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Effects of Long-Term Daily Administration of Prostaglandin-E_2 on Maintaining Elevated Proximal Tibial Metaphyseal Cancellous Bone Mass in Male Rats

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Summary. The effects of long-term prostaglandin E_2 (PGE_2) on cancellous bone in proximal tibial metaphysis were studied in 7-month-old male Sprague-Dawley rats given daily subcutaneous injections of 0, 1, 3, and 6 mg PGE_2/kg/day and sacrificed after 60, 120, and 180 days. Histomorphometric analyses were performed on double fluorescent-labeled undecalcified bone specimens. After 60 days of treatment, PGE_2 produced diffusely labeled trabecular bone area, increased trabecular bone area, eroded and labeled trabecular perimeter, mineral apposition rate, and bone formation rate at all dose levels when compared with age-matched controls. In rats given PGE_2 for longer time periods (120 and 180 days), trabecular bone area, diffusely labeled trabecular bone area, labeled perimeter, mineral apposition, and bone formation rates were sustained at the elevated levels achieved earlier at 60-day treatment. The eroded perimeter continued to increase until 120 days, then plateau. The observation that continuous systemic PGE_2 administration to adult male rats elevated metaphyseal cancellous bone mass to 3.5-fold of the control level within 60 days and maintained it for another 120 days indicates that the powerful skeletal anabolic effects of PGE_2 can be sustained with continuous administration.

Key words: Prostaglandin E_2 – Long-term treatment – Cancellous bone – Bone formation – Bone resorption – Bone turnover – Remodeling.

Previous studies in our laboratory demonstrated that systemic administration of prostaglandin E_2 (PGE_2) to normal rats produces large amounts of new bone mass in both cancellous and cortical bone regions, regardless of age and sex [1–7]. A similar cortical bone increase induced by PGE_2 or related compounds was also evidenced by studies done in humans [8–10] and in dogs [11–15]. PGE_2 administration also restored the cancellous bone mass in ovariectomy-induced osteopenic metaphysis to above normal level [6–7]. PGE_2 stimulates osteoclastic resorption as well as osteoblastic recruitment and activity. It activates bone modeling in the formation mode and remodeling with bone formation exceeding bone resorption to create a positive bone balance. These studies indicate that PGE_2 is a powerful skeletal anabolic agent when administered systemically and locally. However, it is not clear whether the skeletal anabolic effect of PGE_2 would be sustained with long-term administration as the treatment periods of previous studies were short compared with the skeletal remodeling period (sigma). Therefore, in this study we have prolonged the treatment periods to 60, 120, and 180 days to investigate the long-term effects of PGE_2 on metaphyseal cancellous bone in adult male rats. The longest treatment period used in this study equals at least four bone remodeling cycles for controls and five for treated rats.

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Materials and Methods

Seventy-four male 7-month-old Sprague-Dawley rats, weighing approximately 622 g (Charles River Laboratory, Inc., Portage, MI) were acclimated to local vivarium conditions for 16 days. Five rats were sacrificed at day 0 as beginning controls. The remaining rats were divided into four groups for daily administration of 0, 1, 3, and 6 mg/kg/day of PGE_2. Five to 7 rats from each group were serial sacrificed at days 60, 120, and 180. During the experimental period, the rats were allowed free access to water and pelleted commercial natural product diet (Rodent Laboratory Chow 5001, Ralston-Purina Co., St. Louis, MO).

All rats, except those killed at day 0, received injections of 1 ml/kg PGE_2 solution. Powdered PGE_2 (Upjohn, Kalamazoo, MI) was first dissolved in 100% ethanol and further diluted with deionized water into desired PGE_2 concentrations (final ethanol concentration was 20%) for 0, 1, 3, or 6 mg/kg/day groups. The daily injections were given subcutaneously on the back. The rats were weighed weekly and the volume of the injection solution was adjusted accordingly. All rats received subcutaneous injection of 25 mg/kg of tetracycline (achromycin-tetracycline hydrochloride; Lederle Laboratory, Pearl River, NY) 12 days before sacrifice, and 10 mg/kg of calcein (Sigma Chemical Co., St. Louis, MO) 2 days before sacrifice. All animals were sacrificed by cardiac puncture under ketamine anesthesia. The lungs, liver, adrenal glands, thymus, spleen, and kidneys were removed and weighed.

The left tibia was removed and stored in 70% ethanol prior to measuring of bone mineral density. Using single photon absorptiometry (Norland Corporation, Fort Atkinson, WI), the proximal tibia was scanned transversely at the mid-point between the distal end of the soleal line and the proximal end of the tibiae. During the measurement, bone mineral content and bone width were obtained and bone mineral density was calculated [16].

