Journal of Animal and Veterinary Advances 10 (22): 2943-2955, 2011
ISSN: 1680-5593

Effect of Long-Term Dietary Lipids on Femur Mineral Content, *Ex vivo* Prostaglandin E_2 Release and Bone Growth in Growing Rabbits

D.M. Al-Nouri and A.S. Al-Khalifa
Department of Food Sciences and Nutrition, Faculty of Food Sciences and Agriculture,
King Saud University, Riyadh, Saudi Arabia

Abstract: The long-term effects of different dietary oil sources with varying omega-6/omega-3 (ω-6/ω-3) Polyunsaturated Fatty Acids (PUFAs) ratios on the bone marrow fatty acids level, bone growth and *ex vivo* Prostaglandin E_2 (PGE_2) release and minerals content in bone were evaluated in rabbits. Weanling male and female New Zealand white rabbits were randomly assigned to five groups and fed *ad libitum* on diets containing 70 g kg^{-1} of added oil for 100 days as follow; Soybean Oil (SBO control), Sesame Oil (SO), Fish Oil (FO), DHA algae oil (DHA) and DHA and ARA algae oils (DHA/ARA). The dietary lipid treatments were formulated to provide the following ratio of ω-6/ω-3 fatty acids: 8.7 (SBO), 21.8 (SO), 0.4 (FO), 0.6 (DHA) and 0.7 (DHA/ARA). The bone marrow fatty acids level of rabbits was significantly influenced by and reflected the dietary level of ω-6 and ω-3 fatty acids fed to rabbits. Rabbits fed the FO diet maintained a lower ω-6/ω-3 ratio and a higher EPA and DHA concentrations in their bone marrow those fed the SO diet maintained a higher ω-6/ω-3 fatty acids ratio while those fed the SBO diet maintained a higher LA and ALA concentrations and intermediate value of ω-6/ω-3 ratio. *Ex vivo* PGE_2 level declined progressively as the ω-6/ω-3 dietary ratio declined. There was a significant main effect of dietary treatment on femur Ca, P, Mg and Zn contents in both genders. These results demonstrate that dietary ω-6/ω-3 ratio modulates bone PGE_2 production in growing rabbits, hence may reduce bone resorption and improve bone mass during growth. In addition, the significant elevation in mineral content and the maintenance of optimal Ca/P ratio in bone of DHA/ARA and DHA fed groups demonstrates that marine algae oils may be promising dietary sources for promoting bone mineralization and formation thus improving bone mass during the growth stage.

Key words: ω-6 and ω-3 PUFAs, bone minerals, prostaglandin E_2, bone growth, growing rabbits, Saudi Arabia

INTRODUCTION

There is increasing evidence that lack of certain fatty acids in the diet contributes to bone loss (Das, 2000; Kettler, 2001) while their presence in the diet helps reducing the risk of osteoporosis and bone fracture (Fernandes *et al*., 2003; Sun *et al*., 2003, 2004). Long-Chain Polyunsaturated Fatty Acids (LCPUFAs) of the ω-6 (arachidonic acid ARA, 20:4) and ω-3 (Eicosapentaenoic Acid EPA, 20:5 and Docosahexaenoic Acid DHA, 22:6) families are synthesized in the liver, brain and retina from their respective Polyunsaturated Fatty Acids (PUFAs), Linoleic Acid (LA, 18:2 ω-6) and α-linolenic acid (ALA, 18:3 ω-3) via a series of alternating desaturation (addition of double bond) and elongation (addition of 2 carbon atoms) steps (Sauerwald *et al*., 1997).

Bone is a living and dynamic multi-functional tissue which consists of a structural framework of mineralized organic matrix including collagen such as Pyridinolines (Pyd) and Deoxypyridinolines (Dpd) and non-collagenous proteins such as osteopontin, osteonectin and Osteocalcin (OC) (Price and Thompson, 1995), plus bone cells (osteoblasts, osteocytes and osteoclasts), cartilage as well as connective tissue (Watkins, 1998; Boskey, 1999; Anderson, 2000). Osteoblasts are mono-nucleated bone-forming cells (Baron, 1999) which are responsible for the production of organic matrix collagen and non-collagenous proteins (Heinegard and Oldberg, 1989); their plasma membrane is rich in Alkaline Phosphatase (ALP), an enzyme that plays an important role during the mineralization process (deposition of hydroxyapatite crystals) (Baron, 1999; Anderson, 2000). Osteoclasts are multi-nucleated cells responsible for bone resorption (Baron *et al*., 1993; Baron, 1999) their plasma membrane is rich in Acid Phosphatase (ACP), an enzyme that plays an important role during this process (Baron *et al*., 1985). They synthesize lysosomal enzymes

Corresponding Author: D.M. Al-Nouri, Department of Food Sciences and Nutrition, Faculty of Food Sciences and Agriculture, King Saud University, Riyadh, Saudi Arabia

and secrete several metalloproteinases such as collagenase and gelatinase which in combination with the acidic environment from ACP, degrade the organic matrix to release minerals (Baron *et al*., 1985; Lian and Stein, 1999; Baron, 1999). However, osteoblasts and osteoclasts both originate from bone marrow (Hattersley *et al*., 1991; Malaval *et al*., 1994).

