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Moderate/subclinical calcium deficiency attenuates trabecular mass, microarchitecture and bone growth in growing rats

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ABSTRACT

Adequate dietary calcium (Ca) intake is essential for bone accretion, peak bone mass (PBM) attainment, bone quality and strength during the mammalian growth period. Severe Ca deficiency during growing age results in secondary hyperparathyroidism (SHPT) and poor bone quality and strength. However, the impact of moderate Ca deficiency during rats early growth period on bone health and the reversibility with supplementing calcium later in adult life remains unclear. Female Sprague-Dawley (SD) rats (postnatal 28th day, P28) were initiated either with a moderate calcium-deficient diet (MCD, 0.25% w/w Ca) or a control diet (0.8% w/w Ca, control group) till P70. Thereafter, MCD rats were continued either with MCD diet or supplemented with calcium diet (0.8% w/w Ca, calcium supplemented group, CaS) till P150. Another group (control rats) were fed 0.8% w/w Ca containing diet from P28 till P150.

MCD group, as compared to the control group, had significantly reduced serum ionized Ca and procollagen type 1 N-terminal propeptide (P1NP) at P70 while no significant change was observed in serum corrected Ca, inorganic phosphate (P), alkaline phosphatase (ALP), 25-hydroxy vitamin D [25(OH)D], intact parathyroid hormone (iPTH), and urinary C-terminal telopeptide of collagen 1 (CTX-1), Ca, and P. Femoral and tibial metaphysis in MCD rats had significantly reduced linear growth, cortical and trabecular volumetric BMD (vBMD), trabecular microarchitecture (BV/TV%, trabecular thickness, separation and number, structural model index and connectivity density), cortical thickness, and bone stiffness despite the absence of secondary hyperparathyroidism (SHPT). Continued MCD at P70–P150 results in persistence of compromised bone strength while calcium supplementation (CaS group) improved all the parameters related to bone strength and microarchitecture. Our results indicate that uncorrected moderate/subclinical calcium deficiency in growing rats can result in poor bone quality and strength despite the absence of SHPT. This finding could have relevance in children with poor calcium intake in childhood and adolescence.

1. Introduction

Dietary calcium (Ca) deficiency is recognized as a cause of nutritional calcipenic rickets in children and adolescents, particularly in the low and middle-income countries of Asia and Africa, despite plentiful sunshine in these regions [1–5]. The most common etiologies related to secondary hyperparathyroidism (SHPT) include renal failure, vitamin D deficiency and low dietary Ca intake [6]. Dietary Ca deficiency is one of the most common causes of SHPT. It is characterized by excessive serum parathyroid hormone (PTH) levels, parathyroid hyperplasia, and imbalance in calcium and phosphorus metabolism. The consequences of SHPT induced by dietary Ca deficiency in growing rats include hypocalcemia, hypophosphatemia, hypocalciuria, hyperphosphaturia and elevated CTX-1 level [7]. Various studies in growing rats [7–15] and other mammals [16,17] also show that severe dietary Ca deficiency (0.1–0.2%), as compared to adequate calcium intake (0.5–0.8%), results in SHPT and compromised bone microarchitecture, quality and strength.

However, unlike a consistent observation of deranged bone growth

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due to severe Ca deficiency in animal models [7,10,16,17], the effect of moderate Ca deficiency during the early growth periods of development is little understood [18–20]. In many developing countries, Ca consumption usually lies below 50% of the specified requirements due to different socio-economic and health factors, leading to moderate Ca deficiency. In animal models, the effect of moderate Ca deficiency (0.25% w/w), which is roughly around half of the adequate calcium intake (0.5–0.8%), has shown contradictory results on calcium homeostasis, bone density and bone microarchitecture [18–20]. Furthermore, the sensitivity of bone growth to suboptimal yet not severely diminished levels of Ca, during development has not been well addressed in the literature.

Therefore, to resolve these pertinent issues, this study aimed to determine the effect of moderate Ca deficiency (0.25% Ca w/w) on vBMD, microarchitecture, growth and bone strength during the growth period (P28–P70), and its reversibility with adequate Ca supplementation (0.8%) from P70 till P150, in female rats. Our results show that moderate/subclinical Ca deficiency significantly impairs bone remodeling, peak bone mass (PBM) attainment, and bone strength during the growth period, despite the absence of SHPT.

2. Materials & methods

2.1. Reagents and kits

The chemicals, reagents (Sigma-Aldrich, USA) and ELISA kits for iPTH (intact parathyroid hormone), P1NP (procollagen type 1 N-terminal peptide) and CTX-1(C-telopeptide of type 1 collagen) were procured from Elabscience, USA. Ca modified diet {(0.8% and 0.25% Ca diet, w/w), AIN-93G diet} [21] was purchased from Research Diet Inc. USA and vitamin D total [25(OH)D] kit from Roach Diagnostics.

