Utility of measuring long bone length in toxicity studies: results of a 14-day repeated dose oral toxicity study of dexamethasone in young, periadolescent and adult rats

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ABSTRACT — Measurement of the bone length is not used routinely in toxicity studies but used to examine growth and bone toxicity. To investigate the utility of measuring the bone length in toxicity studies and to identify the appropriate site, we evaluated femur, tibia, humerus, and sternum, in a 14-day repeated dose oral toxicity study of Dexamethasone (DEX), which is known to cause growth retardation and osteoporosis, in young, periadolescent, and adult rats. To observe the effect of decreased food consumption, we also evaluated the changes in each diet-restricted group in which the food intake restricted to levels corresponding to that consumed by the DEX-treated periadolescent and adult rats. Significant decreases of the bone length at all the measured sites and histopathological findings in growth plates and/or trabecular bone were observed in the DEX-treated young and periadolescent rats. Significant decreases of the femoral length and decreased trabecular bone were observed in the DEX-treated adult rats. No histopathological changes were observed in any of the diet-restricted groups, while decreases of the femoral length, similar to that in the DEX-treated adult rats were observed in the diet-restricted adult rats. The results suggested that measurement of the bone length in femur, tibia, humerus, and sternum was useful in young and periadolescent rats, and measurement of the femoral length was useful in adult rats. Moreover, our results showed that the decreases in the femoral length in the DEX-treated adult rats were not only related to the DEX-treatment, but were also influenced by the decreased food consumption.

Key words: Rats, Bone length, Histopathology, Dexamethasone, Dietary restriction

INTRODUCTION

The skeletal system is composed of a variety of specialized forms of supporting or connective tissue, and bone provides a rigid protective and supportive framework for most of the soft tissues of the body (Young and Heath, 2000). Bone is a dynamic connective tissue and an important organ for postnatal development of the body (Zoetis et al., 2003); it is a metabolically active tissue and is also important as a calcium reservoir in calcium homeostasis. Formation, modeling and remodeling of bone are influenced by many factors and conditions, especially hormonal and dietary factors and conditions (Young and Heath, 2000; Leininger and Riley, 1990). Thus, the skeletal system has important functions, and investigation of changes in the skeletal system is important in drug toxicity studies.

Histopathological evaluation is one of the most commonly used methods to investigate bone toxicity in preclinical general toxicity studies, and histomorphometric and immunohistochemical analyses are occasionally used for additional and special explorations. Measurement of
long bone length is an important examination, not only to investigate overall growth in nonclinical safety testing in the development of pediatric medicines (FDA, 2006; ICH, 2018), but also for the detection of drug-induced bone toxicity (Gropp et al., 2018; Frazier, 2017; Vahle et al., 2004). It is easy to compare measured bone lengths, to investigate growth and skeletal abnormalities, by statistical analyses, although measurement of bone length has not been used routinely in general toxicity studies conducted in periadolescent and adult rats, and further information is required to measure the bone length in general toxicity studies conducted in periadolescent and adult rats. The measurement of one long bone at necropsy is generally sufficient in studies of young animals, as supporting evidence in the development of pediatric medicines (ICH, 2018), although the bone sites for such measurement has not been specified. In other words, the skeletal sites mentioned for length measurement differ among guidelines and/or reports, as follows: (1) the femur is mentioned as a typical site for measurement of the bone length in the ICH guideline (ICH, 2018); (2) the tibia is mentioned as an example site for measurement of the bone length in the FDA guidance (FDA, 2006); (3) tibia, femur, and/or humerus have been used as sites for measurement of the bone length in various kinds of studies performed in rat models (Even-Zohar et al., 2008; Yin et al., 1982; Heinrichs et al., 1997; Xian et al., 2004, 2006; Yoshida et al., 2014).

