● Original Article

Effect of hyperbaric oxygenation on osteopenia in ovariectomized rats

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We investigated how hyperbaric oxygenation (HBO) modified the osteopenic response in osteopenic ovariectomized rats. Female Sprague-Dawley rats of 10 weeks of age were subjected to bilateral ovariectomy (OVX) or sham surgery (control). The rats were divided into four groups (n=10/group) each as follows: OVX+HBO, OVX alone, control+HBO, and control. HBO, which was started 3 days after surgery, provided the rats with 2.8 atm abs of pure oxygen for 1 h, once a day, 5 days per week for a total of 30 h. All rats were sacrificed 90 days after surgery. Their lumbar vertebrae, bilateral femora and tibiae were removed. Effects of HBO were studied on the trabecular bone area, the arrangement of collagen fibrils, bone mineral density (BMD), and the cortical thickness index (CTI). The area of trabecular bone within the region of cancellous bone of the femur and tibia was expressed as a ratio, and the value was significantly higher in the OVX+HBO group than in the OVX group. Collagen fibrils in OVX+HBO group were packed denser than those in the OVX group. BMD and CTI in HBO groups (OVX+HBO and control+HBO) showed a tendency to increase. These findings indicate that HBO has beneficial effects for the prevention of osteopenia.

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INTRODUCTION

Hyperbaric oxygenation (HBO) is used most frequently to supplement oxygen delivery to ischemic tissues. HBO benefits healing of fracture, osteomyelitis, etc.[1)~4)] Previously, HBO has also reduced osteonecrosis of the femoral head, and osteopenia of the vertebra in spontaneously hypertensive rats[5)6)]. Studies on the effect of HBO on bone composition and metabolism in 1-hydroxyethylidene-1, 1-bisphosphonate (HEBP)-induced rachitic rats, revealed that HBO inhibited bone resorption in areas of high osteoclastic activity after cessation of HEBP administration, and that the treatment increased total bone mass[7)8)]. However, it cannot prevent rachitic changes in subjects during HEBP administration. The effect of HBO on estrogen-deficient osteopenia, however, has not been reported.

In the present study, we investigated the

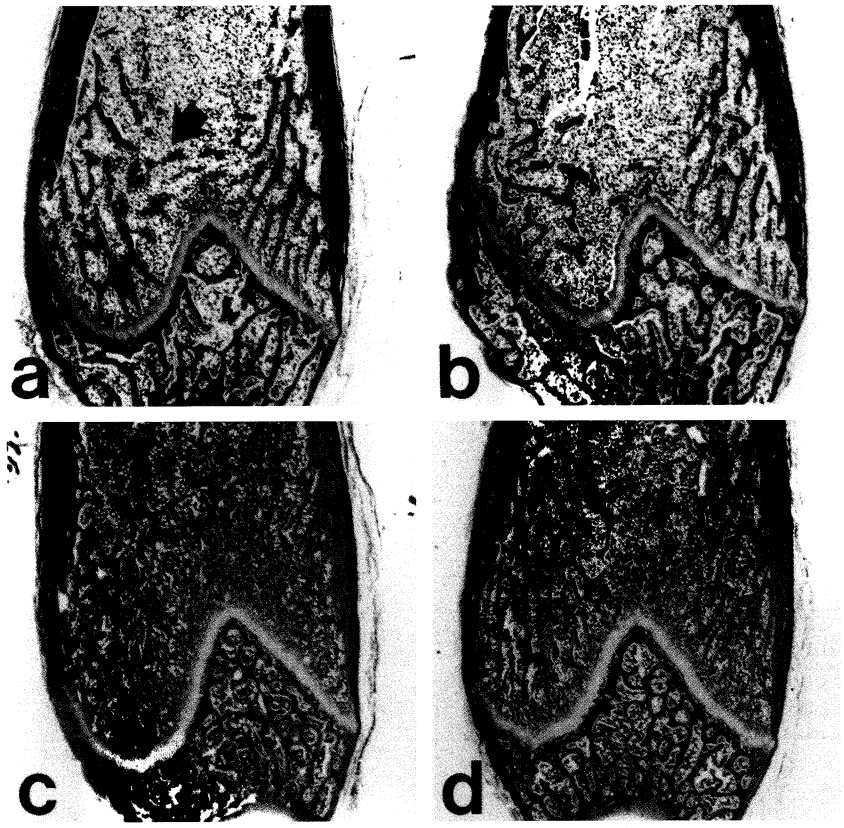

Fig. 1 Light microscopic profiles of rat distal femoral metaphyses in longitudinal sections.
Small trabecular bone area in cancellous bone is prominent in OVX (b). The area increases (arrow) in OVX+HBO group (a). Without OVX, the area of trabecular bone is larger in rats of control+HBO (c) than in control rats (d). Azan stain. Magnification: ×12.

skeletal effects of HBO in ovariectomized (OVX) rats, and studied the microscopical changes in the trabecular bone area and in collagen fibrils. The changes in both the bone mineral density (BMD) and cortical thickness index (CTI) were also examined.