2.2. Experimental design

The Institutional Animal Ethical Committee (IAEC) approval (Ref. No.SGPGIMS/AH/22nd IAEC/PP50/067/2016) was obtained, and the study was performed as per ethical guidelines for the care and use of laboratory animals. Weaning female Sprague-Dawley (SD) rats (21 days old) were kept on a normal diet for seven days for acclimatization before starting the main experiment. Four weeks (28 days) old rats were housed in cages (either 2 or 3 rats/cage) and maintained at 24 ± 2 °C and 50 ± 5% relative humidity, with free access to food and de-ionized water. Food intake and body weight were monitored weekly.

Sixty rats were recruited in the study. The experimental design with the number of animals in each group, is shown in Fig. 1. Ten female SD rats were sacrificed at P28 (baseline) and the remaining rats were randomly divided into two groups: a) rats fed on a control diet (0.8% Ca w/w) and b) rats fed on moderate Ca deficient diet (MCD, 0.25% Ca) till 70 days of life (P70). At P70, rats (n = 10/group) were sacrificed from both the groups. At P70, the MCD group was further sub-divided into two subgroups: one group was continued on the same diet (MCD group), and the other group received control diet [(0.8% Ca), termed here as Ca Supplemented (CaS) group] till P150. At P150, rats (n = 10/group) were sacrificed from each group; Control, MCD and CaS.

2.3. Sample collection and rat autopsy

Twenty-four-hour urine samples of rats were collected from the metabolic cages and centrifuged at 1200 g for 10 min, and the supernatant was stored at −80 °C for further analysis. The rats were euthanized in a fasting state; the blood samples were collected through cardiac puncture, and serum was separated by centrifugation at 1200*g* for 10 min. The serum was stored at −80 °C for further analysis. After the autopsy, the femur and tibia were isolated from each rat and kept at −20 °C, for the analysis of bone parameters.

2.4. Biochemical, bone-turnover markers and calciotropic hormone analysis

Serum Ca^{2+} was measured using an automated EasyLyteanalyzer as per the manufacturer's instructions. Serum total Ca, P, total ALP and albumin, and urinary Ca, P and creatinine were determined by an automated chemistry analyzer (Selectra Pro M Elitech Clinical system). Urinary CTX-1 (# E-EL-R1456), serum PINP (# E-EL-R1414) and serum iPTH (# E-EL-R0535) were measured using an ELISA kit. Serum vitamin D total level (25-OH-D_3 and 25-OH-D_2) was measured using the commercially available kit (Roche, #5894913190) and fully automated electro-chemiluminescence technology.

2.5. Quantitative analysis of bone microarchitectural parameters by micro CT

Trabecular parameters including bone volume fraction (BV/TV), trabecular thickness (Tb.Th.), trabecular spacing (Tb. Sp.), trabecular number (Tb.N.), structural model index (SMI) and connectivity density (Conn. D.) of excised bones (femur and tibia) were analyzed using a high-resolution X-ray μCT scanner (SkyScan1076, Ltd, Belgium). SMI is an indicator of the structure of trabeculae; SMI is 0 for parallel plates and

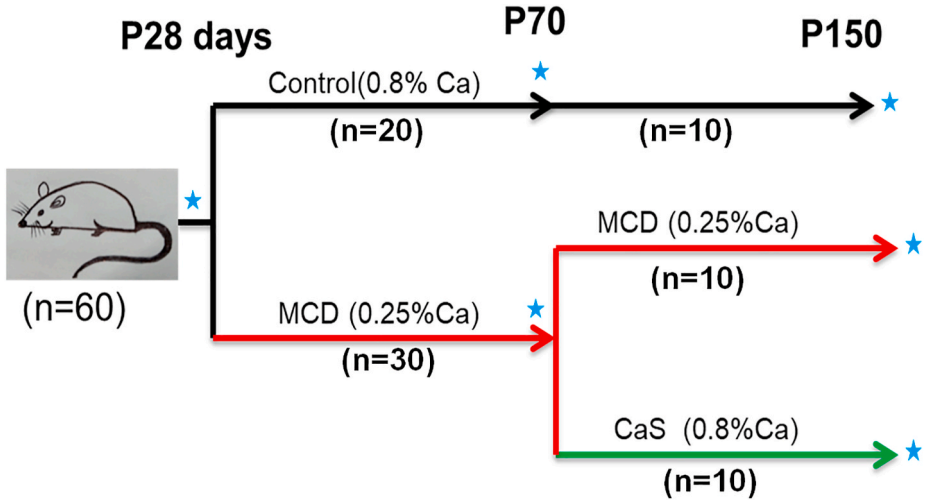

Fig. 1. Showing different groups and experimental time points -postnatal (P)28 (baseline), P70(Control and MCD) and P150 (Control, MCD and CaS). Star indicates sacrificed rats (n = 10). MCD; Moderate calcium deficient group, CaS; Ca Supplemented group.

