Abstract To determine the effects of ammonium chloride (NH₄Cl) dosage and swimming exercise training during 4 weeks on bone metabolic turnover in rats, seven-week-old female 24 Wister-Kyoto (WKY) rats were investigated by bone status including bone mineral density (BMD) and biomechanical markers from blood and urine. Twenty-four rats (initial weight: 191.2±7.6 g) were randomly divided into four groups: baseline (8 weeks old) control group (n=6, BC), 4-week control group (n=6, Con), 4-week swimming exercise loading group (n=6, Swim) and 4-week chronic NH₄Cl dosage group (n=6, Acid). All rats were fed an AIN93M diet (Ca: 0.5%, P: 0.3%), and both Con and Swim groups were pair-fed by feeding volume of the NH₄Cl dosage group. The acid group only received 0.25 M NH₄Cl distilled water ad libitum. At the end of the experimental period, rats were sacrificed with blood drawn and femur and tibia were removed for analysis of bone mineral density (BMD) by dual energy X-ray absorptiometry (DEXA). In the Swim group, 24-hour urinary deoxy-piridinoline (Dpd) excretion, reflecting bone resorption, was significantly increased (p<0.05) with a tendency towards decrease of BMD (N.S.), and body weight and abdominal fat weight were decreased in approximately 7% (p<0.05) and 58% (p<0.001), as compared with age matched Con rats. In the Acid group, 24-hour urinary calcium (Ca) and phosphorus (P) excretion were increased approximately 2.1-fold (p<0.05) and 2.0-fold (p<0.01), respectively, with increase of kidney weight as much as in the Con groups. Serum Ca and P concentration, as well as urinary Dpd excretion were, however, not significantly changed. These results suggest that blood Ca and P concentrations in the chronic acidosis condition during the 4-weeks might be maintained by hypercalciuria and hyperphosphaturia with kidney disorder, and swimming exercise training leads to decrease in BMD with stimulation of bone resorption and reduction of body fat. J Physiol Anthropol Appl Human Sci 24(6): 595–600, 2005 http://www.jstage.jst.go.jp/browse/jpa [DOI: 10.2114/jpa.24.595]

Keywords: bone mineral density (BMD), swimming exercise, acidosis, rat, dual energy X-ray absorptiometry (DEXA)

Introduction

It is generally accepted that osteoporosis is characterized by low bone mass and deranged bone micro-architecture, leading to increased bone fragility and risk of fracture. Bone mass is also influenced by hormonal changes such as puberty and menopause and by lifestyle factors such as physical loading. Weightlessness or skeletal unloading mainly lead to bone loss (Kim et al., 2002; Wronska and Morey, 1982; Turner et al., 1981). Contrary mechanical forces including exercise play a pivotal role in maintaining and increasing bone mass (Colletti et al., 1989; Dalen and Olsson, 1974). Resistance training increases the cancellous bone of rats by stimulating bone formation (Westerlind et al., 1998), and osteoinductive activity in the bone was probably due to an increase of bone morphogenetic proteins (BMPs) following voluntary exercise (Goseki et al., 1995), which decreases bone resorption and increases bone formation (Iwamoto et al., 1998). There is disagreement, however, in the past studies which report the effects of exercise loading on the bone. Positive effects of increased bone mass, as well as the negative effects of a decreased and/or unchanged bone mass (Bilanin et al., 1989; Bourrinn et al., 1992; Matsuda et al., 1986; Myburgh et al., 1989; Risser et al., 1990) were reported in the literature. Our previous study found that prolonged swimming exercise in stroke-prone spontaneously hypertensive rats induced osteopenia with a tendency towards hypophosphatemia (Kim et al., 2000). Particularly over-training and compulsive exercise induced marked bone loss (Matsumoto et al., 1997; Orwoll et al., 1989) but these mechanisms were not clear.

