Measurement of Serum [^{3}H]Tetracycline Kinetics and Indices of Kidney Function Facilitate Study of the Activity and Toxic Effects of Bisphosphonates in Bone Resorption

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The [^{3}H]tetracycline ([^{3}H]TC) model is based on the observation that TC is released from the bones of rats prelabeled with [^{3}H]TC via first-order kinetics, a factor directly reflecting the kinetics of bone resorption. In the present paper we applied the [^{3}H]TC elimination model to rats treated with antiresorptive drugs. The validity of this model was evaluated by examining the effect of the bisphosphonate, 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (ABP), and a novel bisphosphonate, dihydrogen disodium adipoylbisphosphonate (AdBP), on serum TC levels and the elimination rate constant. ABP and AdBP significantly inhibited the TC elimination rate. However, ABP treatment caused impairment of bone mineralization, renal dysfunction, and inhibition of somatic growth. It is concluded that antiresorptive effects of bisphosphonates could be evaluated by the [^{3}H]TC model, but this model is limited to animals with normal kidney function. The experimental conditions provide a technically simple method which is sensitive enough to examine antiresorptive properties in a healthy animal and to detect adverse effects on the kidney. The activity of the novel bisacylphosphonate, AdBP, and lack of its adverse effects indicate the potential of this drug for clinical applications.

KEY WORDS: bisphosphonates (diphosphonates); bisacylphosphonates; bone resorption; tetracycline; mineralization; nephrotoxicity.

INTRODUCTION

Klein and co-workers (1–4) presented a pharmacokinetic model in animals, of the direct quantification of bone resorption by monitoring [^{3}H]tetracycline ([^{3}H]TC) in serum or urine of rats pretreated with the labeled drug. This model is based on the observation that TC is released from bone by first-order kinetics, a factor directly reflecting the kinetics of bone resorption (4–6). The resorption rate can therefore be quantitated by its first-order elimination constant (K_e). The determination of [^{3}H]TC in serum or urine of prelabeled animals has been used to assess bone resorption during different experimental conditions such as effects of vitamin D deficiency or hypervitaminosis D (2), age and species of animals (3,4), and bone type (3).

There is a need for a quantitative, technically simple model for bone resorption in healthy animals, for the evaluation of antiresorptive drugs. Recently it was shown that rats' urinary [^{3}H]TC levels are reduced following treatment with dichloromethylenebisphosphonate (ClMBP) (7). However, an evaluation of the effect of antiresorptive drugs on TC elimination kinetics has not yet been done. Since treatment with bisphosphonates is frequently associated with nephrotoxicity (8–10), measurements of kidney function are required to verify whether the reduced [^{3}H]TC elimination rate is due to inhibited bone resorption and/or suppressed renal clearance. Serum, rather than urine, measurement of [^{3}H]TC kinetics is preferable since it represents total-body clearance (i.e., renal and nonrenal pathways).

In the present paper, the validity of the TC elimination model was evaluated by examining the effect of the bisphosphonate, 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (ABP; also known as AHPrBP), on serum [^{3}H]TC levels and the elimination rate constant, in a dose–response manner. ABP, an extensively studied antiresorptive agent, was selected because it is one of the most effective inhibitors of bone resorption (11,12) and is nephrotoxic (10).

Recently, we have reported on the synthesis and properties of new bisacylphosphonates (13,14). These compounds represent a new class of nongeminal bisphosphonates [P-(C)n-P, $n \geq 2$] which inhibit hydroxyapatite formation and dissolution *in vitro* and the pathological calcification of bioprosthetic heart valve tissue cusps *in vivo* (14). However, the antiresorptive efficacy of these compounds has not been examined. *In vivo* testing of new bisphosphonate homologues would enhance the understanding of the relationship between chemical structure and biological activity and might stimulate the development of a new, improved generation of such drugs. In addition, preliminary toxicological studies indicate that these compounds are far less toxic than ABP (15). Therefore, it is interesting to compare the antiresorptive and nephrotoxic effects of ABP and AdBP in the new model.

