Zinc-Deficient Rats Have More Limited Bone Recovery During Repletion Than Diet-Restricted Rats

HEATHER J. HOSEA,* CARLA G. TAYLOR,*,[1] TRISHA WOOD,* REBECCA MOLLARD,* AND HOPE A. WEILER*,†

Departments of Human Nutritional Sciences and †Pediatrics, University of Manitoba, Winnipeg, MB, R3T 2N2, Canada

The objective of this study was to investigate the effects of dietary zinc deficiency and diet restriction on bone development in growing rats, and to determine whether any adverse effects could be reversed by dietary repletion. Weanling rats were fed either a zinc-deficient diet *ad libitum* (ZD; <1 mg zinc/kg) or nutritionally complete diet (30 mg zinc/kg) either *ad libitum* (CTL) or pair-fed to the intake of the ZD group (DR; diet-restricted) for 3 weeks (deficiency phase) and then all groups were fed the zinc-adequate diet *ad libitum* for 3, 7, or 23 days (repletion phase). Excised femurs were analyzed for bone mineral density (BMD) using dual-energy x-ray absorptiometry, and plasma was analyzed for markers of bone formation (osteocalcin) and resorption (Ratlaps). After the deficiency phase, ZD had lower body weight and reduced femur BMD, zinc, and phosphorus concentrations compared with DR; and these parameters were lower in DR compared with CTL. Femur calcium concentrations were unchanged among the groups. Reduced plasma osteocalcin in ZD and elevated plasma Ratlaps in DR suggested that zinc deficiency limits bone formation while diet restriction accelerates bone resorption activity. After 23 days of repletion, femur size, BMD, and zinc concentrations remained lower in ZD compared with DR and CTL. Body weight and femur phosphorus concentrations remained lower in both ZD and DR compared with CTL after repletion. There were no differences in plasma osteocalcin concentrations after the repletion phase, but the plasma Ratlaps concentrations remained elevated in DR compared with CTL. In summary, both ZD and DR lead to osteopenia during rapid growth, but the mechanisms appear to be due to reduced modeling in ZD and higher turnover in DR. Zinc deficiency was associated with a greater impairment in bone development than diet restriction, and both deficiencies limited bone recovery during repletion in growing rats.** Exp Biol Med 229:303–311, 2004

Key words: bone; zinc deficiency; malnutrition; repletion; rats

Bone has one of the highest concentrations of zinc of all tissues, and has been shown to release zinc during deficiency for soft tissue metabolism (1). Several zinc-dependent enzymes and hormones are involved in bone metabolism (2). For example, zinc has been shown to stimulate the activity of alkaline phosphatase (ALP), which is involved in bone mineral deposition (3). Rossi and colleagues (4) have shown that zinc deficiency in growing rats results in reduced bone growth, bone volume, and force required before breaking. Atik (5) reported that men suffering from senile osteoporosis had lower serum zinc and lower femur zinc compared with control patients. Epidemiological human studies suggest that bone loss in osteoporosis could be due in part to reduced IGF-1 concentrations due to low zinc intake (6, 7).

Investigators have found a direct relationship among serum zinc, growth velocity, and bone maturation in short children (8). A low rate of childhood growth is associated with increased risk of hip fracture later in life (9). The risk of osteoporotic fracture increases as bone mineral density (BMD) decreases, therefore, BMD is used as an indicator of bone mass and to diagnose osteoporosis (9). The two main factors that affect adult bone mass and the risk of osteoporosis are the level of peak bone mass achieved during growth and the rate of bone loss later in life (9). Therefore, methods of maximizing childhood growth and bone mineral acquisition early in life are likely to decrease the risk of developing osteoporosis later in life.

It has been estimated that nearly half the world's population may not be getting sufficient zinc from their diet (10). According to the Third National Health and Nutrition Examination Survey (1988–94), 81% of children aged 1–3 years had intakes lower than 77% of the 1989 RDA,

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[1] To whom requests for reprints should be addressed at Department of Human Nutritional Sciences, University of Manitoba, H507 Duff Roblin Building, 190 Dysart Road, Winnipeg, MB R3T 2N2, Canada. E-mail: ctaylor@ms.umanitoba.ca

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indicating a potential risk of not meeting their individual requirement (11). It has also been reported that short, but otherwise healthy, Ontario boys benefited from a zinc supplement (increased growth and attention span; Ref. 12). Case studies have shown that children who have been exclusively breast-fed for longer than 6 months present symptoms of zinc deficiency (13–16). Although previous studies have examined the effects of zinc deficiency on bone development, the extent of recovery from deficiency requires more investigation.

The objective of this study was to investigate the effects of dietary zinc deficiency and diet restriction on bone development in growing rats and to determine whether any adverse effects could be repaired by dietary repletion at the level of zinc used and for the duration of the present study. The growing rat model was chosen because previous research has shown that severe dietary zinc deficiency can be achieved in 3 weeks (17). A diet-restricted group (representing energy malnutrition) was included as a control for the reduced-feed intake associated with dietary zinc deficiency. Three time points were chosen to assess repletion: 3 days to identify any rapid changes, 7 days as an intermediate time point, and 23 days as the longest time point.