Bone contains many minerals including Calcium (Ca), Phosphorus (P), Magnesium (Mg) and Zinc (Zn) each of which is essential to growth and bone mineralization. Ca and P provide structural integrity in the skeleton where they are deposited in the organic matrix and which in combination with hydroxyl ions, mature into hydroxyapatite crystals $[Ca_{10}(PO_4)_6(OH)_2]$ (Anderson, 2000; Sherwood, 2004). About 99% of Ca and 85% of P is found in the skeleton in the form of hydroxyapatite. Approximately, 67% of Mg is located on the hydroxyapatite crystal surface (Czajka-Narins, 1996; Broadus, 1999) and approximately 29% of Zn appears in the crystalline structure of bone (Czajka-Narins, 1996). Mg is involved in bone mineral homeostasis (Wallach, 1990) where it plays an important role in Ca metabolism, since it is a cofactor of enzymes required for the synthesis of calcitriol (vitamin D_3) and parathyroid hormone PTH (Connor *et al*., 1972). In addition, Mg affects bone cell function. During Mg deficiency, the number of osteoblasts is decreased and the number of osteoclasts is increased (Rude *et al*., 2003) however, excess Mg inhibits bone calcification (Czajka-Narins, 1996). Zn is essential in bone metabolism as a cofactor of enzymes (Saltman and Strause, 1993) including ALP. In addition, Zn is needed for adequate osteoblastic activity (Czajka-Narins, 1996). The biological importance of ARA, EPA and DHA originated in part from their role as constituents of structural lipids that influence the activities of membrane-linked functional molecules (receptors, enzymes and transporters) (Fernstrom, 2000) and signal transduction mechanisms (Decsi and Koletzko, 1994). ARA and its precursor LA are important for growth, dermal integrity, wound healing, liver and kidney function and protection against infection whereas DHA and its precursors ALA and EPA are important in sensory and other neural-based behaviors (Carlson, 1997). In addition to their structural role, ARA, EPA and DHA play an important direct role in bone metabolism (Watkins *et al*., 2003) as they have been shown to inhibit the activity of osteoclasts and enhance the activity of osteoblasts in animals thus the optimal quantities of these fatty acids appear to inhibit bone resorption and promote bone formation (Sun *et al*., 2003; Watkins *et al*., 2003). Emerging evidence from human and animal research as well as tissue culture studies has revealed the positive role of ω-3 and ω-6 polyunsaturated fatty acids in bone integrity. The evidence confirmed that these fatty acids modified the fatty acid composition of tissues and cells; increased ALP activity; an indicator of bone formation and altered the histomorphometric measurements of bone formation parameters (Li *et al*., 1999; Fernandes *et al*., 2003; Sun *et al*., 2003; Mollard *et al*., 2005a; Coetzee *et al*., 2007). In growing rats, dietary EPA and DHA supplementation increased Ca absorption and balance and bone Ca content (Claassen *et al*., 1995; Lobo *et al*., 2009). In previous studies involving piglets, supplementation with ARA and DHA elevates bone mass (Weiler, 2000; Weiler and Fitzpatrick-Wong, 2002; Blanaru *et al*., 2004). A proposed mechanism for elevations in bone mass in response to ω-6 and ω-3 LCPUFA supplementation is an alteration in mineral metabolism (Claassen *et al*., 1995). Elevations in bone Ca, P, Mg or Zn contents could enhance bone mineralization and potentially may explain the higher bone mass seen with supplementation of LCPUFA.

To the best of the knowledge, there were no studies on how different dietary lipids affect bone growth, metabolism and mineralization following long-term supplementation in male and female growing rabbits. Therefore, the objectives of the present study were to investigate the long-term effects of different dietary oil sources, varying in their ω-6/ω-3 fatty acid ratios on bone marrow specific fatty acids level, bone content of specific minerals and *Ex vivo* PGE_2 release in bone and determine whether the variation in dietary ω-6/ω-3 fatty acid ratios well affect bone growth following long-term supplementation; determine whether males and females respond differently or similarly to this variation.

MATERIALS AND METHODS

Diets and animals: Five oils were used in this study; Soy Bean Oil (SBO), Sesame Oil (SO), Fish Oil (FO) (DHA 40% + EPA 30%) and two types of marine brown micro algae oils of the genus *Crypthecodinium cohnii* that are DHA 40% (40 g/100 g of fatty acids) and ARA 40% (40 g/100 g of fatty acids). SBO oil was purchased from a local market in Cairo, Arab Republic of Egypt, SO oil was procured from Horse Factory for Food Products, Riyadh, Saudi Arabia, FO and DHA oils were purchased from Huatai Biopharm inc., Deyang, China and ARA oil was from Nutrakey Industries inc., Qingdao, China. All oils were kept refrigerated at 4°C until used in the preparation of diets. Basal diet was purchased from the Arabian Agricultural Services Company (ARASCO), Riyadh, Saudi Arabia which has been prepared in accordance with its specification for rabbit feed (47152-Rabbit 18/14 Pellet

without fat). The experimental diets were prepared by adding the oil blend to the basal diet (70 g kg^{-1} diet) as follows; SBO diet, 70 g soybean oil kg^{-1} diet; SO diet, 50 g sesame oil +20 g soybean oil; FO diet, 50 g fish oil +20 g soybean oil; DHA diet, 50 g DHA oil +20 g soybean oil; DHA/ARA diet, 25 g DHA oil+25 g ARA oil +20 g soybean oil. Oils were added into the basal diet by spraying under pressure with continuous mixing during the spraying.

Fresh diets were mixed weekly to avoid oil oxidation and kept refrigerated at 4°C until fed. Oils were checked periodically for peroxidation by measuring their Peroxide Value (PV) according to the AOAC (1962, 2002). The diets provided 7% fat (70 g kg^{-1} diet) which covers the needs of the growing rabbits (Reeves, 1997).

About 45 weanling male (n = 25) and female (n = 20) New Zealand white rabbits (6 weeks old, weighing 500-1000 g) were obtained from Experimental Animal Care and Experimental Surgery Center at the Faculty of Medicine, King Saud University, Riyadh, Saudi Arabia and randomly divided by weight into five groups where the weight difference between the members of each group did not exceed±100 g. All rabbits were individually housed in stainless steel cages under controlled temperature (25±2°C) and relative humidity (50±5%) with a 12 h light/dark cycle. Food and tap water were offered *ad libitum* throughout the experimental period (100 days). Food cups were re-filled every 2nd day and food provided and the remaining were weighed to calculate daily food consumption. Body weight (wt) (kg) was recorded in the non-fed state at the beginning of the study (initial wt) and at time before slaughter (final wt). Weight gain (g final body weight-g initial body weight), food efficiency (total g weight gain/total g food consumed) and growth rate (total g weight gain/100 days study period) were calculated.

Samples collection: After 100 days, rabbits were food deprived over-night then immediately slaughtered from the neck by a sharp knife; bones (femur, tibia, humerus and forearm) were obtained as previously described by Dekel *et al*. (1981). Briefly, bone was excised and carefully freed of soft tissue by gentle scraping with a scalpel, rinsed with normal saline (0.9% NaCl), dried using a lint-free paper towel and stored in a plastic container at -20°C until elicitation of bone marrow.

All procedures were accepted by the Experimental Animal Care and Experimental Surgery Center at the Faculty of Medicine, King Saud University, Riyadh, Saudi Arabia. The bone was thawed at room temperature; bone growth measurements [Weight (Wt.), Length (Lt.) and Width (Wd.)] were taken as follow; Wt. (g) of femur, tibia,

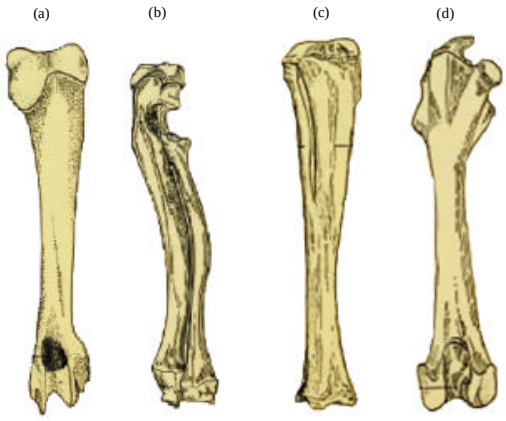

Fig. 1: Method of taking bone length measurements; a) Humerus; b) Forearm; c) Tibia and d) Femur

