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HIGH LEVELS OF SERUM INSULIN PREVENTS BONE RESORPTION OF THE OFFSPRING BORN TO CALCIUM DEFICIENT WISTAR RATS

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ABSTRACT
Importance of calcium is well established in glucose and bone metabolism. Calcium is substantially required during gestation for proper fetal growth and development. Hence, we investigated the role of chronic maternal calcium deficiency per se on calcium homeostasis, glucose metabolism and bone structure of the offspring at 30 weeks of age. Six virgin female Wistar rats were fed on calcium deficient diet for three months for induction of calcium deficiency. These calcium deficient (CaD) females were mated with normal Wistar male rats and the offspring(male & female) born to CaD and control dams were followed for 30 weeks. Post-weaning all the offspring were fed on control diet containing adequate amount of calcium. Fasting blood glucose levels, serum calcium, vitamin D, Parathyroid Hormone (PTH) and insulin levels of the offspring were estimated using Enzyme Linked Immunosorbent Assay (ELISA). Bone calcium content of the offspring was analyzed by Atomic Absorption Spectroscopy (AAS). Bone histology was examined using H & E stain. The offspring born to CaD dams showed aberration in calcium homeostasis and increase in HOMA-IR along with an increase in serum insulin levels of the offspring born to CaD dams. Bone morphology and bone calcium levels of CaD offspring remained unaffected. This study reports that maternal calcium deficiency per se programs altered calcium homeostasis in the offspring while their bone morphology is rescued by higher insulin levels at 30 weeks of age.

KEYWORDS: Calcium, Vitamin D, Bone, Parathyroid Hormone (PTH), Insulin.

INTRODUCTION
Calcium is an important mineral for the metabolism of bone and regulation of glucose metabolism.[1,2] Calcium homeostasis in blood is mainly regulated through an integrated hormonal system. Hormonal regulation of calcium homeostasis involves two important hormones that is Parathyroid Hormone (PTH) and Vitamin D.[3,4] Studies suggest that defects in calcium metabolism in adults and their associated hormonal and intracellular alterations leads to diabetes.[5,6]

Calcium and vitamin D are substantially required during pregnancy and lactation for proper fetal growth and development.[7] It is known that maternal vitamin D deficiency is a risk factor for several metabolic aberrations affecting maternal and fetal health.[8] A study in humans has shown that maternal vitamin D insufficiency is associated with reduced bone-mineral accrual in the offspring during childhood and supplementation of vitamin D during pregnancy reduces the risk of osteoporotic fractures in their offspring.[9] Another study in Sprague-Dawley rats has reported that maternal vitamin D deficiency during pregnancy leads to increased inflammation and insulin resistance in the offspring.[10]

An association between maternal vitamin D deficiency and its effect on offspring bone and glucose metabolism is well established. But, the role of maternal calcium deficiency per se on glucose metabolism and bone health of the offspring is yet to be explored.

In this study, we investigated the role of chronic maternal calcium deficiency per se on calcium homeostasis, glucose metabolism and bone structure of the offspring at 30 weeks of age.

MATERIALS AND METHODS
All the procedures and techniques related to the use, care of animals for research and the experimental design were approved by the Institutional Animal Ethical Committee (IAEC) of the Agharkar Research Institute, Pune, India, established under the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Approval No.: 101/GO/c/99/CPCSEA.

Development of inter-generational calcium deficient model
The diets were prepared manually as per the recommendations of American Institute of Nutrition

(AIN) guidelines for rodents.[11] Control female rats (n=6) were fed with control diet (AIN93G) and CaD female rats (n=6) were fed with calcium deficient diet (Table 1). The CaD diet samples contained 0.025gm calcium/kg diet while control diet had 6.025 gm calcium. Control and CaD female rats were then mated with normal male Wistar rats and were fed on their respective diets during gestation, lactation and weaning. Litter size and the birth weight of the pups born to both the dams were recorded. Post weaning, the offspring were divided into four groups (n=6 each) viz. control male, control female, CaD male and CaD female. All the offspring were then kept on control diet containing recommended amounts of vitamin D and calcium throughout the study.