The right tibia was removed, dissected, and cut into three equal parts. The proximal third was fixed in formalin, dehydrated in ethanol, and defatted in acetone, then embedded in methyl methacrylate (Eastman Organic Chemicals, Rochester, NY). Frontal sections were cut and ground to 100 μm and microradiographed. Thereafter, sections were mounted on plastic slides and further ground to a thickness of 20 μm and stained with 0.1% toluidine blue 0 (Fisher

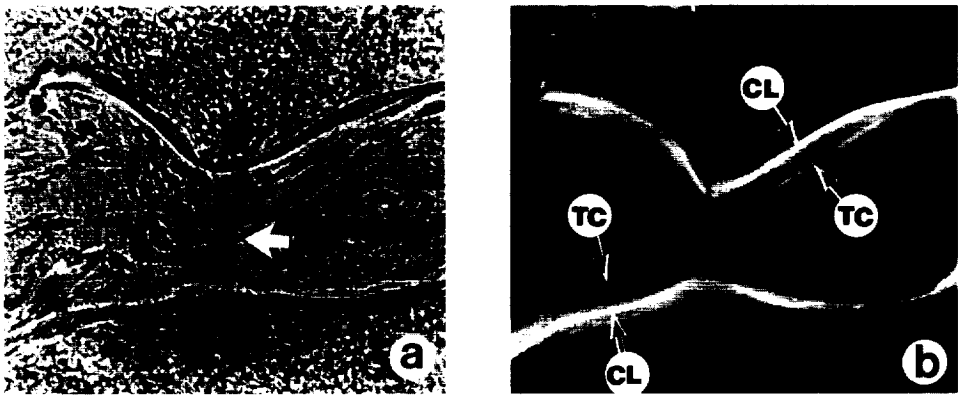

Fig. 1. Morphologic evidence of remodeled trabecular packets in rat proximal tibial metaphyseal cancellous bone under **(a)** light and **(b)** fluorescent microscopy. **(a)** The 20 μm undecalcified section shows two trabecular packets with reversal lines (black arrows) located on both upper and lower surfaces separated by interstitial lamellae (white arrow). **(b)** The appearance of tetracycline (TC) and calcein (CL) labels in both trabecular packets indicates that they are newly formed trabecular bone remodeling units. × 250.

Table 1. Histomorphometric measurements and calculations

Measured parameters	Abbreviations	Definitions	Unit
Total tissue area	T.Ar	A area of (2.4 × 1.8 mm) in central metaphysis	mm^2
Total trabecular area	TTb.Ar	Total cancellous bone area within total area	mm^2
Total trabecular perimeter	TTb.Pm	The perimeter of TTb.Ar	mm
Diffuse labeled new bone area	DLNB.Ar	Cancellous bone area diffusely labeled with tetracycline and/or calcein	mm^2
Lamellar trabecular bone area	LTb.Ar	Cancellous bone area with lamellar bone structure	mm^2
Lamellar trabecular bone perimeter	LTb.Pm	The perimeter of LTb.Ar	mm
Single label perimeter	sL.Pm	The length of trabecular surface labeled with tetracycline or calcein	mm
Double label perimeter	dL.Pm	The length of trabecular surface labeled with both tetracycline and calcein	mm
Interlabel width	Ir.L.Wi	The distance between tetracycline and calcein labels	μm
Interlabel width (growth)	Ir.L.Wi.-G	The distance between tetracycline and calcein labels in growth plate metaphyseal junction region	μm
Eroded perimeter	E.Pm	The length of trabecular surface with Howship's lacuna	μm
Osteoid perimeter	O.Pm	The length of trabecular surface covered with osteoid	mm
Wall width	W.Wi	The distance between reversal line and trabecular surface	μm
Calculated parameters	**Abbreviations**	**Formulae**	**Unit**
Percent total trabecular area	%TTb.Ar	TTb.Ar/T.Ar × 100	%
Percent diffuse labeled new bone area	%DLNB.Ar	DLNB.Ar/T.Ar × 100	%
Trabecular width	Tb.Wi	(2000/1.199)*TTb.Ar/TTb.Pm	μm
Trabecular number	Tb.N	1.199/2*TTb.Pm/T.Ar	#/mm
Trabecular separation	Tb.Sp	(2000*1.199)*(T.Ar TTb.Ar)/TTb.Pm	μm
Percent eroded perimeter	%E.Pm	E.Pm/LTb.Pm × 100	%
Percent osteoid perimeter	%O.Pm	O.Pm/LTb.Pm × 100	%
Percent labeled perimeter	%L.Pm	(dL.Pm + sL.Pm/2)/LTb.Pm*100	%
Mineral apposition rate	MAR	Ir.L.Wi/Interval	μm/day
Bone formation rates (bone area based)	BFR	(dL.Pm + sL.Pm/2)*MAR/LTb.Ar*365*100	%/year
Formation period	F.P	W.Wi/MAR	days
Resorption period	R.P	F.P × E.Pm/O.Pm	days
Remodeling period	Rm.P	F.P + R.P	days
Longitudinal growth rate	LGR	Ir.L.Wi-G/Interval	μm/day

