Bone and lipid profile changes in anandamide injected and high fat fed rats

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Abstract: Background: Endocannabinoid system has recently attracted the attention not only for the physiological functions, but also for the promising therapeutic potentials as drugs. Bone has been identified as major target for endocannabinoids in which anandamide and 2-arachidonoylglycerol are present at high levels. Also, obesity represents a risk factor for many health disorders including cardiovascular disease, diabetes and cancer. **Aim**: to explore the effect of high fat diet and anandamide on lipid profile and bone in normal and high fat fed rats. **Design**: A total number of forty healthy, adult, male albino rats were divided into 4 equal groups each (n=10): Group I: lean control group, Group II: lean injected group, injected with anandamide (0.02 mg/kg i.p., daily for 2 weeks), Group III: high fat-fed group(high fat diet control group) fed with (58% fat) for 12 weeks and Group IV: high fat-fed injected group fed with (58% fat) for12 weeks and then injected with anandamide (0.02 mg/kg i.p., daily for 2 weeks) , in all groups initial body weight, final body weight, cholesterol, triglycerides, high density lipoproteins, serum calcium, serum phosphate, serum alkaline phosphatase, dry femur weight, ash weight, bone calcium content, bone phosphorus content were measured and histopathological studies for bone sections were done. **Results**: high fat diet fed rats showed significant increase in final body weight, total cholesterol (TC), triglycerides (TG), alkaline phosphatase activity, dry and ash femur weight, as well as significant decrease in serum level of high density lipoproteins (HDL), bone calcium and phosphorus content and corrected dry and ash femur in comparison to lean control group. While, anandamide injected groups (lean and high fat fed) showed significant increase in final body weight, total cholesterol (TC), triglycerides (TG), dry and ash femur weight, bone calcium and phosphorus content with significant decrease in serum level of high density lipoproteins (HDL) in comparison to their corresponding control groups. Alkaline phosphatase activity was significantly increased in lean injected, but significantly decreased in high fat injected rats in comparison to their control groups. The histopathological study showed normal bone architecture in lean control group, foci of new bone formation in lean injected group while high fat fed groups showed thinning in bone trabeculea with mild correction and foci of new bone formation in high fat fed injected group. **Conclusion**: High fat diet and anandamide caused disturbances in body weight and lipid profile. High fat diet was detrimental to bone health while, anandamide was able to produce beneficial effects on bone.

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Introduction:

The endocannabinoid system is present in some mammalian organs and tissues, the endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) have been demonstrated in a variety of tissues (**Mechoulam et al., 1995, Howlett, 2002**). The endocannabinoids bind to and activate two G protein coupled seven-transmembrane domain receptors, the cannabinoid receptors CB1 and CB2 (**Devane et al., 1992**). CB1 and CB2 receptors couple primarily to the Gi/o subtypes of G protein, although coupling to adenylate cyclase through Gi/o usually results in inhibition of cyclase activity, cannabinoids can also stimulate isoforms of adenylate cyclase **(Rhee et al., 1998)**. CB1 is present in the brain and in the peripheral neurons (**Herkenham et al., 1990, Zimmer et al., 1999)**. CB2 has been reported in the immune system, liver cirrhosis and atherosclerotic plaques (**Munro et al., 1993, Steffens et al., 2005)**.

Endocannabinoids and their receptors have been discovered in bone (**Tam et al., 2008)**. The main endocannabinoids, anandamide and 2-AG, are present in bone at levels similar to those found in the brain. Because the blood endocannabinoid levels are several orders of magnitude lower, it is very likely that anandamide and 2-AG are synthesized locally in bone (**Bab et al., 2008**). Indeed, both ligands are produced by osteoblasts and osteoclasts in culture (**Tam et al., 2008**), In line with the occurrence of the endocannabinoid ligands in bone, both CB1 and CB2 cannabinoid receptors are also present in bone (**Bab et al., 2008**). The endocannabinoid system has aroused enormous interest not only for the physiological functions, but also for the promising therapeutic potentials against numerous diseases **(Berry and Mechoulam, 2002)** or the use of drugs interfering with the activity of cannabinoid receptors **(Pagotto et al., 2006)**.