In this study, we measured the bone lengths of various bones (femur, tibia, humerus, and sternum) in a 14-day repeated dose toxicity study in young, periadolescent and adult rats, to investigate the utility of measuring the bone length in toxicity studies and to identify the most appropriate site for measurement of the bone length. In the young rat, animals at 2 weeks of age at the start of dosing and 4 weeks of age at necropsy (the end of dosing) were used to observe the changes in the bone length in juvenile animal studies in support of development of pediatric medicines (e.g., exploratory 14-day repeated dose toxicity study). In the periadolescent rat, animals at 6 weeks of age at the start of dosing and 8 weeks of age at necropsy were used to observe the changes in subacute toxicity studies (e.g., 14-day repeated dose toxicity study). In the adult rat, animals were 28 weeks of age at the start of dosing and 30 weeks of age at necropsy, that is similar to the age of terminal necropsy in chronic toxicity studies (e.g., 6-month repeated dose toxicity study). We also evaluated the histopathological changes in these bones to confirm the effects of dexamethasone (DEX) on the bone. DEX was administered orally in this study, as it is known to be one of the synthetic glucocorticoids (Malkawi et al., 2018), and to cause glucocorticoid-induced growth retardation and osteoporosis in rats (Wood et al., 2018). The dose of DEX was determined from the results of an exploratory dose-finding study conducted in periadolescent male rats, in which we observed DEX-induced histopathological changes in the bone (data not shown). We used male rats in this study to eliminate the sex difference, because the responses and effective doses of glucocorticoid are known to differ by sex (Wood et al., 2018), and longitudinal bone growth is influenced by various hormones, including estrogens and androgens (Emons et al., 2011). In drug toxicity studies, moderate to severe body weight loss and decreased food consumption are often observed, and it is important, although sometimes difficult, to distinguish body weight loss caused by the direct effects of a drug and secondary changes caused by a significant reduction of food consumption. In addition, we investigated the effects of the drug as well as of the associated food restriction on the bone, using diet-restricted groups not treated with DEX, in which food intake was restricted to levels corresponding to those consumed by the rats in the DEX-treated groups of periadolescent and adult animals. We did not examine a diet-restricted group in young rat, because the age at which the rats were used for this study was the age at which rats are usually switched from milk to standard laboratory animal diet.

MATERIALS AND METHODS

Experimental animals

Three pregnant female Crl:CD (SD) rats (gestation day 15) were purchased from Charles River Japan (Kanagawa, Japan), and the day of birth was designated as post-natal day (PD) 0. Naturally delivered 2-week-old (PD 14, young) male pups from these dams, a total of 10 in number and weighing 33-36 g, were used as the young group in this study. Six-week-old male (periadolescent) Crl:CD (SD) rats, a total of 15 in number and weighing 190-226 g, and 28-week-old (adult) rats, a total of each 15 in number and weighing 606-743 g were also purchased from Charles River Japan and used as the periadolescent and adult groups in this study. These animals were maintained under the following conditions: temperature of 20°C-26°C, relative humidity of 30%-70%, 12-hr light/dark cycle, and ventilation frequency of 5-40 air exchanges/hr.

Study design

All rats were divided into the following eight groups (n=5 per group): (1) vehicle-treated control young group; (2) DEX-treated young group; (3) vehicle-treated con-
trol periadolescent group; (4) DEX-treated periadolescent group; (5) diet-restricted periadolescent group; (6) vehicle-treated control adult group; (7) DEX-treated adult group; and (8) diet-restricted adult group. Animals in the DEX-treated young, periadolescent and adult groups were treated orally once a day with DEX for 14 days (Wako Pure Chemical Industries Ltd., Osaka, Japan), at the dose of 0.3 mg/kg/day. Animals in the other groups were treated orally once a day with 0.5% methylcellulose in distilled water for 14 days (Wako Pure Chemical Industries Ltd.). The dosing volume was 5 mL/kg body weight, and was calculated based on the most recent body weight. Animals in the control and DEX-treated young animal groups were allowed natural suckling before being weaned and separated from the dams, and independent feeding was started on Day 9 (PD 22). In the independent feeding phase, animals in the control and DEX-treated young animal groups had free access to a standard laboratory animal diet (MF; Oriental Yeast Co., Ltd., Tokyo, Japan). Animals in the control and DEX-treated periadolescent and adult animal groups had free access to a standard laboratory animal diet (MF; Oriental Yeast Co., Ltd.). Animals in the diet-restricted groups were not treated with DEX, but instead fed a restricted amount of food, corresponding to the amounts of food consumed by the rats in the DEX-treated groups. The day of start of DEX administration was designated as Day 1, and that of pair-feeding as Day 2 in this study. The animals were euthanized and necropsied at 4 weeks, 8 weeks and 30 weeks of age, at the end of the administration period. All the animals were treated in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Taisho Pharmaceutical Co., Ltd.

