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BRIEFPAPER

Assessment of changes due to the long-term effect of estrogen and calcium deficiency in the trabecular bone structure in rats

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ABSTRACT

Objective. *To assess structural changes, especially structural anisotropy, of rat bone trabecular system 6 months after ovariectomy followed by low-calcium diet.*

Method. *The study was carried out on the group of 32 female rats, half of which were ovariectomized at the age of 75 days. The animals were divided into 4 groups: one receiving a normal diet (N), another receiving a low-calcium diet (LCa), ovariectomized rats receiving a normal diet (OVX), and ovariectomized animals receiving a low-calcium diet (OVX+LCa). After 6 months the animals were killed, bone specimens were collected and cut into sections of 6 μm thickness. Digital images of the sections were analyzed using a software package enabling analysis of the transversal and longitudinal trabeculae.*

Results. *Significant changes in trabecular structure due to a low-calcium diet (trabecular bone volume loss of 19%), ovariectomy (53%) and ovariectomy combined with low-calcium diet (71%) were observed. In all the analyzed groups, the percentage loss (as compared with controls) of transversal trabeculae was more significant than the loss of longitudinal trabeculae. In the LCa group, transversal trabecular loss was 39%, longitudinal 25%, in (Ovx): 63% and 54%, respectively, and in OVX + LCa: 77% and 72%. The structural anisotropy coefficient, defined as the ratio of transversal to longitudinal trabecular surface area was 0.64 for (N), 0.50 for LCa, 0.49 for OVX, and 0.54 for OVX+LCa groups.*

Conclusions. *The effect of ovariectomy and low-calcium diet on trabecular structure can be assessed quantitatively by means of analysis of transversal and longitudinal trabeculae associated with the main direction of strain. The degree of transversal trabecular loss is much higher than the longitudinal trabecular loss; the difference becomes smaller with the progress of bone destruction, being greatest in the LCa group, the smallest in the (OVX+LCa) group.*

Introduction

Mechanical strength of trabecular bone is dependent not only on bone density, but also on the spatial pattern of the trabecular structure (1-5). Considerable bone mass loss and structural destruction – characteristic of osteoporosis – result in deterioration of mechanical bone parameters and, consequently, in fractures. Therefore, qualitative assessment of the trabecular microarchitecture is very important to determine the extent of osteoporotic lesions and the fracture risk prognosis.

There are many known methods to assess trabecular structure, both two-dimensional (6-9) and three-dimensional (10-14). The method used in this study has been previously described (9) and is based on mechanical anisotropy of the trabecular bone (15,16). Mechanical anisotropy refers to the differing mechanical resistance of the bone to strain depending upon its orientation in relation to the direction of strain. Thus, the strength of the longitudinal trabeculae parallel to the main strain direction, will be different from that of the transversal trabeculae which are perpendicular to it, and the degrees of destruction in osteoporosis will also be different. Therefore, mechanical anisotropy is reflected in the trabecular bone structure. The software developed by us analyzes transverse and longitudinal trabeculae.

The presented study assessed structural bone anisotropy due to prolonged, simultaneous effect of hypogonadism and low-calcium diet. The influence of an ovariectomy and low calcium diet on the trabecular structure was shown by several studies (17-21). These studies were performed on rats during early periods following an ovariectomy and low calcium diet. The questions about long-term influence of these factors and character structural changes arises. The aim of those studies was to assess transverse and longitudinal trabecular loss and structural anisotropy of the trabecular bone in rats exposed to the prolonged effects of estrogen- and calcium deficiency.

Material and methods

Bone specimens

This study focused on the trabecular structure of the proximal tibia and was performed on 32 female Wistar rats. Half of them underwent an ovariectomy on the 75th day of life. The rats were divided into 4 groups: 1) ovariectomized rats on a standard diet (22) (OVX); 2) ovariectomized rats on a low-calcium diet OVX + LCa; 3) non-ovariectomized rats on a low-calcium diet (LCa); and 4) non-ovariectomized rats on a standard diet (N). The low-calcium diet contained 0.25% of calcium After 6 months, the animals were killed and the metaphysical tibia samples were collected, stained in methyl methacrylate and fixed. Next, the 6 μm thick specimens (3 on average per rat) were prepared. The study was approved by local ethics committee.

