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Original Article

Role of Low Calcium and High Calcium Diet on Adipocyte Metabolism with Respect to Serum Parathyroid Hormone (PTH) Levels in Male Wistar Rats

Das Sandeep and Choudhuri Dipayan*

Department of Human Physiology,
Tripura University (A Central University),
Suryamaninagar, Agartala, Tripura – 799 022, India

Abstract

Dietary calcium has recently emerged as a potential candidate for therapeutic use against obesity, the major health hazard of the present century. However, there are controversies regarding the role of calcium diet on adipocyte metabolism. We used healthy male wistar rats and fed them with the control diet, low and high calcium diet for three consecutive months. Low calcium diet group showed significantly higher weight gain, accumulation of adipose tissue and increased adiposity index. High calcium diet provided a protection against dyslipidemia by reducing the serum cholesterol and triglyceride. Serum calcium level and PTH level varied significantly in low calcium diet group compared to other two groups. The results of our study revealed that dietary calcium regulates the adiposity of male wistar rats when fed with low and high calcium diet for a long period by altering both lipogenesis and lipolysis pathways within the adipocytes along with changes in serum PTH level.

Introduction

Obesity has emerged as a major epidemic in the present century and is projected to become a major cause of disability and premature deaths in near future all over the world including in Asian countries (1). A substantially large body of evidence has emerged since the beginning of this century that support the view that dietary calcium plays an important role in the regulation of energy metabolism and in modulating obesity risk (2). The relationship between calcium intake and body weight was first noted by MaCannon et al. (3) which was further substantiated by Zemel et al. through series of experiments, especially by using agouti mouse model (4). Various epidemiological studies over the period have confirmed the role of calcium diet in body weight regulation in different groups of the population (5). An inverse relationship between calcium intake and obesity in terms of body weight gain and accumulation of fat and its mechanism has been discussed by several authors during the period (6). A careful analysis of the information indicates contradicting results and lack of consensus owing

***Corresponding author :**

Dr. Dipayan Choudhuri, Associate Professor, Department of Human Physiology, Tripura University (A Central University), Suryamaninagar, Agartala, Tripura – 799 022, India, Ph.: 9436461010, E-mail address: dipayanchoudhuri@gmail.com

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mainly due to the use of different experimental models to establish the calcium and obesity hypothesis. Therefore, it was proposed that further studies are needed to address the mechanisms involved in calcium intake induced changes in adipocyte metabolism keeping in view the limitations of previous study designs (7).

The serum calcium level is tightly regulated by the secretion of PTH from parathyroid gland which in turn regulates the calcium reabsorption in kidney and bones. At the same time, PTH also stimulates the secretion of 1, 25-dihydroxyvitamin D [1, 25-$(OH)_2$ D] from kidney which activates the vitamin D receptor (VDR) in the gut for calcium absorption. Thus, both PTH and [1, 25-$(OH)_2$ D] seems to monitor the calcium homeostasis with respect to calcium level in our body (8). Researchers have suggested that low dietary calcium affects both lipolytic and lipogenic process in adipocytes (9). Low dietary calcium is proposed to act via regulation of parathyroid hormone and 1, 25-dihydroxyvitamin D that induces an elevation in adipocyte cytosolic calcium level (10). High calcium diet was found to be associated with higher rate of fat oxidation in non-obese healthy adults (11). However, rest of the experimental and epidemiological studies suggested that this might happen only in the energy-restricted diet (12).

Keeping the above facts in view we planned to evaluate the effect of chronic exposure to both low and high calcium diet on adipocyte metabolism in normal non-obese male rats who are neither on a calorie restricted nor on a high calorie diet.

Materials and Methods

Animals

Thirty (n=30) healthy male Wistar albino rats (145-160 gm) were used for the study. The rats were purchased from CPCSEA, registered animal supplier. The animals were maintained in the animal house of Tripura University Tripura, India, at a constant temperature of (23±3°C) with 12-hour light/dark cycle. The animals were supplied with a balanced diet based on AIN-93G diet (13) and water *ad libitum*. The animals were acclimatized to their living conditions in the university animal house for 1 week before inclusion in their study group. The entire study protocol was approved by the Institutional Animal Ethical Committee (Ref.No: TU/IAEC/2014/VIII/2-6 dated 12.09.2014).

Chemicals and kits

Kits for the measurement of serum triacylglycerol (TAG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), serum calcium and glucose level were purchased from Erba Mannheim (Germany). PTH Intact ELISA kit was purchased from RayBiotech, Inc., (USA). Hematoxylin, eosin, ethanol, paraffin, chloroform, acetyl coenzyme lithium salt and Nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH) were purchased from Himedia Laboratories (India). Malonyl coenzyme A lithium salt was purchased from Sigma-Aldrich (USA). Glycerol quantification kit purchased from Abcam (UK).