MATERIALS AND METHODS

Ten-wk-old female Sprague-Dawley rats (Japan SLC, Inc.) weighing 210 to 225g were randomly divided into the following four groups (n=10/group): (a) OVX with HBO (OVX+HBO); (b) OVX without HBO (OVX); (c) sham surgery with HBO (control+HBO); (d) sham surgery without HBO (control). During the experimental period, the rats were provided with free access to laboratory food (Ca content, 1.4%) (CLEA Japan, Inc., Tokyo) and water. All rats were housed in groups of two at 23-24℃ with a 12 hr/12 hr -light/dark cycle. Care of the animals in this investigation was according to the Guide for Animal Research, Nagoya University School of Medicine.

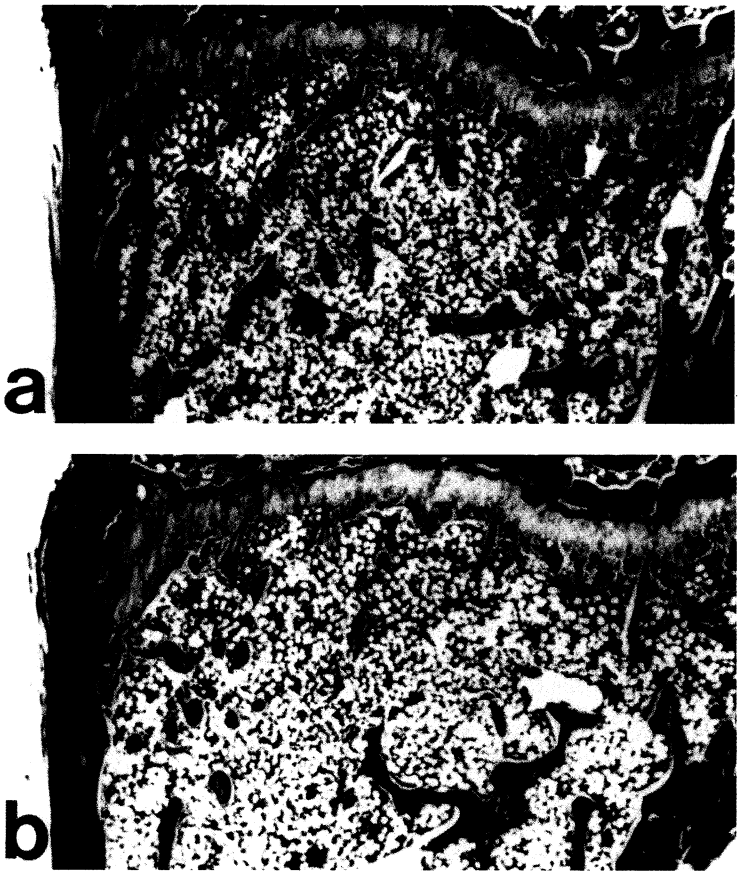

Fig. 2 Longitudinal sections of proximal tibial metaphyses of OVX rats.
HBO-exposed (a) and unexposed (b) profiles are shown.
Trabecular bone area increases (arrow) in OVX+HBO rats (a).
Azan stain. Magnification : ×32.

All rats were anesthetized with an IP injection of pentobarbital sodium at doses of 25 mg/kg BW. Bilateral ovariectomies were performed in half of the rats from an abdominal approach[9]. The remainder were subjected to sham surgeries in which the ovaries were exteriorized. Ninety days after surgery, all rats were sacrificed by exsanguination under ether anesthesia. Their lumbar vertebrae, bilateral femora and bilateral tibiae were collected for the designed studies.

HBO Treatment. In group (a) and (c), HBO was started from postoperative day 3 and provided for 1 h, once a day, at 2.8 atm abs of pure oxygen in an HBO chamber. HBO exposure was 5 days per wk for a total of 30 h, using the same method reported previously by Matsuda et al.[5] and Kataoka et al.[6].