3 for cylindrical rods. For scanning, the X-ray source was set at 70 kV and 142 mA, pixel size of 18 μm, capturing image after every 0.6 rotation. Cross-sectional reconstruction was carried out using N-recon software which was based on a modified Feldkamp algorithm. CTAn software was used for manually selecting the trabecular bone as a region of interest (ROI) using ellipsoid contours on reconstructed images. For trabecular femur and tibia regions, an ROI of 100 slices was drawn in secondary spongiosa situated 1.5 mm (50 slices) away from the distal border of the growth plate. We have measured cortical thickness (Ct.Th.) and endosteal perimeter (E.Pm.) from the mid-diaphysis of femur and tibia. For cortical bone assessment, ROI was drawn on total 100 slices in the mid diaphysis situated 15 mm (500 slices) away from the same point as for trabecular bones. The ROI quantification of trabecular 3D parameters and cortical bone 2D parameters was performed using the Batman software. The 3-D images from ROI were constructed using the CTAn software.

2.6. Analysis of volumetric bone mineral density

The volumetric bone mineral density (vBMD) of the femur and tibia was determined using μCT. The standard protocol was used, as provided by Skyscan, Belgium. Phantom rods with 4 mm diameter of low density (0.25 gHA/cm3) and high density (0.75 gHA/cm3) were used as a reference to determine vBMD values of the isolated bones. For calibration, phantoms were placed in a tube filled with water and scanned similar to those used for the bone scan (X-ray source was set at 70 kV and 142 mA, and the pixel size was 18 μm), to obtain values in the Hounsfield unit. These values were then entered into the calibration panel of CTAn software to determine the vBMD values of the different bone samples.

2.7. Bone strength analysis

Bone mechanical strength was determined by 3-point bending strength of the femur mid diaphysis and the femur head's compressive strength was evaluated using a bone strength tester (Model TK-252C, Muromachi Kikai Co. Ltd, Japan). Femur bone was positioned in the anteroposterior direction for the three-point bending test, and force was applied at middle part of the bone at a deformation rate of 20 mm/min, as reported earlier [22,23]. For the femur head's compressive test; 0.8 cm distal femur (femur head) was cut out from the whole femur. Femur head was placed between the faces of a compression jig and a constant force was applied in the cranio-caudal direction at a deformation rate of 20 mm/min, as reported earlier [23,24]. For both tests, the load-displacement curves generated were used to calculate the ultimate load or max power (N), stiffness (N/mm), and work to fracture (mJ).

2.8. Statistical analysis

Results are expressed as mean ± SEM. The statistical significance of differences was assessed by unpaired student t-test (2 groups) or one-way ANOVA (>2 groups) followed by Tukey's post-test analysis using Graph Pad Prism 5.

3. Result

3.1. Bodyweight and food intake

There was no difference in body weight of control and MCD rats at baseline (P28). There was no significant difference in the weight gain at P70 (control 208.4 ± 11.35 *vs.* MCD 214.3 ± 8.63 g) and P150 (control 284.3 ± 14.55 *vs.* MCD 285.1 ± 14.46 *vs.* CaS 292.8 ± 10.71 g) between the groups. The food intake was also not significantly different between the groups at both time points (P70: control 14 ± 0.94 *vs.* MCD 13.8 ± 1.03 g; P150: control 15.9 ± 1.29 *vs.* MCD 15.8 ± 1.32 *vs.* CaS 15.71 ± 0.95 g). Thus, 0.25% Ca diet did not affect body weight and food intake in growing rats. However, along with food consumption, animal body weights were progressively increased throughout the experiment with no significant difference between respective groups at P70 and P150. We did not observe any behavioural change in MCD rats as compared to control rats.

3.2. Biochemical parameters, calciotropic hormones and bone turn over markers

At P70, serum ionized Ca (Ca^{2+}) was significantly lower in the MCD intake group than in control rats, while serum corrected Ca and P were not considerably different (Table 1). There was no significant difference in serum P, 25(OH) D, iPTH and ALP levels, and 24 h urinary Ca, P, and Ca/Creatinine ratio. Bone turnover marker analysis showed a significant reduction in bone formation marker (P1NP) with no significant difference in bone resorption marker CTX-1.

At P150, however, there were no significant differences in all these parameters (Table 1). Although the MCD group showed lower serum Ca^{2+}, lower urinary Ca excretion, urinary Ca/Creatinine ratio, and higher urinary P excretion, whereas the MCD and CaS group showed higher ALP levels than controls (Table 1), however the differences were not statistically significant. The nutritional status of vitamin D was analyzed by measuring the total serum vitamin D (25-OH-D_3 and 25-OH-D_2) level. There was a progressive increase in serum 25(OH) D levels in rats from baseline to end of the study, in all the groups, due to consumption of vitamin D fortified rat diet. Furthermore, there was no vitamin D deficiency in any group, as serum 25(OH)D levels were >30 ng/mL, reflecting its normal level as previously described [25,26].