Experimental design

After the acclimatization period, animals were divided randomly into three groups consisting of 10 animals in each group. Group 1 received standard diet based on AIN-93G purified diet (13) and was considered as the control group. Group 2 received the low calcium diet and Group 3 received the high calcium diet. The compositions of different diets are mentioned in (Table I). The low calcium group received 0.25% of calcium in the diet and the high calcium group received 1% of calcium in diet expressed in (mg Ca/g dry mass), the source of calcium was in calcium carbonate form. These calcium levels represented 50% and 200% of the National Research Council's calcium requirements for rats, respectively (14). The following diets were isocaloric in nature and differed only in calcium content. These special diets were purchased from National Centre of Laboratory and Animal Services, National Institute of Nutrition (Indian Council Medical Research Unit, Govt. of India), Hyderabad, India. The animals were fed with these three different types of diets for three consecutive months. Body weight of the animal was recorded weekly and food consumption was recorded daily during the whole period of treatment.

TABLE I : Diet Ingredients.

Sl. No.	*Ingredients*	*Standard Diet- Group I (%)/(gm/ 100 gm diet)*	*Low Calcium Diet- Group II (%)/(gm/100 gm diet)*	*High Calcium Diet- Group III (%)/(gm/ 100 gm diet)*
1	Roasted Bengal gram	60.0	60.0	60.0
2	Wheat	22.5	22.5	22.5
3	Skim Milk Powder	5.0	5.0	5.0
4	Casein	4.0	4.0	4.0
5	Ground nut oil	4.0	4.0	4.0
6	Mineral Mix (AIN93)	4.0	4.0[A]	4.0[B]
7	Vitamin Mix (AIN93)	0.5	0.5	0.5

A- Mineral Mix- The calcium content is 0.25% of calcium in diet expressed in (mg Ca/g dry mass).
B- Mineral Mix- The calcium content is 1% of calcium in diet expressed in (mg Ca/g dry mass) [14].
The source of calcium is in the form of calcium carbonate ($CaCO_3$).

At the end of treatment period, all the animals were fasted overnight and sacrificed the next day following Indian Council of Medical Research (ICMR) guide lines (15). The blood was collected immediately by cardiac puncture in a clotted vial and left to coagulate at 4°C and then centrifuged at 3000 x g for 15 minutes and the clear serum was collected for biochemical analysis. The adipose tissue including epididymal, perirenal and omental fat tissues was dissected out from each rat and the tissues were weighed immediately to avoid evaporative weight loss and kept at –80°C for downstream analysis.

Anthropometric Measurements and Daily Food Intake:

The final body weight was calculated at the end of the study. The adiposity index of each animal was calculated as: 100 x (epididymal + perirenal + omental fat tissue)/ final body weight (16). Lee index was also calculated using the equation: cube root of body weight (g)/nose to anal length (cm) and was considered an anthropometric marker of obesity. When the value of Lee index is greater than 0.30 it indicates the phenotype of obesity (17). Food consumption (gm/day/rat) was calculated daily by subtracting the amount of food left over in each cage for each rat from the measured amount of food provided at the previous day. The mean food consumption for each rat was calculated by dividing the amount of food eaten in a week divided by seven. The average food consumptions were represented in gm/day/rat. The body weight (gm) for each rat was determined once a week.

Biochemical analysis of serum:

Serum triglyceride (TG), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) levels were assayed by enzymatic colorimetric method using standard commercial kits (Erba, Mannheim) and with a semi auto-analyzer (Analytica, Biochemistry Analyzer, RMS). Low-density lipoprotein cholesterol (LDL-C) levels were calculated using the equation presented in the study by Friedewald et al. (18). Serum calcium levels were measured by the Arsenazo dye method using a commercial calcium kit (Erba, Mannheim).

The calcium related hormone, PTH was measured from serum using an Intact PTH ELISA kit (RayBiotech, Inc., USA) according to the manufacturer's instruction.

Preparation of Adipose Tissue Homogenate and determination of Fatty Acid Synthase activity (FAS):

The epididymal adipose tissue samples were immediately homogenized in ice-cold buffer containing 0.25 mol/L sucrose, 1 mmol/L dithiothreitol and 1 mmol/L EDTA, pH 7.4. The cytosolic fractions were obtained by centrifugation at 1,00,000 x g for 1 h at 4°C. The FAS activity in the epididymal adipose tissue was measured in duplicate by measuring the malonyl CoA–dependent oxidation of NADPH at 37°C (19). One unit of enzyme activity represents 1 mmol of NADPH oxidized per minute at 37°C. The results were expressed as mU/g of adipose tissue.