Examinations and methods

Each animal was checked for clinical signs at least three times daily (before dosing, immediately after dosing, and three hours after dosing). The body weights were measured every day from Day 1 (PD 14), and food consumption were measured every day from Day 10 (PD 23) in the young animal groups. In the periadolescent and adult animal groups, the body weights and food consumption were measured every day. The bone length of the left femur, left tibia, left humerus, and sternum was measured by the same person, using a digital caliper (Mitsutoyo Co., Kanagawa, Japan) before the trimming for histopathology. For the histopathological evaluation, the left femur, left tibia, left humerus, and sternum were fixed in 10% neutral buffered formalin, and decalcified using the ethylenediaminetetraacetic acid (EDTA)-decalcification protocol (Wako Pure Chemical Industries Ltd.). After fixation and decalcification, hematoxylin and eosin (HE)-stained specimens were prepared and subjected to microscopic observation.

Statistical analysis

The mean values and standard deviations of the numerical data obtained in regard to the food consumption, body weight, and bone length were calculated for each group. Data on the body weight and bone length were analyzed for statistically significant differences by multiple comparisons of the data obtained in all of the periadolescent and all of the adult groups. Statistical analyses were performed using one-way ANOVA, followed by Tukey’s test (if homogeneous) or the Steel-Dwass (if heterogeneous) test. Statistical analyses were also performed using the F-test followed by the Student’s t-test (if homogeneous) or Aspin-Welch’s t-test (if heterogeneous), to determine the differences in food consumption between the DEX-treated and control periadolescent and adult animal groups, and the differences in all the data between the DEX-treated and control young animal groups. For all the statistical analyses, we used the EXSUS Version 7.7.1 Statistical Analysis System (CAC Croit Corporation, Tokyo, Japan), which is based on the SAS software program (version 9.2; SAS Institute Inc., Cary, NC, USA).

RESULTS

Clinical signs and mortality

In the young animals, one premature death was observed in the DEX-treated group on Day 4 prior to the start of dosing. This animal showed a decrease in locomotor activity on Day 3, and macroscopic observation revealed perforation of the esophagus. Therefore, the cause of death in this animal was considered to be the dosing procedure. No deaths and no abnormal clinical signs were observed in any of the other young animal groups. Because of the premature death on Day 4, the assessments, except for the in-life examinations in the DEX-treated group, were only conducted for four animals.

In the periadolescent animals, no deaths and no abnormal clinical signs were observed in any of the groups.

In the adult animals, three premature deaths were observed in the DEX-treated group (one on Day 10 and the others on Day 13). These animals showed a decrease in locomotor activity, incomplete eyelid opening, reddish tears, and/or chromaturia. No abnormalities were observed in the diet-restricted groups. Because of the premature deaths, the assessments, except for the in-
life examinations and histopathology in the DEX-treated group, were only conducted for two animals.

Food consumption (Figs. 1-3)

In the young animals, statistically significant (p < 0.05, p < 0.01, and/or p < 0.001) decreases in food consumption were observed in the DEX-treated as compared to the control young animal group (Fig. 1). At the end of the drug administration period, the percentage reduction in the food intake in the DEX-treated group as compared to...

![Fig. 1](image1.png)

Fig. 1. Food consumption in the young animals. Statistically significant (p < 0.05, p < 0.01, and/or p < 0.001) decreases in food consumption were observed in the DEX-treated as compared to the control young animal group. Values are means ± SD, n = 4-5 animals/group. * Significantly different from the control group (p < 0.05). ** Significantly different from the control group (p < 0.01). *** Significantly different from the control group (p < 0.001). DEX = dexamethasone.

![Fig. 2](image2.png)

Fig. 2. Food consumption in the periadolescent animals. Statistically significant (p < 0.05 and p < 0.01) decreases in food consumption were observed in the DEX-treated as compared to the control periadolescent group from Day 3 to Day 5. Values are means ± SD, n = 5 animals/group. * Significantly different from the control group (p < 0.05). ** Significantly different from the control group (p < 0.001). DEX = dexamethasone.

![Fig. 3](image3.png)

Fig. 3. Food consumption in the adult animals. Statistically significant (p < 0.05, p < 0.01, and/or p < 0.001) decreases in food consumption were observed in the DEX-treated as compared to the control adult animal group from Day 3 to Day 10, and on Day 12. Values are means ± SD, n = 2-5 animals/group. * Significantly different from the control group (p < 0.05). ** Significantly different from the control group (p < 0.01). *** Significantly different from the control group (p < 0.001). DEX = dexamethasone.