Light Microscopy. Whole bones of the right femur and right tibia were fixed in neutralized 10% formalin and decalcified, then dehydrated and embedded in paraffin. Longitudinal sections of 5μm thickness were mounted on glass slides and stained with Azan.

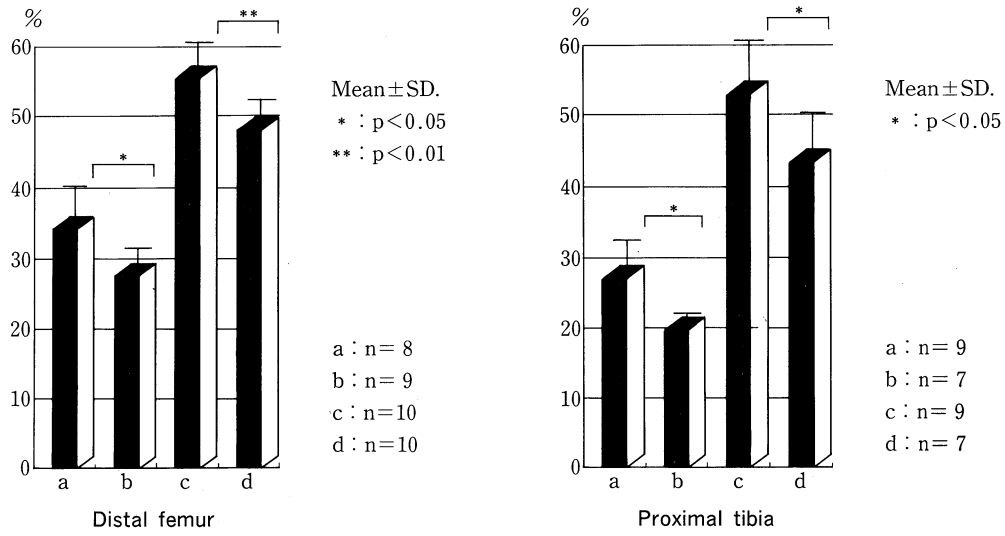

Fig. 3 Measurement of bone area with the light microscopy of distal femoral metaphysis and proximal tibial metaphysis in longitudinal sections by computer analysis.
Ratio of trabecular bone area to cancellous bone expressed as a percent. a, OVX+HBO; b, OVX alone; c, control+HBO; d, control. Values of trabecular bone area of both femur and tibia are statistically significant in HBO-treated bone.

Morphometric Study. Light microscopic images of rat distal femoral and proximal tibial metaphyses in longitudinal sections were entered into a personal computer and the areas of trabecular bone were analyzed with computer image analyzing software NIH Image 1.58. A similar range was covered in each group (**Fig. 1, 2**). The trabecular bone area/total cancellous bone area of the metaphysis x 100 was calculated.

Electron Microscopy. Small cortical bone specimens with decalcification were fixed in Karnovsky's fixative at 4℃ for 3 days. After washing in phosphate buffered saline (PBS), they were post-fixed in 1% osmium tetroxide buffered with PBS at room temperature for 90 min, then dehydrated in a graded series of ethanol and embedded in Quetol 812 (Nissin EM, Tokyo). Ultra-thin sections were cut with an ultramicrotome (Porter-Blum MT-6000), double-stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope H-7100 (Hitachi, Tokyo).

Bone Mineral Density (BMD). Lumbar vertebrae were fixed in 70% ethanol, and then submitted to dual energy X-ray absorptiometry (DXA), (DCS-600 System, Aloka Co., Tokyo, Japan). Left femora and left tibiae were kept in a deep freezer (-80℃). Then the soft tissue was removed from the bone, and submitted to DXA study (Hologic QDR-1000 Plus., U.S.A.; Scan type whole body v5.67p).

Cortical Thickness Index (CTI). The microdensitometry (MD) method[10] was applied onto the midpoint of the femoral length. The femoral length denotes the distance from the top of the major trochanter to the distal end of the femur. (Microdensitometer: Multi-Pen recorder, Rikadenki Co.; 2405 type-B