Table 1: Composition of diet

	Nutrient gm/kg	
Diet component	**Control**	**CaD**
Corn starch	397.48	397.48
Casein	200.00	200.00
Dextrinized cornstarch	132.00	132.00
Sucrose	100.00	100.00
Soybean oil	70.00	70.00
Fiber	50.00	50.00
AIN-93G-MX	35.00	35.00
Calcium[added as carbonate in AIN-93G-MX]	05.00	00.00
AIN-93G-VX	10.00	10.00
Vitamin D in AIN-93G-VX	1000 IU	1000 IU
L-cysteine	03.00	03.00
Choline bitartarate	02.00	02.00
tert-butylhydroquinone	0.014	0.014

All the nutrients are in accordance to the recommendations of AIN93G diet for rodents.
Abbreviations: AIN-93G-MX; American Institute of Nutrition 93 diet for growth-Mineral mix. AIN-93G-VX; AIN-93G- Vitamin Mix, CaD; calcium deficient diet.

Biochemical parameters of mother rats

All the animals were kept on overnight fasting (12 hours) prior to blood withdrawal. Aliquots of serum were stored at -80°C and were not thawed until analysis. Blood was withdrawn by retro-orbital puncture technique. Serum calcium was estimated using Arsenazo method involving specific binding of Arsenazo III to calcium, forming a colored complex which is measured at 650 nm in the Randox autoanalyzer against controls (Assayed human control level 2, HN 1530 and Assayed human control level 3, HE1532) provided by Randox.The minimum detectable concentration of calcium was 0.14mmol/l and the inter-assay CV was < 4%. Serum 25-Hydroxy vitamin D3 (vitamin D) level was measured by competitive inhibition method using a commercially available ELISA kit (CEA915Ge; USCN, China) and following the manufacturer's instructions. The minimum detectable concentration of 25-Hydroxy vitamin D3 was 0.09 IU and inter-assay CV was < 10%.

Biochemical parameters of the offspring

Fasting blood glucose of the offspring was measured using glucometer. The serum concentrations of calcium, vitamin D were determined using the procedures described earlier. Serum level of intact PTH was detected using rat specific PTH sandwich ELISA kit (Cat# 60-2700, Immunotopics USA) by following manufacturer's instructions at 30th week of age. The minimum detectable concentration of intact PTH was 3 pg/ml and inter-assay CV was < 4%. Serum Insulin was determined using Ultra-Sensitive Rat Insulin ELISA kit (Cat#90060, Crystal Chem Inc. USA) by following manufacturer's instructions. Minimum detectable concentration of Insulin was 0.1ng/ml and Inter-assay precision CV was ≤ 11.0%. Homeostatic Model Assessment for Insulin Resistance (HOMA- IR) was calculated for control and CaD groups using the formula:
HOMA-IR = Glucose (mmol/L) x Insulin / 22.5

Bone parameters

Wet weights of the femur bone was determined immediately after sacrifice. Bone samples were dried at 100°C for 24 hours and ashed in a muffle furnace at 580°C–600°C for another 24 hours and the ashes were weighed. The ash was powdered and was hydrolyzed using 6 M HCl. The hydrolysate solution was filtered using 42micron whatman filter paper. The filtrate was then used to determine the calcium content using AAS at a wavelength of 422.7 nm with 100% lamp current at 4 seconds measurement time.

Bone histological examination was carried out using Hematoxylin and Eosin (H&E) staining. Briefly, head portion of the femur bones were fixed in 10% neutral formalin for 3 days. The bones were then decalcified using 80% formic acid for 1 week and were embedded in paraffin to cut into longitudinal sections of 5 µm thickness. The sections were stained with H&E and were observed under a light microscope.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism software version 5.0 (GraphPad Software Inc., CA, USA) for windows. The data were expressed as mean ± SEM, and statistical analysis was carried out by two tailed unpaired t-test. Differences were considered significant if the P- value was less than 0.01 (**) or less than 0.05 (*).

RESULTS AND DISCUSSION

In this study, we developed an inter-generational chronic maternal calcium deficient Wistar rat model and investigated its effect on calcium, glucose and bone metabolism of adult offspring.

Development of calcium deficient model

Female virgin Wistar rats were kept on calcium deficient diets (CaD) for 3 months for induction of calcium deficiency. Serum calcium levels were estimated to confirm the calcium deficiency in CaD group. It was found that serum calcium levels of CaD group were significantly ($P < 0.05$) lower than the control females (Table 2). Serum vitamin D levels was also reduced in CaD group ($P < 0.01$) when compared to control group (Table 2). After confirming the induction of calcium deficiency in CaD females, they were mated with normal male Wistar rats. The respective diets of CaD and control group were continued throughout gestation and lactation. The reproductive performance of the CaD and control dams was also recorded. The litter size of the CaD dams was found to be significantly ($P < 0.01$) smaller than the control dams. While no difference in birth weights of the CaD offspring were observed when compared to the offspring born to control dams (Table 2). A similar observation was reported in a study in which maternal calcium deficiency lead to yielding of smaller litter size.[12] Another study reported no change in birth weight of the offspring born to calcium deficient dams.[13] The anthropometrical measurements of mother rats showed that the food intake and body weights of the both of the dams was not affected due to calcium deficiency (Table 2).