However, serum 25(OH) D levels were not significantly different between the groups, at the corresponding time points (Table 1). The PTH level remained unchanged in the MCD group at P70 and P150, as compared to corresponding control groups (Table 1). Thus, decreased Ca^{2+} due to moderate Ca intake in early growing rats at P70 is maintained with available dietary Ca without inducing SHPT.

Table 1
Serum and urinary biochemical parameters, calciotropic hormone and bone turnover markers. Data are presented as mean ± SEM.n ≥ 6/group, *P < 0.05 control vs. MCD group. The statistical significance was assessed by unpaired student t-test (2 groups) at P70 or one-way ANOVA (>2 groups) followed by Tukey's post-test analysis at P150. (Abbr: MCD, Moderate calcium deficient group; CaS, Ca Supplemented group; U. Ca, Urinary Calcium; U.P., Urinary Phosphate).

Parameters	P28	P70		P150		
	Baseline	Control	MCD	Control	MCD	CaS
Ca^{2+} (mmol/L)	1.23 ± 0.03	1.20 ± 0.02	1.06 ± 0.05*	1.16 ± 0.05	1.13 ± 0.05	1.19 ± 0.04
Corr.Ca (mg/d)	9.47 ± 0.34	11.00 ± 0.13	11.24 ± 0.12	11.30 ± 0.21	11.48 ± 0.15	10.95 ± 0.22
P (mg/dL)	8.56 ± 0.93	8.13 ± 0.3	7.95 ± 0.73	4.89 ± 0.47	5.80 ± 0.18	5.02 ± 0.46
U.Ca (mg/dL)	27.55 ± 1.61	1.60 ± 0.30	1.17 ± 0.15	2.26 ± 0.42	2.13 ± 0.41	2.37 ± 0.64
Ca/Creatinin ratio	0.73 ± 0.09	0.06 ± 0.02	0.04 ± 0.02	0.06 ± 0.02	0.04 ± 0.01	0.06 ± 0.02
U.P (mg/dL)	16.13 ± 6.94	138.3 ± 22.3	198.2 ± 22.6	69.97 ± 9.24	99.03 ± 18.79	86.20 ± 9.04
25(OH)D (ng/ml)	31.89 ± 3.69	42.19 ± 2.51	41.08 ± 2.62	49.47 ± 3.46	51.57 ± 5.33	52.33 ± 3.07
iPTH (pg/ml)	233.6 ± 5.41	212.3 ± 8.61	217.45 ± 7.90	187.32 ± 4.02	187.49 ± 6.78	175.07 ± 4.71
ALP (U/L)	677.43 ± 34.2	452.6 ± 22.9	395.2 ± 15.9	175.1 ± 19.7	197.9 ± 16.3	198.0 ± 22.1
P1NP (ng/ml)	35. 31 ± 2.37	51.60 ± 4.70	37.22 ± 2.15*	53.54 ± 7.30	53.28 ± 4.85	49.91 ± 4.9
CTX-1 (ng/ml)	9.74 ± 1.82	8.02 ± 1.44	8.94 ± 1.54	14.23 ± 1.7	13.82 ± 1.40	15.00 ± 0.75

The total serum ALP levels in control rats were highest at the baseline (P28) and declined progressively till P150 days. However, there was no significant difference between the control and MCD groups. The P1NP levels were significantly lower in the MCD group than the control group at P70, showing comparatively decreased bone formation in the MCD group (Table 1). However, at P150, the P1NP levels were similar in the control, MCD and CaS groups. There was no difference in bone resorption marker, CTX-1 at P70 and P150 time points.

3.3. Length and weight of femur and tibia

At P70, the length of the femur was non-significantly shorter (control 31.13 ± 0.83 *vs.* MCD 30.75 ± 0.71 mm, $p > 0.05$) and tibia was significantly shorter in the MCD group (control 34.00 ± 0.30 *vs.* MCD 33.13 ± 0.22 mm, $p < 0.05$). The weights of both femur (control 0.775 ± 0.07 *vs.* MCD 0.699 ± 0.03 g, $p < 0.01$) and tibia (control 0.649 ± 0.07 *vs.* MCD 0.573 ± 0.04 g, $p < 0.05$) were significantly decreased in the MCD group compared to age-matched controls. At P150, there was no significant difference in length [tibia length (control 36.86 ± 0.38 *vs.* MCD36.38 ± 0.74 *vs.* CaS 36.50 ± 0.76 mm) and femur length (control 33.13 ± 0.64 *vs.* MCD 33.13 ± 0.64 *vs.* CaS 33.25 ± 0.71 mm);] and weights [tibia weight (control 0.696 ± 0.03 *vs.* MCD0.691 ± 0.03 *vs.* CaS 0.693 ± 0.02 g); and femur weight (control 0.848 ± 0.05 *vs.* MCD 0.817 ± 0.02 *vs.* CaS 0.838 ± 0.03 g);] of tibia and femur among groups.