![Fig. 4](image4.png)

Fig. 4. Body weight in the young animals. Statistically significant (p < 0.05 and p < 0.001) decreases in the body weight were observed in the DEX-treated as compared to the control young animal group from Day 2 to Day 14. Values are means ± SD, n = 4-5 animals/group. * Significantly different from the control group (p < 0.05). ** Significantly different from the control group (p < 0.001). DEX = dexamethasone.
the control group was 29.8%.

In the periadolescent animals, statistically significant ($p < 0.05$ and $p < 0.01$) decreases in food consumption were observed in the DEX-treated as compared to the control group (Fig. 2). In the diet-restricted group, the rats ate all the food during the experimental periods (except for an animal that left some food for Day 10 to Day 13). At the end of the drug administration period, the percentage reductions in food intake in the diet-restricted and DEX-treated groups as compared to the control group was 15%.

In the adult animals, statistically significant ($p < 0.05$, $p < 0.01$, and $p < 0.001$) decreases in food consumption were observed in the DEX-treated as compared to the control group (Fig. 3). In the diet-restricted groups, the rats ate all the food during the experimental periods. At the end of the drug administration period, the percentage reductions in food intake in the diet-restricted and DEX-treated groups as compared to the control group was 40.3%.

**Body weight (Fig. 4–6)**

In the young animals, statistically significant ($p < 0.05$ and $p < 0.001$) decreases in the body weight were observed in the DEX-treated group as compared to the control group (Fig. 4).

In the periadolescent animals, statistically significant ($p < 0.05$, $p < 0.01$, and $p < 0.001$) decreases in the body weight were observed in the DEX-treated group as compared to the control and diet-restricted group (Fig. 5). Furthermore, statistically significant ($p < 0.05$) decreases in the body weight were observed in the diet-restricted group as compared to the control group (Fig. 5).

In the adult animals, statistically significant ($p < 0.05$, $p < 0.01$, and $p < 0.001$) decreases in the body weight were observed in the DEX-treated group as compared to the control group (Fig. 6).
the control and diet-restricted groups (Fig. 6). Statistically significant (p < 0.05) decreases in the body weight were observed in the diet-restricted group as compared to the control group (Fig. 6).

Bone length (Fig. 7-9)

In the young animals, statistically significant (p < 0.05 and p < 0.001) decreases in the lengths of all the measured bones were observed in the DEX-treated young animal group as compared to the control young animal group (Fig. 7).

In the periadolescent animals, statistically significant (p < 0.05, p < 0.01, and p < 0.001) decreases in the lengths of the tibia, humerus and sternum were observed in the DEX-treated as compared to the control and diet-restricted groups (Fig. 8). A tendency towards decrease or a statistically significant (p < 0.01) decrease in the length of the femur was observed in the DEX-treated as compared to the diet-restricted periadolescent group. Furthermore, statistically significant decreases in the lengths of all the measured bones (p < 0.05, p < 0.01, and p < 0.001) were observed in the DEX-treated as compared to the diet-restricted periadolescent group. Values are means ± SD, n = 5 animals/group. ** Significantly different from the control group (p < 0.01). *** Significantly different from the control group (p < 0.001). †Significantly different from the diet-restricted group (p < 0.05). ††Significantly different from the diet-restricted group (p < 0.01). †††Significantly different from the diet-restricted group (p < 0.001). DEX = dexamethasone, DR = diet-restricted.

In the adult animals, no statistically significant changes in the lengths of the tibia, humerus, and sternum were observed in the DEX-treated as compared to the control and diet-restricted groups (Fig. 9). The length of the femur in DEX-treated group was not statistically significantly different, but was obviously decreased as compared with that in the control group (Fig. 9). The femoral length was statistically significantly (p < 0.05) decreased in the diet-restricted as compared to the control group (Fig. 9). No statistically significant difference in the length of the femur was observed between the DEX-treated group and the diet-restricted group (Fig. 9).