Table 2: Anthropometric, biochemical parameters and reproductive performance of control and CaD mother rats.

	Control	CaD
Serum calcium [mmol/l]	12.21±0.20	7.31±1.62 *
Serum 25-hydroxy vitamin D3 (IU)	20.13 ± 0.92	16.12 ± 0.68**
Food Intake (gms)	13.4 ± 0.86	12.8 ± 0.49
Body weight (gms)	196.6 ± 7.52	232.1 ± 40.0
Litter size	7.75 ± 0.28	6 ± 0.32**
Offspring birth weight (gms)	6.5 ± 0.43	5.77 ± 0.38

Values shown as mean ± SE for control (AIN93G) and Calcium deficient (CaD) dams (* P value < 0.05, ** P<0.01). Comparison is done between the values obtained for CaD female offspring against control female offspring at 30th week of their age by using two tailed unpaired t-test.

Effect of maternal calcium deficiency on the offspring

Calcium homeostasis of the offspring

The offspring born to CaD and control group were followed till 30 weeks of age. Post -weaning, the offspring of both the groups were fed on control diet with adequate amounts of calcium and vitamin D. Comparisons were made between control male vs CaD male and control female vs CaD female.

An aberration in calcium homeostasis was observed in CaD male and female offspring as evidenced by their significantly ($P<0.05$) low serum calcium levels (Table 3). Vitamin D levels of CaD offspring were also found to be low ($P<0.05$, Table 3). PTH is considered to be a regulatory hormone of calcium homeostasis. [14]. In our study the serum PTH levels of CaD male and female offspring were significantly higher ($P < 0.05$) than control male and female offspring respectively (Table 3).

Bone calcium and morphology of the offspring

To investigate the effect of maternal calcium deficiency on the bone metabolism we examined their bone morphology and determined the bone calcium content at 30th week of age.

No differences in the wet weights of the femur bones of CaD offspring was observed when compared to control offspring. The bones were then dried and ashed and the ashes were weighed. The bone ash weight of the CaD offspring also did not show any difference when compared to control offspring. Bone calcium content was determined in these ashes and no differences in bone calcium content of the offspring born to the control and calcium deficient dams was observed at 30th week of age ($P < 0.05$, Table 1). Further bone histological analysis showed no bone deformities in CaD offspring when compared to control offspring as evidenced by their normal trabecular structure (Fig.1). These observations suggest that bone calcium content of the offspring is not affected due to maternal calcium deficiency at 30 weeks of age.

Surprisingly, no bone resorption was observed in spite of higher PTH levels in the CaD offspring(Fig.1 and Table 1). Although it is known that a deregulation of calcium homeostasis and higher PTH levels cause alterations in bone mineral content and morphology.[15] Higher PTH

levels are known to increase bone resorption in adults.[16] An inter-generational study in humans reported that parental calcium deficiency or vitamin D deficiency may also lead to decreased bone mineral density of their children.[17] These results further prompted us to identify the causal mechanism behind maintenance of bone structure despite of impaired calcium homeostasis.

Glucose metabolism of the offspring

Impaired calcium metabolism is known to cause higher insulin production or Insulin Resistance(IR).[2] It is also postulated that metabolic syndrome may prevent bone resorption and thus maintains the bone mineral density and its structure.[18] A study has shown positive association between Type 2 diabetes mellitus and bone structure measured by quantitative ultrasound and suggested that metabolic abnormalities leads to healthy bone structure in elderly subjects.[19] Thus, we hypothesized that CaD offspring might have higher insulin levels that are rescuing their bone deformities, despite of their altered calcium homeostasis. We therefore investigated the glucose metabolism of the offspring.

We observed no difference in fasting blood glucose levels of both male and female CaD offspring when compared to control male and female offspring respectively (Table 3). But, the Insulin levels of the both male and female CaD offspring were found to be significantly (P<0.01) higher than the control male and female offspring (Table 3). This observation indicates that maternal calcium deficiency programs impaired calcium homeostasis in CaD offspring which further leads to hyperinsulenimia, despite feeding the adequate amounts of calcium post-weaning. Further calculation of HOMA-IR showed that HOMA-IR values of CaD offspring was significantly (P<0.01) higher than the control offspring indicating an onset of insulin resistance in CaD offspring (Table 3). These observations suggest that higher insulin levels plays a role in maintenance of bone structure in the offspring born to calcium deficient mothers.