3.4. The cortical and trabecular bone microarchitecture

The μ-CT analysis of trabecular bone at femoral distal metaphysis and proximal metaphysis of tibia at P70 revealed that rats in the MCD group, compared to control rats, showed significantly deteriorated rats trabecular microarchitecture (Fig. 2A and Supp. Figure 1A). The rats in MCD group, as compared to control rats, had significantly reduced vBMD, bone volume fraction (BV/TV), trabecular thickness (Tb.Th.), and trabecular number (Tb.N.), and connectivity density (Conn.D). Also, the structure model index (SMI) and trabecular spacing (Tb.Sp.) were significantly increased (Fig. 2B and Suppl Figure 1B). This implies that there is a predominance of cylindrical rods as compared to parallel plates in MCD rats. Trabecular spacing (Tb.Sp.) is the mean distance between trabeculae. A high value of SMI and Tb.Sp., indicates the poor geometrical arrangement of trabeculae (Fig. 2B and Suppl Figure 1B). All the trabecular bone micro-CT parameters in MCD group were not significantly different from control group and CaS at P150 (Fig. 2B and Suppl. Figure 1B).

The μ-CT analysis of cortical bone at mid-diaphysis of femur and tibia showed that cortical volumetric bone mineral density (vBMD) and cortical thickness (Ct.Th.) increased progressively with age from P28 till P150 (Table 2). At P70, vBMD and Ct.Th. at tibia were significantly lower in the MCD group as compared to the control group. However, the lowering in both the parameters was not statistically significant. Endosteal perimeter (E.Pm.) was lower in both femur and tibia ($p > 0.05$). Interestingly, at P150, cortical vBMD, thickness and endosteal