Scientific Co., Fair Lawn, NJ), then coverslipped [6, 17]. Trabecular packets were readily observed in these ground sections (Fig. 1).

Microradiographs and 20 μm thick undecalcified sections were viewed qualitatively and/or quantitatively. Static and kinetic measurements were performed on 20 μm thick undecalcified sections using a digitizing image analyzing system, which consists of a light or epifluorescent microscope, an Apple Macintosh Computer with a digitizing pad, and a morphometry program named "Stereology" (KSS Computer Engineers, Magna, UT). Except for longitudinal bone growth, all measurements were performed using 156 × or 312 × magnifications on a 4.32 mm^2 (2.4 × 1.8 mm) area of central metaphysis located 0.6 mm distal from the growth cartilage metaphyseal junction. The 0.6 mm metaphyseal region was omitted in order to focus on cancellous bone changes in the secondary spongiosa. All static and dynamic histomorphometric measurements and calculations are summarized in Table 1 according to Jee et al. [17] and Parfitt et al. [18]. We have included an additional measurement, the amount of diffusely labeled trabecular bone (Fig. 2), representing woven bone formed at 12 and 2 days before sacrifice when fluorescent markers were injected. This parameter underestimated the total amount of woven bone.

Statistical differences between age-matched control and treat-

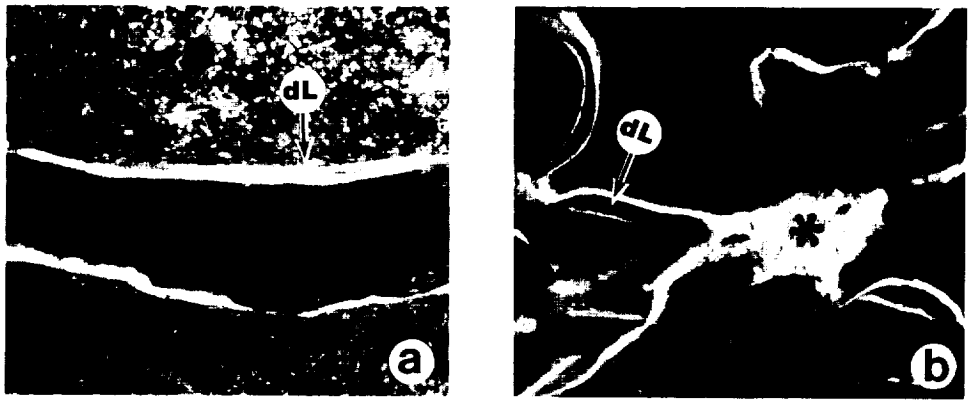

Fig. 2. Fluorescent micrographs of trabeculae from proximal tibial metaphyses of adult male rats as (**a**) age-matched control and (**b**) treated with 6 mg PGE_2/kg/day for 60 days. PGE_2-treated rat exhibits greater interlabeling distance of double labels (dL) than control rat (a versus b). Also diffusely labeled woven trabecula (*) is observed in treated rat only (**b**). × 250.

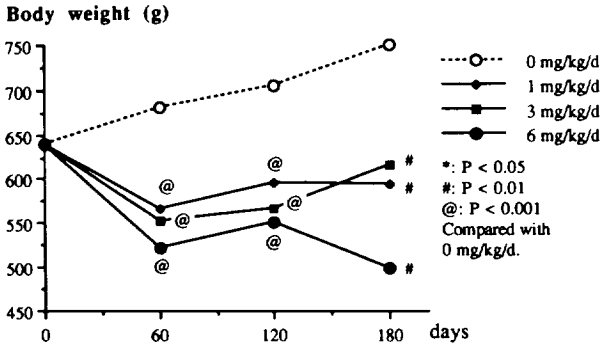

Fig. 3. Time course of the body weight changes in control and PGE_2-treated rats during the experimental period.

ment groups were evaluated for each time period using the Kruskal-Wallis test [19].