The mammalian skeleton undergoes a continuous remodeling process whereby the mineralized matrix is being continuously removed and subsequently replaced with newly-formed bone tissue. This renewal process has a key role in maintenance of the skeleton and in its physiologic function (**Bab et al., 2008**). Also, obesity affects more than 300 million adults worldwide, and its prevalence is predicted to rise over the next decades (**Haslam and James, 2005**). Although obesity is associated with increased risk of many chronic diseases, including cardiovascular disease, diabetes, hypertension and cancer, some reports suggest that it may protect against osteoporosis (**Wang et al., 2005**) and it may increase bone mineral density (**Villareal et al., 2005**), others suggest that marrow adipogenesis is negatively correlated with osteoblastogenesis (**David et al., 2007, Sen et al., 2008**) and obesity is detrimental to bone in animals (**Cao et al., 2009, 2010**) and humans (**Pollock et al., 2007**).In view of all previous reports this study was designed to explore the effect of anandamide and high fat diet on lipid profile and some bone parameters.

Materials and methods

This study was conducted on 40 healthy, adult, male albino rats weighing 120–140 gm (animals were obtained from animal house in faculty of medicine and the animal experiments were approved by the local ethics committee) divided into four equal groups (n=10). The rats had free access to water and chow and are kept at room temperature, 12 hours day and night cycles for 2 weeks, then the following protocols were performed: **Group I:** lean control group, rats received standard chow [25.8 % protein, 62.8 % carbohydrate and 11.4 % fat (**Ahren and Scheurink, 1998**)]. The animals were fed standard chow for 12 weeks and injected (i.p) with a single daily dose of 0.1 mL/rat of the vehicle [ethyl alcohol, propylene glycol (ADWIC Laboratory Chemicals, Egypt) and normal saline in a ratio of 1:1:2 respectively] **(da Veiga et al., 2008)** for another 2 weeks. **Group II:** lean injected group, rats received standard chow for 12 weeks and then injected with anandamide (arachidoneal ethanolamide, approx.98% TLC, Sigma Chemical, St. Louis, MO) in a dose of 0.02 mg/kg i.p. (dissolved in the same vehicle and equalized in 0.1mL/rat) (**da Veiga et al., 2008**) daily for another 2 weeks. **Group III:** high fat-fed group, rats received high-fat chow (16.4% protein, 25.6% carbohydrate, and 58.0% fat (**Ahren and Scheurink, 1998**) in the form of cotton seed oil added to the laboratory chow diet. The animals were fed high fat diet for 12 weeks and injected (i.p) with a single daily dose of 0.1 mL/rat of the same vehicle for another 2 weeks. **Group IV:** high fat-fed injected group fed with high-fat chow for 12 weeks and injected with anandamide in a dose of 0.02 mg/kg i.p. (dissolved in the vehicle and equalized in 0.1mL/rat) daily for another 2 weeks. Diets were obtained from faculty of agriculture, Zagazig University.

At the end of the experimental period; animals were sacrificed, the blood was collected in clean centrifuge tubes. Serum was separated by centrifugation of blood for 10 minutes at 3000 rpm. The supernatant serum was pipetted off using fine tipped pipette and then kept frozen at -20 C° for subsequent determination of:

- **Serum cholesterol levels:** Cholesterol was estimated according to the method described by **Tietz (1995).**
- **Serum Triglycerides:** triglycerides were estimated according to the method described by **(Fossati, 1982 and Nauck et al., 1997).**
- **Serum High density lipoproteins (HDL**): HDL was estimated according to the method described by **(Nauck et al., 1997).**
- **Serum calcium level:** was estimated according to the method described by **(Gindler et al., 1972)**.
- **Serum phosphate level:** was estimated according to the method described by **(Goldenberg and Fernfindez 1966).**
- **Serum alkaline phosphatase activity (SAP):** was determined by alkaline phosphatase assay kits (Biochain, USA) according to **(Eaton, 1977)**.

Bone tissue samples (right and left femur); right femora were removed and cleaned of from adhering tissue. The whole bone was extracted two times with a 1:1 mixture of absolute ethanol and diethyl ether for 48 h and one time with diethyl ether for 24 h. The dehydrated and defatted bones were dried in 80 C° oven for 48 h to measure the dry weight. The bones were ashed in a muffle furnace at 600°C for 48 hours after putting each bone in a clean porcelain dish, after that bone ash weights were measured then bones were hydrolyzed in 6 N hydrochloride solution for determination of calcium and phosphorus concentrations in bone.