Lipolysis Assay

The rate of glycerol release from adipose tissue is considered to be an index of lipolytic activity. Around 100 mg of adipose tissue from each animal of three groups was taken and with the help of sterilized scissor it was chopped into small pieces and washed with ice cold PBS (pH=7.4). Now the tissue was placed in a 10 ml flask containing 2 ml of a solution containing Krebs/Ringer/phosphate buffer KRB buffer (pH 7.4), 40 mg of BSA, and 0.2 mg of glucose, under an atmosphere of carbogen (O_2:CO_2,95:5) at a

shaking rate of 90 cycles/min; the incubations were maintained at 37°C for 1 hour. The reaction was stopped on ice and medium was carefully collected for measurement of glycerol released (20). The glycerol content in the media was measured at 570 nm colorimetrically by a glycerol quantification kit (Abcam, UK) in a microplate reader. The lipolytic rate was estimated by the time course of glycerol release and the value of glycerol content was expressed in nmol/100 mg of tissue/hour.

Triglyceride Quantification from Adipose Tissue:

Lipids were extracted from white adipose tissue according to Folch et al. (21). Around 50 mg of white adipose tissue was homogenized in 2 ml of chloroform: methanol solution (v/v, 2:1), shaken for 20 minutes and centrifuged at 1800 rpm for 10 min. The supernatant was separated, mixed with 0.9% NaCl and centrifuged at 2000 rpm for an additional 20 min. The organic phase was dried and dissolved in 3% Triton X-100 out of which tissue triglyceride was estimated using the same above kit used for plasma triglyceride quantification and the value was presented as milligrams of triglyceride per gram (mg/g) of adipose tissue.

Histological analysis of Adipose Tissue:

The isolated adipose tissue was washed gently in phosphate buffer saline (PBS) and fixed in 10% neutral buffered formalin. After that the adipose tissue was passed through the dehydration and infiltration steps followed by embedding in paraffin, then 5 (µm) microtome sections from paraffin block were taken for Hematoxylin–Eosin (H & E) staining. The stained slides were viewed under a microscope (Leica DM400 B LED, Germany) using 20X magnification. Now for morphometric analyses, at least 100 adipocytes were randomly selected and captured for each animal. The adipocyte area was calculated using Image J software, (National Institute of Health, Bethesda, MD, USA) and expressed as average cell surface area (5 μm^2).

Statistical analysis

The results were presented as Means±SEM. The differences between the groups were analyzed by one way ANOVA followed by Tukey HSD posthoc test using SPSS (Statistical Program for the Social Sciences) 16.0 for Windows. Statistical significance was considered at $p<0.05$.

Results

Effect of different calcium diet on anthropometric data and food intake:

The body weight increased in all the groups through out the entire period of study. However, the different types of calcium diet with changes in calcium content in the diet found to have a significant role in body composition at the end of the study. Rats fed with low calcium diet (0.25%) showed significantly higher ($p<0.01$; Table II) final body weight and weight gain while the high calcium diet (1.0%) fed rats showed significantly ($p<0.05$; Table II) reduced final body weight and weight gain in comparison to the control group rats. Lee index, an anthropometric marker for detecting obese phenotype in rodents (17), did not show any significant difference among the animals from different groups. This implies that in spite of having a greater body weight in group 2 animals compared to other two groups; these animals cannot be considered obese. The adipose tissue and adiposity index altered significantly at the end of the study by the different types of calcium diet. The adipose tissue and adiposity index was significantly

TABLE II: Anthropometric data and food intake.

Parameters	*Group 1 (Control Diet)*	*Group 2 (Low Calcium Diet)*	*Group 3 (High Calcium Diet)*
Initial body weight (gm)	142.5±2.1	146.8±3.2	148.3±3.06
Final body weight (gm)	339.8±6.8	387.3±4.7^{a**}	321.3±4.4^{b*}
Body Weight gain (gm)	197.3±8.3	240.5±3.3^{a**}	172.5±5.5^{b*}
Lee Index	0.295±0.0042	0.306±0.0033	0.288±0.0030
Adipose tissue mass (gm)	14.08±0.69	19.1±0.52^{a**}	11.16±0.22^{b**}
Daily food intake (gm/day/rat)	19.3±0.96	18.083±0.93	19.6±0.93

Values represent Mean±SEM of ten (10) rats in each group; a = Group 1 vs Group 2; b = Group 1 vs Group 3. Significant level; * = p<0.05; ** = P<0.01.

higher in-group 2 (p<0.01 and p<0.05; Table II and Fig. 1) and significantly lower in group 3 (p<0.01 and p<0.05; Table II and Fig. 1) rats in comparison to the control group. There was no such significant difference in mean daily food intake among the groups through out the entire period of study (Table II).