Figure 1 shows examples of bone specimen images from the four examined groups.

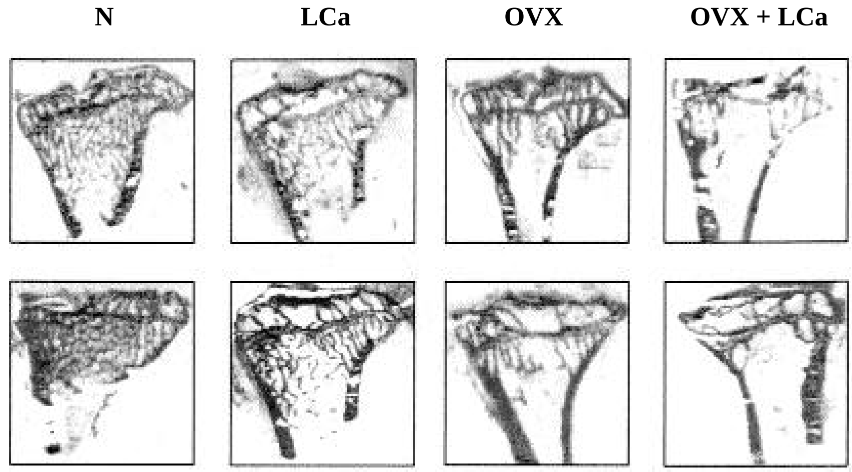

Fig. 1. Examples of images of bone specimens from four examined groups after 6 months of estrogen and calcium deficiency. Two specimens from each group are presented.
N: non-ovariectomized rats on standard diet, **LCa**: non-ovariectomized rats on low-calcium diet, **OVX**: ovariectomized rats on standard diet, **OVX + LCa**: ovariectomized rats on low-calcium diet.

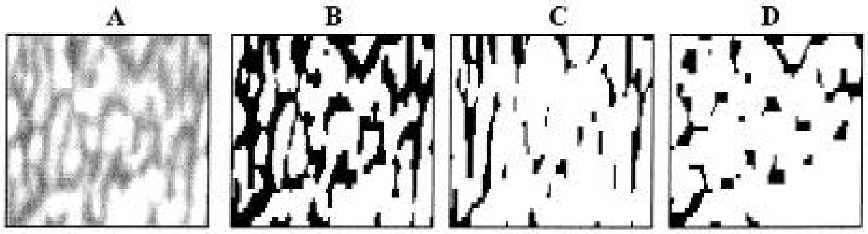

Fig. 2. Illustration of consecutive image processing stages: (**A**) original image of bone specimen; (**B**) the image binarized; (**C**) visualization of transversal elements; (**D**) visualization of longitudinal elements.

Image analysis

Digital images of the specimens (256 shades of gray) were obtained using a TV camera and analog-digital converter. For analysis the area 130 x 150 pixels from the proximal tibia metaphysis just below the growth plate was chosen. The images were further processed using the software described in (9). The software comprised a binarisation procedure to analyse erosion by means of linear transversal and longitudinal structure elements. This enabled us to obtain trabecular structure images with selected longitudinal and transversal elements, which were used for measurement.

Figure 2 illustrates the consecutive stages of image processing. The following parameters were determined: the longitudinal trabecular area S(lt); the transversal trabecular area S(tv); the ratio of the transversal and longitudinal areas d (structural anisotropy coefficient); and the trabecular bone volume BV/TV. Two additional parameters were also calculated: q (lt), the ratio of the mean longitudinal trabecular area in the given group to the mean longitudinal trabecular area in the control group, and q(tv), defined analogically for the transversal trabecular area.

Statistical evaluation

The differences between groups were tested using the non-parametric Mann-Whitney U test.

