk feeding, body weight gain and food intake efficiency ratio were not significantly different among groups except for food intake efficiency ratio between OVX4 and OVX1 ($p > 0.05$). The weights of femur and tibia were not significantly different among all groups. There was no difference in dry weight and ash weight, however, calcium or phosphorus contents in dry weight or ash weight were significantly higher in OVX4. In addition, femoral BMD (bone mineral density) appeared to be greater in OVX4, which supplemented with nano-calcium than those in other groups ($p < 0.05$). Among groups, OVX4 group showed the highest bone strength as stiffness ($p < 0.05$) and slightly higher in maximum energy compared with other groups. Trabecular bone areas (%) in tibia were significantly higher in OVX4 group ($p < 0.05$). The present study indicated that nano-calcium supplementation in milk among other kinds of calcium supplementation showed the higher contents of calcium and phosphorus in femur and tibia, and the greater stiffness compared with other kinds of calcium supplementation. Therefore, nano-calcium supplementation might have a potential role for preventing bone loss and enhancing bone sparing effects in ovariectomized rats.

Key word: nano-calcium, bone mineral metabolism, preventing bone loss.

Rat Test for the Prevention from & Treatment of Osteoporosis by Feeding the NANO Calcium Supplemented Milk

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1. INTRODUCTION

Tendency of extending the national life span resulting big increase of the aged people ratio owing to the development of Economy & Medicine

Importance of Improving Life Quality of the Aged along with the Extension of Life Span

One of the Raised Issue for the National Health Care is Osteoporosis → Increase of Bone FractureWhat is Osteoporosis?

Decrease of Bone Density → Increase of rough Pumice or numerous minor Sponge Holes in the Bone Matrix → Conditions of Weak & Vulnerable to Bone Fracture

Major Causes : Female : Menopause → Decrease of Estrogen

Male : Aging

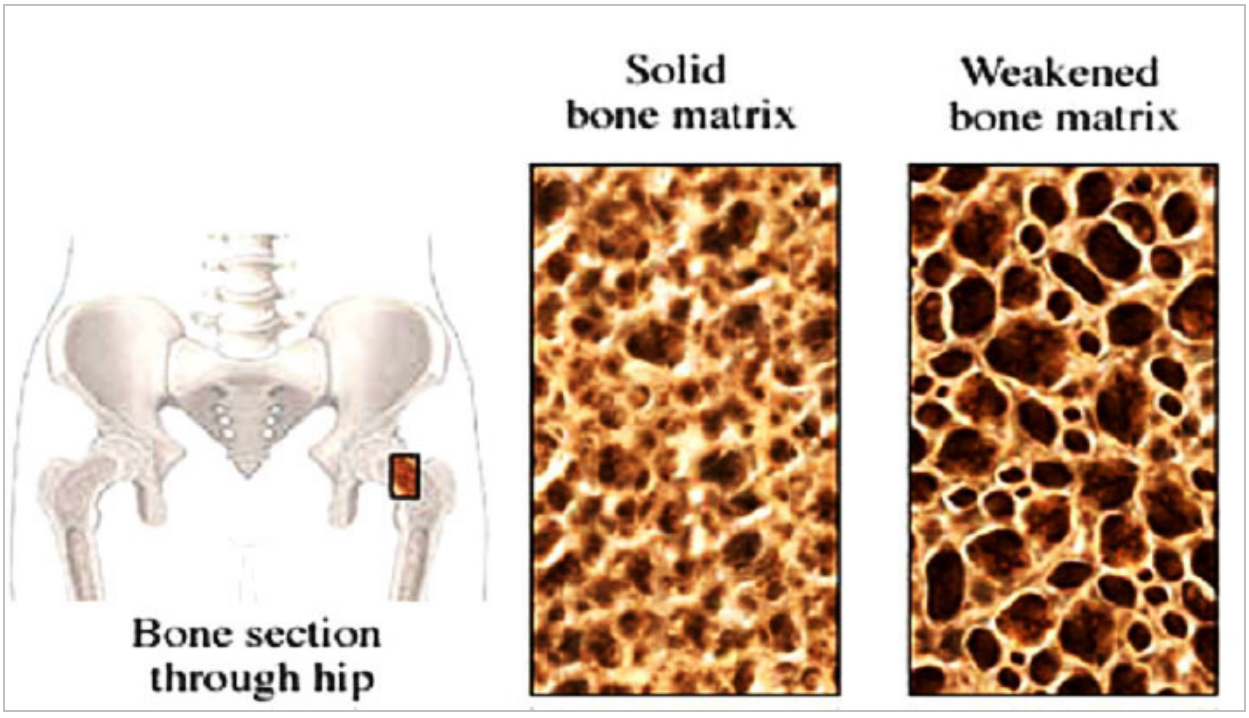

Figure 1. View of Femur Section of the Menopause Female with the Progressed Osteoporosis