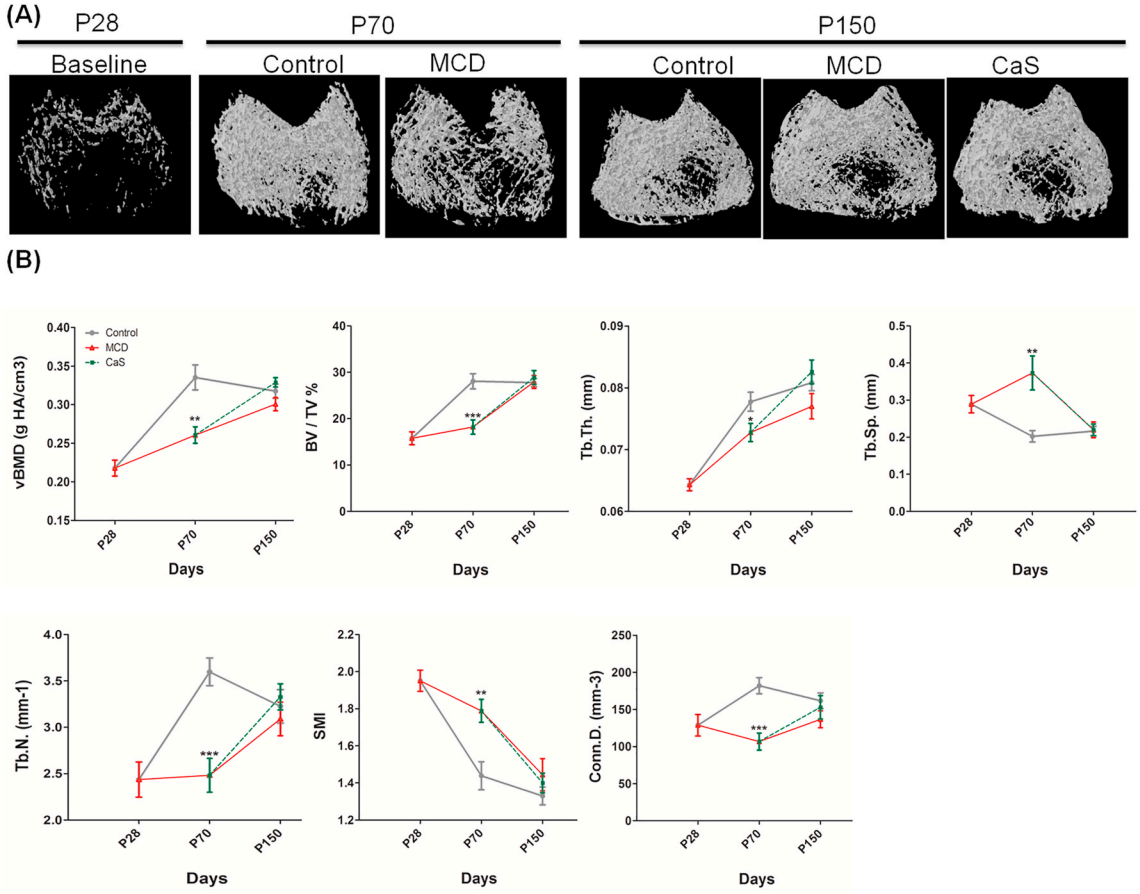

Fig. 2. Micro-CT parameters of trabecular microarchitecture at femur showing significant deterioration at P70 and recovery at P150. (A) Representative trabecular μCT images of femur distal metaphysis (B) Micro-CT trabecular parameters: volumetric bone mineral density (vBMD), bone volume fraction (BV/TV), trabecular thickness (Tb.Th.), trabecular spacing (Tb. Sp.), trabecular number (Tb.N.), structural model index (SMI) and connectivity density (Conn. D.). n ≥ 6/group, Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ control vs. MCD group. The statistical significance was assessed by unpaired student t-test (2 groups) at P70 or one-way ANOVA (>2 groups) followed by Tukey's post-test analysis at P150. MCD; Moderate calcium deficient group, CaS; Ca Supplemented group.

Table 2
Micro-CT parameters of cortical bone at mid-diaphysis of femur and tibia. Data are presented as mean ± SEM. n ≥ 6/group, *$P < 0.05$, **$P < 0.01$ Control vs. MCD group. The statistical significance was assessed by unpaired student t-test (2 groups) at P70 or one-way ANOVA (>2 groups) followed by Tukey's post-test analysis at P150. (Abbr: MCD, Moderate calcium deficient group; CaS, Ca supplemented group; Ct.vBMD, Cortical volumetric bone mineral density; Ct.Th., Cortical thickness; E.Pm., Endosteal perimeter).

	P28	P70		P150		
Parameters	Baseline	Control	MCD	Control	MCD	CaS
Tibia						
Ct.vBMD (gHA/cm^3)	1.103 ± 0.012	1.200 ± 0.03	1.080 ± 0.05*	1.276 ± 0.02	1.227 ± 0.07	1.278 ± 0.02
Ct.Th. (mm)	0.167 ± 0.01	0.231 ± 0.01	0.181 ± 0.01**	0.303 ± 0.02	0.275 ± 0.02	0.298 ± 0.04
E.Pm. (mm)	6.96 ± 0.20	14.16 ± 0.73	14.33 ± 0.87	12.84 ± 0.76	11.58 ± 0.57	11.99 ± 0.35
Femur						
Ct.vBMD (gHA/cm^3)	1.168 ± 0.012	1.252 ± 0.06	1.257 ± 0.04	1.406 ± 0.02	1.388 ± 0.05	1.398 ± 0.03
Ct.Th. (mm)	0.198 ± 0.02	0.305 ± 0.02	0.298 ± 0.01	0.508 ± 0.01	0.460 ± 0.02	0.477 ± 0.02
E.Pm. (mm)	7.33 ± 0.19	10.43 ± 0.19	10.35 ± 0.35	9.89 ± 0.57	9.12 ± 0.20	9.19 ± 0.20

perimeter at tibia and femur were lower, but not significant, in the MCD group compared to control and CaS groups (Table 2).

3.5. Bone strength

The three-point bending test at the femur diaphysis (cortical site) and the femoral head's compressive strength showed a progressive increase in maximum load and work to fracture and stiffness in control rats from P28 until P150. At P70, the MCD group had lower maximal load ($p > 0.05$), work to fracture ($p > 0.05$) and stiffness ($p < 0.05$) in femoral 3-point bending test (Fig. 3A). Similar results were obtained in the femoral head compression strength test (Fig. 3B). At P150, the MCD group had an increase in maximal load, work to fracture and stiffness but remained lower than control rats in femur head compression strength test (maximum load: $p > 0.05$, work to fracture: $p > 0.05$, stiffness: $p < 0.05$). Similar results were obtained in femur 3-point bending test (maximum load: $p > 0.05$, work to fracture: $p > 0.05$, stiffness: $p > 0.05$). There was no difference between the control and CaS group in all these parameters, in both the tests. Thus, the compromised bone stiffness in early growing age is restored at P150 with Ca supplementation.

The linear regression analysis of bone stiffness vs. bone mineral content (BMC) or max load vs. BMC at the femoral head at P150 in MCD, control and CaS groups showed a positive correlation (Fig. 3C and D). There were no significant differences between the slopes among the groups (r^2: control, 0.8737, MCD, 0.8494, CaS, 0.7740:, $p = 0.06734$ for max load vs. BMC, and r^2: control, 0.8742, MCD, 0.9289, CaS 0.9167:, $p = 0.117$ for stiffness vs. BMC). Therefore, we merged all the three groups and observed the coefficient of the determinant (r^2) = 0.5785 for max load vs. BMC, and $r^2 = 0.8261$ for stiffness vs. BMC (Fig. 3C and D). Thus, a positive correlation of max load vs. BMC and stiffness vs. BMC indicate that both max load and bone stiffness increases with an increase in BMC.