Materials and Methods

Animals and Diets. Ninety-nine 3-week-old male Sprague Dawley rats (Charles River Laboratories, St. Constant, Canada) were acclimatized for 5 days and were randomly assigned to the baseline group ($n = 8$) or were fed a zinc-deficient diet *ad libitum* (ZD group; <1 mg zinc/kg; $n = 30$), or a nutritionally complete diet (30 mg zinc/kg) either *ad libitum* (CTL; $n = 30$) or individually pair-fed to the intake of the ZD group (DR; diet restricted; $n = 30$) for 3 weeks (deficiency phase). The initial body weights were not different among baseline (68.8 ± 1.3 g), ZD (66.6 ± 0.7 g), DR (66.4 ± 0.8 g), and CTL (66.3 ± 0.7 g) groups ($P > 0.05$). Eight animals from each dietary treatment group were sacrificed at the end of the deficiency phase, and the remaining 22 rats in each dietary group began the repletion phase of the study. During the repletion phase, all rats were fed the control diet *ad libitum* for 3 ($n = 8$/experimental group), 7 ($n = 8$/experimental group), or 23 ($n = 6$/experimental group) days. The experimental diets, containing egg white and additional biotin (2mg/kg diet) and potassium phosphate (5.4 g/kg diet for the growth formulation), have been previously described (18). Zinc content of the diets was verified by atomic absorption analysis. Care was taken to avoid zinc recycling and contamination by housing the rats in stainless steel hanging cages with mesh bottoms and providing distilled water in plastic bottles with stainless steel sipper tubes. The rats were maintained in an environment of controlled temperature (21°–23°C), humidity (55%), and light cycle (14:10-hr light:dark). Body weights were determined weekly, and feed intake was determined daily. Animal care was provided in accordance with a protocol approved by the Local Animal Care Committee (University of Manitoba).

Tissue Collection. At baseline, after the 3-week deficiency phase, and after 3, 7, or 23 days of repletion, rats were sacrificed by CO_2 asphyxiation and cervical dislocation. Trunk blood was collected in EDTA vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ), centrifuged to obtain plasma, and stored at –80°C until analysis. Femurs were excised and cleaned of soft tissue. Femur measurements (length, diaphysis, hip- and knee-joint widths) were obtained using calipers (to the nearest 0.1 mm).

High Resolution Scans. Femurs were analyzed for bone mineral content (BMC), bone area (BA), and bone mineral density (BMD) *in situ* using dual energy x-ray absorptiometry (DEXA, 4500A; Hologic Inc., Bedford, MA; small animal software high resolution option for excised femurs). Femurs were placed in a plastic water bath (tested for interference with the scan accuracy) with 2 cm of water above the bone and aligned in an anterior-posterior position. The femur was selected to represent a common long bone that is easily excised, positioned, and stabilized in a water bath to enable accurate measurements. The average precision error (as CV%) for femur BMD was 0.64% without repositioning and 0.70% with repositioning.

Mineral Analyses. After wet and dry weights were obtained, whole femurs and diet samples were wet-washed using trace-element–grade nitric acid as previously described (18). All glassware was acid-washed to prevent contamination. After appropriate dilution of digests, zinc concentration was determined in triplicate by atomic absorption spectroscopy using a Spectra AA-30 Spectrophotometer (Varian Canada, Georgetown, Canada). Quality control was monitored using bovine liver standard reference material 1577b (U.S. Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD). Calcium and phosphorus concentrations were analyzed in triplicate via emission spectrometry (Varian Liberty 200 ICP; Varian Canada, Mississauga, Canada).

Biochemical Assays. Plasma total ALP activity was determined using a kinetic colorimetric assay (procedure 245; Sigma Diagnostics, St. Louis, MO). Plasma osteocalcin and Ratlaps were measured by radioimmunoassay (Dia-Sorin, Stillwater, MN) and ELISA (Osteometer BioTech, Herlev Hovedgade, Denmark), respectively. Ratlaps is designed specifically for use in rats, and it measures a fragment of Type I collagen, a marker for bone resorption (19). Samples were analyzed in duplicate; agreement was >85%.

Statistical Analysis. Data were analyzed by one-way ANOVA using the general linear models procedure (SAS software release 8.2; SAS Institute, Cary, NC). When necessary, data were normalized by log transformation for statistical analyses, but nontransformed means are reported. Significant differences between means were determined using Duncan's multiple range test. Differences were considered significant at $P < 0.05$. Changes over time are

described in the text and differences due to dietary treatment are indicated in the tables and figures.

Results

Feed Intake and Body Weight. The total feed intake of ZD and DR during the 3-week deficiency phase was 50% of CTL (205 ± 7, 213 ± 6, and 427 ± 8 g, respectively). The ZD group weighed 14% less than DR, and DR weighed 41% less than CTL after the deficiency phase (Fig. 1a). After 23 days of repletion, ZD and DR weighed 21% and 11% less, respectively, than CTL, but total feed intake was not different (463 ± 17, 508 ± 16, and 518 ± 23 g, respectively).

Bone Morphology. ZD and DR had lighter femurs than CTL at the end of the deficiency phase (Fig. 1b). After 23 days of repletion, the femur weight of ZD was 15% less than DR, and DR was 13% less than CTL.