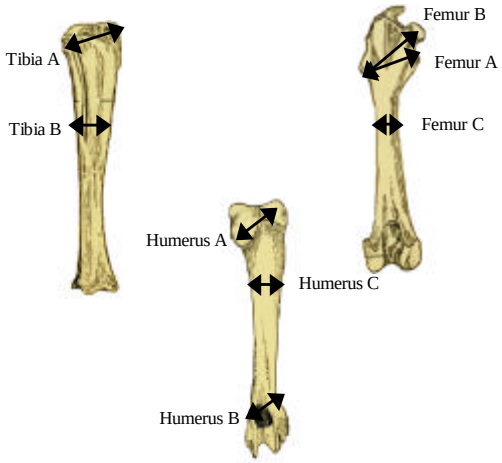

Fig. 2: Method of taking bone width measurements. Tibia A, proximal tibia; B, tibia diaphysis; Femur A, proximal epiphysis femur; B, proximal femur; C, femur diaphysis; humerus A, proximal humerus; B, humerus deltoid tuberosity and C, humerus diaphysis

humerus and forearm (Wt. A) (bone wt.+bone marrow wt.) was measured using a sensitive digital balance (PJ600 Mettler-Toledo Inc., Switzerland) to the nearest 0.01 g. Lt (cm) of femur, tibia, humerus and forearm (Fig. 1) and Wd (cm) of femur, tibia and humerus (Fig. 2) were measured as described by Reichling and German (2000) using a vernier caliper to the nearest 0.01 mm. After the completion of bone measurements, both bone epiphysis of tibia, femur and humerus were removed using a fine saw incision was made along the bone using a sharp non-serrated knife, bone marrow was removed and weighed separately (Wt. B) to the nearest 0.0001 g (AL204, Mettler-Toledo Inc., Shanghai, China) and then stored in a plastic tube at

-20°C until used for lipid extraction and fatty acid analysis. Bone samples were rinsed again with normal saline, dried using a lint-free paper towel and then stored separately in a plastic tube at -20°C until used for minerals and PGE_2 analysis. Bone wt. (g) was calculated by subtracting wt. B from wt. A.

Analysis: Lipids from bone marrow samples were homogenized at 4°C (Bench top Homogenizer 300 DS PRO Scientific Inc., Oxford, CT, USA). Total lipids from 0.8 g aliquots of the homogenized tissue were extracted according to Folch *et al.* (1957). Extracted lipids and oils blend samples were transmethylated using 14% boron trifloride in methanol to Fatty Acid Methyl Esters (FAMEs) according to Bligh and Dyer (1959). FAMEs were separated by gas chromatography (GC Clarus 500, Perkin Elmer, Shelton, WA, USA), equipped with an Omegawax™ 320 capillary column (30 m×0.32 mm i.d×0.25 μm film thickness, Cat. No. 24152, Supelco Inc., Bellefonte, PA, USA) and operated as follow; oven temperature, 200°C; carrier gas, helium 25 cm sec^{-1} at 200°C; detector, Flame Ionization (FID) 260°C; injection, 1 μL split 100:1 at 250°C. FAMEs (C14-C22) from bone marrow samples and oils blend were identified by comparison with retention times of standard fatty acids (Supelco Inc., Bellefonte, PA, USA) PUFA-2, animal source and FAME Mix RM-1, Oil Reference, Sigma-Aldrich, St. Louis, MO, USA, respectively and fatty acid concentration was expressed as % wt./wt. of fatty acids (g/100 g of total fatty acids).

Bone organ culture was performed as previously described (Blanaru *et al.*, 2004; Mollard *et al.*, 2005b). Briefly, tibia samples (1 g) were incubated in 10 mL of Hank's balanced salt solution (Mediatech Inc., Manassas, VA, USA) for 2 h at 37°C in a shaking water bath followed by the removal of bone and rapid freezing of solution at -20°C until duplicate analysis of PGE_2 by a competitive Enzyme-Linked Immunosorbent Assay (ELISA) technique using a rabbit polyclonal antibody PGE_2 kit (Oxford Biomedical Research Inc., Oxford, MI, USA). PGE_2 levels were expressed as ng g^{-1} bone.

To determine Calcium (Ca), Phosphorous (P), Magnesium (Mg) and Zinc (Zn) content, femur samples (300 mg) were digested in 6 mL of concentrated nitric acid (HNO_3) and 2 mL hydrogen peroxide (H_2O_2) for 30 min in a microwave digestion system (START D, Milestone, Italy). Ca, Mg and Zn concentrations were measured using an inductively coupled plasma mass spectrometer (ICP-MS 7500A, Agilent Technologies Inc., Santa Clara, CA, USA) whereas P concentration was measured using molybdo-vanadate colorimetric method according to the AOAC (1962, 2002) and the optical density was measured at 410 nm using a spectrophotometer (UV/Visible 160A, Shimadzu, Kyoto, Japan). Minerals' concentration was expressed as mg g^{-1} bone.

Statistical analysis: Data were analyzed using SAS statistical software package (SAS Institute Cary, NC, USA) and expressed as mean±Standard Error of the Means (SEM). The differences among the dietary treatment groups were analyzed by one-way ANOVA whereas the differences between males and females were analyzed by two-way ANOVA both at a significance level of $p \leq 0.05$ if significant differences were found, a Post-hoc analysis using Duncan's multiple range test was performed.

RESULTS AND DISCUSSION

Analyzed fatty acid value for the formulated dietary treatments ranged from 0.4-21.8 for the ratio of ω-6/ω-3 fatty acids (Table 1). The SO diet had the highest ω-6/ω-3 ratio and the FO diet had the lowest and contained highest amounts of EPA and DHA (20.4 and 29.8 g/100 g total fatty acids, respectively) whereas the SBO diet had an intermediate ω-6/ω-3 ratio and contained highest

Table 1: Fatty acid composition (g/100 g total fatty acids) of the experimental diets

	Experimental diets[1]				
Fatty acid[2]	SBO	SO	FO	DHA	DHA/ARA
SAT					
C14:0	0.05	0.03	0.09	2.51	2.910
C16:0	5.87	10.16	1.96	18.94	8.370
C18:0	2.79	6.04	1.75	1.95	1.150
C20:0	0.38	0.59	-	0.19	0.240
Total SAT	9.09	16.82	3.80	23.59	12.67
MUFA					
C16:1 ω-7	0.02	0.12	0.12	0.67	0.550
C18:1 ω-9	24.33	35.40	11.35	0.02	4.990
C18:1 ω-7	-	-	1.04	7.33	5.060
C20:1 ω-9	0.23	0.15	1.31	0.07	ND
Total MUFA	24.58	35.67	13.82	8.09	10.60
ω-6 (PUFA)					
C18:2 LA	61.55	45.02	18.89	18.24	17.00
C18:3 GLA	-	-	-	0.14	0.100
C20:4 ARA	-	-	1.49	1.16	0.890
C22:4 DTA	-	-	-	-	-
Total ω-6	61.55	45.02	20.38	19.54	17.99
ω-3 (PUFA)					
C18:3 ALA	7.09	2.07	2.41	2.14	3.020
C20:5 EPA	-	-	20.44	0.67	0.520
C22:6 DHA	-	-	29.77	28.31	22.80
Total ω-3	7.09	2.07	52.62	31.12	26.34
Ratios					
ω-6/ω-3	8.68	21.75	0.39	0.63	0.680
ARA/DHA	-	-	0.05	0.04	0.040