- **Dry weight and ash weight of femur:** were measured according to **(Doster et al., 1969)**.
- **Calcium and inorganic phosphorus level in bone:** both Calcium and Phosphorus content was determined by atomic absorptiometry **(Yamazaki and Yamaguchi 1989)**
- **Histopathological study:** The left femur was removed from each animal, cleaned of adhering soft tissue, after proper fixation and staining with Heamatoxylin and Eosin stain (H&E) the specimen were prepared for light microscopy examination according to **(Bancroft and Cook 1984, Raab et al., 1991)**.

Statistical analysis: Data were expressed as mean ± SD, and statistically analyzed by One-way analysis of variance (ANOVA) followed by LSD test using SPSS for widows version 11.5. Differences were considered to be significant at P <0.05.

Results

Table (1): There was significant(P<0.05) increase in final body weight, serum total cholesterol (TC) (P<0.01), triglycerides (TG) (P<0.05), significant (P<0.05) decrease in high density lipoproteins (HDL) in group II, III and IV in comparison to group I, also group IV showed the same effects when compared to group III. **Table (2)**: serum alkaline phosphatase level was significantly (P<0.05) higher in group II, III and IV in comparison to group I, but it was significantly lower (P<0.05) in group IV compared to group III. **Table (3)**: there is significant (P<0.05) increase in dry and ash femur weight, with significant (P<0.05) decrease in corrected dry and ash weight and in bone calcium and phosphorus content in group III and IV compared to group I. Group II showed significant (P<0.05) increase in dry and ash femur weight compared to group I and so was group IV compared to group III.

Figure (1,2,3,4): microscopic appearance of metaphysic of long bone in group I, II, III, IV respectively.

Table (1): Body weight and lipid profile in all studied groups:

	Group I	Group II	Group III	Group IV
Initial BW (gm)	129 ± 6.3	128.3 ± 3.4	127.17 ± 5.8	130.33 ± 7.9
Final BW (gm)	221.7 ± 18.6	244.2 ± 9.5*	317.3 ± 8.7*	344 ± 19*#
TC (mg/dl)	114.7 ± 7.2	142.3 ± 10.1*	185.7±19.2*	214 ± 21.1*#
TG(mg/dl)	51.8±4.1	61.2 ± 5.3 *	75.3 ±6.5*	91.2 ±12.1*#
HDL(mg/dl)	61.7±10.5	50.3 ± 6.3 *	45.5± 6.8 *	35.2±4.7 *#

* Significant (P< 0.05) versus group I # Significant (P< 0.05) group IV versus group III

Table (2): Serum calcium level, serum phosphate level and serum alkaline phosphatase level in all studied groups:

	Group I	Group II	Group III	Group IV
Serum Ca+2 (mg/dL)	9.5 ± 0.89	9.7 ± 0.45	9.4±0.9	9.6 ± 1.01
Serum P (mg/dL)	3.7 ± 0.96	4.7 ± 0.51	3.9 ± 0.76	3.8 ± 0.92
SAP activity (U/L)	151.5 ± 14.1	191 ± 8.7*	283.7 ±33.1*	245 ± 47.9*#

* Significant (P< 0.05) versus group I # Significant (P< 0.05) group IV versus group III

Table (3): bone parameters in all studied groups:

	Group I	Group II	Group III	Group IV
Dry femur weight (mg)	318.3 ±8.2	362.5±12.3*	362.3 ±23.5*	433±51.4*#
Ash weight (mg)	221±8.7	240.2±13.6*	236.8 ± 8.1*	280 ± 6.7*#
Corrected Dry femur weight (mg/100gm B.W.)	144.3± 10.3	148.8± 9.9	114.3± 8.4*	125.6±9.8*
Corrected ash weight (mg/100gm B.W.)	99.1± 8.4	98.4± 3.8	77.9±5.3*	82.5± 5.7*
Femur Ca+2 content (mg/gm ash)	104.8 ±7.1	115.3± 11.4*	82.2 ±5.03*	93.5 ±6.2*#
Femur Pi content (mg/gm ash)	47.8 ± 4.6	53.8 ± 5.1*	35.2 ± 3.2*	41.5 ± 4.6*#

* Significant (P< 0.05) versus group I # Significant (P< 0.05) group IV versus group III