MATERIALS AND METHODS

Materials

Tetracycline-7-^{3}H(N) (0.64 Ci/mmol) was obtained from New England Nuclear (Boston, MA). ABP (disodium salt) was provided by Dr. T. Klenner, The German Cancer Research Institute (Heidelberg, Germany). Dihydrogen disodium adipoylbisphosphonate (AdBP; 1,6-dioxohexane-1,6-bisphosphonic acid, disodium salt) was synthesized in the laboratory of Prof. E. Breuer of our institution (13,14).

[^{3}H]TC Labeling and Bisphosphonate Treatment

The method of rat labeling is based on the work of Li *et al.* (4) with some modifications. The isotope was dissolved in 0.05 M HCl containing 0.05% ascorbic acid. Weanlings, 21-day-old male Sabra rats (Faculty of Medicine, The Hebrew University of Jerusalem, Israel), were used in this study. The rats were labeled with nine subdermal injections for a total dose of 207.9 μCi/rat, over a period of 16 days (see Table I).

Starting on the third day postlabeling the rats were treated for 6 days with subcutaneous injections (1 ml/kg) of

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Table I. Experimental Protocol

Experiment	Number of rats	Labeling period (days)[a]	Treatment period (days)[a]	Data collection time (days)[a]
[^{3}H]TC kinetics	34[b]	−16–0	3–8	3, 7, 12, 17, 24, 31
Biochemical indices	30[c]	—	3–8	7, 12, 24

[a] Zero time = end of labeling.
[b] Twelve control; seven ABP-16; and five in each group of ABP-40, ABP-160, and AdBP-160.
[c] Six in each experimental group.

bisphosphonates (see Table I). The rats were divided into five treatment groups: saline; ABP-treated rats at doses of 16, 40, and 160 μmol/kg/day (ABP-16, ABP-40, and ABP-160, respectively); and with 160 μmol/kg/day of AdBP (AdBP-160). All rats were maintained on a normal diet.

TC Kinetics

Blood samples (see Table I) were withdrawn from the tail artery under mild anesthesia induced with ether, without anticoagulation. The blood samples were centrifuged at 3000 rpm for 10 min, and 0.1 ml of serum was combined with 10 ml of liquid scintillation cocktail (Atomlight, New England Nuclear). The radioactivity in rats serum was quantitated in a Packard liquid scintillation counter (Packard Instrument Co., Meriden, CT). A standard curve was prepared by combining 0.1 ml of [^{3}H]TC at various concentrations with the liquid scintillation cocktail. Nontreated rat serum was used as a "blank" and its background was less than 20 cpm.

Kidney Indices

Serum samples (see Table I) obtained as detailed above were analyzed for creatinine, urea-nitrogen (BUN), alkaline phosphatase (ALP) activity by appropriate analytical kits (Sigma, St. Louis, MO). Serum concentration of the various markers was determined by UV/Vis spectrophotometer (Spectronic 1001, Milton Roy, Rochester, NY) with standard curves having a correlation coefficient of 0.998–1.

Chemical and Histological Bone Analyses

The left femur of each animal was taken at the end of the experiments (days 24 and 31; see Table I). For bone chemistry analyses, the bone was dried for 24 hr at 105°C and weighed (dry weight). The ash content was determined after heating at 600°C for 24 hr. The ash was then dissolved in 25 ml of 1.5 *N* HCl and its calcium content was determined by atomic absorption spectroscopy (16).

For histological examinations, the excised bone was immersed in Karnovsky's buffer (17). The lower halves of some femurs were dehydrated and embedded in glycol methacrylate (18). Longitudal sections (2–3 μm thick) were stained with toluidine blue and examined by light microscopy.

Data Analysis

Data are expressed as mean ± standard error of the mean (SE). The significance of differences between measurements was assessed with the Kruskal–Wallis and Mann–Whitney tests. Results are termed significant at $P < 0.05$.