Table 3: Biochemical and bone parameters of the offspring

	Control female	CaD female	Control male	CaD male
Serum calcium (mmol/L)	2.53 ± 0.24	1.86± 0.08*	2.05± 0.04	2.17± 0.02*
Vitamin D (IU)	19.82 ± 0.83	15.03 ± 0.91**	21.23± 0.99	21.01± 0.89
Parathyroid hormone (pg/ml)	272 ± 22.3	399.32 ± 34.01*	284 ± 18.2	357 ± 26.3*
Insulin(ng/ml)	22.18 ± 1.10	34.69 ± 0.69**	23.79 ± 1.51	35.73 ± 1.04**
Fasting blood glucose(mg/dl)	4.23±0.05	4.16±0.13	3.57±0.17	3.21±0.05
HOMA-IR	4.16 ± 0.25	6.41 ± 0.49**	3.77 ± 0.33	5.09 ± 0.41*
Bone wet weight (gm)	0.53 ± 0.03	0.49 ± 0.02	0.68 ±0.04	0.61 ±0.02
Bone ash weight (mg)	271.7 ± 19.22	228.3 ± 9.45	364 ± 16.27	326 ± 6.90
Bone calcium (mg/L)	2.31 ± 0.10	2.03 ± 0.02	3.03 ± 0.2	2.80 ± 0.07

Values shown as mean ± SE for control offspring and Calcium deficient (CaD) offspring (* P value < 0.05, ** P<0.01). Comparison is done between the values obtained for Control male Vs CaD male offspring and Control female vs CaD female offspring at 30th week of their age by using two tailed unpaired t-test.

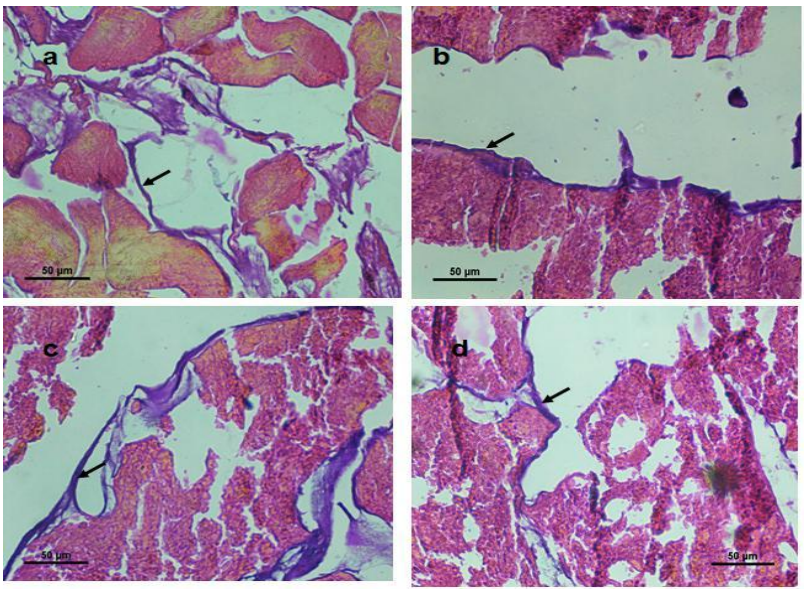

Fig. 1 Bone histology (femur head) of male and female offspring.

Fig.1 Bone histology (femur head region) of offspring. No trabecular changes were observed in (a) CaD female offspring when compared to (b) Control female offspring. Similarly, no trabelcular changes were

observed in (c) CaD male offspring when compared to (d) Control male offspring. The arrow indicates trabeculae in the head portion of femur bone.

CONCLUSION

We conclude that maternal calcium deficiency per se causes altered calcium homeostasis leading to increase in insulin levels and onset of insulin resistance in the offspring. Interestingly, the condition of hyperinsulinemia in CaD offspring could not be reversed by supplementing adequate amount of calcium to them post weaning. The bone structure of the offspring born to calcium deficient mothers is not altered probably due to increased insulin levels in the offspring which might rescue the bone resorption, despite an increase in PTH levels. To our knowledge, this is the first inter-generational study reporting that maternal calcium deficiency per se programs hyperinsulinemia in the offspring which prevents their bone resorption. Further clinical investigations are required to observe the long term effect of maternal calcium deficiency on the metabolic health of their offspring.