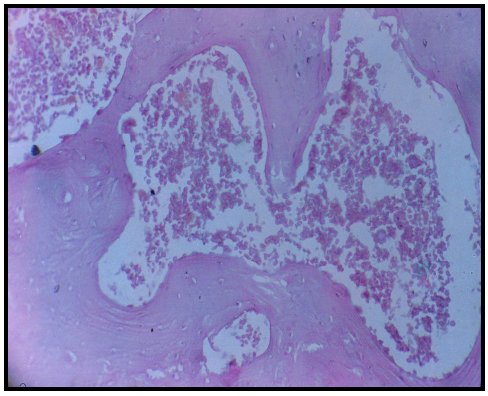

Figure (1): (H&E X 400) metaphysis of long bone showing normal bone trabeculea (a) in group I

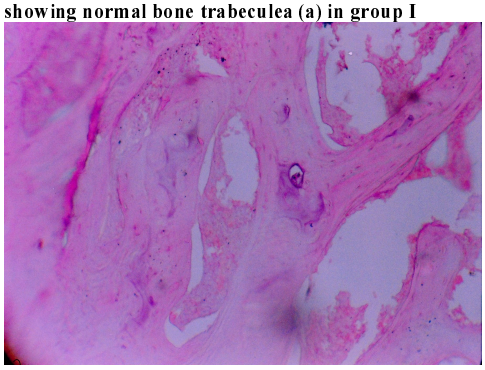

Figure (2): (H&E X 400) metaphysis of long bone showing normal bone trabeculea (a) with new bone (b) in group II.

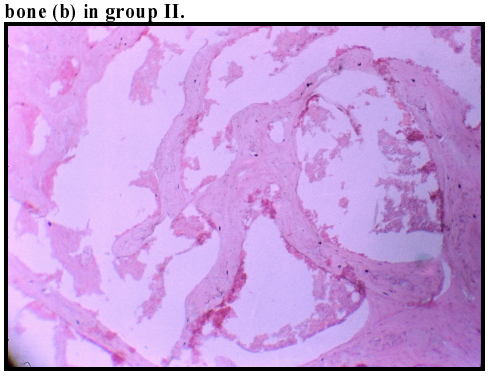

Figure (3): (H&E X 400) metaphysis of long bone showing thin bone trabeculea (a) in group III

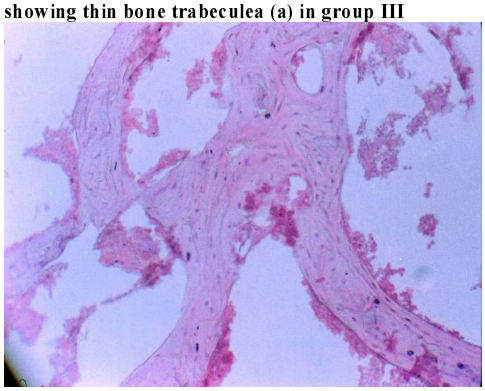

Figure (4): (H&E X 400) metaphysis of long bone showing partially corrected thin bone trabeculea (a) with new bone formation (b) in group IV

Discussion

The skeleton is a dynamic tissue, constantly remodeling itself by the coordinated removal and replacement. A delicate balance between these processes is essential for maintaining both the mechanical and mineral homeostatic functions of the skeleton (**Wong et al., 2008**).

Our results showed that, high fat diet resulted in a significant increase in final body weight, serum levels of total cholesterol, triglycerides and a significant decrease in serum level of high density lipoprotein HDL, these results are in agreement with the results of **Kalaivanisailaja et al. (2003)** in mice and the results of **Woo et al. (2008)** in rats. These dyslipidemic disturbances may be attributed to increased GIT absorption of cholesterol and triglycerides or altered cholesterol and HDL metabolism **(Schaalan et al., 2009)**.These changes in final body weight and lipid profile were significantly exaggerated in anandamide injected rats (lean and high fat) than corresponding control groups. This may be attributed to the effect of anandamide on body weight and lipid profile due to increase in food intake as shown by **Costa et al. (1999)** who reported that anandamide is capable of increasing food intake in rat through central action of CB1 receptors in the hypothalamus **(Berry and Mechoulam, 2002)** or direct effect of anandamide on clearance, hepatic production or catabolism of plasma lipoproteins as reported by **Jeong et al. (2008)** through CB1 receptors **(Ruby et al. 2008)**. In contrast, **Karimi and Hayatghaibi**, **(2006)** reported a significant increase in levels of HDL in rats upon treatment with cannabinoids.