Histopathology (Table 1, Fig. 10)

The following findings were observed only in the bones of the DEX-treated young animals: thickening of the growth plate in the proximal femur, distal humerus, and sternum; thinning of the growth plate in the proximal humerus; and increased trabecular bone in the distal tibia and sternum (Table 1). The incidence of these findings were differed depending on the observed bone site, and no remarkable findings were observed in the control group of young animals, or in the distal femur and proximal tibia of the DEX-treated young animal group.
In the bones of the periadolescent animals, thinning of the growth plate, increased trabecular bone, and decrease in osteoblast were observed only in the DEX-treated group, and the incidence in these findings were differed depending on the observed bone site (Table 1 and Fig. 10). All of the findings mentioned above were observed in the proximal and distal femur, proximal tibia, proximal humerus, and some of them were observed only in the distal tibia and sternum. No remarkable histopathological findings in bone were observed in the control or diet-restricted periadolescent animal groups, or in the distal humerus of the DEX-treated periadolescent group.

In the bones of the adult animals, decreased trabecular bone was observed only in the distal femur, proximal tibia, and sternum of the DEX-treated group (Table 1 and Fig. 10). No remarkable histopathological findings in bone were observed in the control or diet-restricted adult animal groups, or in the other observed sites of the DEX-treated group.

In the bone marrow, decreased cellularity was observed in the DEX-treated animals of all ages in most of the observed bone areas, and this finding was considered to be related to the DEX treatment (Ekblom et al., 1993).

**DISCUSSION**

In this study, we measured the bone length of various bones (femur, tibia, humerus, and sternum) in a 14-day repeated dose oral toxicity study of DEX in young (4-week-old), periadolescent (8-week-old) and adult (30-week-old) rats, to investigate the utility of measuring the bone length in toxicity studies and to identify the appropriate bones for measuring the bone length. The DEX-treated young and periadolescent animal groups showed statistically significant decrease/tendency towards decrease of the bone length of all the measured bones (femur, tibia, humerus, and sternum). On the other hand, in the DEX-treated adult group of animals, only a significant decrease in the bone length of the femur was observed, with no remarkable change in the bone length of any of the tibia, humerus, or sternum. These results suggest that measurement of the bone length is useful in toxicity studies of young, periadolescent as well as adult rats, because decrease of the bone length caused by DEX was observed in all of these DEX-treated groups. On the other hand, the appropriate bone site for conducting this measurement appears to differ with age. While all of the four selected bone sites (femur, tibia, humerus, and sternum) were found to be useful for the analyses in the young and periadolescent animals, femur was the most appropriate site for evaluating bone growth and skeletal changes in the adult animals. The tibia may also be considered to be an appropriate site to detect a tendency towards change of the bone length in adult animals, but changes in the length of the humerus and sternum were rather difficult to detect in adult animals. It was considered that changes in the bone length could be observed in all the selected bones in the DEX-treated young and periadolescent animals, because of the accelerated increase in the length of each long bone of the limbs from 0 to 8 weeks of age in rats (Fukuda and Matsuoka, 1979). In contrast, site-specific differences in the bone length were observed in the DEX-treated adult animals. Longitudinal bone growth is known to be a result of chondrocyte proliferation and subsequent differentiation in the growth plate of the long bones (van der Eerden et al., 2003); in other words, the growth plate is one of the key tissues involved in longitudinal bone growth. Little has been reported on the relationship between longitudinal bone growth and epiphyseal closure in any of the laboratory animals, including the rat (Kilborn et al., 2002); however, the maturation processes of bone and the time of epiphyseal closure...
are known to differ by the site of bone among bones at different sites in the rat (Dawson, 1925; Fukuda and Matsuoka, 1979), which could explain the site-specific differences in the drug-induced changes of the bone length in the rats. Fukuda’s study clearly indicated that the maturation process differs among various bones at the same age. In detail, the time of fusion of the secondary ossification centers in each proximal site was 78-104 weeks in the femur, 104 weeks or longer in the tibia, and 52 weeks in the humerus (Fukuda and Matsuoka, 1979). Regarding the distal sites, the time of fusion of the secondary ossification centers were earlier than that at each proximal site, being 15-17 weeks in the femur, 13-15 weeks in the tibia, and 4 weeks in the humerus (Fukuda and Matsuoka, 1979). The age of the adult rats in this study was 28 weeks at study initiation and 30 weeks at study completion, and each of the proximal sites of the femur, tibia and humerus has a growth plate; however, the time of maturation at the distal site differed among the femur, tibia and humerus. These results suggest that fusion of the secondary ossification centers at the distal site in the bones occurred last in the femur, which is speculated as the reason for the femur being identified as the most sensitive site for determination of the changes in the bone length in adult animals. These results also raise the possibility that the growth plates at the distal sites of the long bones contribute more to longitudinal growth than those at the proximal sites in the rat. Concerning the sternum, the time of fusion of the secondary ossification centers has not been previously reported, and the maturation process has been shown to already be less active in rats at 25 weeks of age (Jasty et al., 1986); therefore, the changes in the sternal bone length were not remarkable. These age- and site-specific results were also considered