Results

In all PGE_2-treated rats, diarrhea was observed 1 hour after injection. Their body weight decreased about 17% during the first 60 days and remained depressed thereafter whereas the body weight of control rats increased continuously throughout the experiment (Fig. 3). In the treatment rats, soft tissue weights showed significant increases in adrenal glands, liver, kidneys, and lungs when normalized to body weight (Fig. 4). No significant change was observed in weights of thymus and spleen (data not shown).

Significant increases in proximal tibial bone mineral density were detected in rats treated with 3 and 6 mg PGE_2/kg/day compared with age-matched controls (Fig. 5). Bone mineral density increased by 22 and 17% at 120 and 180 days for 3 mg/kg/day dose level and by 18, 17, and 17% at 60, 120, and 180 days for 6 mg/kg/day dose level.

The longitudinal growth rate was depressed in a dose-dependent manner; the magnitude of this depression increased with longer treatment period (Fig. 6). Growth plates were partially fused in most rats treated with PGE_2 for 180 days at higher dose groups; a few small pieces of cartilage tissue were scattered along the fused plate.

The obvious cancellous bone changes are apparent in Figure 7. During the treatment period, bone mass declined with aging in control animals (c versus a). But bone mass increased in a dose-dependent manner in animals treated with 1, 3, and 6 mg PGE_2/kg/day for 60 days (d, g, and j). The elevated bone mass was maintained at the same level for each dose group by daily administration for another 60 days (e, h, and k) and 120 days (f, i, and l).

Figure 8 summarizes static morphometric changes for control and PGE_2-treated animals. PGE_2 administration produced diffusely labeled, new woven trabecular bone at all dose levels throughout the experiment. Significant dose-dependent increases were observed in total trabecular bone area, trabecular width, trabecular number, and wall width. Similarly, decreases in trabecular separation after 60-day treatment when compared with age-matched controls were also observed. For 120- and 180-day treatments, these parameters were maintained and the remodeling of woven to lamellar bone was underway at the 60-day treatment levels, except that trabecular width continued to increase until 120 days and then plateau at 6 mg dose levels. Dynamic histomorphometric changes are listed in Figure 9. PGE_2 administration for 60 days increased percent eroded perimeter, osteoid perimeter, percent labeled perimeter, mineral apposition rate, and bone formation rate in a dose-dependent manner; but it decreased the resorption, formation, and remodeling periods. The eroded perimeter continued to increase until 120 days and plateau thereafter, whereas all other parameters were maintained at 60-day treatment levels after 120 and 180 days of daily PGE_2 treatment.

Discussion

The current study demonstrated that the powerful skeletal anabolic effects of PGE_2 can be sustained with daily administration of PGE_2 for 180 days. This equals at least four and five bone remodeling cycles for control and treated rats, respectively. The average time for completing one remodeling cycle was 45 days in control and 35 days in treated animals (Fig. 9h). The skeletal dynamics in the current and previous studies [2, 6, 7] suggest that *in vivo* PGE_2 administration in all rats (regardless of whether they are male or female, young or aged, estrogen-deplete osteopenic or normal) acts in the same manner to increase metaphyseal cancellous bone mass. It stimulates bone modeling by producing new woven trabeculae. It also stimulates bone remodeling by increasing both bone formation and bone resorption on the existing bone surface but it favors bone formation. Thus,