[1]The basal diet contained the following (g kg^{-1}): Corn, 150; Barley, 106; Wheat bran, 200; Soya meal, 162; Lime stone, 2.8; Alfalfa, 370.5; Choline Chloride 60%, 0.6; Methionine (MHA) powder, 1; Di Calcium Phosphate (DCP) 18%, 5.1; Vitamin and mineral mix, 2. The experimental diets included; SBO: Soy Bean Oil (control); SO: Sesame Oil; FO: Fish Oil; DHA: DHA algae oil; DHA/ARA, (DHA+ARA algae oils 1:1 ratio). [2]SAT: Saturated Fatty Acids; MUFA: Monounsaturated Fatty Acids; PUFA: Polyunsaturated Fatty Acids; LA: Linoleic Acid; GLA: Gamma-Linolenic acid; ARA: Arachidonic Acid; DTA: Docosatetraenoic Acid; ALA: Alpha-Linolenic Acid; EPA: Eicosapentaenoic Acid; DHA: Docosahexaenoic Acid; ND: Not Detected

amounts of LA and ALA (61.6 and 7.1 g/100 g total fatty acids, respectively). The amount of LA and ALA ranged from 17-61.6 g and 2.1-7.1 g/100 g of the total fatty acids, respectively. ARA, EPA and DHA ranged from 0.9-1.5, 0.5-20.4 and 22.8-29.8 g/100 g of the total fatty acids, respectively however, the SBO and SO diets were devoid of ω-6 (ARA) and ω-3 (EPA and DHA) LCPUFAs.

The growth of rabbits was significantly affected by the dietary lipid treatments. Long-term dietary lipid supplementation with various ω-6/ω-3 ratios had a pronounced effect on Final Body weight (FB wt.), weight gain (wt. gain) and Growth Rate (GR) in males but not in females (Table 2). These growth indicators were significantly higher in the SBO and SO groups and significantly lower in the DHA group. However, feed efficiency was significantly decreased in male DHA group (0.37±0.01) compared to the other groups (SBO, 0.41±0.01; SO, 0.42±0.01; FO, 0.43±0.01 and DHA/ARA, 0.44±0.01 g wt. gained g^{-1} food consumed) and did not differ among female groups (SBO, 0.42±0.01; SO, 0.39±0.03; FO, 0.41±0.07; DHA, 0.46±0.01 and DHA/ARA, 0.48±0.02).

Furthermore, there were significant differences between males and females in the DHA group where all growth indicators were significantly higher in females compared to males, however no significant differences between males and females in the other dietary treatment groups.

The bone marrow fatty acids of male and female rabbits were significantly influenced by and reflected the dietary lipid treatments (Table 3). There was a significant effect of dietary treatments on all fatty acids measured. Compared to the control (SBO) group, rabbits fed the SO diet had the lowest ARA content in both genders. Those fed the FO diet had the highest EPA and DHA contents in females and EPA in males. Those fed the DHA diet had the highest DHA content in males but not in females and the lowest LA and ALA contents in both genders. Those fed the DHA/ARA diet had the highest content of ARA yet the lowest contents of EPA and DHA in both genders. However, rabbits fed the control diet had the highest amounts of LA and ALA in both genders. Furthermore, significant differences existed in fatty acid ratios between the dietary treatment groups in both genders (Table 3).

Compared to the control (SBO) group, rabbits fed on the SO diet had the highest LA/ALA and LA+ARA/ALA+DHA ratios in both genders and rabbits fed on the FO diet had the lowest ARA/EPA, ARA/DHA and ARA/EPA+DHA ratios in males and LA/ALA,

Table 2: Growth indicators of male and female rabbits fed diets with different dietary oil sources and varying ω-6/ω-3 ratio for 100 days[1]

	[2]Dietary treatment									
	SBO		SO		FO		DHA		DHA/ARA	
[3]Growth indicator	M (n = 5)	F (n = 5)	M (n = 5)	F (n = 3)	M (n = 5)	F (n = 3)	M (n = 5)	F (n = 5)	M (n = 5)	F (n = 4)
FB Weight (g)	3140±91.38^{Aa}	3270±165.53^{Aa}	3325±119.90^{Aa}	2850±332.92^{Aab}	2570±152.15^{Ab}	2300±200^{Ab}	2120±37.42^{Bc}	2666.67±109.29^{Aab}	2350±50^{Abc}	2387.50±132.88^{Ab}
Wt. gain (g)	2280±101.98^{Aab}	2400±178.19^{Aa}	2537.50±131.30^{Aa}	2133.33±290.59^{Aa}	1910±151.16^{Ab}	1675±225^{Aa}	1520±33.91^{Bc}	2066.67±60.09^{Aa}	1950±50^{Ab}	1987.50±135.98^{Aa}
GR (g day^{-1})	22.80±1.02^{Aab}	24.20±1.69^{Aa}	25.50±1.20^{Aa}	21.33±2.91^{Aa}	19.40±1.60^{Ab}	17±2^{Aa}	15.40±0.40^{Bc}	21±0.58^{Aa}	19.50±0.50^{Ab}	20±1.41^{Aa}
FE	0.41±0.01^{Aa}	0.42±0.01^{Aa}	0.42±0.01^{Aa}	0.39±0.03^{Aa}	0.43±0.01^{Aa}	0.41±0.07^{Aa}	0.37±0.01^{Bb}	0.46±0.01^{Aa}	0.44±0.01^{Aa}	0.48±0.02^{Aa}

[1]Data are expressed as mean±SEM. Values within a row having different superscripts are significantly different ($p \leq 0.05$) where the small letters indicate significant among dietary treatment groups for each gender separately as indicated by one-way ANOVA followed by Duncan's multiple range test (a>b>c) while the capital letters indicate significant between males and females as indicated by two-way ANOVA followed by Duncan's multiple range test (A>B), [2]SBO: Soy Bean Oil (control); SO: Sesame Oil; FO: Fish Oil; DHA: DHA algae oil; DHA/ARA, DHA+ARA algae oil 1:1 ratio. [3]FB Wt: Final Body Weight, Wt gain: Weight gain; GR: Growth Rate, FE: Food Efficiency

Table 3: Bone marrow fatty acids of male and female rabbits fed diets with different dietary oil sources and varying ω-6/ω-3 ratios for 100 days[1]