RESULTS

Antiresorptive Effect—Tetracycline Kinetics

A marked decrease in TC plasma concentration was observed in all experimental groups during 20 days postlabeling (Fig. 1 and Table II). Following this period, the elimination rate reached a steady state, and the TC levels were very low in all experimental groups. The plasma TC kinetic parameters revealed a 26 and 21% decrease in the elimination constants of rats treated with ABP-40 and AdBP-160, respectively, in comparison to the control group (see Table II). In all other experimental groups (i.e., ABP-16 and ABP-160) the elimination constant was similar.

Statistically significant differences in the antiresorptive activity of the various treatments were determined by comparing the mean [^{3}H]TC concentration of each experimental group, at each time point, to the mean pretreatment value of all experimental groups (Fig. 1). Significant inhibition of bone resorption was exhibited by both ABP-40 and AdBP-160, 7 and 12 days after the end of labeling. ABP-16 was found active only 12 days postlabeling. The reduction in TC levels observed in the ABP-160-treated animals was not significantly different from the control values (Fig. 1). This was due to the relatively high SD at the 7-day time point and the small number of animals which survived ($n = 4$) at the 12-day time point.

Biochemical Markers and Toxic Effects

Severe inflammation and necrotic tissue was observed at the injection site only in ABP-treated animals (ABP-40 and ABP-160). Death of animals occurred only in the ABP-160 treatment group. Five of 11 rats died, one between day 8 and day 12 and four between day 12 and day 24. The effects of bisphosphonate treatment on certain biochemical indices are presented in Figs. 2 and 3. Renal function parameters, serum creatinine and serum urea nitrogen values, are described in Fig. 2. Significantly higher serum levels of both urea nitrogen and creatinine were detected in the ABP-160 group in comparison to control-group values. No significant differences were observed between all other groups throughout the experimental period.

The effect of the bisphosphonate treatment on alkaline phosphatase (ALP) activity in serum and on animals' weight gain is shown in Fig. 3. Reduced ALP levels were monitored after 7 and 12 days in the ABP-40- and ABP-160-treated rats, but only the latter treatment was significantly different from control-group values.

Animals' weight gain after 12 and 24 days was affected by all ABP treatments, but only the 12- and 24-day data were