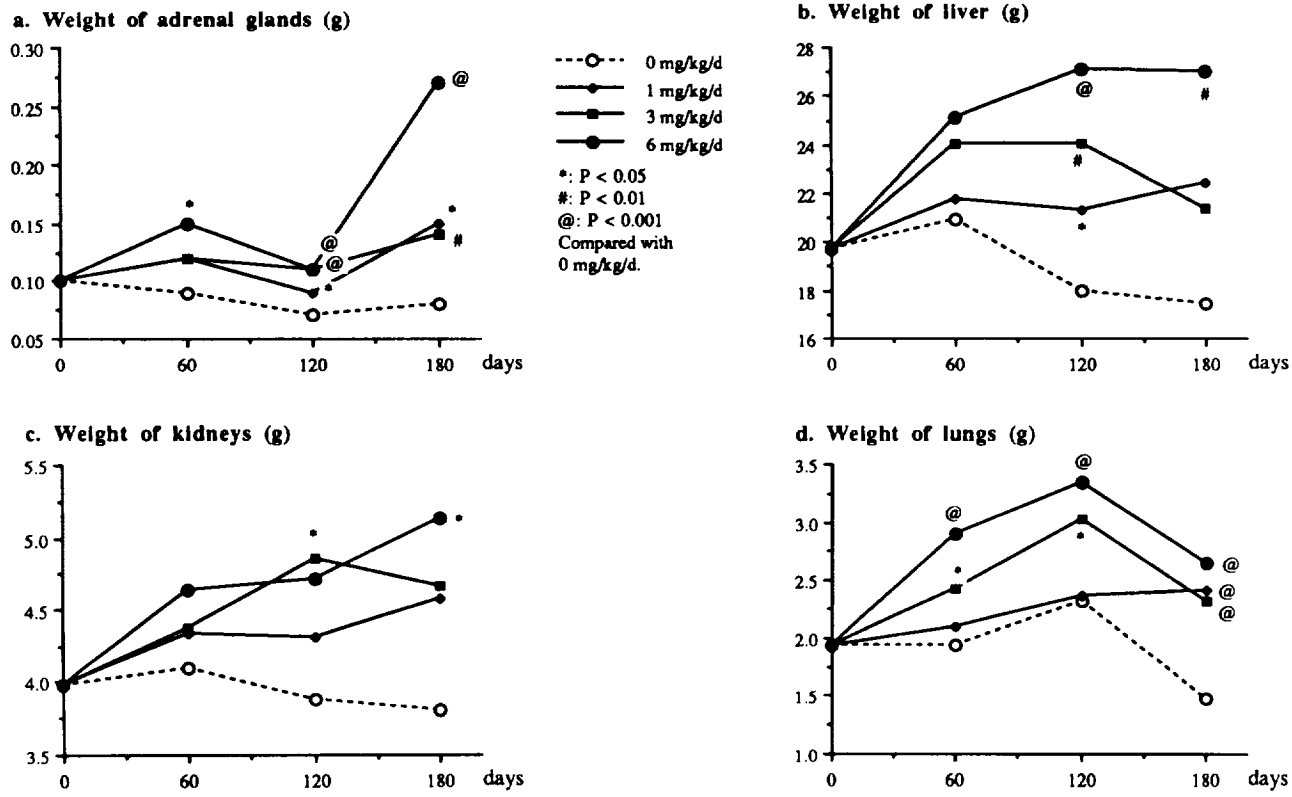

Fig. 4. Time course of the soft tissue weight changes in control and PGE_2-treated rats during the experimental period.

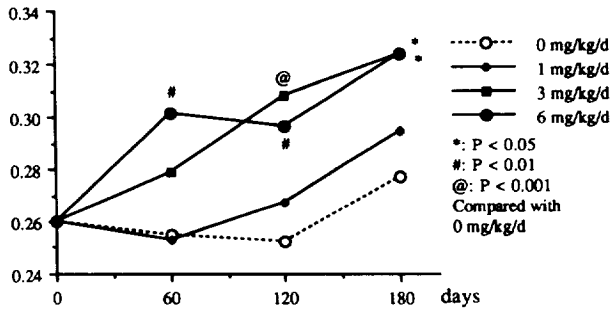

Fig. 5. Time course of proximal tibial bone mineral density changes in control and PGE_2 rats during the experimental period.

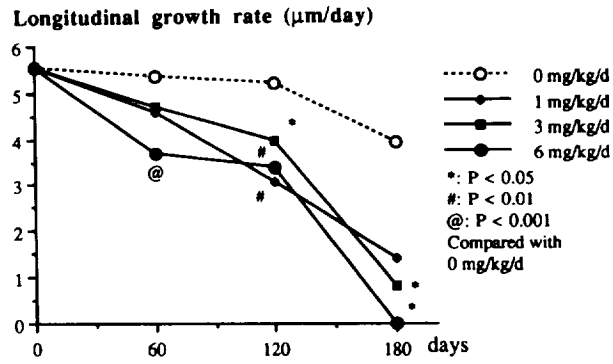

Fig. 6. Time course of proximal tibial longitudinal bone growth rate changes in control and PGE_2 rats during the experimental period.

the bone balance is shifted to the positive direction. Furthermore, PGE_2 shortens the entire bone remodeling period.

During the first 60-day treatment, PGE_2 induced an increase in bone formation involving new woven bone production, labeled trabecular perimeter, mineral apposition rate, and therefore, bone formation rate. All these formative parameters reached peak values sometime before 60 days. However, PGE_2 also induced a minor increase in bone resorption. Despite this minor increase, the imbalance between bone resorption and bone formation grew, because a smaller ratio of eroded to labeled trabecular perimeter was seen in treated rats than in controls (0.21 versus 0.28). These changes led to a positive bone balance so that cancellous bone mass accumulated to the maximal level within the first 60 days. Subsequently, formative parameters were sustained at the elevated levels whereas eroded trabecular perimeter increased progressively until 120 days to a level that offset the new bone formation as the ratio of eroded to labeled perimeter returned to near the control level (0.26). Woven bone formations decreased at all dose levels, but the total trabecular bone mass remained approximately constant, indicating that woven bone was being replaced by lamellar bone. These changes guaranteed the maintenance of the elevated bone mass. Thus, a new equilibrium (steady state) was achieved with an elevated bone mass and an elevated, zero-balanced bone turnover in rats treated with PGE_2 for 120 and 180 days.