	Dietary treatment[2]									
	SBO		SO		FO		DHA		DHA/ARA	
[3]Fatty acid	M (n = 5)	F (n = 5)	M (n = 5)	F (n = 3)	M (n = 5)	F (n = 3)	M (n = 5)	F (n = 5)	M (n = 5)	F (n = 4)
C18:2 ω-6	45.63^{Aa}	44.77^{Aa}	34.07^{Ab}	36.19^{Ab}	20.48^{Ac}	19.79^{Ac}	17.45^{Ac}	13.96^{Ad}	20.93^{Ac}	20.69^{Ac}
C20:4 ω-6	0.41^{Ad}	0.40^{Acd}	0.17^{Ad}	Tr^{Ad}	1.00^{Ac}	1.33^{Abc}	2.15^{Ab}	2.13^{Ab}	8.74^{Aa}	9.19^{Aa}
C18:3 ω-3	3.03^{Aa}	2.89^{Aa}	0.81^{Ac}	0.77^{Ad}	1.27^{Ab}	1.48^{Ab}	0.80^{Ac}	0.75^{Ad}	1.12^{Abc}	1.08^{Ac}
C20:5 ω-3	-	-	-	-	6.10^{Ba}	9.61 ^{Aa}	0.55^{Ab}	0.70^{Ab}	0.28^{Ab}	0.26^{Ab}
C22:6 ω-3	-	-	-	-	15.97^{Aa}	19.55^{Aa}	16.92^{Aa}	17.64^{Ab}	10.13^{Ab}	10.28^{Ac}
LA/ALA	15.07^{Ac}	15.53^{Ac}	42.25^{Aa}	46.74^{Aa}	16.00^{Ac}	13.44^{Ad}	22.27^{Ab}	18.62^{Ab}	18.65^{Abc}	19.12^{Ab}
LA+ARA/ALA+DHA	15.20^{Ab}	15.67^{Ab}	42.34^{Aa}	46.74^{Aa}	1.24^{Ac}	1.00^{Acd}	1.12^{Ac}	0.87^{Bd}	2.64^{Ac}	2.64^{Ac}
ARA/EPA	-	-	-	-	0.13^{Ac}	0.14^{Ab}	4.21^{Ab}	3.08^{Ab}	31.62^{Aa}	35.36^{Aa}
ARA/DHA	-	-	-	-	0.05^{Ac}	0.07^{Ab}	0.13^{Ab}	0.12^{Ab}	0.86^{Aa}	0.89^{Aa}
ARA/EPA+DHA	-	-	-	-	0.03^{Ac}	0.05^{Ac}	0.12^{Ab}	0.12^{Ab}	0.85^{Aa}	0.88^{Aa}

[1]Mean values within a row having different superscripts are significantly different ($p \leq 0.05$) where the small letters indicate significant among dietary treatment groups for each gender separately as indicated by one-way ANOVA followed by Duncan's multiple range test (a>b>c>d) while the capital letters indicate significant between males and females as indicated by two-way ANOVA followed by Duncan's multiple range test (A>B); [2]SBO: Soy Bean Oil (control); SO: Sesame Oil; FO: Fish Oil; DHA: DHA algae oil; DHA/ARA: DHA+ARA algae oils 1:1 ratio; [3]LA: Linoleic Acid; ALA: Alpha-Linolenic Acid; ARA: Arachidonic Acid; EPA: Eicosapentaenoic Acid; DHA: Docosahexaenoic Acid; Tr: Trace

Table 4: Femur mineral content and *Ex vivo* tibia PGE_2 level of male and female rabbits fed diets with different dietary oil sources and varying ω-6/ω-3 ratios for 100 days[1]

	[2]Dietary treatment									
	SBO		SO		FO		DHA		DHA/ARA	
Contents	M (n = 5)	F (n = 5)	M (n = 5)	F (n = 3)	M (n = 5)	F (n = 3)	M (n = 5)	F (n = 5)	M (n = 5)	F (n = 4)
[3]Ca	184.15±2.570^{Bb}	197.56±2.180^{Ac}	213.45±3.740^{Ab}	174.00±4.500^{Bd}	243.79±15.490^{Aa}	250.11±1.010^{Ab}	256.14±6.130^{Aa}	263.44±2.52^{Ab}	271.27±7.690^{Aa}	291.70±6.550^{Aa}
P	114.79±5.080^{Ab}	122.83±1.110^{Ab}	132.16±2.720^{Aa}	135.52±3.400^{Aa}	136.32±2.000^{Aa}	136.44±0.120^{Aa}	131.94±2.440^{Aa}	130.15±1.29^{Aa}	134.21±1.750^{Aa}	134.48±2.660^{Aa}
Mg	2.90±0.120^{Ac}	3.07±0.040^{Ac}	3.24±0.100^{Ac}	2.64±0.040^{Bd}	3.78±0.230^{Ab}	3.91±0.190^{Ab}	3.88±0.140^{Aab}	4.05±0.04^{Ab}	4.37±0.160^{Aa}	4.67±0.110^{Aa}
Zn	0.08±0.004^{Ab}	0.09±0.004^{Aab}	0.10±0.008^{Aa}	0.08±0.003^{Bbc}	0.07±0.005^{Ab}	0.07±0.003^{Ac}	0.07±0.004^{Ab}	0.08±0.01^{Aabc}	0.10±0.009^{Aa}	0.10±0.002^{Aa}
[4]PGE_2 (ng g^{-1})	12.56±0.310^{Aa}	11.49±1.950^{Aa}	13.33±1.050^{Aa}	11.53±0.760^{Aa}	6.91±0.870^{Ac}	9.98±1.580^{Aa}	9.43±0.580^{Ab}	10.55±0.92^{Aa}	12.35±0.350^{Aa}	12.96±0.940^{Aa}

[1]Data are expressed as mean±SEM. Values within a row having different superscripts are significantly different ($p \leq 0.05$) where the small letters indicate significant among dietary treatment groups for each gender separately as indicated by one-way ANOVA followed by Duncan's multiple range test (a>b>c>d) while the capital letters indicate significant between males and females as indicated by two-way ANOVA followed by Duncan's multiple range test (A>B). [2]SBO: Soy Bean Oil (control); SO_1: Sesame Oil; FO: Fish Oil; DHA: DHA algae oil; DHA/ARA: DHA+ARA algae oils 1:1 ratio; [3]Ca: Calcium; P: Phosphorous; Mg: Magnesium; Zn: Zinc (mg g^{-1} bone); [4]Prostaglandin E_2 (ng g^{-1} bone)

ARA/EPA, ARA/DHA and ARA/EPA+DHA ratios in females. The rabbits fed on the DHA diet had the lowest LA+ARA/ALA+DHA ratio in both genders. Those fed the DHA/ARA diet had the highest ARA/EPA, ARA/DHA and ARA/EPA+DHA ratios in both genders. However, rabbits fed the control diet had the lowest LA/ALA ratio in males but not in females. There were significant differences between males and females in EPA concentration in the FO fed group where females had a higher value than males; furthermore, significant differences between males and females in LA+ARA/ALA+DHA ratio were observed in the DHA fed group where males had a higher value than females (Table 3). There was a significant main effect of dietary treatments on femur Ca, P, Mg and Zn contents in both genders (Table 4). Ca content was significantly higher in males and females in the DHA/ARA, DHA and FO groups (271.3±7.7, 291.7±6.6; 256.1±6.1, 263.4±2.5 and 243.8±15.5, 250.1±1.01 mg g^{-1} bone, respectively) compared to the control and the SO groups (184.2±2.6, 197.6±2.2 and 213.5±3.7, 174±4.5 mg g^{-1} bone, respectively). Similarly, Mg content was significantly higher in males and females in the DHA/ARA, DHA and FO groups (4.4±0.2, 4.7±0.1; 3.9±0.1, 4.1±0.04 and 3.8±0.2, 3.9±0.2 mg g^{-1} bone, respectively) compared to the control and the SO groups (2.9±0.1, 3.1±0.04 and 3.2±0.1, 2.6±0.04 mg g^{-1} bone, respectively).