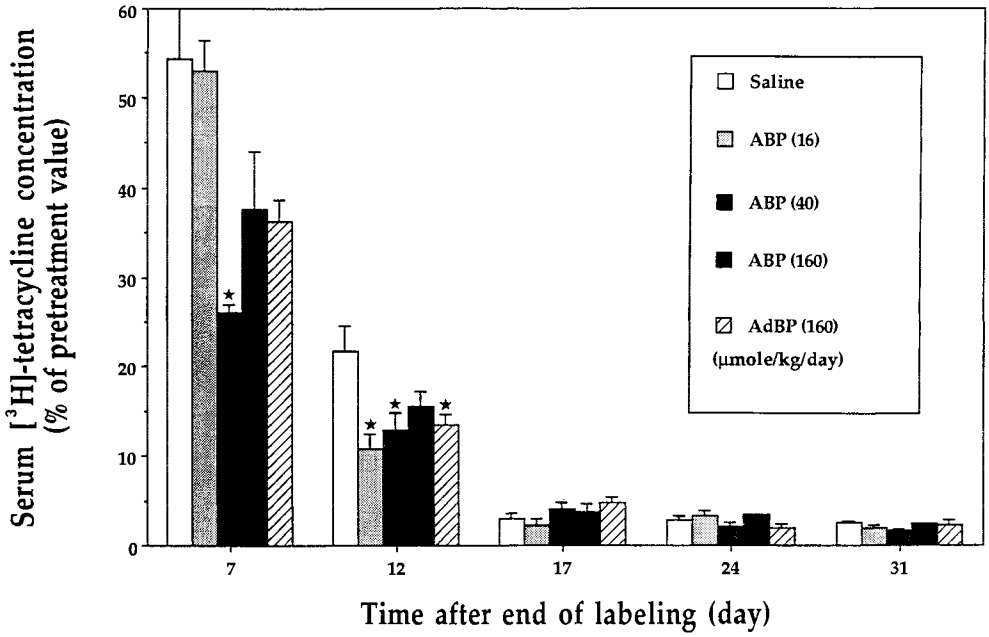

Fig. 1. Serum [^{3}H]tetracycline concentration in animals treated with bisphosphonates as a ratio of the pretreatment mean value of all groups. Each bar represents the mean ± SE. (★) Significantly different from control (saline)-group value at corresponding time point, Kruskal–Wallis and Mann–Whitney test ($P < 0.05$).

Table II. Serum [^{3}H]Tetracycline Kinetic Parameters

	Drug				
	Saline	ABP			AdBP
Dose (μmol/kg/day)	—	16	40	160	160
K_e, elimination constant (day^{-1})[a]	−0.11	−0.12	−0.081	−0.099	−0.087
r, correlation coefficient	0.958	1.000	0.980	0.982	1.000

[a] Calculated by $C_t = C_0 e^{-ke t}$, where C_t and C_0 are concentrations of the isotope at time t and time 0, respectively.

significantly different. The novel bisphosphonate, AdBP, had no effect on weight gain throughout the experimental period.

Bone Chemistry and Histology

The effects of bisphosphonates on calcium content, ash, and dry bone weight are summarized in Table III. Significantly lower ash/dry bone weight and calcium/ash content were found only in rats treated with ABP-16 and ABP-40. The total femur calcium content of these groups was lower than those observed in control and AdBP-160-treated animals. The ABP-160-group data are not presented since only two femures were retrieved at this time point.

Severe rachitic changes were observed following ABP treatment regardless of the dose. This was manifested by a wide epiphyseal plate and persistence of abundant nonmineralized cartilage in the metaphysis (Fig. 4b). As shown in Fig. 4c, similar rachitic changes were observed following AdBP treatment (160 μmol/kg/day) but to a lesser extent than in ABP-treated animals.

DISCUSSION

In addition to the physicochemical interaction with calcium phosphate crystals, bisphosphonates also influence cellular metabolism affecting bone resorption via osteoclasts (12). The mechanism of bone resorption, represented by the loss of TC, is an active process of osteoclastic cells rather than a passive exchange and excretion of the compound (4–6). TC released from the bone in first-order kinetics directly reflects bone resorption (4–6). The serum TC elimination constant in rats treated with ABP-40 was lower than that observed in both untreated and ABP-16-treated animals (Table II). This indicates that the antiresorptive effect of ABP could be detected by the direct measurement of the TC serum elimination rate. In comparing ABP-16 and the control group, a statistically significant difference was observed between serum TC levels of these groups after 12 days (Fig. 1). It is probable that the cumulative effect of low-dose ABP on bone resorption could be detected only after accumulation of a sufficient amount of bisphosphonate in the bone, which occurred in this study after completion of the treatment (the last injection was on the eighth day). This effect was masked in the overall first-order kinetics measurement reflecting the overall kinetics of bone resorption, whereas the TC serum concentrations at each sampling time point represent the temporal steady-state values.

Comparable effects on bone resorption were exhibited by AdBP-160 and ABP-40, as evidenced by the reduced TC levels at 7 and 12 days postlabeling (see Fig. 1), despite the different doses. This result suggests that the geminal bisphosphonate, ABP, is a more potent inhibitor of bone resorption than AdBP, a representative of a new class of drugs (13,14), in agreement with our findings on the antiresorptive efficacy of these compounds *in vitro* (14). However,