Our result showed significant increase in dry and ash weight of femur and alkaline phosphatase activity of high fat diet fed groups in comparison to lean control group.

The increased bone mass in high fat fed rats is in agreement with **Iwaniec et al. (2009)** who reported that increased body mass has a positive effect on bone mass in high fat diet fed mice and with the results of **Ricci et al. (2001)** in postmenopausal women and the results of **Radak (2004)** who showed that obesity is correlated with increased bone mass in premenopausal women. The increased bone mass in obesity may be attributed to systemic factors (e.g., elevated sex steroid and leptin levels) and increased skeletal loading due to increased body mass (**Felson et al., 1993**). This denotes increased bone formation supported by increased alkaline phosphatase activity which is a marker of bone formation **(Yamaguchi and Uchiyama 2003)**.

On the other hand, our result showed a significant decrease in corrected dry and ash femur weight, femur calcium and phosphorus content in high fat diet fed groups in comparison to lean control group indicating bone resorption. This was observed also by **Xiao et al. (2011)** who reported that high fat diet inhibited bone formation and enhanced bone resorption in mice , also **Patsch et al. (2011)** reported that, short-term and

extended high-fat diet–induced obesity caused significant bone loss in male mice mainly because of resorptive changes in trabecular architecture. However, **Tarquini et al. (1997)** found no significant difference between bone mineral density in obese women and control group and **Villareal et al. (2005)** reported that obesity increases bone mineral density is a protective factor for osteoporosis in humans.

Histopathological results showed thinning of bone trabeculea in high fat fed rats indicating bone resorption which may be caused by either low grade inflammation occurring in obesity leading to release of multiple cytokines which cause increased osteoclastic activity (**Khosla 2001, Shoelson et al. 2007)** or increase bone marrow adipogenesis that inhibits osteoblastogenesis **(David et al., 2007, Sen et al., 2008)** or interference with intestinal calcium absorption **(Nelson et al. 1998**) or inhibition of bone formation through central action of leptin (**Shoelson et al. 2007**). Taken together, high fat diet caused increase bone quantity (dry and ash weight) meanwhile bone was of low quality (low mineral content and thin trabeculea).

This study revealed also, significant increase in femur dry, ash weight, calcium and phosphorus content in anandamide injected rats than the corresponding control groups, while alkaline phosphatase activity was significantly higher in lean injected and significantly lower in high fat injected compared to the corresponding control, also the histopathological sections showed foci of new bone formation in both injected groups and partial correction of thin bone trabeculea in high fat injected group showing positive effect of anandamide on bone mineral content and architecture. This is in agreement with the results of **Napimoga et al. (2009)** who found that the administration of cannabidiol (cannabinoid component from Cannabis sativa) significantly inhibited the volume of bone loss in rat, and **Bab et al. (2008)** who demonstrated that both osteoblasts and osteoclasts produce anandamide and express CB2 receptors whose stimulation in these cells decreases bone resorption and enhances bone formation. By contrast, **Idris et al. (2005)** reported the stimulation of osteoclast formation and bone resorption by anandamide and the inhibition of these effects by cannabinoid receptors antagonists in vitro. Anandamide is considered as non-selective CB1 and CB2 agonist; it was found to affect bone cells directly by binding to CB2 receptors **(Bab and Zimmer 2008)** which are expressed in osteoblasts, osteoclasts and their precursors (stromal cells and monocytes respectively) leading to direct stimulation of osteoblasts/ stromal cells; and inhibition of osteoclasts /monocytes **(Ofek et al. 2006)** or indirectly via activation of CB1 receptors that negatively regulate noradrenaline release from sympathetic nerve terminals in the vicinity of osteoblasts, noradrenaline suppresses bone formation by binding to osteoblastic adrenergic receptors (**Takeda et al., 2002**), and this suppression is apparently alleviated by activation of CB1 on sympathetic nerve terminals **(Tam et al., 2008)**. Collectively anandamide effect on bone appears to be biphasic; stimulation of bone formation and inhibition of bone resorption.

Conclusion: High fat diet and anandamide caused increased body weight and dyslipidemia which were detrimental to bone health in high fat diet, but not anandamide treated animals denoting beneficial effects of anandamide on bone in both normal and high fat fed rats. Further studies are needed to evaluate its effect in humans and evaluate the medical use of other cannabinoids agonist as therapeutic agents as regard their effect on bone.

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