Effect of dietary calcium on biochemical parameters of serum:

Low calcium diet seemed to have no significant effect on the lipid profile of group 2 animals in comparison to the control animals at the end of the study. While high calcium diet showed a positive effect by significantly increasing (p<0.05; Table III) the serum HDL and significantly decreasing (p<0.05; Table III) the serum total cholesterol and LDL-cholesterol level of group 3 animals as compared to the control group at the end of the study. The blood glucose level did not show any significant changes among the three groups at any point of time. The serum calcium level as expected was significantly lower (p<0.05; Table III) in group 2 rats and significantly higher in group 3 rats (p<0.05; Table III) as compared to control group. Dietary calcium showed to regulate the PTH secretion by significantly increasing the (p<0.01; Table III) serum PTH level in group 2 animals fed with low calcium diet and significantly decreasing (p<0.05; Table III) in group 3 animals fed with high calcium diet when compared to the control group after three months of study.

TABLE III : Serum biochemical parameters.

Parameters	*Group 1 (Control Diet)*	*Group 2 (Low Calcium Diet)*	*Group 3 (High Calcium Diet)*
Total cholesterol (mg/dl)	98.8±3.9	111.3±4.7	83.3±2.4[b*]
Triglyceride (mg/dl)	77.3±2.6	81±2.7	70.8±2.6
HDL- cholesterol (mg/dl)	35±1.3	33.3±1.2	41.1±1.9[b*]
VLDL- cholesterol (mg/dl)	15.4±0.5	16.2±0.5	14.1±0.5
LDL-cholesterol (mg/dl)	48.3±4.8	61.8±5.2	28.0±3.7[b*]
Blood glucose (mg/dl)	93.0±6.7	107.6±6.3	88.5±4.7
Serum calcium (mmol/l)	2.1±0.09	1.7±0.06[a*]	2.4±0.09[b*]
PTH (pg/ml)	46.5±2.8	65.5±3.3[a**]	36.8±2.4[b*]

Values represent Mean±SEM of ten (10) rats in each group; a = Group 1 vs Group 2; b = Group 1 vs Group 3. Significant level; *=p<0.05; **=p<0.01.

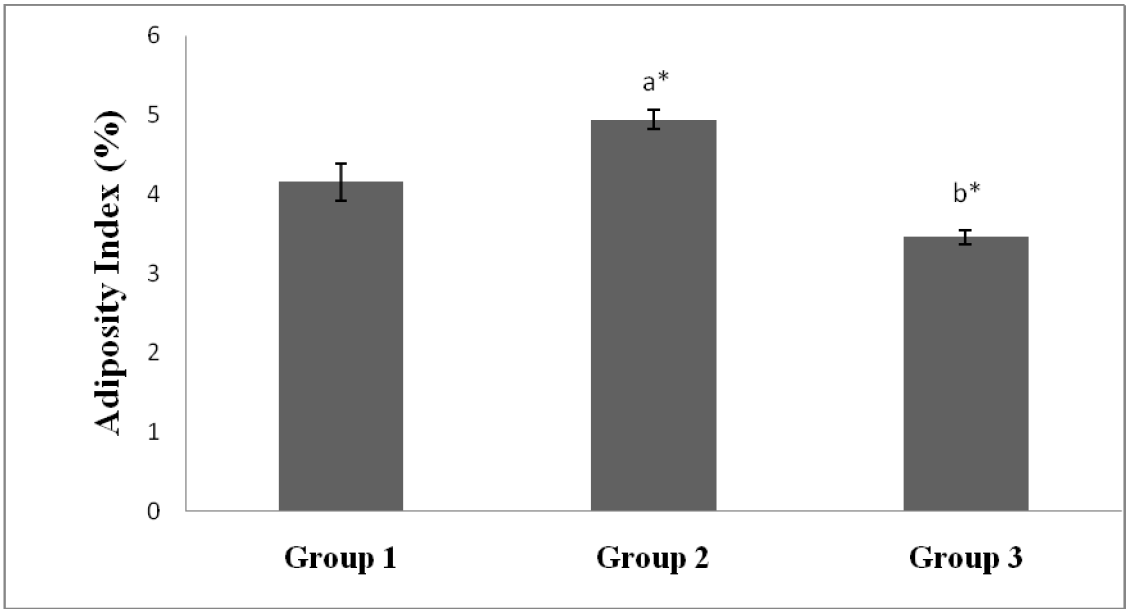


Fig. 1 : Adiposity Index
Values represent Mean±SEM of ten (10) rats in each group; a = Group 1 vs Group 2; b = Group 1 vs Group 3. Significant level; *=p<0.05; **=p<0.01.

