대한정형외과연구학회지 제 14권 제 1호
J. of Korean Orthopaedic Research Society
Volume 14, Number 1, June, 2011

Alendronate와 Calcitriol 고정용량복합제의 장기간의 복용이 난소가 제거된 백서의 해면골의 미세구조에 미치는 효과

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= Abstract =

Effect of a Long-term, Oral Fixed Dose with a Combination of Aledronate and Cacitriol on the Cancellous Bone Microarchitecture in Ovariectomized Rats

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Purpose: To study the effect of a long-term, oral, fixed dose with a combination of alendronate and calcitriol on the cancellous bone microarchitecture in an ovariectomized rat model.

Materials and Methods: Twenty eight female Sprague-Dawley rats were divided into 2 equal groups: a non-medication group (OVX), and a medication group (ALD). The ALD group was treated with an oral daily fixed dose with a combination of alendronate and calcitriol for six months, starting from 4 weeks after ovariectomy, while the OVX group was given only a placebo. After six months, all animals were sacrificed, and an in vitro micro-CT analysis of the the distal femur was performed. The bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), structure model index (SMI), connectivity density (Conn.D), and bone mineral density (BMD) were assessed.

Results: The ALD group had significantly higher BV/TV, Tb N, BMD and Conn.D and it also had significantly lower Tb Sp and SMI than the OVX group.

Conclusion: A long term, daily, oral fixed dose with a combination of alendronate and calcitriol could significantly reduce the osteoporotic changes in this ovariectomized rat model.

Key Words: Microarchitecture, Cancellous bone, Ovariectomized rat, Alendronate, Calcitriol, Long-term, Oral, Combination

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접수일: 2011년 4월 28일, 게재확정일: 2011년 5월 13일
* 본 논문은 (주)유유제약의 임상연구비 지원을 받아 이루어졌음.

Introduction

Post-menopausal osteoporosis is a major health problem in the elderly women, with substantial morbidity and disability. It is characterized by low bone mass, and structural deterioration of bone tissue, leading to increased risk of fragility fractures[1,7,29]. However, pharmacological intervention can curb these osteoporotic changes and reduce the incidence of these fractures. Several agents are available and oral bisphosphonates, with concomitant calcium and vitamin D is currently the most common[1,5,7,14,18,28]. Alendronate, a nitrogen containing bisphosphonate, is a powerful and selective inhibitor of osteoclast mediated bone resorption. It reverses the progression of osteoporosis, increases bone mineral density (BMD), and decreases the incidence of osteoporotic fractures. Many studies have reported that vitamin D analogues like calcitriol and alfacalcidol can be considered for combination treatments with antiresorptives like bisphosphonates[1,7,12,18,24,25]. The rationale for this combination is that, most elderly osteoporotic patients who are prescribed alendronate, tend to have Vit D deficiency, which in turn adversely affects the antiresorptive efficacy of bisphosphonates[7,14].

The objective of our study was to assess the efficacy of a long term, oral, fixed dose with a combination of alendronate and calcitriol in reverting the osteoporotic changes in an ovariectomized rat model, since it represents the most important clinical features of estrogen deficiency-induced or post-menopausal bone loss in the adult human.

Materials and Methods

Twenty eight female Sprague-Dawley rats were selected for the study. At 12 weeks of age, they were anesthetized and an ovariectomy (OVX) was performed. Four weeks after OVX, they were randomly divided into two groups of 14 each; a non-medication group (OVX, n=14), and a medication group (ALD, n=14). The mean body weight of the ALD group was 375g (range, 334~428 g), and of the OVX group was 368g (range, 324~ 416 g). The rats were housed in individual cages, for the convenience of administering the oral medication. The room temperature was maintained at 24°C and humidity maintained at 30%. Food pellets (20% protein, 3.5% lipid, 8% fiber, 8% ash, 5% calcium, and 1.5% phosphorous) and tap water were being given ad libitum, except during the overnight fasting period, before medication. The medication for the ALD group was started 4 weeks after OVX. In our study, we have used a novel method to orally administer medicine, in the form of a sugar coated pill containing a fixed dose with a combination of alendronate and calcitriol. Each pill was reformulated to a rat dose to be administered in 400 microgram of alendronate and 0.04 microgram of calcitriol. It was calculated from the daily recommended dose for a 60 kg adult and adopting it to 373 g, which was the average weight of the rats. After an overnight fasting period, a single pill was kept in a glass dish in each cage, and half an hour after the pill was consumed, the food pellets were put back in the cages. Though initially, the animals were hesitant to consume the pills, within a few days, they readily started taking the

pills as soon as it was being given to them. The pills were being given daily for six months. The OVX group was given only a placebo, which was also administered in a similar manner. All animals were treated according to the practice code of the Ajou University Medical School-Institutional Animal Care and Use Committee (AUSM-IACUC), with the approval number AMC63, dated 14/9/2009.