Femur length (Fig. 1c) was 8% lower in ZD compared with CTL, but not different from DR at the end of the deficiency period. After 23 days of repletion, the femur lengths of ZD and DR were 9% and 6% lower, respectively, than CTL.

For each of the dietary treatment groups, all bone morphometric measurements were shortest at baseline and longest at 23 days of repletion ($P < 0.05$). At the end of the deficiency phase, the diaphysis width of ZD was 10% lower than both DR and CTL (Fig. 2a). After 3 days of repletion, there were no differences among the groups, but after 7 days of repletion ZD and DR had 5%–10% lower diaphysis width compared with CTL. At the end of the study, there were no differences among the dietary treatment groups for diaphysis width.

The hip joint (proximal epiphysis) width in ZD was 8% lower than CTL at the end of the deficiency phase (Fig. 2b). After 23 days of repletion, ZD was 13% lower than CTL. DR did not show any differences from CTL until 7 and 23 days of the repletion phase when it was 9% and 6% lower, respectively, than CTL.

The knee joint width was 15% lower in ZD and DR compared with CTL at the end of the deficiency phase (Fig. 2c). Knee joint width was recovered to CTL levels by 7 days of repletion in DR and by 23 days of repletion in ZD.

Femur Mineral Concentrations. *Calcium.* Femur calcium concentrations increased 13%–16% from baseline in DR and CTL at the end of the deficiency phase ($P < 0.05$) and were unchanged for the rest of the study. There were no differences in femur calcium concentrations among the dietary treatment groups at the end of the deficiency phase (Fig. 3a). During the repletion phase, ZD had a lower femur calcium concentration compared with DR and CTL at 3 days of repletion, but there were no differences among the dietary treatment groups at any other time point.

Phosphorus. During the deficiency phase, all treatment groups had increased femur phosphorus concentrations ($P < 0.05$) by 57%, 85%, and 130% (ZD, DR, and CTL, respectively) from baseline concentrations. However, femur phosphorus concentration was 32% lower in ZD and 19% lower in DR compared with CTL at the end of the deficiency phase (Fig. 3b). During the 23-day repletion phase, the femur phosphorus concentrations increased in all treatment groups ($P < 0.05$) by 66% (ZD), 54% (DR), and 42% (CTL). At 7 days of repletion, ZD and DR femur phosphorus concentrations were no longer different from each other, but they remained 20%–25% lower than CTL. By 23 days of repletion, ZD and DR femur phosphorus concentrations had recovered to only 80% and 88% of CTL, respectively.

Zinc. Femur zinc concentration decreased 63% ($P < 0.05$) from 4.5 ± 0.1 μmole zinc/g dry bone in the baseline group to 1.6 ± 0.1 μmole zinc/g dry bone in ZD at the end of the deficiency phase. During the same period, there was no change in DR, but there was a 17% increase of femur zinc in CTL (4.5 ± 0.1 to 5.3 ± 0.2 μmole zinc/g dry bone). At the end of the deficiency phase, ZD had a 64% lower femur zinc concentration than DR, and DR had a 15% lower femur zinc concentration than CTL (Fig. 3c). The femur zinc concentration of DR recovered to CTL levels by 7 days of repletion, while the femur zinc concentration of ZD was still 20% lower than DR and CTL at the end of the repletion phase. During repletion, femur zinc increased ($P < 0.05$) in the ZD rats at each time point (40% after 3 days, 32% between 3 and 7 days, and 30% between 7 and 23 days). The femur zinc concentrations of DR increased 10% ($P < 0.05$) by the end of the repletion phase, but there were no changes in the femur zinc concentrations of CTL during the repletion phase.

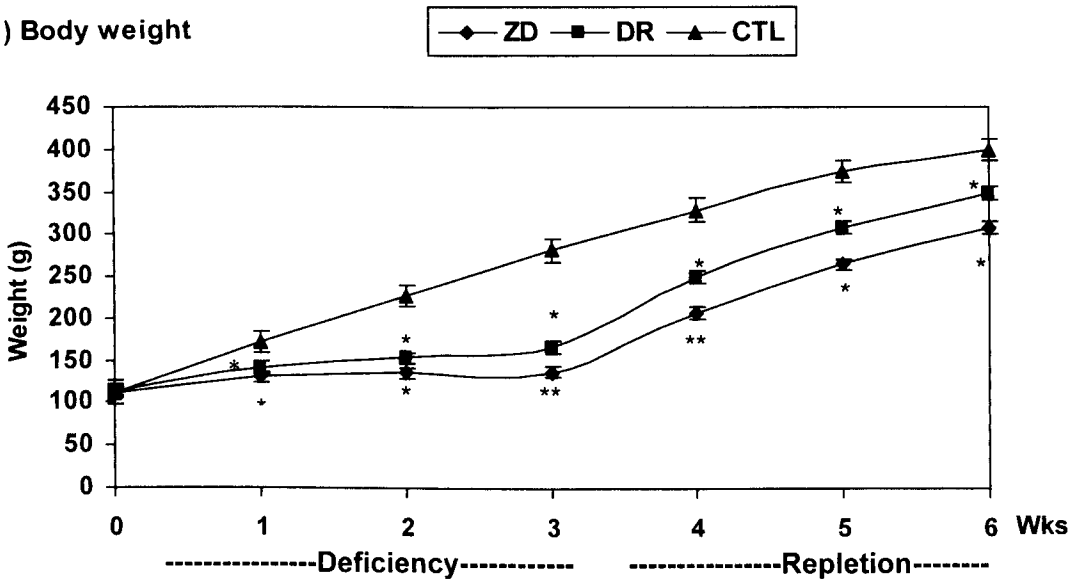
Plasma Markers of Bone Metabolism. Plasma ALP is an indicator of serum zinc and a marker for bone mineralization. Plasma ALP activity of ZD decreased 75% from 51.4 ± 4.7 U/L in the baseline group to 13.1 ± 1.1 U/L at the end of the deficiency phase ($P < 0.05$). At the end of the deficiency phase, the ALP activity of ZD was 67% and 81% lower than DR and CTL, respectively, and DR was 43% lower than CTL (Table 1). From 3 days (data not shown) to 23 days of repletion, there were no differences among the dietary treatment groups.