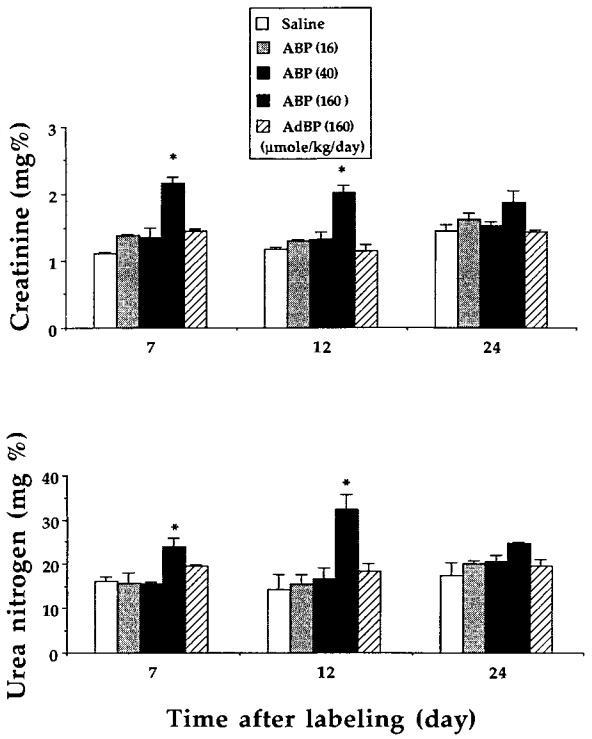

Fig. 2. Creatinine and urea nitrogen levels in rats treated by bisphosphonates. Each bar represents the mean ± SE. (*) Significantly different from control (saline)-group value at corresponding time point, Kruskal–Wallis and Mann–Whitney test ($P < 0.05$).

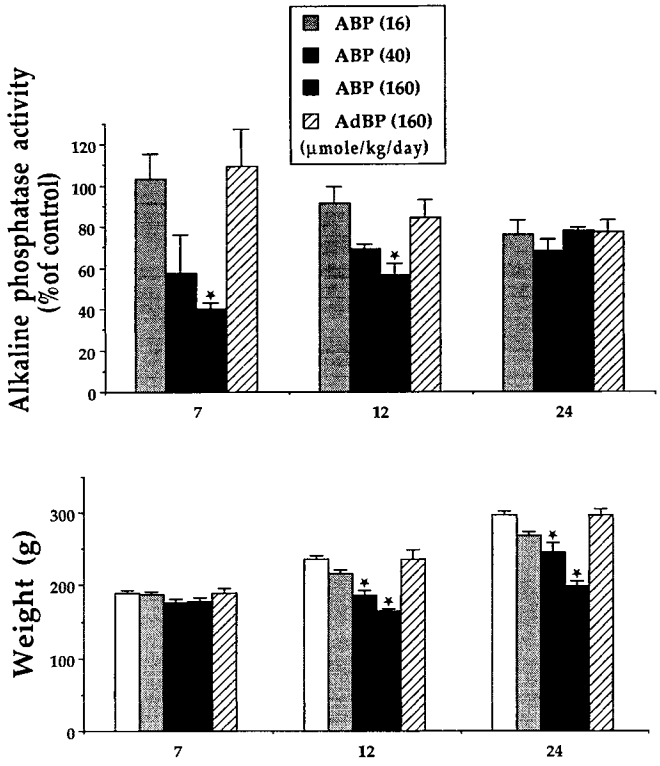

Fig. 3. Alkaline phosphatase activity (saline-treated-group value termed 100%), and rats' weight gain (□, saline-treated group) in rats treated with bisphosphonates. Each bar represents the mean ± SE. (★) Significantly different from control (saline)-group value, Kruskal–Wallis and Mann–Whitney test ($P < 0.05$).

in view of the similar activity exhibited by ABP-16 and ABP-40 after 12 days, it seems that an accurate comparison between drug potencies might be achieved by monitoring [^{3}H]TC elimination in younger animals where the bone resorption rate is enhanced (labeling *in utero* or utilizing 0- to 2-week-old rats). Nevertheless, our experimental conditions provide a technically simple method, sensitive enough to examine antiresorptive properties in a healthy animal.