On the other hand, the maintenance of a stable physiologic systemic pH is critically important to mammals and it is suggested that acidosis, decreases systemic pH as a result of increased blood lactate concentration (Ashizawa et al., 1997), induces Ca efflux from bone (Bushinsky, 1995), and ultimately
produces hypercalciuria (Lemann et al., 1967) which also can cause loss of bone mass (Barzel, 1995; Eiam-Ong and Kurtzman, 1994). Acidosis is one of the side effects induced by exercise (Kristofferson et al., 1995). Intense exercise might cause a decrease in blood pH as a result of increased lactate and blood CO₂ (Ashizawa et al., 1997). In several previous studies (Pan et al., 2004; Green et al., 2005), chronic acidosis has been induced by drinking ammonium chloride (NH₄Cl) water. However, the bone loss mechanism induced by both exercise and acidosis has not been clarified.

The present study was designed to clarify the effects of swimming exercise and chronic acidosis on bone metabolism, with swimming loading and administration of NH₄Cl dosages to the rats over 4-weeks.

**Materials and Methods**

**Animal care**

Twenty-four female Wistar-Kyoto (WKY) rats, aged 7 weeks were purchased from Funabashi Farm Co. Ltd., Chiba, Japan. Rats were individually housed in a room (12 h. light-dark cycle condition), where the temperature and humidity were controlled at 23±1°C and 55±5%, respectively. After a week of stabilization, they were randomly divided into four groups: Baseline (8-week old) control group (n=6, BC), 4-week control group (n=6, Con), 4-week swimming exercise loading group (n=6, Swim) and 4-week chronic acidosis group (n=6, Acid). They were pair-fed (AIN93M diet, Ca: 0.5%, P: 0.3%) by feeding the Acid group, and distilled water was available ad libitum except for the Acid group (NH₄Cl dosage, 0.25 M NH₄Cl distilled water). The swimming exercise in free style was carried out in a circular plastic barrel (diameter, 60 cm; depth, 55 cm) filled with water maintained at a temperature of 34±2°C. The exercise consisted of daily swimming for 60 min., five days a week, for 4 weeks. The water depth level, 40 cm, was set to prevent the rats from resting by supporting their tail on the barrel edge. This exercise corresponds to an intensity of approximately four metabolic equivalents (Dawson and Horvath, 1970).

Body weights were measured once a week, and the daily amount of intake food was also measured. Before sacrifice, urine was directly drawn for 24-hours and frozen at −30°C until assay. At the end of the experimental period, the animals were fast overnight, and put down by exsanguination from the carotid artery, the blood being collected. The serum obtained by centrifugal separation was stored at −30°C until assay. The femur and tibia were removed, and bone status was measured. The hind limbs’ muscles and other organs were weighed.

**Biochemical markers of bone metabolism**

Serum Ca and 24-hour urinary Ca excretion were determined by methods of orthocresol-phthalein complexion (OCPC; Ca-test kit, Wako, Japan), and inorganic phosphate (iP) of both were also measured by methods of molybdenum blue (iP-test kit, Wako, Japan). Twenty-four hours urinary Deoxypyridinoline crosslinks (Dpd) excretion, reflecting bone resorption, was analyzed by a commercially available ELISA kit (Osteolinks, Sumitomo, Japan) according to the manufacturer’s method.

**Statistical analysis**

Statistical analysis was performed using SPSS Statistical Software (ver. 10.0). Data were expressed as Mean±SD. Statistically significant differences among intergroups were determined by the one-way ANOVA. After finding significant differences in univariate tests, Bonferroni correction as a post-hoc analysis were used to determine the location of significant differences. A significance level of P value less than 0.05 was used for all comparisons.

**Results**

**Change of total body weight and organ weight**

The average body weight of all the rats was practically the same at 8 weeks old (mean±SD; 191.2±7.6 g) (Fig. 1(A)).
During the experimental period, the body weight of all the rats gradually increased ($p<0.001$), and no significant difference between the Con group and the Acid group was found at 12 weeks old. That of Swim group was, however, approximately 7% lower than that of Con group at 12 week old ($p<0.05$). The abdominal fat weight of all the rats was also gradually increased ($p<0.01$, $p<0.001$) (Fig. 1(B)), but that of Swim group was approximately 58% lower than that of Con group at the end of experiment ($p<0.001$). The kidney weights during the experimental period in the Con and Swim groups were not changed, but that of the Acid group was significantly increased by 16% at 12 weeks old ($p<0.01$) (Fig. 1(C)). In several other organ weights, no significant difference of the intergroup was found at the end of the experimental period (Table 1).