At the end of the deficiency phase, DR had a 40% higher plasma Ratlaps concentration (used as an indicator of bone resorption) than the other 2 treatment groups. After 23 days of repletion, the Ratlaps concentration was still 35% higher in DR compared with CTL, but not different from ZD.

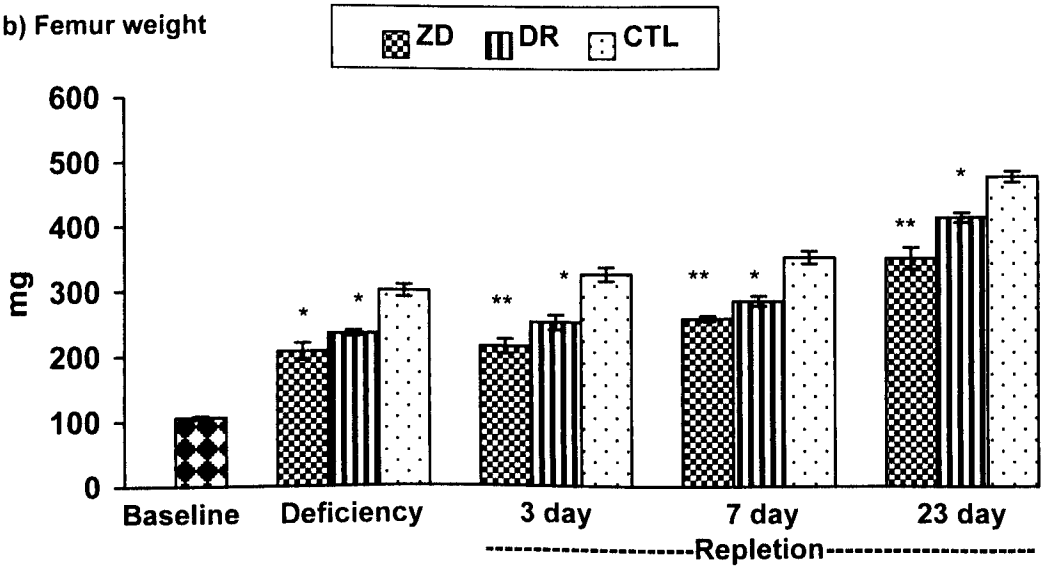
The plasma osteocalcin concentration (used as an indicator of bone formation) in ZD was 29% lower than DR and CTL at the end of the deficiency phase. By 23 days of repletion, plasma osteocalcin was not different among the treatment groups.

Bone Mass. BA, BMC, and BMD followed a similar pattern of change, and both BA and BMC (data not shown) contributed to the changes in BMD. BMD was increased from baseline levels in all treatment groups after the deficiency phase; BMD increased by 137% in ZD, 150% in DR, and 173% in CTL (Fig. 4). As a result, BMD of ZD