- Prevention of the Menopause Female from Osteoporosis Estrogen Administration → Control of the Osteoclast Activity → Reducing the Resorption of Bone, Effective for the Cardiovascular System
- Trouble for Estrogen Administration: Motivating Breast Cancer, Endometrioid Tumor and etc.
- NIH(US) Report related to the Trouble with Estrogen Patient Number for the Clinic Test: 16,600 Patients

  Mastitis: 26%

  Cardiac crisis: 29%

  Cerebral Apoplexy: 41%
- Ban of the Estrogen Administration: 2002. 7. 9

- Major Ingredients of the Osteoplastic Process: Calcium
- Most Effective Absorptance of Calcium: Calcium in Milk
- Calcium Absorption from Milk by the Postmenopausual Female:
  Almost not Effective → Lack of Estrogen
- Conclusion : Prevention from & Treatment of Osteoporosis of the Postmenopausual Female, currently, has been almost Negative: Press release by US NIH (Chosun Daily: 2006. 02. 17)
- Particle Size of Calcium in the Current Market Places: um (1/1,000 mm)
- Particle Size of the newly Developed NANO Calcium: nm (1/1,000um = 1/1,000,000mm)
- Purpose of the Study:
  - To Research & Verify the possibility of the Prevention from & Treatment of Osteoporosis with the Postmenopausual Female by feeding the NANO Calcium through Rat Test.

2. THE MATERIALS AND METHOD OF TEST

1. Testing Animal

- 24 White Female Rats of SD race, aged 7 weeks
- Initial 1 week: Experiment Feed for Animal (for adaptation)
- Testing Room Temperature: 22± 2℃
  Testing Room Humidity: RH 40-60%
  Testing Room Light & Dark: 12hrs' shift
  Water Supply: Free without Limit

2. Ovariectomy

- Administered the Ketamine Hydrochloride (1ml/kg) to Abdominal Cavity with 8 weeks aged white Rats → Anesthesia
- Antiseptic Surgery
- Recovery Period: 1 Weeks after the Surgery

3. Rat Grouping & Milk feeding :

① OVX 1: normal milk without Calcium Supplement
② OVX 2: calcium carbonate supplemented milk
③ OVX 3: ionized calcium supplemented milk
④ OVX 4: NANO CALCIUM supplemented Milk

- Daily feeding of 20mg of the pure NANO CALCIUM supplemented milk for 18 weeks
- Particle size of NANO CLACIUM: 30-900nm
- Source of Supply: Nano-Techworld Co., Ltd.

4. Sampling

- Stopped feeding for 24 hours in the last day of test

① Blood Sampling

- Taken from Eye Ball of RATS
- Centrifugation for 15 minutes at 3,000rpm
- Serum Sampling from Supernatant, then stored in a freezer at -20℃ temperature.

② Tibia Sampling

- Taken from Tibia Area of the Right Leg
- Stored in the 10% formalin liquid

③ Femur Sampling

- Stored in the 70% ethanol
- To check the Bone Density

④ Sampling the Liver & Kidney

- Extracted from the sacrificed animal, weighed the Osteogenic Tissue from an electronic scale.
- Sample production of Liver and Kidney being decomposed in acid to analysis the Ca & P Property Values.
- Measurement of Ca & P Properties out of the produced sample through ICP (Inductively Coupled P lasma) Emission Spectrometer (Shimadzu, ICPS-1000IV, Japan)

5. Biochemical Test

① Biochemical Examination

- Ca & P Property Value in the Serum & Tissue Atomic absorption spectrophotometer
- Serum P

  Fiske-Subarow method

② Bone Examination

- Ca & P Property Values in the Dried Femur Inductively Coupled Plasma
- Bone Density Test of Femur X-ray Bone Densitometer
- Bone Strength and maximum Load Test Bone strength meter
- Bony Trabecula Area: Epiphyseal Plate of the low part of Tibia

