Bone composition and metabolism after hyperbaric oxygenation in rats with 1-hydroxyethylidene-1,1-bisphosphonate-induced rickets

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Iwase T, Hasegawa Y, Ito T, Makihara N, Takahashi H, Iwata H. Bone composition and metabolism after hyperbaric oxygenation (HBO) in rats with 1-hydroxyethylidene-1,1-bisphosphate-induced rickets. Undersea Hyperbaric Med 1996; 23(1):5–9. We examined bone composition and metabolism after hyperbaric oxygenation (HBO) in Wistar rats with 1-hydroxyethylidene-1,1-bisphosphonate (HEBP)-induced rickets. Twenty rats at 4 wk of age were divided into four groups of five rats each. The HEBP+HBO group received high dose (50 mg · kg⁻¹ · day⁻¹) HEBP injections subcutaneously for 7 days and were then exposed to HBO for 7 days. The HEBP group received only high dose HEBP injection for the first 7 days. Control group A received neither HEBP nor HBO. Control group B received no HEBP injection and was exposed to HBO only for the second 7 days. Both bone mineral and hydroxyproline contents significantly increased in rats in the HEBP+HBO group as compared with the HEBP group. Alkaline phosphatase activity of bone, which is a marker of osteoblastic activity and bone formation, was high in the HEBP+HBO and HEBP groups compared with control groups A and B, although there was no difference between the former two groups. On the other hand, tartrate-resistant acid phosphatase activity, which is a marker of bone resorption, was lower in the HEBP+HBO group than in the HEBP group. These findings suggest that HBO suppresses bone resorption in high osteoblastic activity after the cessation of HEBP administration, and this phenomenon increases total bone mass.

hyperbaric oxygenation, 1-hydroxyethylidene-1,1-bisphosphonate, bone metabolism, alkaline phosphatase, tartrate-resistant acid phosphatase

Several investigators have reported that hyperbaric oxygenation (HBO) therapy has some beneficial effects on bone metabolism; for example, fracture healing (1,2), enhancement of new bone formation (3,4), and healing of osteoradionecrosis (5,6).

Recently we found that HBO can prevent femoral head osteonecrosis (7) and osteopenia (8) in the spontaneously hypertensive rat (SHR), but its influence on bone metabolism has not been specifically studied.

High dose administration (more than 10–20 mg · kg⁻¹ body weight) of 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) to young rats induces rickets-like changes and osteomalacia within 1 wk (9,10). This HEBP-induced rickets occurs primarily as a result of inhibition in the rate of mineralization and is reversible on withdrawal of HEBP administration. HEBP also inhibits bone resorption because it suppresses osteoclastic activity. Although those influences on the bone metabolism gradually disappear after cessation of HEBP administration, it takes several months to be washed out completely. Therefore this animal model is useful to examine the influence of various drugs or agents against disturbed mineralization or bone metabolism. Makihara et al. recently found that HBO stimulates calcification of the enlarged physis of tibia in this animal model (unpublished). In the present study we examined bone composition and turnover of bone metabolism after HBO treatment in rats with HEBP-induced rickets.

MATERIALS AND METHODS

Animals and grouping: Male weanling Wistar rats at 4 wk of age, initially weighing 100–110 g each were randomly divided into four groups of five animals each. All rats were bred in the laboratory of Nagoaya University School of Medicine and were housed in cages and fed a standard laboratory diet (Nippon Clea Inc.) ad libitum throughout the experiment. The rats in the HEBP+HBO group received HEBP injection daily subcutaneously for 7 days and were then subjected to hyperbaric oxygenation therapy (HBO) twice a day for the next 7 days, and those in the HEBP group received the same dose of HEBP daily for 7 days but no HBO for the next 7 days. The rats in control group A received no treatments (no HEBP, no HBO) during the experiment, and the rats in control group B received no HEBP for the first 7 days and then HBO twice a day for the next 7 days.

1-Hydroxyethylidene-1,1-bisphosphonate (HEBP) injec-
tion: HEBP (Sumitomo Pharmaceutical Co., Osaka, Japan) was dissolved in physiologic saline solution and was injected subcutaneously at a dose of 50 mg \( \cdot \) kg\(^{-1} \) body weight once a day for the first 7 days in the HEBP group and HEBP+HBO group.