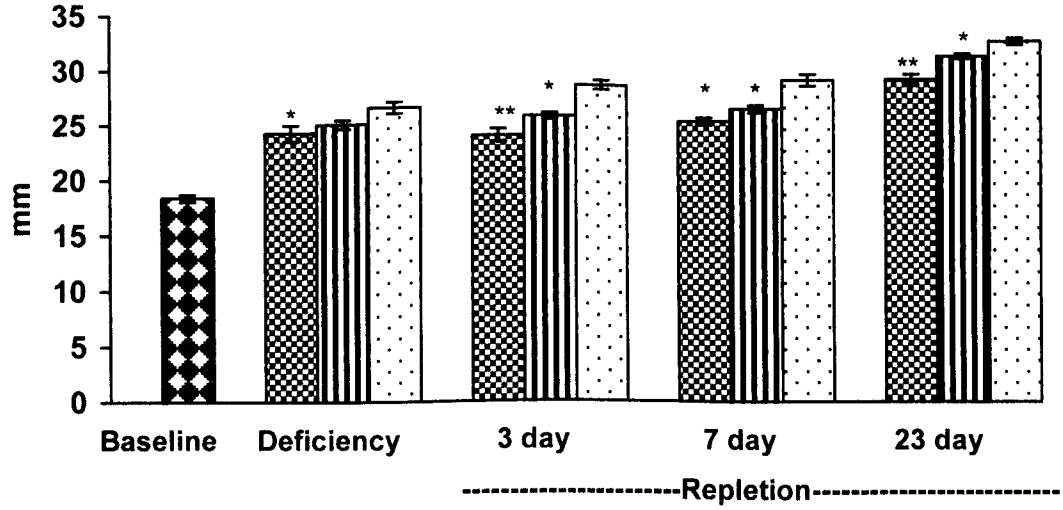
a) Body weight



b) Femur weight



c) Femur length



and DR was 80% and 87%, respectively, of CTL at the end of the deficiency phase. Throughout the repletion phase, BMD in ZD was lower than DR, and DR was lower than CTL. By the end of the study, the BMD of neither the ZD nor DR was able to catch up to control levels (86% and 91% of CTL, respectively).

Discussion

Previous studies have examined the effect of zinc deficiency on bone in the young rat (4, 20, 21), but this is the first study to evaluate bone recovery during dietary repletion. This study shows that dietary zinc deficiency and diet restriction in young growing rats have a negative effect on bone development that cannot be repaired by an equal period of repletion with a nutritionally complete diet. Furthermore, zinc deficiency has a greater negative impact than diet restriction. At the end of the deficiency phase, ZD had lower body weight and femur BMD, zinc, and phosphorus concentrations compared with DR; these parameters were lower in DR compared with CTL. By the end of the repletion phase, all measurements remained lower in ZD, and only femur zinc recovered in DR. We conclude that repletion for 23 days was not long enough to repair the damage to bone metabolism and/or that additional nutrient supplementation is required above a normal diet. These results suggest that bone growth and mineralization in early life are vulnerable to zinc deficiency and suboptimal dietary intake, emphasizing the importance of proper nutrition.

ZD had lower plasma osteocalcin concentrations compared with both DR and CTL at the end of the deficiency phase, suggesting that there was less bone formation. This is consistent with ZD having shorter femurs that weighed less. DR had greater bone resorption compared with both ZD and CTL as indicated by elevated plasma Ratlaps concentration at the end of the deficiency phase. Furthermore, maintenance of femur length in DR, despite lower femur weight and BMD, is also consistent with greater bone remodeling. Similarly, Rossi and colleagues (4) reported lower bone volume in both zinc deficient and pair-fed rats, but greater osteoclast size and numbers only in pair-fed rats compared with both the zinc-deficient and control groups. Thus, both bone histomorphometry (4) and serum bone markers of bone metabolism (the present study) indicate that the mechanism for the osteopenia may involve reduced modeling in zinc deficiency and higher turnover in diet restriction.

During dietary repletion, elevated plasma Ratlaps concentrations in DR indicated more bone resorption that could be in support of modeling for growth or release of minerals for other tissues. Plasma osteocalcin concentrations at the end of the repletion phase were similar among the dietary treatment groups, suggesting that repletion normalized bone formation rates. Also, there was an age-related decline in both plasma Ratlaps and osteocalcin concentrations in 6-week-old rats (end of deficiency phase) versus 9-week-old rats (after 23 days of repletion) ($P < 0.05$), suggesting that there is a critical period for bone development and that ZD might have missed the window of opportunity for accelerated bone formation early in life. While the rats in our study were not followed to maturity, it has been reported that young men born with low birth weight were able to achieve appropriate height and bone mass; however, they had accelerated bone turnover entering adulthood (18–21 years), suggesting that the early insult results in altered cell programming (22). Future studies need to address the role(s) of dietary deficiencies on cell programming during bone development *in utero* and early in life.

ALP is a zinc-dependent enzyme released by osteoblasts involved in bone mineralization (2). We measured total ALP activity in the serum, not bone-specific ALP. However, bone and liver represent the major sources of ALP (19). The lower plasma ALP activity in ZD and DR at the end of the deficiency phase was associated with lower femur BMC, the ZD group being affected more severely. Plasma ALP activity of ZD and DR responded rapidly to intervention with a nutritionally complete diet (Table 1), which is in agreement with zinc treatment increasing ALP activity (3). ALP activity is also an indicator of short-term zinc status (23), whereas femur zinc reflects longer term zinc status, as evidenced by the reduced values of the ZD group throughout the repletion phase (Fig. 2c).

Although both ZD and DR had lower BMDs and different alterations in bone morphology, ZD was more severely affected. At the end of the deficiency phase, DR maintained diaphysis width, but ZD did not. In DR, bone formation was maintained (osteocalcin), but mineralization (ALP) was not able to match resorption (Ratlaps), whereas ZD had less bone formation and mineralization. Thus, it is not surprising that, during repletion, there was better bone recovery in DR compared with ZD, which likely required deposition and mineralization of matrix. These results, along with previous work showing decreased bone trabeculae thickness and reduced activity of the growth plate in dietary zinc deficiency (4, 20), support the hypothesis that zinc deficiency during early life may increase the risk of developing osteoporosis in adulthood by limiting the peak bone mass achieved early in life.