**Changes of BMD and bone status in the femur and tibia**

The femoral BMD (g/cm$^2$, mean±SD) of the BC, Con, Swim and Acid groups was 0.162±0.011, 0.192±0.006, 0.189±0.015 and 0.184±0.009, respectively, and that of all the groups was gradually increased ($p<0.001$, $p<0.01$) during the experimental period (Fig. 2(A)). The tibial BMD of all the groups also showed a similar increase ($p<0.01$, $p<0.05$) during the experimental period (Fig. 2(B)). In the femoral and tibial BMD, although no significant difference of the intergroups was found, there was a tendency towards decrease in both the Swim and Acid groups at the end of the experimental period. The femoral and tibial BMC, weight and length of bone in all the groups showed a similar tendency with BMD (Table 2).

**Biochemical markers of bone**

Although all the rats showed a similar serum Ca level (no significant difference) during the experimental period (Fig. 3(A)), serum P in all groups showed a tendency towards decrease ($p<0.05$, $p<0.01$) (Fig. 3(B)). In the serum Ca and P, no significant difference of the intergroup was found at the end of the experimental period. Twenty-four hours urinary Ca and P decreased in all the groups during the experimental period (Fig. 3(C)).

### Table 1 Change of the several organ weight after swimming exercise and chronic acidosis during 4 weeks

<table>
<thead>
<tr>
<th></th>
<th>8 weeks</th>
<th>12 weeks old</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>BC</td>
<td>Con</td>
</tr>
<tr>
<td>Heart (mg)</td>
<td>795±63</td>
<td>932±50**</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>6.3±0.5</td>
<td>6.8±1.0</td>
</tr>
<tr>
<td>Uterus (mg)</td>
<td>559±214</td>
<td>604±423</td>
</tr>
<tr>
<td>Spleen (mg)</td>
<td>631±73</td>
<td>833±148*</td>
</tr>
<tr>
<td>Adrenal (mg)</td>
<td>104±44</td>
<td>203±32</td>
</tr>
</tbody>
</table>

Values are means±SD. Statistical significance was evaluated with the one-way ANOVA. BC, baseline control group; Con., control group; Swim, Swimming exercise group; Acid, Acidosis group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs BC group.

**Fig. 2** Change of femoral BMD (A), tibial BMD (B) and urinary Dpd excretion (C) in Con, Swim and Acid group. Con., control group; Swim, Swimming exercise group; Acid, Acidosis group. BMD, Bone mineral density; Dpd, Deoxypyridinolin. Statistical significance was evaluated with the one-way ANOVA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs 8 weeks old control group; # $p<0.05$ vs 12 weeks old control group.

### Table 2 Change of the BMC, bone weight and length after swimming exercise and chronic acidosis during 4 weeks

<table>
<thead>
<tr>
<th></th>
<th>8 weeks</th>
<th>12 weeks old</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>BC</td>
<td>Con</td>
</tr>
<tr>
<td>Femoral bone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMC (mg)</td>
<td>58±26</td>
<td>119±16***</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>0.56±0.03</td>
<td>0.72±0.02***</td>
</tr>
<tr>
<td>Length (mm)</td>
<td>31.7±0.4</td>
<td>34.6±0.74***</td>
</tr>
<tr>
<td>Tibial bone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMC (mg)</td>
<td>32±20</td>
<td>80±27**</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>0.43±0.03</td>
<td>0.54±0.02***</td>
</tr>
<tr>
<td>Length (mm)</td>
<td>35.2±0.5</td>
<td>37.6±0.88***</td>
</tr>
</tbody>
</table>

Values are means±SD. Statistical significance was evaluated with the one-way ANOVA. BMC, bone mineral content; BC, baseline control group; Con., control group; Swim, Swimming exercise group; Acid, Acidosis group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs BC group.
Discussion