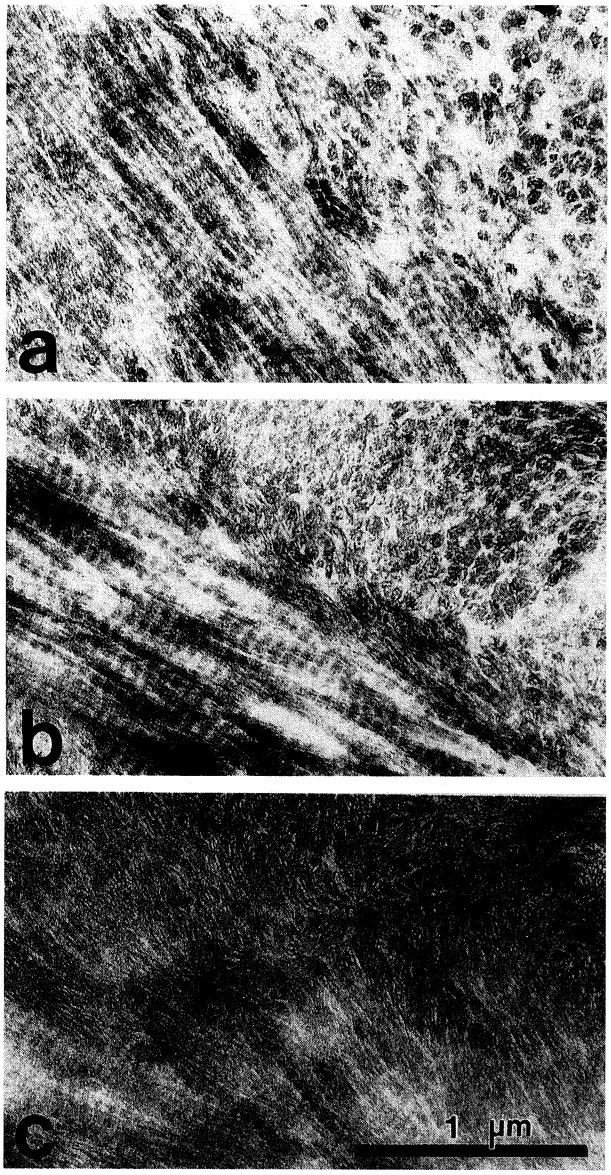

Fig. 4 Electron micrographs showing cortical bone matrix of rat tibia.

The arrangement of collagen fibrils is shown. The spaces between collagen fibrils are considerably larger in OVX (a) than those in OVX+HBO bone (b). In the control bone matrix (c), collagen fibrils are very dense. Double electron staining. Magnification: × 40,000.

Table. 1 Bone Density of HBO Rats

	OVX+HBO	OVX	Control+HBO	Control
BMD (Lumbar) (mg/cm²)	130.2±9.6[b]	127.0±4.4[b]	154.5±9.7	147.0±9.5
BMD (Femur) (mg/cm²)	124.7±12.5	121.1±5.8[a]	136.5±10.2	134.0±10.9
BMD (Tibia) (mg/cm²)	106.3±8.7[a]	105.7±8.1[a]	119.7±6.3	116.0±9.0
CTI (Femur)	0.40±0.02	0.38±0.02	0.42±0.01	0.40±0.02

BMD : bone mineral density ; CTI : cortical thickness index ;
All values are the mean ± SD. n=10.
[a]$p<0.05$ vs. control group, [b]$p<0.01$ vs. control group.
The values of OVX group were significantly lower than those of control group.

microdensitometer, Abe Co., Japan).

Statistical Analysis. Data are expressed as the mean ± SD for each group. Continuous variable data were analyzed for statistical differences ($p<0.05$) by F-test for homogeneity of variance and then subjected to t-test.

RESULTS

Histological examination of trabecular bone after HBO in OVX rats : Light micrographs of the distal femoral metaphysis of the OVX rats showed a notable increase in trabecular bone area after HBO (**Fig. 1a**). This was also shown in control rats (**Fig. 1c**). In HBO rats, trabecular bones connected with each other were frequently observed, and the width of the trabecula seemed to increase in HBO rats. In the proximal tibial metaphysis, the trabecular bone area increased in OVX+HBO rats (**Fig. 2**). Those areas were measured by computer analysis. **Fig. 3** shows a ratio of trabecular area to cancellous area of femur and tibia. The HBO treated animals had significant increases in the ratio of trabecular bone area to cancellous bone as compared to the controls without ovariectomies ($p<0.01$) and with the ovariectomized animals ($p<0.05$).

Electron microscopic observation of bone matrix : Since a significant increase in trabecular bone was observed after HBO, the arrangement of bone matrix, chiefly collagen fibrils, was examined in the cortical bone of the tibia by electron microscopy (**Fig. 4a, b, c**). There was a space between collagen fibrils in the bone of OVX rats (**Fig. 4a**), but this space decreased in OVX+HBO rats (**Fig. 4b**). In control rats, collagen fibrils were densely packed (**Fig. 4c**).