Scanning was done using the micro-CT scanning machine (Skyscan model 1173, Skyscan, Belgium)(Fig. 1). Image acquisition was done with a source voltage of 130 KV and a source current of 30 μA. The aluminum filter used for beam hardening artifact reduction was 1 mm thick. The image pixel size was 12.16 μm, the number of rows and columns was 1120 each, vertical object position was 35 mm, and averaging frames 3. The exposure time was 250 ms, and the rotation step was 0.3°. The total scanning time was 20 min. The scanning region was centered on the distal femur including the femoral condyles and metaphyseo-diaphyseal region. After scanning, the images were reconstructed using the reconstruction software (NRecon, V1.4.1 Skyscan, Kontich, Belgium)(Fig. 2). After image recon-

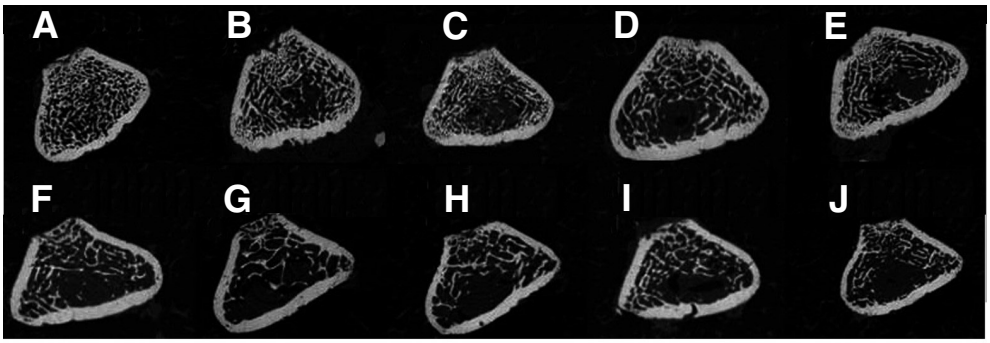

Fig. 1. 2D cross sectional images of the cancellous area of distal femur of five rats each, in the ALD group **(A, B, C, D, E)**, and the OVX group **(F, G, H, I, J)**. The OVX group is showing a much lesser number of trabeculae, with increased trabecular separation than the ALD group.

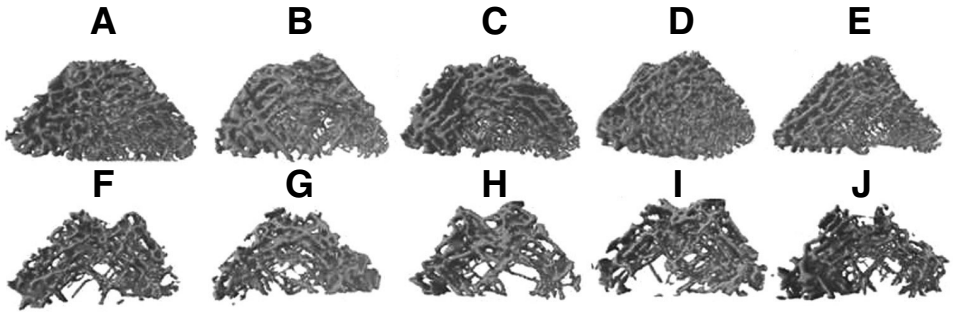

Fig. 2. 3D micro-CT reconstruction models of the volume of interest (VOI) of cancellous bone of distal femur of five rats each, in the ALD group **(A, B, C, D, E)** shown in blue, and the OVX group **(F, G, H, I, J)** shown in green. It is obvious from the 3D models that the ALD group is having a higher trabecular bone volume, with increased number of trabeculae, and lesser trabecular separation than the OVX group.

struction, using the image analysis software (CTAn V1.10.1.3, Skyscan, Kontich, Belgium), analysis of the reconstructed images were done after selecting a volume of interest (VOI) containing exclusively cancellous bone. The VOI included 200 cross sections extending from 1 mm above the proximal end of the growth plate, proximally for a height of 2 mm.