  Grid point counting technique

  % = point on bone / points on tissue inside the measurement area
- Bony Trabecular Area Photo×400

6. Statistic Process

- ANOVA
- Duncan's multiple test

3. TEST RESULT

Table 1. Weight Gaining, Daily Food Intake Volume & Intake Efficiency [1] with the Osteoporosis induced Rats Group

Group	Total weight gain (g)	Daily weight gain (g/d)	Daily food intake (g/d)	FER (WG/FI) (%)
OVX1[2]	140.5±12.0^{ab}	1.1±0.1^{ab}	12.1±0.8^{ab}	9.3±0.7^{a}
OVX2[3]	147.3±20.8^{a}	1.2±0.2^{a}	12.4±0.9^{a}	9.5±1.6^{a}
OVX3[4]	128.4±28.1^{ab}	1.0±0.2^{ab}	11.2±0.5^{b}	9.1±1.9^{ab}
OVX4[5]	**117.7±4.5^{b}**	**0.9±0.0^{b}**	**12.3±0.6^{a}**	**7.6±0.5^{b}**

[1]Values within the same column with different superscripts are significantly different at p<0.05 by Duncan's multiple-test.

[2]OVX1 : ovariectomized + no supplemented milk

[3]OVX2 : ovariectomized + calcium carbonate supplemented milk

[4]OVX3 : ovariectomized + ionized calcium supplemented milk

[5]OVX4 : ovariectomized + nano-sized calcium supplemented milk

Table 2. Ca & P values, Intake Volume & Intake Efficiency1with the Osteoporosis induced Rats Group

Group	Group Serum Ca(mg/dl)	Serum P(mg/dl)
OVX1[2]	10.2 ± 1.3^{a}	4.4 ± 0.4^{a}
OVX2[3]	10.2 ± 1.5^{a}	4.5 ± 0.9^{a}
OVX3[4]	9.9 ± 0.7^{a}	4.5 ± 0.9^{a}
OVX4[5]	**9.8 ± 0.4^{a}**	**4.5 ± 0.1^{a}**

[1]Values within the same column with different superscripts are significantly different at p<0.05 by Duncan's multiple-test.

[2]OVX1 : ovariectomized + no supplemented milk

[3]OVX2 : ovariectomized + calcium carbonate supplemented milk

[4]OVX3 : ovariectomized + ionized calcium supplemented milk

[5]OVX4 : ovariectomized + nano-sized calcium supplemented milk

Table 3. Ca & P values 1in the Liver & Kidney with the Osteoporosis induced Rats Group

Group	Liver		Kidney	
	Ca(㎍/g)	P(mg/g)	Ca(㎍/g)	P(mg/g)
OVX1[2]	12.2 ± 8.4[b]	2.0 ± 0.3[a]	38.7 ± 7.1[b]	1.7 ± 0.3[a]
OVX2[3]	12.6 ± 6.5[b]	1.2 ± 0.2[a]	44.1 ± 15.5[a]	2.0 ± 0.4[a]
OVX3[4]	9.6 ± 5.6[b]	1.7 ± 0.3[a]	42.0 ± 25.3[b]	1.8 ± 0.3[a]
OVX4[5]	**25.9 ± 15.5[a]**	**1.9 ± 0.3[a]**	**77.8 ± 23.4[a]**	**1.8 ± 0.3[a]**

[1]Values within the same column with different superscripts are significantly different at p<0.05 by Duncan's multiple-test.

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[3]OVX2 : ovariectomized + calcium carbonate supplemented milk

[4]OVX3 : ovariectomized + ionized calcium supplemented milk

[5]OVX4 : ovariectomized + nano-sized calcium supplemented milk

Table 4. Length[1] of the Femur and the Tibia with the Osteoporosis induced Rats Group

Group	Femur length (mm)	Tibia length (mm)
OVX1[2]	35.8 ± 0.8[b]	40.7 ± 0.5[ab]
OVX2[3]	36.8 ± 0.6[a]	41.3 ± 0.4[a]
OVX3[4]	35.8 ± 0.6[b]	40.2 ± 0.8[b]
OVX4[5]	**35.5 ± 0.5[b]**	**40.3 ± 0.4[b]**

[1]Values within the same column with different superscripts are significantly different at p<0.05 by Duncan's multiple-test.