Bone density of HBO rats : BMD values of the lumbar vertebra, femur and tibia in the four groups of rats are listed in **Table 1**. The OVX group values were significantly lower than those of the control group. After exposure to HBO, the BMD in OVX+HBO group showed higher BMD values compared to the OVX group, though the difference was not statistically significant. Higher values were also noted on CTI of femora.

DISCUSSION

Our data warrant discussion of the following observations : 1) Trabecular bone areas of the femur and the tibia increased. 2)

The density of collagen fibrils in the cortical bone of the tibia increased. 3) The bone mineral density and the cortical thickness index increased. 4) The trabecular bone area increased after exposure to HBO even in the control rats.

Others have reported that rapid bone loss occurred in the proximal tibial metaphysis of OVX rats aged 75 days, and that a twofold decrease in trabecular bone volume was noted at 5 weeks postovariectomy[11]. We feel that the most important observation from our study was that the trabecular bone area increased in OVX rats after exposure to HBO. The two-dimensional observation of the longitudinal microscopic bone section is generally used to assess the trabecular bone volume change. The results obtained by an area-ratio calculating device in this case agreed with the point-system used previously; measurement of the sections of 36 points and 6 semicircular lines within a square[11].

In discussing bone volume, we do not include the osteoid tissue, and the ratio between the calcified bone and the osteoid is reported to be rather constant[11]. We, therefore, followed Mosekilde et al.[12], using decalcified samples for evaluation. In contrast to previous reports[4)8)], we found that the trabecular bone volume also increased in the control+HBO group. Since the ratio of osteoid to calcified bone is reported to be constant, we did not feel it was necessary to consider osteoid tissue when discussing bone volume. Moreover, the trabecular bone volume increase in our study was observed when rats were exposed to HBO regardless of ovariectomy. The increase in trabecular bone volume even in rats with intact ovaries calls attention to the mechanisms at work here. HBO stimulates angiogenesis and proliferation of osteoblasts and in turn, accelerates the excretion and synthesis of collagen[4], and eventually enhances calcifying activities (bone formation).

Moreover, our study strongly points to estrogen as one of these factors. It is said that estrogen-deficiency induces interleukin-1 (IL-1) which induces bone absorbing cytokine production[13], and that the bone marrow B-lymphocyte increase also promotes bone absorption[14]. On the other hand, HBO exposure was found to reduce the IL-1 from macrophage[15], and the B-cell activity[16]. The finding concerning the effect of HBO suggests that HBO may reduce bone absorption in OVX rats.

The contribution of collagen to the mechanical strength of bone is obvious. As to collagen, the interstices of such a trabecular network are filled with collagen type-I. Hydroxyapatite deposits on the collagen fibrils. The synthesis of bone collagen is enhanced due to the presence of estrogen in vitro[17]. Tuncay et al.[18] reported in their in vitro study that collagen-I increased when oxygen tension was high (90%); this supports the present result. Our findings of an increase in the cortical bone index (though not statistically significant) also clearly support more densely packed collagen fibrils with the HBO treated group. Moreover, the beneficial effect of HBO exposure on preserving bone in the lumbar vertebrae was evidenced by BMD. The femora and tibiae of the HBO treated animals also indicated an increase in the trabeculae of cancellous bones.

The increase in bone density was not as obvious as the increase indicated by photomicroscopic data in the trabecular area, suggesting the rats were somewhat immature for this kind of study, especially due to their hormonal state. To evaluate the long-term effects of OVX on the rat skeleton, studies were conducted in OVX rats beginning at about 3 months of age (mature rat model). The term 'mature' is used loosely to differentiate them from the 'aged rat model' (12

months) of postmenopausal bone loss[19)20)]. The use of precision instruments regarding the trabeculum and bone density, peripheral quantitative computed tomography (pQCT) and/or micro computed tomography (μCT) will also improve the accuracy of the data on bone density[21)22)].

The biomechanical competence of trabecular bone that was found by Kleerekoper et al.[23)] is dependent not only on the absolute amount of bone present but also on the trabecular microstructure; moreover, the same amount of trabecular bone is biomechanically less competent when the trabecular microstructure is weak due to a thinning of the cortical portion and/or porosity. The osteoporotic process is characterized by this hysteretic pathological process. Study of the trabecular area is crucial where bone strength is involved.

We believe this is the first reported attempt to determine the HBO effect on osteopenia in ovariectomized rats. HBO will help prevent estrogen-deficient osteopenia, and it will also contribute to the cure of estrogen-deficient osteopenia in the near future.

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