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Figure 1. Effects of zinc deficiency and diet restriction followed by repletion on a) body weight, b) femur weight, and c) femur length in young, growing rats. Values are means ± standard error for $n = 8$ (baseline, deficiency, 3- and 7-day repletion) and $n = 6$ rats (23-day repletion). Statistical differences ($P < 0.05$) between groups (ZD, DR, and CTL) at each time point are indicated by asterisks: * indicates ZD or DR are significantly different from CTL. ** indicates ZD is significantly different from both DR and CTL. Abbreviations: ZD, zinc-deficient group; DR, diet-restricted group; CTL, control group.

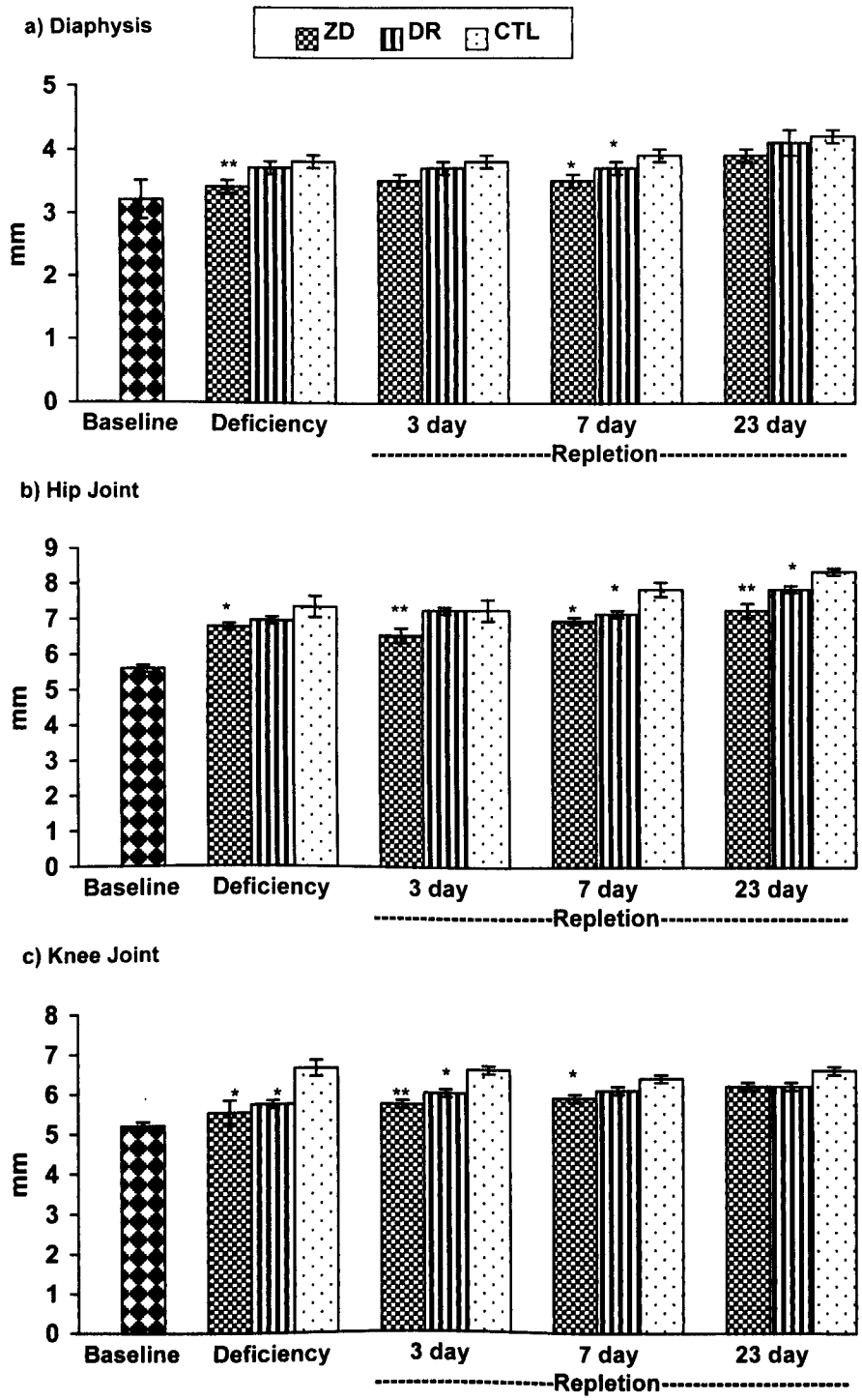

Figure 2. Effects of zinc deficiency and diet restriction followed by repletion on a) diaphysis width, b) hip joint width, and c) knee joint width in young, growing rats. Values are means ± standard error for $n = 8$ (baseline, deficiency, 3- and 7-day repletion) and $n = 6$ rats (23-day repletion). Statistical differences ($P < 0.05$) between groups (ZD, DR, and CTL) at each time point are indicated by asterisks: * indicates ZD or DR are significantly different from CTL. ** indicates ZD is significantly different from both DR and CTL. Diaphysis, width at narrowest point of femur. Hip joint, width of anteriormost point to posteriormost point. Knee joint, width at knee joint. Abbreviations: ZD, zinc-deficient group; DR, diet-restricted group; CTL, control group.