Results

Six months after the ovariectomy and low calcium diet, we observed the following trabecular structure changes (Table I). In animals following an ovariectomy (OVX), the mean transversal trabecular area was 63% lower, and the longitudinal trabecular area was 54% lower than in the control group. Also in animals following an ovariectomy and on a low calcium diet (OVX + LCa), transversal S(tv) and longitudinal trabecular area values S(lt) were different from the control group ($p < 0.005$). The mean value of S(tv) was 77% lower and S(lt) was 72% lower in comparison with the control group. In non-ovariectomized animals on a low calcium diet (LCa), the transversal trabecular area was 39% lower, and the longitudinal trabecular area was 25% lower in comparison with the control group.

The differences were found, on the basis of the Mann-Whitney U-test, to reach statistical significance ($p< 0.005$) for the transversal trabecular area values S(tv) in all three examined groups compared with the controls. The same was found for the longitudinal trabeculae with one exception: no statistically significant difference was found between LCa and controls.

The ratio of transversal to longitudinal trabecular areas, referred to as the anisotropy coefficient, was 0.61 for the control group and 0.50, 0.49, 0.54 for the LCa, OVX and LCa + OVX groups, respectively. A statistically significant difference was found in the values of the anisotropy coefficient d between the OVX and control groups, as well as between the LCa and control groups ($p < 0.005$). No such difference was found in the coefficient values between the OVX+LCa and control groups.

The values for BV/TV in the OVX, OVX + LCa and LCa groups were lower than those obtained in the control group and statistically significant dif-

Table I. Values of measured parameters in the 4 study groups.

	BV/TV mean ± SD (%)	S(lt) mean ± SD (pixels)	S(tv) mean ± SD (pixels)	q(lt)	q(tv)
N	35.6 ± 1.5	663 ± 53	409 ± 20	1	1
LCa	28.1 ± 2.8	495 ± 55	249 ± 38	0.75	0.61
OVX	16.8 ± 1.7	306 ± 33	152 ± 21	0.46	0.37
OVX + LCa	10.4 ± 1.4	182 ± 33	95 ± 17	0.28	0.23

ferences between groups were found ($p < 0.005$). The mean value of BV/TV in the OVX + LCa group was 71%, in the OVX group 53%, and in the LCa group 19% lower than in the control group (Table I).

Discussion

The analysis of trabecular structure images, based on the assessment of trabeculae parallel and perpendicular to the main direction of strain, allowed us to characterize the changes of trabecular structure resulting from estrogen-and/or calcium deficiency. Advanced changes in trabecular structure were observed. Trabecular bone volume loss BV/TV in the OVX + LCa group amounted to 71% and was 1.3-fold higher than in the OVX and 3.7-fold higher than in the LCa group. In all the experimental groups, transversal trabecular area loss was found to be more pronounced than longitudinal trabecular area loss in comparison with the control group.

Several dozen preparations were examined and in every case the finding was confirmed. In the LCa group, transversal trabecular loss as compared with controls was 38% and longitudinal trabecular loss as compared with controls was 25%. In the OVX group losses were 63% and 54% respectively, and in the OVX + LCa group 77% and 72%.

It is noteworthy that the percentage of transversal trabecular area loss compared with controls was always higher than the total trabecular area loss. Thus, total trabecular area loss after ovariectomy amounted to 53%, whereas that of transversal trabecular area reached 61%. After a low-calcium diet, the respective percentage loss values were 19% and 35%, and after ovariectomy combined with low-calcium diet the percentage loss values were 71% and 77%. Transversal trabeculae are the site where the earliest osteoporotic changes appear. Therefore, measurements of the transversal trabeculae may provide important information.

The fact that the transversal trabeculae are more susceptible to destruction than the longitudinal trabeculae results from their lower mechanical strength; the values of mechanical parameters for the transversal trabeculae are lower than those for the longitudinal trabeculae.