P content was significantly higher in males and females in the FO, DHA/ARA, SO and DHA groups (136.3±2, 136.4±0.1; 134.2±1.8, 134.5±2.7; 132.2±2.7, 135.5±3.4 and 131.9±2.4, 130.2±1.3 mg g^{-1} bone, respectively) than the control (114.8±5.1 and 122.8±1.1 mg g^{-1} bone). Further, Zn content was significantly higher in the DHA/ARA and the SO male groups (0.10±0.009 and 0.10±0.008 mg g^{-1} bone, respectively) compared to the control (0.08±0.004 mg g^{-1} bone) and the FO and the DHA groups had intermediate values (0.07±0.005 and 0.07±0.004 mg g^{-1} bone, respectively). In females, Zn content was significantly lower in the FO group (0.07±0.003 mg g^{-1} bone) and slightly increased in the DHA/ARA group (0.10±0.002 mg g^{-1} bone) compared to the control (0.09±0.004 mg g^{-1} bone) and the SO and the DHA groups had intermediate values (0.08±0.003 and 0.08±0.01 mg g^{-1} bone, respectively). In addition, significant differences between males and females were observed in Ca, Mg and Zn but not in P content. Males fed the SO diet had significantly higher Ca, Mg and Zn contents than females whereas females fed the SBO diet had significantly higher Ca content than males, however there were no significant differences between males and females in the other groups. Furthermore, long-term dietary lipid supplementation with various ω-6/ω-3 ratios had a pronounced effect on *Ex vivo* PGE_2 release from tibia in males but not in females (Table 4). *Ex vivo* PGE_2 level was significantly lower in male rabbits fed the FO or the DHA diets (6.9±0.9 and 9.4±0.6 ng g^{-1} bone, respectively) compared to the other groups (SBO, 12.6±0.3; SO, 13.3±1.1 and DHA/ARA, 12.4±0.4 ng g^{-1} bone) while in females, no significant differences were found among the dietary treatment groups (SBO, 11.5±2; SO, 11.5±0.8; FO, 10±1.6; DHA, 10.6 ±0.9 and DHA/ARA, 13±0.9 ng g^{-1} bone), however no significant differences were observed between males and females in *Ex vivo* PGE_2 level in all groups.

In addition, long-term dietary lipid supplementation with various ω-6/ω-3 ratios significantly affects bone Width (Wd.) and Weight (Wt) but not Length (Lt.) in both genders (Table 5). The effect of dietary lipid supplementation was pronounced in proximal femur and femur diaphysis widths in males and in humerus diaphysis, proximal femur and proximal tibia and tibia diaphysis widths in females but not in the other bone widths.

Compared to control group, proximal femur and femur diaphysis widths were significantly narrower in the DHA male group. In females, humerus diaphysis width was significantly narrower in the SO, FO and DHA/ARA

Table 5: Bone growth indicators of male and female rabbits fed diets with different dietary oil sources and varying ω-6/ω-3 ratio for 100 days[1]