**Hyperbaric oxygen:** HBO provided 284 kPa (2.8 atm abs) of pure oxygen for 1 h twice a day in an environment test chamber for an animal model (Tabai Mfg. Co., Osaka, Japan), and the pressure was increased and reduced gradually for over 30 min to prevent pressure injury. The temperature within the hyperbaric chamber was maintained near 21°C during the experiment by venting a continuous flow of pure \( \text{O}_2 \) through the chamber. This venting procedure also prevents the build-up of expired \( \text{CO}_2 \). The venting flow rate was set at a flow that could maintain the \( \text{O}_2 \) content of the vented gases at greater than 95%.

**Analysis of bones:** Two weeks from the beginning of the experiment, all rats were given a lethal dose of ether, and the bilateral femur and tibia of each animal were carefully dissected from soft tissue. After taking radiographs with a soft x-ray apparatus (Softex, ES-M) and Fuji HS film (Fuji Photo Film Co., Ltd., Tokyo Japan) with a setting of 60 kV, 3 mA, 45 s, and a focus to film distance of 45 cm, all bones were frozen and stored at \(-80°C\) in a deep freezer until analysis.

The dry weight of each femur was measured in a drying machine at 120°C for 6 h and subsequently ashed in porcelain crucibles at 800°C for 6 h. After the total ash weight was measured, ash was dissolved in 10 ml of 6N-HCl. When the calcium content was measured colorimetrically by the o-cresolphthalein-complexon method, and phosphate content by Goldenberg's method using a kit from Iatron, Tokyo, Japan.

Each frozen tibia was crushed in liquid nitrogen and was homogenized in 10 ml of 0.25 M saccharose fluid, and then centrifuged at 10,000 \( \times \) g for 20 min (11). The supernatant fluid was used for alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) assay. Each enzyme activity was determined by a kit from Iatron, utilizing phenylphosphate as a substrate (12). The concentration of the protein in the supernatant was determined according to the procedure of Bradford (13). The enzyme activity was expressed as international units per milligram of protein in the supernatant fluid. The sediments were dehydrated with 10 ml of 6N-HCl in 125°C for 18 h and used for measurements of hydroxyproline (OH-proline) content by the colorimetric method described by Firschein and Shill (14) and of calcium content and phosphate contents by the same methods as for the femur.

**Statistical analysis:** All values were expressed as mean \( \pm \) standard deviation. Statistical differences between groups were assessed using one-way analysis of variance, followed by Scheffe's test for multiple comparisons. \( P < 0.05 \) was considered to indicate a significant difference.

**RESULTS**

Treatment was well tolerated by all the rats except one rat in the HEBP + HBO group. That rat died of unknown causes during the experiment and so was excluded from the analysis. The weight gain of animals in the HEBP + HBO and HEBP groups did not differ significantly, but the final weight of the rats in these groups was less than that of the rats in control groups A (no HEBP, no HBO) and B (no HEBP, +HBO) (HEBP + HBO group, 166 \( \pm \) 4 g; HEBP group, 168 \( \pm \) 7 g; control group A, 224 \( \pm \) 7 g; and control group B, 228 \( \pm \) 5 g). The final number of femur and tibia in each group was as follows: HEBP + HBO group, \( n = 8 \); HEBP group, \( n = 10 \); control group A, \( n = 10 \); control group B, \( n = 10 \).

Radiographs indicate enlargement of the physis in the HEBP + HBO and HEBP groups due to suppression of mineral deposits by HEBP, and calcification occurred in this enlarged physis in all the rats in the HEBP + HBO group (Fig. 1).

Ash weight, calcium, and phosphate contents of the femur, which were expressed as a percentage of the dry weight of each bone, were markedly decreased in the rats receiving HEBP, but recovered significantly in the rats in the HEBP + HBO group as compared with the HEBP group (Table 1). Calcium and phosphate contents of the tibia, which were expressed as a percentage of wet weight of each bone, were similar to those of the femur (Table 2).

**FIG. 1—** Soft x-ray photographs of tibia in each group. From left to right, HEBP+HBO group, HEBP group, control group A (no HEBP, no HBO); control group B (no HEBP, +HBO). The proximal physis of the tibia in HEBP + HBO group (left) and HEBP group (second from left) was enlarged because of HEBP treatment. Linear calcification was seen in the enlarged physis in HEBP + HBO group (left, arrows). There is no apparent difference between the control group A and B (two right).
INFLUENCE OF HBO IN RATS WITH HEBP-INDUCED RICKETS