Fig. 10. Histopathological changes in the distal femur of young (a-b), periadolescent (c-e), and adult (f-h) animals (HE staining). In the young animals, no remarkable histopathological changes were observed at this site in either the control (a) or DEX-treated (b) young animals. In the periadolescent animals, no remarkable histopathological changes were observed in the control (c) and diet-restricted (e) groups; however, in the DEX-treated (d) periadolescent animals, thinning of the growth plate, increase in trabecular bone, and decreased cellularity of the bone marrow were observed. In the adult animals, while no remarkable histopathological changes were observed in the control (f) or diet-restricted (h) groups, the DEX-treated (g) adult animals showed a decrease in trabecular bone and decrease in bone marrow cellularity. Bar = 500 μm.
to be related to the maturation processes of the bones as determined by the histopathological changes described in a previous report (Noguchi et al., 2011).

We conducted histopathologic evaluation of the bones to confirm the effects of DEX on the bone by the age. In the DEX-treated young animal group, thickening/thinning of the growth plate and/or increased trabecular bone were observed in the proximal femur, distal tibia, proximal/distal humerus, and sternum. In the DEX-treated periadolescent animal group, thinning of the growth plate, increase of trabecular bone, and/or decrease of osteoblasts were observed in almost all the observed bone sites, except for the distal humerus; however, in the DEX-treated adult animal group, these changes were not observed, and decrease of trabecular bone was observed only in the distal femur, proximal tibia, and sternum among the

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<th>Table 1. Histopathological changes in young, periadolescent, and adult animals.</th>
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<td>Decreased, Osteoblast</td>
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<td>0 0</td>
<td>5 0 4</td>
</tr>
<tr>
<td>Decreased, Cellularity, Bone marrow</td>
<td>5 0 4</td>
<td>0 0</td>
<td>5 0 4</td>
</tr>
</tbody>
</table>

DEX = dexamethasone; DR = diet-restricted
<>: Not remarkable/Number of animals examined
-: None/Negligible, ±: Minimal/Slight, +: Mild, 2+: Moderate
1): Growth plates of a few animals were not examined because of loss of growth plate/epiphyseal closing.
2): All dead animals in the DEX-treated adult group were included.
examined bone areas. Thus, the histopathological changes in the DEX-treated animals differed by the age, but were still all considered to be induced by DEX, because all of the changes have been reported previously following DEX administration in animals. The following have been proposed as the mechanisms underlying the skeletal changes induced by DEX: (1) inhibition of chondrocyte proliferation and matrix synthesis (van der Eerden et al., 2003), (2) apoptosis of chondrocytes in the growth plate (Chrysis et al., 2003; Silvestrini et al., 2000), (3) suppression of VEGF-mediated angiogenesis in the osteoblasts and osteocytes (Weinstein et al., 2010), and (4) increased apoptosis of osteoblasts (Silvestrini et al., 2000). Comparing these histopathological findings with the changes in the bone length observed in the young and periadolescent animals, we observed histopathological changes in the growth plate and/or trabecular bone in the proximal and/or distal femur, tibia, humerus and sternum, all of which showed significant decreases of the bone length. In the adult animals, only the bone length of the femur was significantly decreased and decrease of trabecular bone was observed histopathologically most frequently in the distal femur. These results indicate the existence of a relationship between the changes in the bone length and the histopathological changes.