Bone density is of essential importance for mechanical properties. Trabecular bone strength is generally known to depend on the square of bone density. However, as demonstrated by Sugita (15), mechanical parameters (elasticity modules, maximal stress) decrease in a linear manner together with the decrease of bone density square value, but more rapidly for the longitudinal than for transversal trabeculae. Thus, as the bone density decreases, the difference between the degrees of transversal and longitudinal trabeculae destruction is reduced and structural anisotropy decreases. The results obtained in our study may provide a confirmation of this correlation. In the LCa group, the loss of transversal trabecular area was 41% higher than that of the longitudinal ones, in the OVX group the difference amounted to ca. 14%, and in the OVX + LCa – to ca. 7%. The mean structural anisotropy coefficient d in the OVX + LCa group was lower than in the controls and higher than in the OVX and LCa group.

The microarchitecture of trabecular bone in ovariectomized rats has been analyzed and described by numerous investigators. The findings included reduction of such parameters as the bone volume fraction BV/TV (17-24), trabecular number Tb.N (18,19, 21, 23, 24), reduction of trabecular thickness Tb.Th (21,24), no significant changes in trabecular thickness Tb.Th (18,23), and increased trabecular separation Tb.Sp (18,19,21,24). The magnitude of changes in the above parameters is a function of the time elapsed from ovariectomy. The change in the bone volume fraction BV/TV can serve as an example. Eight days after surgery a BV/TV reduction by 25% was observed (18), and after 4 weeks by 34-41% according to certain studies (19, 23, 24). After 7 weeks BV/TVwas reduced by 38% (17), after 12 weeks by 62% (21), and after 16 weeks by 60.5% (19). In ovariectomized rats on a low-calcium diet, a BV/TV decrease by 64% was found one month after ovariectomy (24). In our study, a decrease of 71% was demonstrated 6 months after the surgery.

In conclusion, in all three analyzed animal groups (OVX, LCa, and OVX + LCa) transversal trabecular area loss was found to be more pronounced than longitudinal trabecular loss. The difference between the percentage loss of transversal and longitudinal trabeculae was most significant in the LCa group, and least significant in the OVX + LCa group, where bone loss was the highest. The structural anisotropy of cancellous bone increases with trabecular loss; however, in cases of extensive bone destruction the bone became more isotropic. The obtained results seem to confirm the fact that in cases of bone mass (density) reduction, the strength of the longitudinal trabeculae is reduced more rapidly than that of transversal trabeculae.

References

1. ULRICH D, VAN RIETBERGEN B, LAIB A *et al.*: The ability of three-dimensional structural indices to reflect mechanical aspects of trabecular bone. *Bone* 1999; 25: 55-60.
2. UCHIYAMA T, TANIZAWA T, MURAMATSU H *et al.*: Three-dimensional microstructural analysis of human trabecular bone in relation to its mechanical properties. *Bone* 1999; 25: 487-91.
3. MAJUMDAR S, KOTHARI M, AUGAT P *et al.*: High-resolution magnetic resonance imaging: three-dimensional trabecular bone architecture and biomechanical properties. *Bone* 1998; 22: 445-54.
4. JIANG Y, ZHAO J, AUGAT P *et al.*: Trabecular bone mineral and calculated structure of hu-