As discussed by Klein and co-workers (4) and by Muhlbauer and Fleisch (7), the [^{3}H]TC model to assess bone resorption is more accurate than the ^{45}Ca technique, because the efficient reuse of ^{45}Ca impedes accurate assessment of bone resorption. The elimination rate constant of TC is a hybrid parameter which reflects the mean value of different resorption kinetics derived from various types of bones (3,4). Therefore, only direct bone measurements provide specific drug–bone effects, as opposed to overall effects represented by K_e, which is the mean of various effects on different bones.

The relatively high TC serum concentrations observed in the ABP-160 group are unexpected in view of this high dosage. It seems that the relatively small decrease in TC levels observed in this group was due to suppressed clearance of the isotope, rather than diminished antiresorptive activity. Serum TC levels at each sampling point are the product of the input rate of TC from the bone into the serum and the elimination rate of TC, i.e., concentration (at steady state) = input rate/clearance. Reduced clearance as a result of renal dysfunction could cause higher steady-state concentrations, as was indeed observed (Fig. 1). The significant increase in both creatinine and serum urea levels, on the one hand (Fig. 2), and the diminished ALP activity, on the other (Fig. 3), indicate that the clearance of TC was reduced in this highest dose-treated group because of impairment of renal function. Thus, this model is limited to data obtained from animals with normal kidney function, when the kinetics of TC elimination will directly reflect bone resorption kinetics.

This limitation of the model cannot be circumvented by measuring urine rather than serum TC levels, because a decrease in glomerular filtration rate due to a drug's nephrotoxicity (10) will result in concomitant changes in blood and urine TC concentrations, i.e., lower urine and higher blood levels of TC. Thus, similar effects on both renal and serum elimination rates should be obtained since the rate of TC excretion into the urine is directly proportional to its serum concentration. Moreover, only 74–76% of the administered tetracycline dose is excreted by the kidney, and some is excreted into the feces (4,7). In addition, the ratios of serum elimination rates (total-body elimination constants) to urinary excretion rates (kidney elimination rate constants) of [^{3}H]TC, in weanling, adolescent, and mature rats are 1.31–1.35 (4), indicating a nonrenal pathway of isotope elimination. Thus, the elimination rate of TC via a nonrenal pathway in kidney-impaired animals would be relatively increased. It is suggested, therefore, that the measurement of serum, rather than urine TC kinetics is more appropriate for evaluating the antiresorptive effect of drugs, because the former is

Table III. Effects of Bisphosphonate Treatment on Calcium Content, Ash Weight, and Dry Bone Weight of Rat Femur (Mean ± SD of 4 Rats)

Compound (μmol/kg/day)	Dry weight (mg)	Ash weight (mg)	Ash/dry weight	Calcium (mg)	Calcium/ash (mg/mg)
Saline	328.87 ± 34.8	245.25 ± 25.71	0.75 ± 0.043	62.00 ± 9.77	0.25 ± 0.016
ABP (16)	358.25 ± 29.1	229.01 ± 24.72	0.64 ± 0.049*	51.93 ± 9.18	0.22 ± 0.024*
ABP (40)	366.26 ± 26.5	226.73 ± 12.71	0.62 ± 0.064*	51.32 ± 8.02	0.22 ± 0.036*
AdBP (160)	327.85 ± 26.9	246.01 ± 25.81	0.75 ± 0.036	67.42 ± 17.31	0.27 ± 0.029

* Significantly different from control (saline)-group values, Kruskal–Wallis and Mann–Whitney tests, $P < 0.02$.