We also evaluated the changes in the bone length and histopathology in diet-restricted groups, that is, in animals in which the food intake restricted to levels corresponding to those in the DEX-treated periadolescent and adult animals, to observe the effect of food restriction per se on the bone. While no histopathological changes were observed in the diet-restricted periadolescent and adult animal groups, a decrease in the femoral length, similar to that in DEX-treated adult animal group, was observed in the diet-restricted adult group in this study. The percentage reduction in food intake in the 14-day diet-restricted periadolescent animal group was 15%, and no changes in the bone length or histopathology were observed in these animals. The percent reduction in food intake in the 14-day diet-restricted adult animal group was 40.3%, and a statistically significant decrease in the bone length of the femur was observed in this group without any associated histopathological changes, consistent with previous reports (Levin et al., 1993; Moriyama et al., 2008; Noguchi et al., 2019). These results also suggest that the femur is a more sensitive site for measurement of the bone length than the tibia, humerus or sternum in adult animals, similar to the results obtained in the DEX-treated adult animal group. Furthermore, a significant decrease in the bone length of the femur was observed in the DEX-treated adult animal group, which was similar in degree to that in the matched diet-restricted group, with no statistically significant difference in the femoral bone length observed between the DEX-treated group and the matched diet-restricted group. Hence, the decrease in the bone length observed in the DEX-treated adult group in this study was considered to be mainly attributable to the decrease in food consumption in these animals, since a similar change was observed in the 14-day diet-restricted group in this study; on the contrary, the observed histopathological findings in the DEX-treated adult animals were considered to be induced by DEX alone and not to be related to the secondary decrease in food consumption in these animals. No obvious changes in the bone length were observed in the diet-restricted periadolescent animal group in this study, and the percentage reduction in food intake of 15% in this group was considered to be insufficient for inducing changes in the bone length.

In this study, we showed that measurement of the bone length is useful in toxicity studies of young, periadolescent and adult rats, because decrease of the bone length was observed in all the DEX-treated groups in this study, regardless of the age of the animals. Moreover, the most appropriate site of measurement of the bone length in toxicity studies appears to vary with age: while all of the four selected bone sites (femur, tibia, humerus, and sternum) were found to be useful for the analyses in the young and periadolescent animals, femur was the most appropriate site for evaluating bone growth and skeletal changes in the adult animals. These results will be useful when undertaking measurement of the bone length for investigating the effects on bone growth and skeletal examination in general toxicity studies of periadolescent and adult animals in the field of medical therapeutics, as well as in studies of young animals undertaken for the development of pediatric medicines, because few general toxicity studies until date have reported examining the bone length or determining the site-specific and/or age-related differences in the bone length. We also revealed that 14-day diet restriction caused a statistically significant decrease in the bone length of the femur in adult animals. Several reports have demonstrated the effect of diet restriction, fasting or malnutrition on the growth plates; however, the results are still inconclusive (Brighton, 1987; van Leeuwen et al., 2003; Heinrichs et al., 1997; Even-Zohar et al., 2008) and they are difficult to extrapolate to toxicity studies, because of differences in the ages of the animals used, the study duration, level of food restriction, and the sites assessed. For this reason, we believe that our findings will be useful to determine whether the observed changes in bone length and histopathology in toxicity studies represent the direct effects of drug toxicity or the effects of
secondary decrease in food consumption in the animals given the test drugs.

Our conclusions were obtained from measurements of the bone lengths and routine histopathological evaluations; further information is needed to consider site-specific differences in the bone length and histopathological changes. Therefore, additional examinations, including histomorphometric analyses to examine the morphological changes in greater detail (e.g., measurement of the widths of the growth plate, etc.), and/or immunohistochemical analyses for cell proliferation markers (e.g., proliferating cell nuclear antigen, etc.) may be needed to confirm the present findings, especially since these specialized techniques have been shown to be useful for determining the mechanisms underlying skeletal changes (Fossey et al., 2016).

In conclusion, we showed that measurement of the bone length is useful in toxicity studies of young, peridolescent and adult rats, because decrease of the bone length was observed in all the DEX-treated groups in this study, regardless of the age of the animals. Moreover, the most appropriate site of measurement of the bone length in toxicity studies appears to vary with age. While measurement of the bone lengths of all of the femur, tibia, humerus, and sternum appeared to be valid to detect changes in the DEX-treated young and peridolescent animals, measurement of the bone length of the femur rather than that of the tibia, humerus or sternum appeared to be more sensitive and useful in the adult animals. In addition, the change in the length of the femur in the DEX-treated adult animals were considered to not only be attributable to the DEX treatment, but to also be influenced by the decreased food consumption of the animals associated with the DEX treatment.

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES


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