PGE_2-treated male rats exhibited a reduced rate of lon-

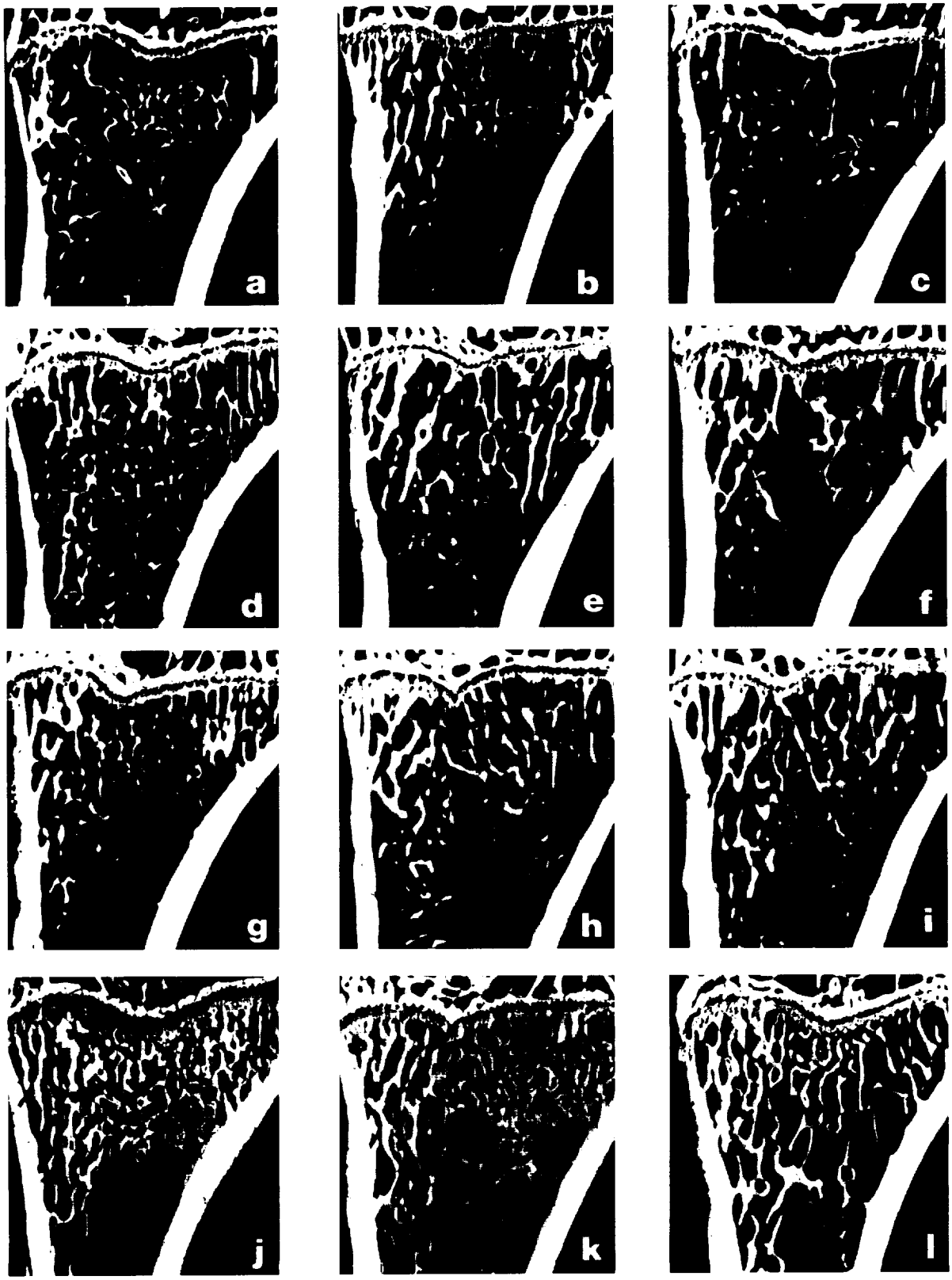

Fig. 7. Microradiographs showing cancellous bone changes in proximal tibial metaphyses from age-matched controls at day 60 (**a**), 120 (**b**) and 180 (**c**); from rats treated daily with PGE_2 at 1 mg/kg/day dose level for 60 (**d**), 120 (**e**), and 180 (**f**) days; at 3 mg/kg/day dose level for 60 (**g**), 120 (**h**), and 180 (**i**) days; at 6 mg/kg/day dose level for 60 (**j**), 120 (**k**), and 180 (**l**) days.