4. Discussion

This study shows isolated moderate Ca deficiency in growing rats (P28–P70) results in significantly reduced volumetric BMD, poor microarchitecture and bone strength despite absence of SHPT.

Skeletal growth including microarchitecture and strength during growth periods depends on adequate nutrients viz., vitamin D, Ca, phosphorus, protein and other micro- and macronutrients. Deficiency of any of these nutrients results in reduced bone mass, low peak bone mass, and bone quality [7,16,18,27]. Severe Ca deficiency (<75% of standard Ca requirements) in various animal models have shown pronounced effects on bone growth and architecture [7,17].

The physiological correlation of rats in periods P28 and P70 corresponds to the human age of approximately one year and 13 years respectively, while P150 corresponds to the human age of 19 years [7]. Female rats are more susceptible to dietary Ca deficiency-induced bone remodeling than male rats [28]. Furthermore, sexual maturation (puberty) occurs at approximately P32–P34 in female rats, whereas in male rats, it varies considerably, ranging from P40 to P76 [29]. As the early growing age is the most critical time for bone formation, development, growth and bone mineralization, inadequate dietary Ca intake during this period has a severe adverse effect on bone [7]. Therefore, the post-weaning female rats with age P28 were chosen to induce Ca deficiency by feeding a moderate Ca-deficient diet, up to post sexual maturity (P70).

In this study, a diet containing 0.25% w/w Ca was fed to post-weaning female SD rats, to induce moderate Ca deficiency. This diet was chosen as it has roughly half of the Ca requirements for growing rats [30]. Interestingly, we found significant deterioration of bone microarchitecture despite absence of SHPT in rats on MCD at P70. We also observed a reduction in the serum P1NP, an indicator of bone formation.

The three-point bending test and compressive strength provide three important parameters of bone strength: maximum load (maximum power attained by bone until the fracture occurs), work to fracture (energy required to cause bone fracture) and stiffness (resistance offered by the bone) [31]. Bone stiffness is defined by the degree of deformation of the bone when a load is applied. As the bone strength directly correlates with the bone stiffness [32], the reduced stiffness in the MCD group indicates compromised bone strength at early growing age (P70).

We observed recovery in vBMD, trabecular and cortical microarchitecture, and bone strength (maximum load, work to fracture) in MCD group at P150 despite continuing moderately Ca deficient diet. This improvement is accompanied by normalization of serum P1NP levels in our study (Table 1). The improvement in trabecular parameters at P150, despite the continuation of a moderate Ca diet (0.25%) in the age group P70–P150, indicates that this Ca content was sufficient for this age of rats [20,33].

Our results are consistent with an earlier study; four days old female rats fed with 0.25% Ca diet up to 12 weeks had significantly lower bone weight, total bone Ca, BMC, bone volume, mineralizing surface (MS) to eroded surface ratio, bone mineral apposition rate (MAR), bone formation rate (BFR), trabecular number, and cartilage cell production as compared to the those fed with 0.5% or 1.0% Ca diet [18]. Furthermore, this study [18] also showed that switching a diet containing either 0.5% or 1.0% Ca after 12 weeks did not improve PBM, bone volume, and bone length, showing the irreversibility of poor bone quality. However, this study lacked serum/urinary biochemical and calciotropic hormone measurements, three–dimensional microarchitecture and strength analysis; thus not providing comprehensive information on subclinical Ca deficiency in rats [18].

Another study in female rats showed that non-fat dry milk solids (NFDM) fed rats from four to ten weeks had greater femur length, width, weight, BMD, bone strength and Ca/bone content as compared to those on CaCO3; however, both the groups were fed the same amount of Ca (0.4% Ca) from different source [34]. Furthermore, changes in femoral width, Ca per bone, and ultimate load were not different in these two groups after 20 weeks of diet intake. This study did not include the Ca-deficient group at initial time points. Also, it lacked calciotropic hormone and measurement of Ca metabolism-related serum and urine biochemical parameters, thus unable to elucidate the comprehensive information on subclinical Ca deficiency.