In our previous study, we found bone loss through prolonged swimming exercise in SHRSP (Kim et al., 2000), and it is known that chronic metabolic acidosis results in osteomalacic and osteopenic bone diseases with hypercalciuria (Bushinsky and Frick, 2000). Although intensive exercise may induce acidosis by increased lactate and blood CO₂ in humans (Ashizawa et al., 1997; Kristofferson et al., 1995), details of these relationships were not clarified. The aim of this study was to examine the differentiative bone loss in conditions of swimming exercise and chronic acidosis. The present study confirmed that swimming exercise increased u-Dpd, both swimming exercise and chronic acidosis. The present study confirmed that swimming exercise increased u-Dpd, reflecting dynamically stimulated bone resorption, and the femoral and tibial BMD showed a tendency towards decrease as compared with age matched control rats. In short, it was indicated that bone loss by the stimulation of bone resorption had occurred. Previously several studies reported conflicting results about the effects of exercise on bone mass. For example, it was reported that voluntary exercise (Goseki et al., 1995; Iwamoto et al., 1998) and resistance training (Westerlind et al., 1998) increased BMD and that decreased bone resorption and increased formation in the rats. In contrast, bone loss induced by exercise has also been reported in the rat. Rico et al. (1999) reported that femoral and vertebral BMD and BMC were lower in the strenuous treadmill exercise rats than those seen in the control. Wheeler et al. (1995) also reported decreases in tibial and femoral torque as well as in bone strength by high intensity exercise. The peculiarities of these exercises are strenuous intensity exercise. Precise intensity exercise might disturb bone metabolic turnover, resulting in bone loss. In the present study, the Swim group showed a significant decrease in the body weight and abdominal fat weight, and these results may indicate a disproof against previous studies. Additionally, it was suggested that BMD and BMC in the Swim group was not significantly different from the Con group because the experimental period in this study was too short. Iwamoto et al. (1998) reported that although BMD of exercised rats showed no significant difference at 4 or 8 weeks from the start of exercise training, it began to show at 12 weeks. This finding might be the reason for no change of BMD in our swimming exercise group. Further investigation, including a longer period of experiment and/or various intensities of exercise will be required to confirm the results of this study.

On the other hand, although the present study observed similar BMD and bone status in the chronic acidosis rats, urinary Ca and urinary P excretions were dynamically increased while kidney weight was markedly increased, as compared to the age matched control rats. A balance of the intestinal absorption and urinary excretion maintains Ca and P concentrations in the circulation. The serum Ca and P concentrations are regulated mainly by 1,25(OH)₂D₃ and PTH in humans (Domingues et al., 1976), and metabolic acidosis induces marked bone loss with an increase of urinary Ca and P excretions in rats (Ambuhl et al., 1998). These results indicated that the disturbed urinary Ca and P excretions may occur in bone loss, and this disturbance might be one of the reasons for acidosis-induced bone loss. For instance, acidosis induced kidney disorder and increased urinary Ca and P excretion, and stimulated Ca and P efflux from bone for maintenance of concentration in the blood.

In the present study, it is an interesting point that both the blood biochemical markers and the change of body fats were different between the Swim and the Acid groups. It was thought that swimming might be carried out by aerobic exercise rather than anaerobic exercise, and/or the production of lactate was low because the exercise intensity of free style swimming was low. Also, the exercise intensity might be insufficient to induce a metabolic acidosis, resulting in the difference between the Swim and Acid groups. Unfortunately, since blood pH or lactate was not assessed in the current study, we are unable to prove whether changing bone metabolic...
turnover caused was by acidosis or not. Further study on the differences of intensity, mode and duration in exercise will be necessary to confirm the results of the study in the future.

In conclusion, the results obtained from the present investigation suggested that blood Ca and P concentrations in chronic acidosis conditions over 4 weeks might be maintained by hypercalciuria and hyperphosphaturia with kidney disorder, and swimming exercise training leads to a decrease in BMD with both the stimulation of bone resorption and the reduction of body fat.

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