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[4]OVX3 : ovariectomized + ionized calcium supplemented milk

[5]OVX4 : ovariectomized + nano-sized calcium supplemented milk

Table 5. Ash Weight of the Tibia, the Ratio 1between Ca & P with the Osteoporosis induced Rats Group

Group	Ash weight (g)	Ca/Ash (%)	P/Ash (%)
OVX1[2]	0.309 ± 0.012[b]	24.42 ± 2.49[c]	12.67 ± 1.83[b]
OVX2[3]	0.332 ± 0.020[ab]	28,22 ± 1.67[ab]	15.14 ± 0.88[a]
OVX3[4]	0.314 ± 0.027[ab]	26.13 ± 0.93[bc]	13.63 ± 1.05[ab]
OVX4[5]	**0.336 ± 0.017[a]**	**29.75 ± 4.13[a]**	**15.58 ± 2.08[a]**

[1]Values within the same column with different superscripts are significantly different at p<0.05 by Duncan's multiple-test.

[2]OVX1 : ovariectomized + no supplemented milk

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[4]OVX3 : ovariectomized + ionized calcium supplemented milk

[5]OVX4 : ovariectomized + nano-sized calcium supplemented milk

Table 6. The Bone Stiffness and the Maximum Load 1of Femur with the Osteoporosis induced Rats Group

Group	Stiffness (N/mm)	Maximum energy (N)
OVX1[2]	115.0 ± 17.8 [b]	83.5 ± 7.7[b]
OVX2[3]	120.8 ± 7.2[b]	88.2 ± 9.4[b]
OVX3[4]	119.5 ± 16.1[b]	88.4 ± 11.6[b]
OVX4[5]	**143.7 ± 12.4[a]**	**101.5 ± 4.8[a]**

[1]Values within the same column with different superscripts are significantly different at p<0.05 by Duncan's multiple-test.

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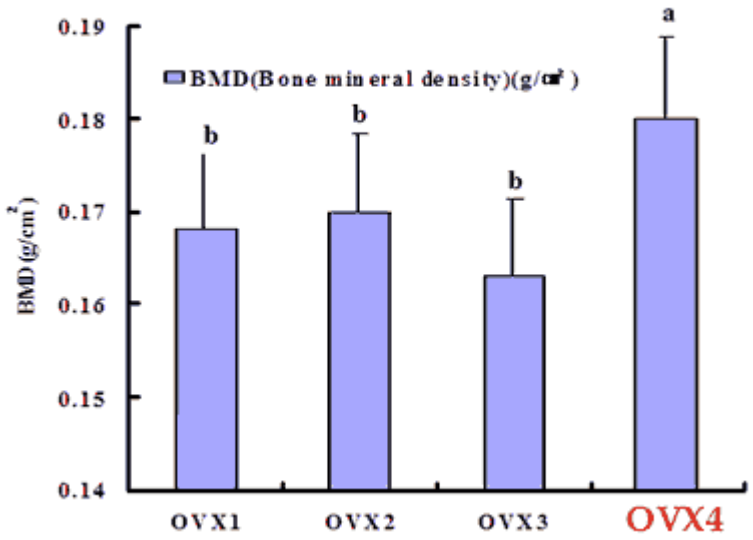


Figure 2. Bone Density of the Osteoporosis induced Rat Groups

ovx1: no calcium supplemented milk fed, **ovx2** : calcium carbonate supplemented milk fed

ovx3: ionized calcium supplemented milk fed **ovx4** : nanoCalcium supplemented milk fed.