	[2]Dietary treatment									
	SBO		SO		FO		DHA		DHA/ARA	
Bone growth indicator	M (n = 5)	F (n = 5)	M (n = 5)	F (n = 3)	M (n = 5)	F (n = 3)	M (n = 5)	F (n = 5)	M (n = 5)	F (n = 4)
Length (cm)										
Humerus	7.01±0.04^{Aa}	7.02±0.07^{Aa}	7.10±0.09^{Aa}	7.00±0.12^{Aa}	7.03±0.10^{Aa}	6.88±0.03^{Aa}	7.11±0.09^{Aa}	6.93±0.14^{Aa}	6.88±0.08^{Aa}	6.85±0.15^{Aa}
Forearm	7.97±0.04^{Aa}	7.92±0.06^{Aa}	8.06±0.07^{Aa}	7.92±0.07^{Aa}	8.03±0.13^{Aa}	7.75±0.05^{Aa}	7.92±0.07^{Aa}	7.90±0.13^{Aa}	7.85±0.00^{Aa}	7.85±0.15^{Aa}
Tibia	10.35±0.09^{Aa}	10.27±0.11^{Aa}	10.44±0.16^{Aa}	10.25±0.18^{Aa}	10.41±0.16^{Aa}	10.20±0.10^{Aa}	10.58±0.12^{Aa}	10.38±0.16^{Aa}	10.33±0.08^{Aa}	10.18±0.22^{Aa}
Femur	9.68±0.08^{Aa}	9.66±0.05^{Aa}	9.68±0.08^{Aa}	9.57±0.12^{Aa}	9.72±0.14^{Aa}	9.40±0.05^{Aa}	9.81±0.11^{Aa}	9.68±0.13^{Aa}	9.58±0.03^{Aa}	9.40±0.28^{Aa}
[3]Width (cm)										
Humerus A	1.23±0.06^{Aa}	1.29±0.02^{Aa}	1.31±0.01^{Aa}	1.27±0.03^{Aa}	1.28±0.02^{Aa}	1.25±0.05^{Aa}	1.26±0.02^{Aa}	1.25±0.00^{Aa}	1.33±0.03^{Aa}	1.29±0.03^{Aa}
Humerus B	0.92±0.01^{Aa}	0.93±0.02^{Aa}	0.96±0.01^{Aa}	0.93±0.03^{Aa}	0.92±0.03^{Aa}	0.88±0.03^{Aa}	0.90±0.02^{Aa}	0.92±0.02^{Aa}	0.93±0.03^{Aa}	0.90±0.02^{Aa}
Humerus C	0.77±0.01^{Ba}	0.80±0.00^{Aa}	0.78±0.01^{Aa}	0.68±0.04^{Ab}	0.72±0.03^{Aa}	0.70±0.00^{Ab}	0.70±0.02^{Aa}	0.73±0.02^{Aab}	0.75±0.05^{Aa}	0.70±0.02^{Ab}
Femur A	1.66±0.02^{Aa}	1.64±0.03^{Aa}	1.60±0.04^{Aa}	1.68±0.07^{Aa}	1.65±0.04^{Aa}	1.55±0.05^{Aa}	1.54±0.04^{Aa}	1.62±0.04^{Aa}	1.63±0.03^{Aa}	1.61±0.06^{Aa}
Femur B	2.41±0.01^{Aab}	2.42±0.03^{Aab}	2.43±0.01^{Aa}	2.45±0.05^{Aa}	2.36±0.04^{Aabc}	2.30±0.00^{Ac}	2.29±0.02^{Ac}	2.35±0.03^{Aabc}	2.33±0.08^{Abc}	2.34±0.02^{Abc}
Femur C	0.63±0.03^{Aa}	0.60±0.02^{Aa}	0.64±0.03^{Aa}	0.55±0.05^{Aa}	0.58±0.03^{Aab}	0.53±0.08^{Aa}	0.49±0.01^{Ab}	0.53±0.02^{Aa}	0.58±0.03^{Aab}	0.51±0.03^{Aa}
Tibia A	1.52±0.01^{Aa}	1.53±0.02^{Aab}	1.55±0.02^{Aa}	1.57±0.03^{Aa}	1.45±0.04^{Aa}	1.38±0.03^{Ad}	1.46±0.02^{Aa}	1.47±0.02^{Abc}	1.45±0.05^{Aa}	1.44±0.02^{Acd}
Tibia B	0.63±0.02^{Aa}	0.62±0.03^{Aa}	0.68±0.01^{Aa}	0.60±0.00^{Bab}	0.59±0.02^{Aa}	0.50±0.00^{Ac}	0.57±0.03^{Aa}	0.55±0.00^{Abc}	0.63±0.03^{Aa}	0.56±0.01^{Aabc}
Weight (g)										
Humerus	4.51±0.14^{Aa}	4.47±0.10^{Aa}	4.51±0.15^{Aa}	4.02±0.15^{Ab}	4.13±0.21^{Aab}	3.61±0.13^{Ab}	3.80±0.07^{Ab}	3.86±0.05^{Ab}	3.81±0.07^{Ab}	4.01±0.16^{Ab}
Forearm	3.81±0.11^{Aa}	3.74±0.06^{Aa}	3.84±0.09^{Aa}	3.42±0.19^{Aa}	3.71±0.23^{Aab}	3.08±0.10^{Ab}	3.24±0.09^{Ab}	3.43±0.09^{Aa}	3.39±0.04^{Aab}	3.63±0.08^{Aa}
Tibia	7.83±0.28^{Aa}	7.67±0.15^{Aa}	7.92±0.27^{Aa}	7.14±0.30^{Aab}	6.94±0.43^{Aab}	5.94±0.33^{Ac}	6.34±0.18^{Ab}	6.55±0.21^{Abc}	6.30±0.25^{Ab}	6.02±0.29^{Ac}
Femur	9.49±0.31^{Aa}	9.18±0.18^{Aa}	9.55±0.24^{Aa}	8.60±0.36^{Aab}	8.54±0.45^{Aab}	7.06±0.52^{Ac}	7.58±0.18^{Ab}	7.85±0.23^{Abc}	7.83±0.06^{Ab}	7.64±0.24^{Ac}

[1]Data are expressed as mean±SEM. Values within a row having different superscripts are significantly different ($p \leq 0.05$) where the small letters indicate significant among dietary treatment groups for each gender separately as indicated by one-way ANOVA followed by Duncan's multiple range test (a>b>c>d) while the capital letters indicate significant between males and females as indicated by two-way ANOVA followed by Duncan's multiple range test (A> B); [2]SBO: Soy Bean Oil (control); SO: Sesame Oil; FO,: Fish Oil; DHA: DHA algae oil; DHA/ARA: DHA+ARA algae oils 1:1 ratio. [3]Humerus A, proximal humerus; B, humerus deltoid tuberosity; C, humerus diaphysis; Femur A, proximal epiphysis femur; B, proximal femur; C, femur diaphysis; Tibia A, proximal tibia; B, tibia diaphysis

groups; proximal femur width was significantly narrower in the FO group; proximal tibia width was significantly narrower in the FO and DHA/ARA groups and tibia diaphysis width was significantly narrower in the FO and DHA groups. In regard to bone weights and compared to control group, the DHA male group and the FO female group had significantly lighter forearm weight. The DHA and the DHA/ARA male groups and the FO, DHA and DHA/ARA female groups had significantly lighter humerus, tibia and femur weights. In contrast, there was no effect of dietary lipid treatments on the lengths of all bones in both genders. However, significant differences between males and females were observed in bone widths but not in weights or lengths. The SBO female group had a wider humerus diaphysis width than males whereas the SO male group had a wider tibia diaphysis width than females. This is the first study to the best of the knowledge, reporting the long-term effect of different dietary oil sources with varying ω-6/ω-3 ratios on bone growth, bone marrow fatty acids and *ex vivo* tibia PGE_2 levels, femur mineral content including Ca, P, Mg and Zn in growing male and female rabbits.

There was a pronounced effect of diets varying from 0.4-21.8 in their ω-6/ω-3 fatty acids ratio on growth measured by final body weight, weight gain, growth rate and food efficiency of male rabbits. Previously in growing male rats, several studies found no effect (Li, 1999; Sirois *et al*., 2003; Kelly *et al*., 2003; Green *et al*., 2004; Mollard *et al*., 2005b; Lobo *et al*., 2009) but one study in male rabbits reported decreased final body weight (Judex *et al*., 2000), this discrepancy might be related to short duration of these studies, 15-63 days versus 100 days for the present study. Further, it might be related to the variation in oils blend that used and the amount added. However, the detrimental effect that was observed in the study of Judex *et al*. (2000) was due to the high fat diet (100 g kg^{-1} diet) compared to the current study (70 g kg^{-1}). Nevertheless, two studies done by Liu *et al*. (2003, 2004) found no effect after 210 days of feeding in male quails.