In addition to reduced BMD using DEXA, specific bone mineral concentrations of ZD and DR were different from each other as well as from CTL. Zhuo and colleagues (1) showed that 10%–20% of zinc from rat femurs could be released during mild zinc deficiency (6 mg zinc/kg diet) to maintain zinc concentrations in soft tissues, whereas more severe zinc deficiency (<1 mg zinc/kg diet) in the present experiment decreased femur zinc concentration by 63% from

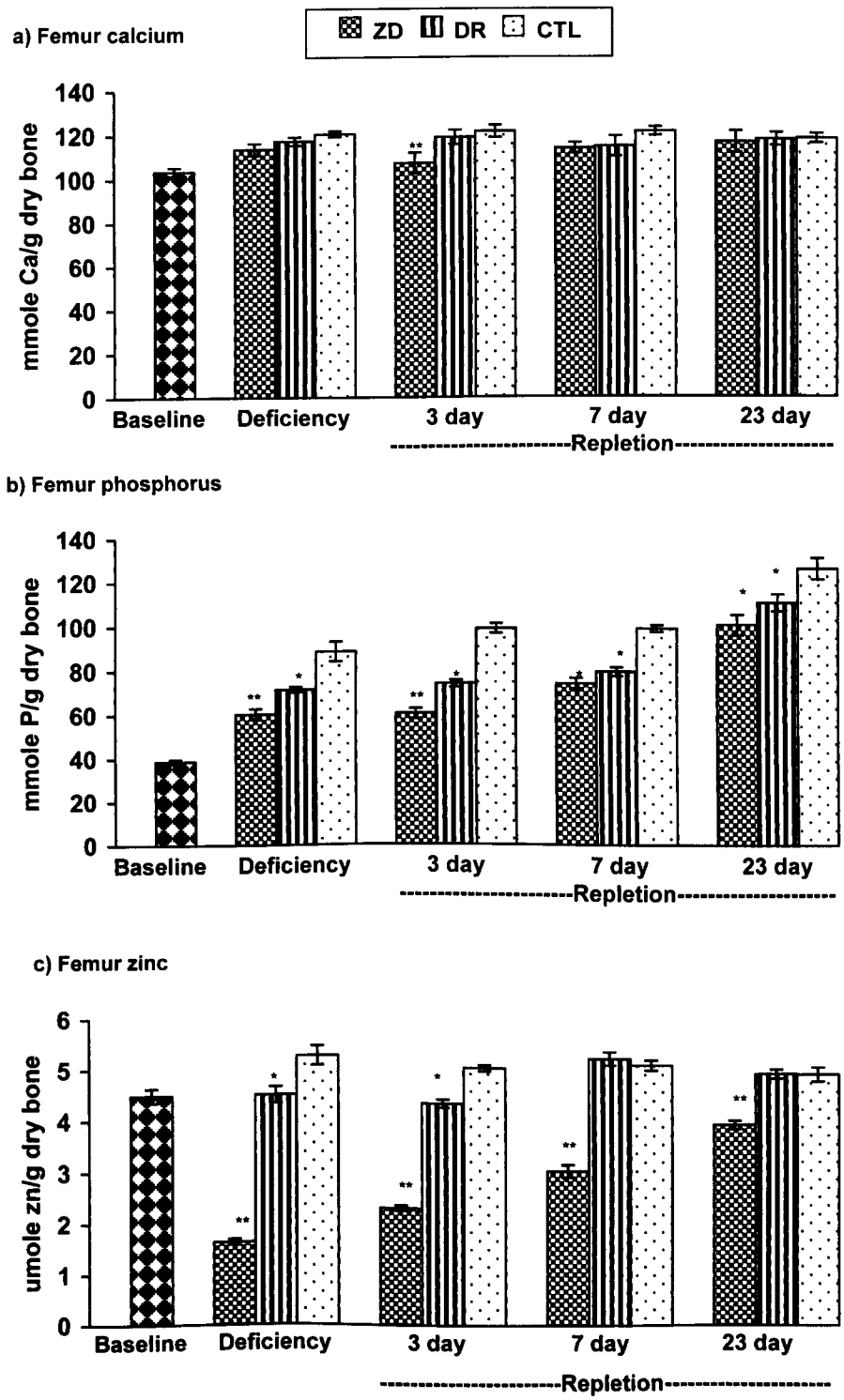

Figure 3. Effects of zinc deficiency and diet restriction followed by repletion on a) femur calcium concentration, b) femur phosphorus concentration, and c) femur zinc concentration in young, growing rats. Values are means ± standard error for $n = 8$ (baseline, deficiency, 3- and 7-day repletion) and $n = 6$ rats (23-day repletion). Statistical differences ($P < 0.05$) between groups (ZD, DR, and CTL) at each time point are indicated by asterisks: * indicates ZD or DR are significantly different from CTL. ** indicates ZD is significantly different from both DR and CTL. Abbreviations: ZD, zinc-deficient group; DR, diet-restricted group; CTL, control group.

baseline. To date there have not been any published studies with a longer repletion time (to indicate whether complete recovery is possible) or repletion with higher dietary zinc concentrations (to determine whether higher concentrations would be beneficial), but both of these issues need to be addressed in future studies. In CTL, the femur zinc and calcium concentrations were relatively constant in 3-, 6-, and 9-week-old rats, but phosphorus concentrations tripled