man bone specimens scanned by peripheral quantitative computed tomography: relation to mechanical properties. *J Bone Miner Res* 1998; 13: 1783-9.
5. YEH OC, KEAVENY TM: Biomechanical effects of intraspecimen variations in trabecular architecture: A three-dimensional finite element study. *Bone* 1999; 25: 223-8.
6. GERAETS WG, VAN DER STELT PF, NETELENBOS CJ, ELDERS PJ: A new method for automatic recognition of radiographic trabecular pattern. *J Bone Miner Res* 1990; 5: 227-33.
7. JAKUBAS-PRZEWŁOCKA J, SAWICKI A: Computer analysis of radiograms of normal and osteoporotic trabecular bone. *Med Sci Mon* 1999; 5: 989-90.
8. CHAPPARD D. CHENNEBAULT A, MOREAU M, LEGRAND E, AUDRAN M, BASLE MF: Texture analysis of X-ray radiographs is a more reliable descriptor of bone loss than mineral content in a rat model of localized disuse induced by clostridium botulinum toxin. *Bone* 2001; 28: 72-9.
9. JAKUBAS-PRZEWŁOCKA J. SAWICKI A, PRZEWŁOCKI P: Assessment of trabecular bone structure in postmenopausal and senile osteoporosis in women by image analysis. *Scand J Rheumatol* 2003; 32: 295-99.
10. OODGARD A: Three-dimensional methods for quantification of cancellous bone architecture. *Bone* 1997; 20: 315-28.
11. DEMPSTER DW: The contributions of trabecular architecture to cancellous bone quality. *J Bone Miner Res* 2000; 15: 20-2.
12. NEWITT DC, MAJUMDAR S, VAN RIETBERGEN B *et al*.: In vivo assessment of architecture and micro-finite element analysis derived indices of mechanical properties of trabecular bone in the radius. *Osteoporosis Int* 2002; 13: 6-17.
13. MAWATARI T, MIURA H, HIGAKI H *et al*.: Quantitative analysis of three-dimensional complexity and connectivity changes in trabecular microarchitecture in relation to aging, menopause and inflammation. *J Orthop Sci* 1999; 4: 431-43.
14. VAN DER LINDEN JC, HOMMINGA J, VERHAAR JAN, WEINANS H: Mechanical sequences of bone loss in cancellous bone. *J Bone Miner Res* 2001; 16: 457-65.
15. SUGITA H, OKA M, TOGUCHIDA J, NAKAMURA T, UBO T, HAYAMI T: Anisotropy of osteoporotic cancellous bone. *Bone* 1999; 24: 513-16.
16. KEAVENY TM, NIEBUR GL, YEH OC *et al*.: Micromechanics and trabecular bone strength. *In* PRENDERGAST PJ, LEE TC and CARR AJ (Eds.): *Proceedings of the 12th Conference of the European Society of Biomechanics 2000*. Dublin, Royal Academy of Medicine in Ireland, 2005: 5.
17. DEMPSTER DW, BIRCHMAN R, XU R, LINDSAY R, SHEN V: Temporal changes in cancellous bone structure of rats immediately after ovariectomy. *Bone* 1995; 16: 157-61.
18. LANE NE, THOMPSON JM, HAUPT D, KIMMELDB, MODIN G, KINNEY J: Acute changes in trabecular bone connectivity and osteoclast activity in the ovariectomised rat in vitro. *J Bone Miner Res* 1998; 13: 219-36.
19. AUDRAN M, CHAPPARD D, LEGRAND E, LIBOUBAN H, BASLE MF: Bone microarchitecture and bone fragility in men: DXA and histomorphometry in human and in the orchidectomised rat model. *Calcif Tissue Int* 2001; 60: 214-7.
20. GIAVARESI G, FINI M, GNUDI S, DE TERLIZZI F, CAPRI A, GIARDINO R: The femoral distal epiphysis of ovariectomized rats as a site for studies on osteoporosis: structural and mechanical evaluations. *Clin Exp Rheumatol* 2002; 20: 171-8.
21. ITO M, NISHIDA A, NAKAMURA T, UETANI M, HAYASHI K: Diffferences of three-dimensional trabecular microstructure in osteopenic rat models caused by ovariectomy and neurectomy. *Bone* 2002; 30: 594-8.
22. DĘBIŃSKI A, SAWICKI A, SZYMAŃSKA-DĘBIŃSKA T: Standard diets for studies on rat bone metabolism. *Progr in Osteoartr* 1996; 8: 65-8.
23. SHEN V, BIRCHMAN R, XU R, LINDSAY R, DEMPSTER DW: Short-term changes in histomorphometric and biochemical turnover markers and bone mineral density in estrogen- and/or dietary calcium- deficient rats. *Bone* 1995; 16: 149-56.
24. ABE T, SATO K, MIYAKOSHI N *et al*.: Trabecular remodeling processes in the ovariectomized rat: Modified node-strut analysis *Bone* 1999; 24: 591:7.
25. SETO H, AOKI K, KASUGAI S, OHYA K, OHYA K: Trabecular bone turnover, bone marrow cell development and gene expression of bone matrix proteins after low calcium feeding in rats. *Bone* 1999; 25: 687-695.