During analysis, the BMD, and the following structural parameters were calculated over each VOI of cancellous bone: Bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), and structure model index (SMI), and connectivity density (Conn.D). The BV/TV was assessed using the marching cubes method, calculating the bone volume (BV) over the volume of the VOI i.e, the tissue volume (TV)[16]. The Tb.Th and Tb.Sp, are measures of the average thickness of the cancellous bone, and the average diameter of the marrow cavities, respectively. They were calculated using the local sphere-fitting method[8]. The Tb.N, which gives the number of trabecular plates per unit length was calculated using the formula Tb.N=(BV/TV)/Tb.Th[20,21]. SMI is a parameter calculated using a differential analysis of the triangulated surface of a structure, which gives an estimate of the ratio of the number of plates to the number of rods constituting the 3D structure[9,22]. Its values range from 0 to 3, with 0 indicating an ideal plate-like structure and 3 indicating an ideal rod-like structure, and the intermediate values representing a structure composed of both plates and rods. Conn.D is a topological parameter that estimates the number of trabecular connections per cubic millimeter[19].

Statistical analysis was done using SPSS version 12 (Chicago, IL, USA). All the parameters with the exception of Tb.N and Conn.D, had a normal distribution (Shapiro-Wilk test, $p>0.05$ for all the examined parameters). So, the variables BV/TV, SMI, Tb.Th, Tb.Sp, and BMD were tested by independent T-Test, while the variables Tb.N and Conn.D variables were tested by Nonparametric Test. For all the comparisons, differences were deemed to be statistically significant at $p<0.05$.

Results

The mean and standard deviations of the various microstructural parameters and the BMD, of the 2 groups are given in table1. All the 28 rats had completed the study. The mean body weight at the end of the treatment period, when the animals were sacrificed was 428 g (range, 388~455 g) in the OVX group, and 368 g (range, 382~562 g) in the ALD group. Thus the OVX group had higher mean weight gain (90 g), in comparison to the ALD group (53 g) (p value-0.04). Both the OVX and the ALD groups showed significant difference with respect to the BV/TV, Tb N, Tb Sp, BMD, SMI, as well as the Conn.D. The ALD group had significantly higher BV/TV, Tb N, BMD and Conn.D and a significantly lower Tb Sp and SMI, in comparison with the OVX group. This indicated that the ALD group had a higher bone mass, increased thickness of cancellous bone, increased number of trabecular plates per unit length, as well as higher number of trabecular connections per unit length, when compared

Table 1. Summary of the comparison of the various microstuctural parameters, and the BMD, between the two groups with the corresponding p values.

*Parameter	ALD group (14)	OVX group (14)	P value
BV/TV	15.029 (±2.978)	7.939(±2.320)	0.0000
SMI	1.379(±0.292)	1.869(±0.337)	0.0004
Tb.Th	8.264(±0.449)	8.372(±0.397)	0.6638
Tb.N	0.018(±0.004)	0.009(±0.003)	0.0000
Tb.Sp	55.027±(7.816)	70.260(±9.959)	0.0001
Conn.D	0.00021(±6.57041E-05)	0.00009(±6.08728E-05)	0.0003
BMD	0.042(±0.037)	0.005(±0.009)	0.0009

Depicted are the mean values, with the standard deviation (SD) in parentheses (±SD).
*TV-total volume ($pixel^2$), BV-bone volume ($pixel^2$), BV/TV-Bone volume fraction (%), SMI-structure model index, Tb.Th-trabecular thickness (pixel), Tb.N-trabecular number (1/pixel), Tb.Sp-trabecular separation (pixel), and Conn.D-connectivity density ($1/pixel^2$) and BMD-bone mineral density (g/cm^2).
One pixel size=12.16 micrometer.

to the OVX group. Though both the groups had intermediate values for the SMI (between 0 and 3), the lower SMI value for the ALD group meant that it had a more plate-like structure when compared to the OVX group. However, there was no significant difference in the Tb Th between the two groups.

Discussion

Many recent reports have appeared in literature on the early and late effects of Zoledronic acid injections in ovariectomized rats, assessed by in vivo micro-CT[3,23]. There have also been previous reports on the combined, as well as comparative effects of alendronate and alfacalcidol in ovariectomized rats[11,12], where mechanical testing and bone histomorphometry were used to assess the mechanical properties of bone, alendronate had been administered subcutaneously, and medication was administered for not more than three months. This is the first study to determine the effect of a long-term (6 months medication period), oral, fixed dose with a combination of alendronate and calcitriol, administered daily, in improving the deterioration of the cancellous bone microstructure, assessed by micro-CT, in an ovariectomized rat model.

The rat OVX model represents the most important clinical features of estrogen deficiency-induced or post-menopausal bone loss in the adult human[13,27]. Two weeks post OVX, there is increased bone turnover and bone resorption with significant decrease in trabecular bone volume in OVX rats. Though the rat OVX model is suitable for evaluating agents for osteoporosis, it has limitations that restrict the evaluation to cancellous bone sites[13,27]. The evaluation of the cortical bone response may not be appropriate since the rat OVX model does not mimic postmenopausal women in this respect[27]. We did the study assessing the effect of alendronate and calcitriol on the cancellous bone of rat distal femur.