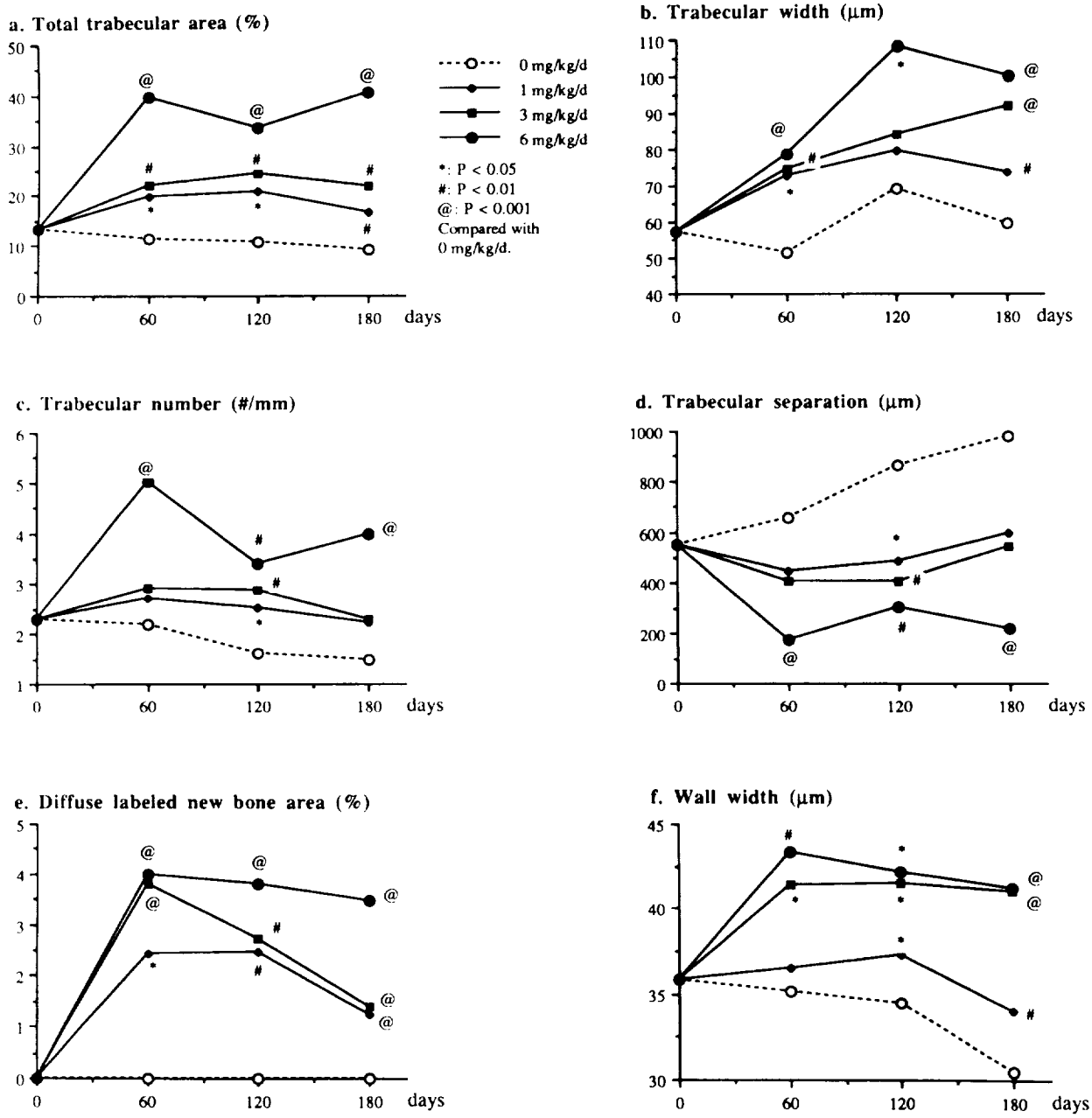

Fig. 8. Time course of cancellous bone static morphometric changes in proximal tibial metaphyses of control and PGE_2-treated rats.

gitudinal bone growth in proximal tibiae. Similar results have been reported by others [20] on PGE_2-treated, rapidly growing male rats in our earlier studies [2–4] and on PGE_1-treated young male rats. Interestingly, longitudinal bone growth rate is increased in a dose-dependent manner in PGE_2-treated normal female rats [6–7]. The mechanism of this sex-dependent bone elongation effect is very intricate as it is not estrogen dependent. Enhanced bone elongation was also seen in estrogen-depleted, ovariectomized rats [6–7]. Thus, leaving the possibility that it may be androgen dependent, the stimulatory effect on bone elongation of PGE_2 may be blocked in an androgen-rich environment. Unfortunately, very little is known about the effects of androgen on the skeleton [21], especially on bone elongation. However, the hypothesis could be tested by treating gonadectomized male rats with PGE_2.