Effect of dietary calcium on fatty acid synthase (FAS) activity and triglyceride amount in adipose tissue:

The results of our study showed that the different types of calcium diet effected the lipogenic pathway by significantly regulating the fatty acid synthase (FAS) activity, an important lipogenic enzyme and triglyceride accumulation in adipose tissue. The fatty acid synthase (FAS) activity and triglyceride level in adipose tissue was significantly higher ($p<0.01$; Fig. 2 and 3) in group 2 animals and significantly lower ($p<0.01$, $p<0.05$; Fig. 2 and 3) in group 3 rats as compared to the control group.

Effect of dietary calcium intake on lipolytic activity:

The different amount of calcium content in our various diets seemed to affect the lipolytic activity thereby regulating the energy metabolism of our animals. The high calcium diet fed rats of group 3 found

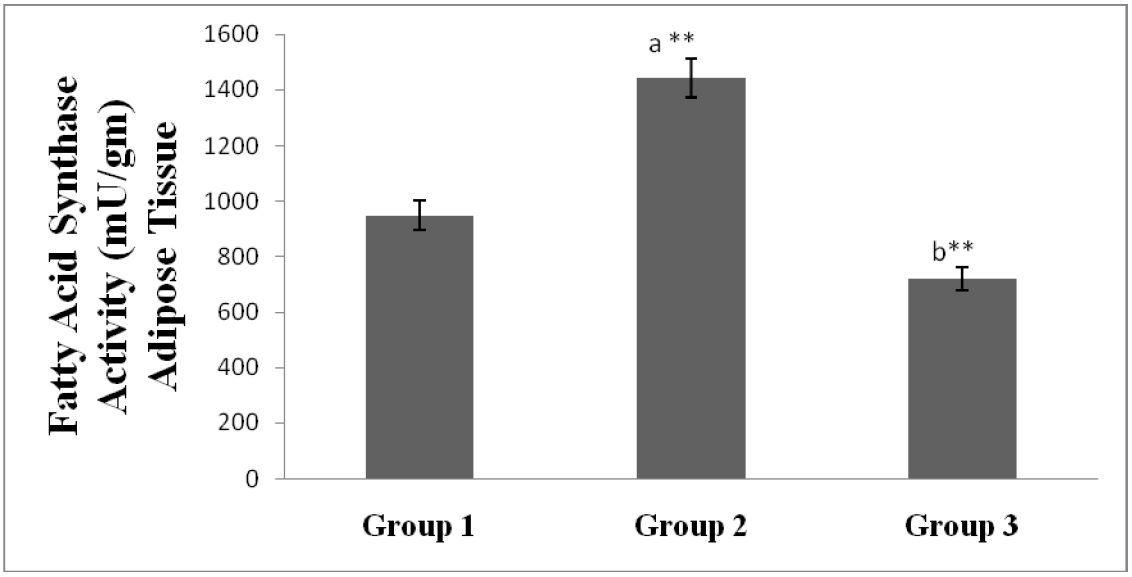


Fig. 2: Fatty Acid synthase activity (mU/gm) in Adipose tissue
Values represent Mean±SEM of ten (10) rats in each group; a = Group 1 vs Group 2; b = Group 1 vs Group 3. Significant level; * = $p<0.05$; ** = $p<0.01$.