There are studies showing that there is a short time window during which the

microstructural parameters undergo the largest magnitude of change, post-ovariectomy[2,4]. Moreover, since this happens within the first three months post-ovariectomy, they suggest, it is preferable to start antiresorptive treatment early. Perilli et al[23], in a longitudinal study using in vivo micro-CT on ovariectomized rats, have shown that full restoration of the microstructural parameters to the baseline values is possible even after starting treatment 2 weeks post-ovariectomy. In our study, treatment was started 4 weeks after ovariectomy, when most of the microstructural changes following ovariectomy would be quite well established according to the previous studies. We decided to give the medication for a period of only six months because, with longer evaluation periods, cancellous bone turnover indices are found to return to the value of sham controls, with a new steady state in bone remodeling[27].

The OVX group had significantly higher mean gain in body weight (90 g) than the ALD group (53 g) ($p < 0.05$), which might probably be due to a significantly higher body fat content[27]. The ALD group had a significantly higher BV/TV, Tb.N, and a significantly lower Tb.Sp and SMI in comparison to the OVX group. The BV/TV plays an important role in the mechanical properties of subchondral trabecular bone[29]. The higher BV/TV seen in the treatment group in our study, may be attributed to an increase in the Tb.N along with a reduction in the Tb.Sp[6,29]. Though BV/TV has been found to positively correlate with Tb.Th also[29], in our study, there was no significant difference between the two groups in Tb.Th. Post-ovariectomy, a reduction in trabecular thickness is normally expected, associated with a significant reduction in BV/TV. However variable findings on trabecular thickness in OVX rats have been reported in literature. While some studies have found a reduction in Tb.Th over time following ovariectomy[2,4], some others have reported either no change, or an increase[3,6,10]. But since all of these studies have not used the same strain of animal, which could also possibily influence the change in Tb.Th, more studies may be needed to clarify this issue. It is possible that, in the OVX rats in our study, the bone loss seen was probably due to a preferential removal of the thinner trabeculae[2,4], resulting in a reduction in the trabecular number and increased trabecular separation, without any significant change in Tb.Th.

The SMI gives an idea about the proportion of the number of plates to that of rods in the three dimensional structure of trabecular bone, and is a good indicator of the mechanical strength of trabecular bone. Siu et al[26], in a study on ovariectomized goats, found that osteoporotic bone tends to have higher SMI values, and that SMI also had a negative correlation with BMD. Though the mean SMI value in our study was significantly higher in the OVX rats, both groups had intermediate values (between 0 and 3); with a mean value of 1.38 for the medication group, and 1.87 for the OVX group ($p=0.0004$). This meant that the ALD group had a larger fraction of trabecular plates, which in turn has been found to make a much larger contribution to the bone's elastic behavior than trabecular rods[15]. Specimens with predominant trabecular plates have been shown experi-

mentally to have lesser deformation than those with predominant rods[17].

The Conn.D, which represents the number of trabecular connections per cubic mm, has a positive association with the Tb.N and correlates with mechanical strength and mass of trabecular bone[6,29]. It is found to be decreased in the osteoporotic ovariectomized rat model and has a role in predicting bone quality of human being. The medication group in our study had a significantly higher Conn.D (p=0.0003) than the OVX group, representing a higher bone mass and strength.

Our study has limitations. We did not have a sham control group, to provide us baseline values to determine the actual degree of bone recovery following medication. Besides, we also did not have comparison groups, either for assessing different dosage schedules, or for the simultaneous assessment of the individual effects of alendronate and calcitriol. Further longitudinal studies using in-vivo micro-CT, and with more comparison groups, would definitely help derive more distinct conclusions.

Conclusion

To conclude, in this study, we have attempted to assess with micro-CT, the improvement in cancellous bone microarchitecture, in ovariectomized rats, following long-term administration of an oral fixed dose with a combination of alendronate and calcitriol. Starting medication 4 weeks following ovariectomy, we could find a significant reduction in the deterioration of cancellous bone microarchitecture, as well as a significant improvement in the BMD after 6 months of treatment.

Disclosure

The authors have received outside funding in support of this research from Yuyu Pharma Inc, Suwon, South Korea. Authors have full control of all primary data and agree to allow the journal to review data if requested.

Acknowledgements

The authors thank Jung, Sun Jang of Genoss Co. Ltd, Korea, for the technical help in scanning and analysis of the specimens, and Ms Bak, Jean Kyung for her help with the statistical analysis.

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