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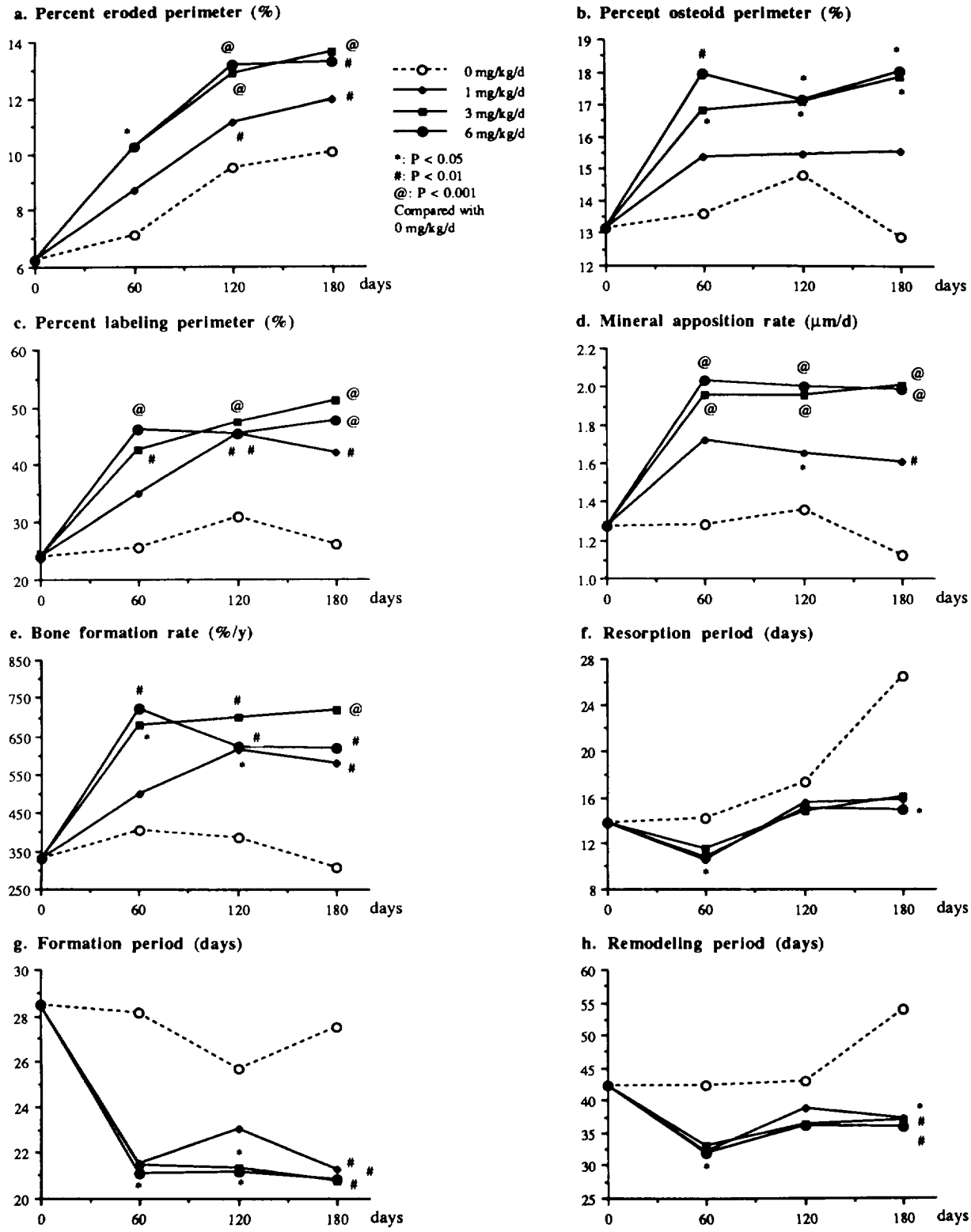

Fig. 9. Time course of cancellous bone dynamic histomorphometric changes in proximal tibial metaphyses of control and PGE_2 treated rats.

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