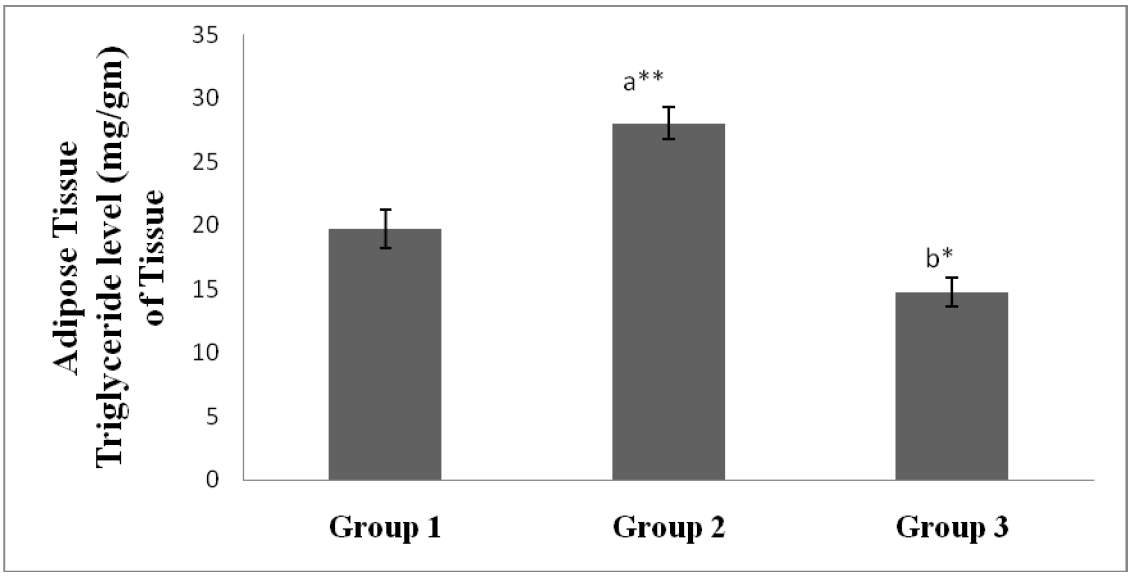


Fig. 3: Adipose tissue triglyceride (mg/gm) quantification
Values represent Mean±SEM of ten (10) rats in each group; a = Group 1 vs Group 2; b = Group 1 vs Group 3; Significant level; * = $p<0.05$; ** = $P<0.01$.

to have significantly greater ($p<0.01$; Fig. 4) amount of glycerol content while the low calcium fed rats of group 2 found to have significantly lower ($p<0.05$; Fig. 4) level of glycerol content in the adipose tissue incubated medium as compared to control group.

Effect of different calcium and control diet on adipocyte size of rats:

The size of adipocytes was found to be altered by the different types of calcium diet. The low calcium diet fed animals of group 2 found to have significantly greater ($p<0.05$; Fig. 5-A and B) adipocyte area while the high calcium diet fed animals of group 3 showed significantly lower ($p<0.05$; Fig.5-A and B) adipocyte area as compared to the control group at the end of the treatment period.

Discussion

The results of present study revealed that dietary calcium plays a significant role in regulating adiposity and body weight in rats exposed to different levels of calcium. The inverse association of dietary calcium with body weight gain and adipose tissue mass observed in our study was reported by several workers in both humans and in rodents (22, 16). Zemel et al. from their study with transgenic mice over expressing agouti protein showed that weight gain and fat pad mass was decreased in animals fed with high calcium diet in comparison to the animals fed with low calcium diet (23). Epidemiological evidence provided by both Zemel et al. (4) and Davis et al. (22) confirmed that weight gain in human subjects was inversely related to calcium intake. Recently He et al. (16) observed that when Sprague-Dawley rats were fed with low (0.30%) and very low calcium diet (0.15 %) for several weeks it leads to higher accumulation of visceral fat mass as compared to the control group due to the anti lipolytic activity of low calcium diet in adipocytes of rats.

In this study, though we observed that increase in weight gain and increase in adipose tissue mass in rats fed with low calcium diet, the rat had not become obese as observed from Lee index. This might be due to the fact that the treatment period was less in comparison to many other studies or the animals were not genetically predisposed to become obese.

Dyslipidemia is often associated with obesity by altering the lipid profile during obese condition. There are very few studies regarding the effect of calcium