Table 1: Mineral Contents of Femur

<table>
<thead>
<tr>
<th>Group</th>
<th>Ash/Dry Weight, %</th>
<th>Calcium/Dry Weight, %</th>
<th>Phosphate/Dry Weight, %</th>
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</thead>
<tbody>
<tr>
<td>HEBP+HBO, n = 8</td>
<td>47.1 ± 1.9&lt;sup&gt;b,c,d&lt;/sup&gt;</td>
<td>13.6 ± 0.4&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>9.8 ± 0.2&lt;sup&gt;c,d&lt;/sup&gt;</td>
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<tr>
<td>HEBP, n = 10</td>
<td>43.5 ± 0.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.8 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.1 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control group A (no HEBP, no HBO), n = 10</td>
<td>52.4 ± 0.6</td>
<td>20.0 ± 1.1</td>
<td>11.3 ± 0.4</td>
</tr>
<tr>
<td>Control group B (no HEBP, +HBO), n = 10</td>
<td>53.1 ± 1.3</td>
<td>19.1 ± 0.9</td>
<td>11.1 ± 0.1</td>
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</tbody>
</table>

Values are expressed as mean ±SD.
<sup>a</sup>P < 0.05 vs. HEBP group; <sup>b</sup>P < 0.01 vs. HEBP group; <sup>c</sup>P < 0.001 vs. control group A; <sup>d</sup>P < 0.001 vs. control group B.

Table 2: Mineral and Hydroxyproline Contents of Tibia

<table>
<thead>
<tr>
<th>Group</th>
<th>Calcium/Wet Weight, %</th>
<th>Phosphate/Wet Weight, %</th>
<th>Hydroxyproline/Wet Weight, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEBP+HBO, n = 8</td>
<td>4.7 ± 0.1&lt;sup&gt;b,d/f&lt;/sup&gt;</td>
<td>2.9 ± 0.1&lt;sup&gt;b,d/f&lt;/sup&gt;</td>
<td>2.5 ± 0.1&lt;sup&gt;b,d/f&lt;/sup&gt;</td>
</tr>
<tr>
<td>HEBP, n = 10</td>
<td>3.8 ± 0.1&lt;sup&gt;c/f&lt;/sup&gt;</td>
<td>2.2 ± 0.1&lt;sup&gt;c/f&lt;/sup&gt;</td>
<td>2.1 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control group A (no HEBP, no HBO), n = 10</td>
<td>8.5 ± 0.2</td>
<td>4.4 ± 0.2</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Control group B (no HEBP, +HBO), n = 10</td>
<td>8.3 ± 0.2</td>
<td>4.2 ± 0.2</td>
<td>1.8 ± 0.1</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD.
<sup>e</sup>P < 0.01 vs. HEBP group; <sup>f</sup>P < 0.001 vs. HEBP group; <sup>g</sup>P < 0.05 vs. control group A; <sup>h</sup>P < 0.001. control group A; <sup>i</sup>P < 0.01 vs. control group B; <sup>j</sup>P < 0.001 vs. Control group B.

The OH-proline content of the tibia, which was expressed as a percentage of the wet weight of each bone, was significantly increased in the rats in the HEBP + HBO group and compared with the HEBP group, control groups A and B. However, there was no difference in OH-proline content between the rats in control groups A and B (Table 2).

Although there was no difference in ALP activity per protein weight in the supernatant fluid of the homogenized tibia between the HEBP + HBO and HEBP groups, or between control groups A and B, its activity in the former two groups was higher than in the latter two groups. On the other hand, tartrate-resistant acid phosphatase activity per protein weight in the same fluid, in which ALP activity was measured, as the HEBP + HBO group was significantly decreased as compared with the HEBP group. There was no difference in this enzyme activity between control groups A and B (Table 3).

DISCUSSION

Many beneficial effects of HBO have been reported, and a few reports have described the effects of HBO on the skeletal system (5,15–17). Osteomyelitis and osteonecrosis have been the main targets of this treatment for skeletal diseases, because improvement of O<sub>2</sub> content in the affected region or critical zone might activate metabolism in such places, and thus enhance healing. However, these reports have mostly described clinical treatment, and there are few basic reports about the effects of HBO on the skeletal system in animal models.

In 1966, Coulson et al.(1) reported that HBO enhanced calcium incorporation into the callus and improved strength against breaking force, and suggested that this might be due to improved osteogenic action by HBO. Matsuda et al.(8) reported that intermittent hyperoxygen conditions stimulated both aerobic and anaerobic metabolism of bone formation. Nilsson et al.(3,4) suggested that HBO influences bone metabolism even in non-ischemic lesions using "a bone harvest chamber." All these reports suggest that HBO accelerates bone formation in pathologic conditions, although the authors did not define the exact metabolic mechanism underlying these phenomena.