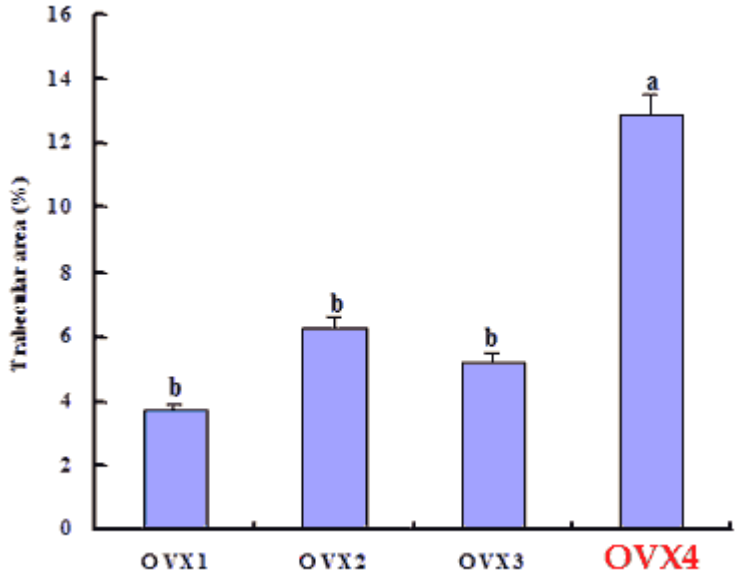
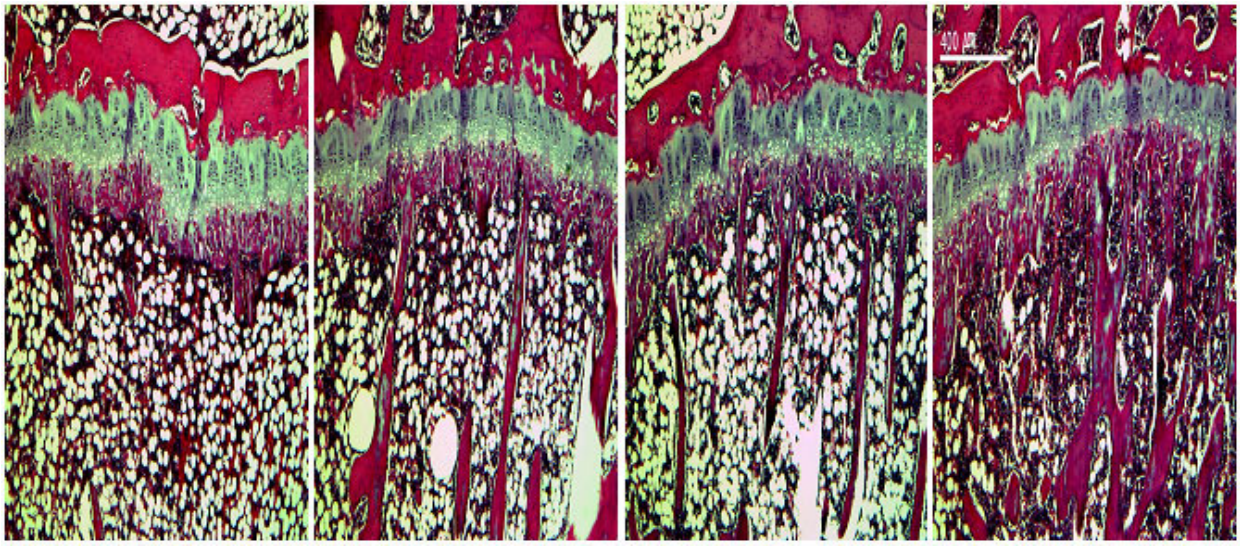


Figure 3. TrabecularAreas of the Osteoporosis induced Rat Groups

ovx1: no calcium supplemented milk fed, **ovx2** : calcium carbonate supplemented milk fed

ovx3: ionized calcium supplemented milk fed **ovx4** : nanoCalcium supplemented milk fed.



OVX1 OVX2 OVX3 **OVX4**

Figure 4. Trabecularof the Tibia with the Osteoporosis induced Rat Groups

ovx1: no calcium supplemented milk fed, **ovx2** : calcium carbonate supplemented milk fed

ovx3: ionized calcium supplemented milk fed **ovx4** : nanoCalcium supplemented milk fed.

1. CONCLUSION

This research has been aimed at verifying the improvement effect of Osteoporosis symptoms when feeding the newly produced NANO CALCIUMsupplemented milk to the ovariectomizedRATS being induced the Osteoporosis; the result summary follows.

① Significant difference was shown with OVX2 and OVX4 in weight gaining rate as well as food intake efficiency after 18 weeks offeeding.

② No change was monitored with Ca & P property values in SERUM among all the test Rat Groups

③ However, Ca & P property values of the ash of FEMUR with OVX4 group was found significantly hihgerthan those of OVX1 and OVX3 groups.

④ Bone Density in FEMUR with OVX4 was found very higher than those of other groups.

⑤ Stiffness, the Maximum Load as well as TrabecularArea of FEMUR were all found highest with OVX4 group.

As a conclusion of this study, NANO CALCIUM supplemented milk is verified as the mosteffective for the improvement of and prevention from OSTEOPOROSIS being induced from bone loss.

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