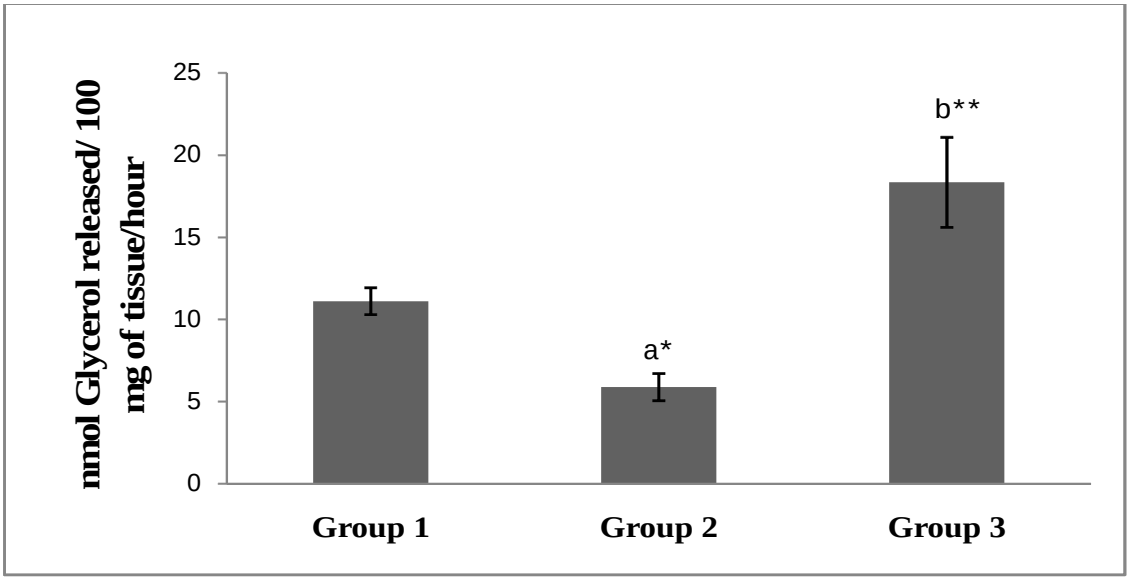
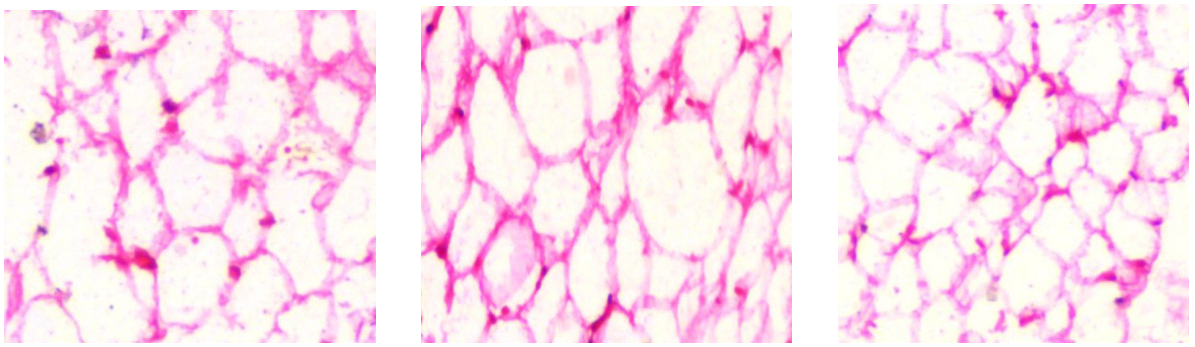


Fig. 4 : Ex vivo lipolytic activity of Adipose tissues
Values represent Mean±SEM of ten (10) rats in each group; a = Group 1 vs Group 2; b = Group 1 vs Group 3; Significant level; * = p<0.05; ** = p<0.01.

(A)



(B)

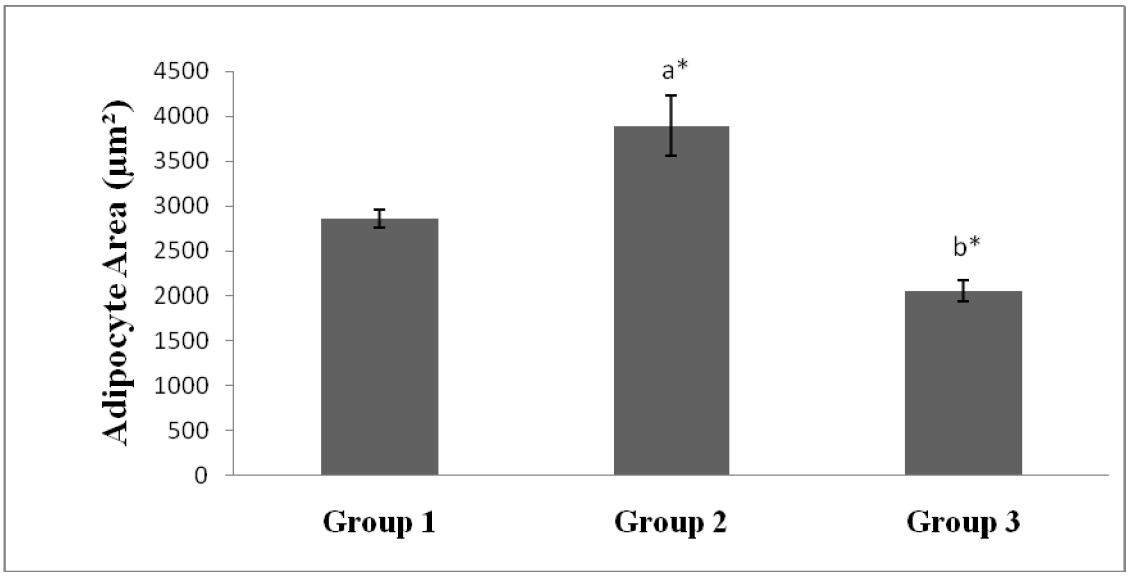


Fig. 5 : Morphological analysis of adipose tissues

Fig. (A): Shows histological sections of epididymal adipose tissue viewed under 20X magnification of three different groups fed with three different diets as: Group 1-control diet; Group-2 low calcium diet; Group 3- high calcium diet for three months.