This study has few limitations: we could not perform dynamic histomorphometry and bio-material parameters such as mineral-to-matrix

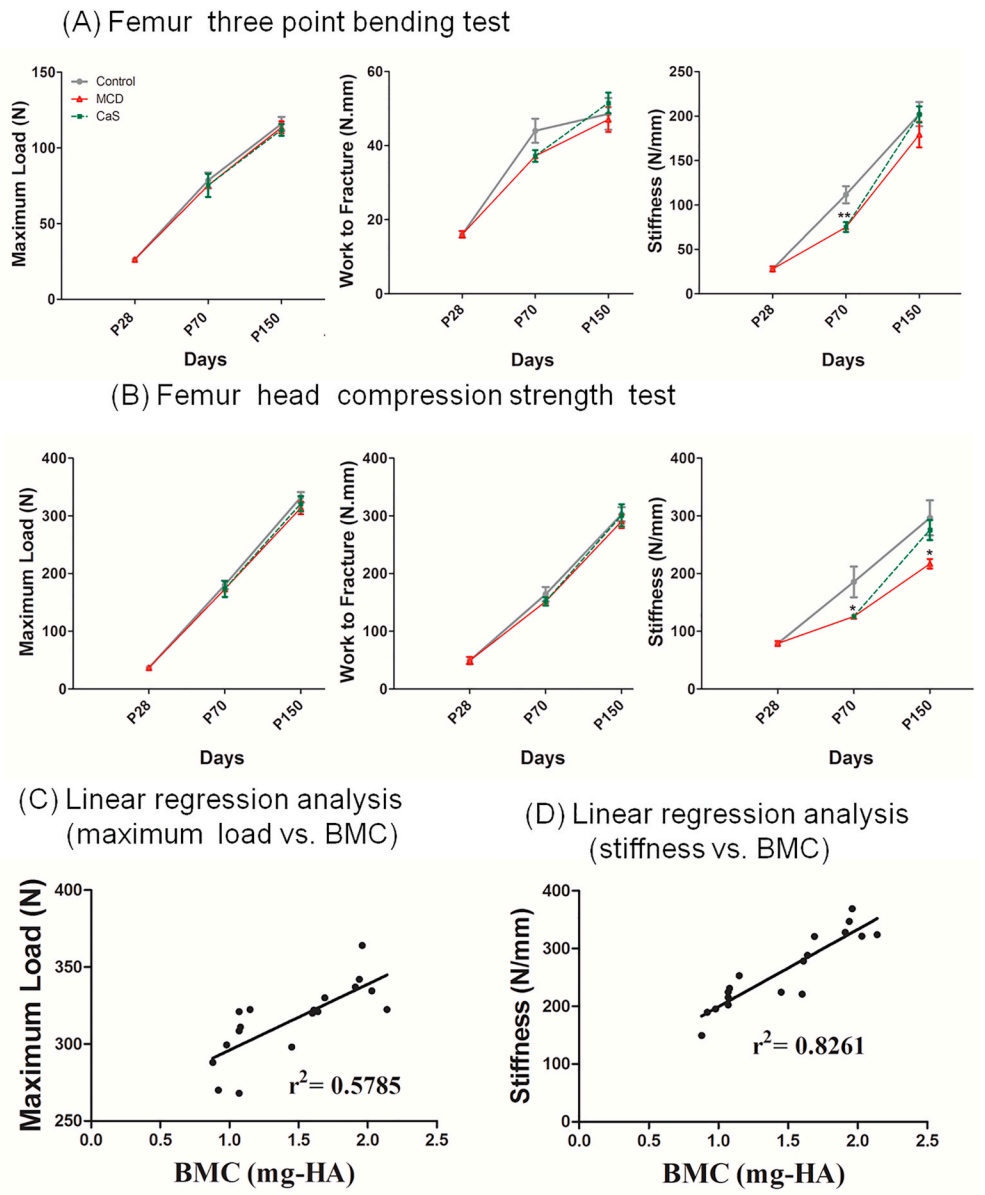

Fig. 3. Moderate calcium intake in early growing female rats decreases bone stiffness. (A) Maximum (max.) power, work to fracture and stiffness. (B) Femur compression test showing maximum load, work to fracture and stiffness. Linear regression analyses were performed across various groups between (C) BMC and maximum load, and (D) BMC and stiffness at P150 days on the femur head. The coefficient of the determinant (r2) is indicated on the graph. n ≥ 6/group, Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ Control vs. MCD group. The statistical significance was assessed by unpaired student t-test (2 groups) at P70 or one-way ANOVA (>2 groups) followed by Tukey's post-test analysis at P150.MCD; Moderate calcium deficient group, CaS; Ca Supplemented group.

ratio, assembly and alignment of collagen fibrils in bone, and crystal packaging (crystallinity). Measuring these parameters would have shed light on pathophysiological alterations of bone in subclinical Ca deficiency. Studies have reported that 0.25% Ca diet supplied adequate Ca to adult rats [19,20] but not to younger rats. Hence, an additional group (negative control), supplemented with 0.8% Ca until P70 and then fed with reduced Ca (0.25%) would have better clarified if Ca deficiency induction after attaining adulthood adversely impacted the skeleton.

Our findings collectively emphasize the importance of adequate Ca intake after weaning to acquire healthy bone mass and quality, and underscores the importance of correcting moderate/subclinical Ca deficiency during early growing periods of life. This study could have a relevance to calcium deficiency induced skeletal changes in children and adolescents in developing countries.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101033.

Credit Author Statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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