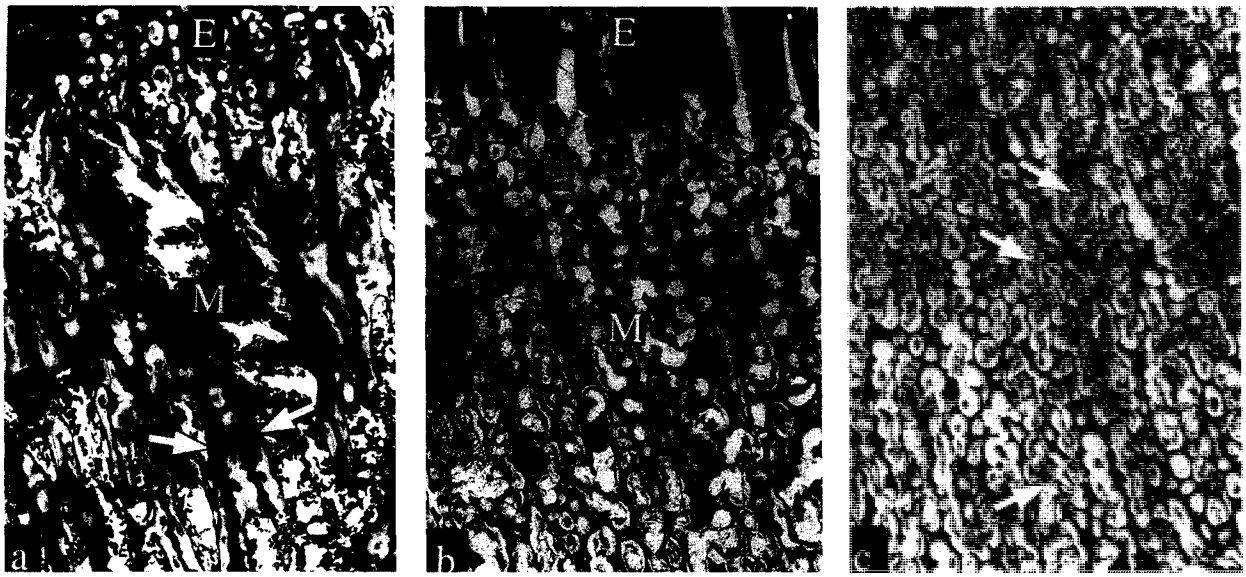

Fig. 4. Micrographs of rat femura following treatment with bisphosphonates: (a) control (untreated rat), (b) ABP-treated rat (40 and μmol/kg/day), and (c) AdBP-treated rat (160 μmol/kg/day). (a) A longitudinal section throughout the femur of untreated rats to show the lower part of the ephyphysis (E) and the metaphysis (M). Note the few metaphyseal bone trabecular (arrows). (b) Femur of ABP-treated rat demonstrating persistence of nonmineralized cartilage throughout the metaphysis. (c) Upper metaphysis of AdBP-treated rat's femur showing persistence of nonmineralized cartilage similar to b. Toluidine blue, ×400; reduced to 85% for reproduction.

less affected by renal dysfunction and represents total-body clearance (i.e., renal and nonrenal pathways). In addition, the steeper elimination slopes obtained by serum measurements facilitate a more sensitive detection of inhibitory effect on bone resorption.

The fall in ALP activity (Fig. 3) could be attributed, in addition to nephrotoxicity, to another known adverse effect of geminal bisphosphonates, i.e., inhibition of normal mineralization (19,20). This is supported by the finding of reduced weight gain in animals treated with 40 and 160 μmol/kg/day ABP. Impairment of bone mineralization was observed in both ABP-16- and ABP-40-treated animals as evidenced from the reduced ash/dry bone weight and calcium/ash content. These findings correlated well with the histological results demonstrating rachitic changes in ABP-treated animals and, to a lesser extent, in AdBP-treated animals, despite the high dose. The toxic and adverse effects accompanying ABP treatment observed in this study are in accordance with the known toxic effects of bisphosphonates in general (19,20) and ABP in particular (10,12). The lack of adverse effects in AdBP-treated animals, despite the high dose of 160 μmol/kg/day, is an advantage that clearly indicates its potential for clinical applications. In this context it is important to note the results of preliminary acute toxicological studies on bisacylphosphonates, studied in Walker carcinosarcoma-bearing female rats. The tolerated dose found for AdBP (613.2 μmol/kg for 2 days of i.v. injections, with neither deaths nor weight loss) was markedly higher than for ABP (50% deaths at a single dose of 200 μmol/kg) (15).

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