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REFERENCES

1. Munro Peacock, Calcium Metabolism in Health and Disease. Clin J Am Soc Nephrol, 2010; 5: S23–S30.
2. Sun G, Vasdev S,Martin GR,Gadag V,Zhang H. Altered calcium homeostasis is correlated with abnormalities of fasting serum glucose, insulin resistance, and beta-cell function in the Newfoundland population., 2005; 54: 3336-9
3. Potts JT, Gardella TJ: Progress, paradox, and potential: Parathyroid hormone research over five decades. Ann N Y Acad Sci., 2007; 1117: 196–208.
4. Jurutka PW, Whitfield GK, Hsieh JC, Thompson PD, Haussler CA, Haussler MR: Molecular nature of the vitamin D receptor and its role in regulation of gene expression. Rev Endocr Metab Disord, 2001; 2: 203–216.
5. Resnick LM.Hypertension and abnormal glucose homeostasis. Possible role of divalent ion metabolism. Am J Med., 1989; 87: 17S–22S.
6. Saltevo J, Niskanen L, Kautiainen H, Teittinen J, Oksa H, Korpi-Hyövälti E, Sundvall J, Männistö S, Peltonen M, Mäntyselkä P, Vanhala M.Serum calcium level is associated with metabolic syndrome in the general population: FIN-D2D study. Eur J Endocrinol, 2011; 165: 429-34.
7. Christopher S. Kovacs. Calcium and Bone Metabolism in Pregnancy and Lactation. The Journal of Clinical Endocrinology & Metabolism, 2001; 86: 2344-2348.
8. Karras SN, Anagnostis P, Bili E, Naughton D, Petroczi A, Papadopoulou F, Goulis DG. Maternal vitamin D status in pregnancy and offspring bone development: the unmet needs of vitamin D era. Osteoporos Int., 2014; 25: 795-805.
9. MK Javaid, SR Crozier, NC Harvey, CR Gale, EM Dennison, BJ Boucher, NK Arden, KM Godfrey, C Cooper. Maternal vitamin D status during pregnancy and childhood bone mass at age 9 years: a longitudinal study. The LANCET, 2006; 367: 36–43.
10. Zhang H,Chu X,Huang Y,Li G,Wang Y,Li Y,Sun C.Maternal vitamin D deficiency during pregnancy results in insulin resistance in rat offspring, which is associated with inflammation and IKbα methylation., 2014; 57: 2165-72.
11. Reeves PG, Nielsen FH, Fahey GC Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr, 1993; 123: 1939–1951.
12. Schmidt CM & Hood WR. Calcium Availability Influences Litter Size and Sex Ratio in White-Footed Mice (Peromyscus leucopus). PLoS ONE, 2012; 7: 41402. doi:10.1371/journal.pone.0041402.
13. Bergel E and Belizán JM. A deficient maternal calcium intake during pregnancy increases blood pressure of the offspring in adult rats. BJOG, 2002; 109: 540-5.
14. Potts JT, Gardella TJ: Progress, paradox, and potential: Parathyroid hormone research over five decades. Ann N Y Acad Sci., 2007; 1117: 196 –208.
15. H. Chen, D. Hayakawa, S. Emura, Y. Ozawa, T. Okumura and S. Shoumura. Effect of low or high dietary calcium on the morphology of the rat femur. Histol Histopathol, 2002; 17: 1129-1135.
16. Kroll MH. Parathyroid hormone temporal effects on bone formation and resorption.Bull Math Biol., 2000; 62: 163-88.
17. A. Ganpule, C. S. Yajnik, C. H. D. Fall, S. Rao, D. J. Fisher, A. Kanade, C. Cooper, S. Naik, N. Joshi, H. Lubree, V. Deshpande, and C. Joglekar. Bone Mass in Indian Children—Relationships to Maternal Nutritional Status and Diet during Pregnancy: the Pune Maternal Nutrition Study. The Journal of Clinical Endocrinology & Metabolism, 2006; 91: 2994 –3001.
18. Peng Xue • Ping Gao • Yukun Li. The association between metabolic syndrome and bone mineral density: a meta-analysis. Endocrine, 2012; 42: 546–554.
19. M. Bullo, M. Garcia-Aloy, J. Basora, M.I. Covas, J. Salas-Salvado. Bone Quantitative Ultrasound Measurements In Relation To The Metabolic Syndrome And Type 2 Diabetes Mellitus In A Cohort Of Elderly Subjects At High risk Of Cardiovascular Disease From The Predimed Study. The Journal of Nutrition, Health & Aging, 2011; 15: 939-944.