Fig. (B): Shows the adipocyte area (µm^2) from the histological sections of the above three groups. Values represent Mean±SEM of ten (10) rats in each group; a = Group 1 vs Group 2; b = Group 1 vs Group 3. Significant level; *=p<0.05; **=p<0.01.

on lipid profile where some show that dietary or calcium supplementations cause a significant decrease in total cholesterol and low density lipoprotein (LDL) cholesterol and an increase in high density lipoprotein (HDL) cholesterol (24) while other reports do not support this theory (25). In our study, high calcium diet showed a beneficial effect on lipid profile reducing the total cholesterol level, triglyceride level, LDL level and increased the HDL level. However, low calcium fed rats of group 2 did not show any significant changes in lipid profile compared to the control group. The saponification of free fatty acids in presence of calcium decreases the intestinal absorption of lipids in gut thereby regulating the lipid profile (24). Similar to our study, Shifdar et al. showed calcium supplementation in overweight men reduced the risk of cardiovascular diseases by decreasing the serum cholesterol, LDL-cholesterol level and systolic blood pressure (26). So the inhibition of absorption of fatty acids could reduce the serum

cholesterol level by decreasing the synthesis of very low density lipoproteins (VLDL) and by up regulating the entry of low density lipoprotein (LDL) inside the liver. Calcium may also bind to the bile acids increasing the fecal excretion through conversion of cholesterol into bile acids (27). Since the lipolysis pathway is inhibited in the low calcium diet fed rats, which in turn may reduce the amount of free fatty acids causing a stable serum triglyceride concentration in them.

In our study the animals fed with low calcium diet were found to have lower serum calcium level and which in turn raised the PTH level in blood while the high calcium diet fed rats showed the opposite effect. This raised PTH level in our study might have altered the intra cellular calcium level $[Ca^{2+}]_i$ which in turn affected the lipogenesis and lipolysis pathway thereby regulating the fat accumulation, body weight and adiposity. Bell et al. (28) in his study showed significantly higher concentrations of immunoreactive PTH and 1, 25-dihydroxyvitamin D in obese (106±6 kg; n= 12) subjects than in normal-weight (68±2 kg; n= 14) control subjects. This was also reported in animal study by He et al. (16) where he showed that when male Sprague-Dawley rats were fed with low calcium (0.30% wt/wt) and very low calcium diet (0.15% wt/wt) for several weeks consecutively the animal showed a significant rise in plasma PTH level and 1, 25-dihydroxyvitamin D level.

Fatty acid synthase (FAS), is a key enzyme in de novo lipogenesis (29) and is primarily regulated by hormonal and nutritional factors at the transcriptional level. The results of our study showed that rats exposed to low calcium diet had greater adipose FAS activity leading to a greater accumulation of triglyceride in the adipose tissue while animals fed with the high calcium diet showed the reverse effect. It has been observed that increased intra cellular calcium level $[Ca^{2+}]_i$ up regulate the gene expression and activity of lipogenic enzyme FAS (30). At the same time, it also exerts an anti lipolytic effect via direct activation of the enzyme phosphodiesterase 3B, resulting in a reduced level of intra cellular cyclic AMP, which in turn inhibits the phosphorylation required for the initiation of lipolysis (31). Thus, we can say that low calcium diet might have reduced the lipolytic activity in adipose tissue producing a low amount of glycerol, an end product of lipolytic pathway from the adipose tissue in the incubated medium during lipolysis assay. The reverse effect was shown by the high calcium diet favoring the lipolysis activity.

Our morphological observations showed that the surface area of the adipocytes of low calcium fed rats was greater compared to other groups, thus showing the features of adipocyte hypertrophy, a common condition during obesity where the size of adipocytes increases to conserve the excess amount of energy in the form of triglycerides (32). Due to the enhanced activity of FAS within the adipose tissue of low calcium fed rats, there was greater lipid deposition thereby increasing the size of the adipocytes. On the other hand, high calcium diet reduced the size of the adipocytes by increasing the lipolytic activity. Thus, it prevents the accumulation of excess lipid within the adipocytes of high calcium diet fed rats.

In conclusion, we can say that dietary calcium regulates the fat accumulation and body weight by regulating the two major metabolic events lipogenesis and lipolysis pathway within the adipose tissue in non-obese rats consuming isocaloric diet. Serum PTH level modulates the process. As the consequences of obesity and obesity related complications are growing rapidly all over the globe and as calcium is an important and common nutrient in our daily diet so further studies regarding its efficiency in the control of adipocyte metabolism may provide effective information for future development of preventive strategies against obesity.

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