In this study we examined the influence of HBO on bone composition and metabolism during the recovery phase after cessation of HEBP injections. We found that bone mineral deposits and hydroxyproline content, which reflects the collagen content of bone was increased in the HOB + HEBP group. These findings mean that HBO can increase not only bone mineral contents in osteoid tissue, which was induced by HEBP injection, but also bone matrix. However, such an effect was not seen in the normal control groups. This means...
that HBO increases bone mass only in a pathologic condition of bone.

To understand the metabolic background of the bone remodeling, it is useful to analyze the enzyme activity that is concerned with bone metabolism (18). Although the exact role of ALP and TRAP is not known, these enzymes are typical markers of bone metabolism. ALP is considered as a marker of bone formation (19). Although this enzyme is located mainly in osteoblast and liver cells, the present findings reflect osteoblastic activity (bone formation activity) because enzyme activity was detected in the supernatant of homogenized bone. TRAP is specifically abundant in mature osteoclasts and is considered as a marker of osteoclast activity participating in bone resorption (20).

Although ALP activity of bone in the HEBP-treated rats (HEBP + HBO group and HEBP group) was high as compared with bone in rats without HEBP treatment (control groups A and B), there was no difference in this enzyme activity regardless of HBO. These findings indicate first that high osteoblastic activity in the recovery phase after HEBP injections ceased, and second that HBO had no effect on this enzyme activity in the HEBP-treated and untreated rats.

On the other hand, TRAP activity of bone in the HEBP-treated groups (HEBP + HBO group and HEBP group) was lower than in untreated control groups A and B. Moreover, its activity in the HBCO+HEBP group was lower than in the HEBP group. As suppression of osteoclast activity is one of the main effects of HEBP (21), these findings indicate that suppression of bone resorption in the HEBP-treated groups (HEBP+HBO and HEBP groups) still remains at the end of the experiment. Furthermore, these findings suggest that HBO inhibits bone resorption under suppressed osteoclast activity due to HEBP.

In summary, the mechanism of increased bone mass in the HBO-exposed, HEBP-induced rachitic rat was caused by the balance of relatively decreased osteoclastic activity due to HBO in high osteoblastic activity. In other words, the bone mass in the HEBP+HBO group increased because bone formation was superior to bone resorption, and such a phenomenon was not seen in the normal bone.

In this experiment, although the influence of HBO against osteoclastic activity was seen only in the HEBP-injected rats, it is unclear whether this type of influence by HBO on bone metabolism is specific to this animal model. Moreover it is also unknown how HBO suppresses osteoclast activity. As the metabolic background of calcium and phosphate is influenced by other factors, i.e., vitamin D and renal function, interpretation of the present study may be limited. Therefore, further study will be necessary to better understand the mechanism of bone metabolism under HBO exposure.

The authors are grateful to Mr. Yukimasa for his technical assistance.

References


10. Sehenk R, Merz WA, Mühlbauer R, Fleisch H. Effect of ethane-1,1-diphosphonate (EHDP) and dichloromethylene diphosphonate (Cl₂ MDP) on the calcification and resorption of

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**Table 3: Enzyme Activity in Homogenized Tibia**

<table>
<thead>
<tr>
<th>Group</th>
<th>Alkaline Phosphate, $\times 10^{-2}$ IU/mg Protein</th>
<th>Tartrate-resistant Acid Phosphatase, $\times 10^{-3}$ IU/mg Protein</th>
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<tbody>
<tr>
<td>HEBP+HBO, $n = 8$</td>
<td>62.5 ± 5.2&lt;sup&gt;c d&lt;/sup&gt;</td>
<td>22.1 ± 3.3&lt;sup&gt;c d&lt;/sup&gt;</td>
</tr>
<tr>
<td>HEBP, $n = 10$</td>
<td>61.5 ± 8.9&lt;sup&gt;c d&lt;/sup&gt;</td>
<td>39.0 ± 4.9&lt;sup&gt;c d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control group A (no HEBP, no HBO), $n = 10$</td>
<td>40.4 ± 2.3</td>
<td>55.4 ± 9.1</td>
</tr>
<tr>
<td>Control group B (no HEBP, +HBO), $n = 10$</td>
<td>40.0 ± 3.8</td>
<td>61.0 ± 10.4</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD.

<sup>a</sup>P < 0.05 vs. HEBP group; <sup>b</sup>P < 0.05 vs. control group A; <sup>c</sup>P < 0.01 vs. control group A; <sup>d</sup>P < 0.01 vs. control group B.
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