Table 1. Effects of Dietary Zinc Deficiency and Diet Restriction Followed by Repletion on Bone Biochemistry[a]

		Dietary group[b]		
	Time	ZD	DR	CTL
Plasma ALP, U/L	Deficiency	13.1 ± 1.2***	39.5 ± 4.5**	69.8 ± 8.0
	23-Day repletion	34.3 ± 4.6	36.9 ± 7.9	30.2 ± 3.5
Plasma Ratlaps, mmole/L	Deficiency	40.2 ± 3.2	56.4 ± 5.8**	38.7 ± 1.9
	23-Day repletion	23.3 ± 1.9	24.9 ± 1.6*	18.5 ± 1.8
Plasma osteocalcin, mmole/L	Deficiency	79.9 ± 6.5***	111.4 ± 7.3	113.7 ± 7.5
	23-Day repletion	78.4 ± 3.8	77.3 ± 8.4	69.5 ± 5.6

[a] Values are means ± standard error for $n = 8$ (deficiency) and $n = 6$ rats (23-day repletion).
[b] Statistical differences ($P < 0.05$) between groups are indicated by asterisks: * indicates DR is significantly different from CTL but not ZD. ** indicates DR is significantly different from both ZD and CTL. *** indicates ZD is different from both DR and CTL. Abbreviations: ZD, zinc-deficient group; DR, diet-restricted group; CTL, control group; ALP, alkaline phosphatase.

between 3 and 9 weeks of age. Bone mineral is in the form of hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$). Our data indicate that between 3 and 9 weeks of age the femur accumulates phosphorus while calcium concentrations remain constant. Bone mineral is also in the form of amorphous calcium, and perhaps the calcium-to-phosphorus ratio changes as the bone matures and becomes more cortical relative to trabecular. The lower BMD in ZD and DR at the end of the deficiency phase was due to lower phosphorus and zinc—but not calcium—concentrations. After repletion, the lower BMD in DR was due to reduced phosphorus concentration, while the lower BMD in ZD was due to reduced phosphorus and zinc concentrations. As well, BMD was likely lower as a function of bone size due to the limitations of DEXA.

The risk of osteoporotic fracture increases as BMD decreases; therefore, BMD is used to indicate bone mass and diagnose osteoporosis (9). The 2 main factors that affect adult bone mass and the risk of osteoporosis are the level of peak bone mass achieved during growth and the rate of bone loss later in life (9). The reduction in BMD due to zinc deficiency and diet restriction in growing rats and the subsequent failure of repletion with control diet for 23 days to recover BMD indicate a potential problem for bone health later in life for these animals. Short-term, severe zinc deficiency or diet restriction followed by short- term dietary repletion in a rodent model was used to investigate the role of zinc in bone development and repair. Caution must be employed in extrapolating these results to humans, as less severe deficiencies for shorter or longer periods during different stages of the life cycle are likely to influence outcomes. This research does suggest that provision of adequate zinc and energy intakes during

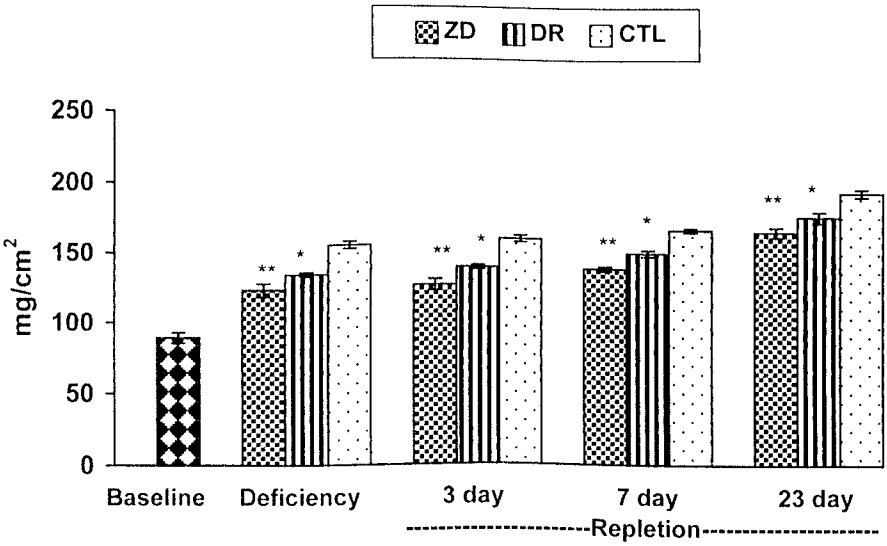

Figure 4. Effects of zinc deficiency and diet restriction followed by repletion on femur bone mineral density (BMD) in young, growing rats. Values are means ± standard error for $n = 8$ (baseline, deficiency, 3- and 7-day repletion) and $n = 6$ rats (23-day repletion). Statistical differences ($P < 0.05$) between groups (ZD, DR, and CTL) at each time point are indicated by asterisks: * indicates ZD or DR are significantly different from CTL. ** indicates ZD is significantly different from both DR and CTL. Abbreviations: ZD, zinc-deficient group; DR, diet-restricted group; CTL, control group.

growth may be important for prevention of bone disease later in life.

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