The reduction in growth indicators of male rabbits fed the DHA diet in the current study was due to low food intake. Whether the decrease in food intake was because of decreased palatability of the algae oils diet or intake of particular fatty acids remain unknown and need further investigation. In contrast, there was no effect of diets varying from 0.4-21.8 in their ω-6/ω-3 fatty acids ratio on the growth of female rabbits. This result was in agreement with a previous study that found no effect on final body weight of female rats (Sirois *et al*., 2003). However, the mechanism(s) for having significant differences in growth indicators of male rabbits but not in females using similar diets in the present study is unclear and further studies in this area are warranted. Interestingly, the slight increase in FE observed in male and female rabbits fed the DHA/ARA diet compared to the control in the current

study, despite their low food consumption and weight gain is elusive thus further studies are needed to determine how these fatty acids affect rabbits FE and whether they have a specific role to play in growth. This study confirmed that different dietary oil sources varying in their ω-6/ω-3 ratios significantly altered the fatty acids level of bone marrow; moreover, bone marrow fatty acid profile reflected the dietary level of ω-6 and ω-3 fatty acids fed to rabbits. Rabbits fed the FO diet (lower in ω-6/ω-3 ratio and higher in EPA and DHA) maintained lower ω-6/ω-3 ratio and higher EPA and DHA concentrations in their bone marrow. Those fed the SO diet (higher in ω-6/ω-3 ratio) maintained higher ω-6/ω-3 ratio. Those fed the SBO control diet (higher in LA and ALA) maintained higher LA and ALA concentrations and intermediate value of ω-6/ω-3 ratio. Furthermore, this study showed that bone marrow fatty acid profile in the different groups reflected the effects of different dietary treatments as reported previously by Li (1999) and Watkins *et al.* (2005, 2006). Furthermore, there was a main effect of different dietary ω-6/ω-3 ratios on bone marrow ARA and EPA concentrations, ARA/EPA, ARA/DHA and ARA/EPA+DHA ratios but not on LA, ALA or DHA concentrations. As the dietary ω-6/ω-3 ratio declined, the concentration of EPA increased while ARA decreased further as the dietary ω-6/ω-3 ratio declined, so did the ARA/EPA, ARA/DHA and ARA/EPA+DHA ratios.

Male and female rabbits fed SBO or SO diets containing high LA and ALA concentrations with no ARA, EPA and DHA had higher levels of LA and ALA yet a lower level of ARA and no EPA and DHA in bone marrow; in contrast, those fed FO, DHA or DHA/ARA diets supplemented with additional ARA, EPA and DHA had higher levels of these fatty acids in bone marrow compared to SBO and SO groups. The concentrations of ARA, EPA and DHA found in tissue phospholipids are the net result of the rates of endogenous synthesis from LA and ALA and the amount of preformed ARA, EPA and DHA in the diet (Innis, 2000). Furthermore, there were preferential uptake of preformed ARA, EPA and DHA compared with the biosynthetic route (Crawford, 2000). Therefore, the absence of EPA and DHA in bone marrow of rabbits fed SBO or SO diets and the depletion in ARA level was presumably due to low activity of desaturation and elongation enzymes in the growing rabbits, although high concentrations of the precursors LA and ALA or low capacity of the bone marrow to synthesize ARA, EPA and DHA from their precursors compared to other organs such as liver thus inclusion of the diet with preformed ARA, EPA and DHA is more effective to enrich bone marrow tissue with these essential fatty acids than to supplement with the precursors. Moreover, results from the present study showed that supplementation with high concentrations of EPA and DHA from fish oil resulted in increased EPA and DHA and decreased ARA concentrations in bone marrow, supplementation with high concentration of DHA from DHA algae oil although, low EPA concentration resulted in increased DHA and decreased ARA concentrations while supplementation with both ARA and DHA from DHA and ARA algae oil blend although, low in EPA concentration resulted in a balance between ARA and DHA concentrations. The concentrations of EPA and DHA were negatively correlated ($r = -0.51$ and $r = -0.85$, respectively) with ARA concentration. ARA and EPA compete for the same biosynthetic enzyme systems (Whelan, 1996; James *et al.*, 2000) with substrate preference of ARA over EPA (Lands, 1992). However, Croft *et al.* (1988) reported an antagonistic effect of EPA on ARA in rat leukocytes. Studies competitive interaction of dietary ω-6 and ω-3 in different animals showed that excess DHA intake competitively inhibit ω-6 fatty acid metabolism, reducing their incorporation into tissue phospholipids and vice versa (Boyle *et al.*, 1998; Presa-Owens *et al.*, 1998; Ward *et al.*, 1998). Presa-Owens *et al.* (1998) demonstrated that feeding piglets for 18 days a formula with 0.8% of total fatty acids ARA with no DHA, resulted in a higher ARA content in plasma, liver, heart and kidney tissue and lower plasma DHA and reduced EPA level in the same tissues. In contrast, piglets fed formula containing 0.3% DHA with no ARA had higher levels of DHA and EPA in plasma, liver, heart and kidney yet, lower levels of ARA in plasma and brain (Presa-Owens *et al.*, 1998). Similarly, Ward *et al.* (1998) fed new born rats, a formula with three levels of ARA and DHA (0, 0.4 and 2.4%) for 13 days. They found that rats fed formula supplemented with additional ARA (2.4%) had higher levels of ARA but lower levels of DHA in red blood cells and brain tissue and vice versa when the rats fed formula with additional DHA. Thus, the result of the current study confirmed the competition between ARA and EPA and revealed that the competition between ARA and DHA was in a substantially greater degree than the competition between ARA and EPA.

In addition, results from the present study found that the level of EPA was significantly different between male and female rabbits; females had significantly higher concentrations compared to males. This finding suggests that the two genders incorporate ω-3 PUFA into bone marrow at a different rate and/or through a different mechanism. A study has suggested that females tend to have higher plasma levels and synthesis of ω-3 PUFA

than males (Burdge, 2006). The finding of the current study was on the contrary with Lau *et al.* (2009) where they found that male mice had higher level of EPA than female in the femur. Male and female rabbits fed on the FO diet low in ω-6/ω-3 ratio had significantly higher Ca, P and Mg contents; despite their low intake of these minerals as a consequence of low food intake than those fed on the control diet with high ω-6/ω-3 ratio. The reduction in ω-6/ω-3 ratio through the inclusion of ω-3 LCPUFA in the diet from fish oil in the present study has been shown to increase mineral content in the bone of growing rabbits. This result disagrees with previous studies where they found no effect in growing rats (Li, 1999; Kelly *et al*., 2003; Green *et al*., 2004; Mollard *et al*., 2005b; Lobo *et al*., 2009) while agrees with the study of Liu *et al.* (2003) where they found that the inclusion of ω-3 LCPUFA in the diet from fish oil significantly increased Ca and P contents in the bone of quails. This discrepancy might be related to short duration of these studies, 15-63 days versus 100 days for the present study and 210 days for the study of Liu *et al.* (2003). Although, results are conflicting, the present study does support a beneficial role of fish oil in bone Ca, P and Mg contents.

Male and female rabbits fed DHA or DHA/ARA diets low in ω-6/ω-3 ratio had significantly higher Ca, P and Mg contents; despite their low intake of these minerals as a consequence of low food intake, than those fed on the control diet with high ω-6/ω-3 ratio. Interestingly, male and female rabbits fed DHA or DHA/ARA diets maintained Ca/P ratio of 2:1 (1.94 and 2; 2 and 2.2, respectively) compared to the other groups. The reduction in ω-6/ω-3 ratio through the inclusion of ω-3 LCPUFA in the diet from marine algae oils in the present study has been shown to increase mineral content and maintain optimal Ca/P ratio in the bone of growing rabbits thus, it is possible that these novel oils may be promising dietary sources for promoting bone mineralization during the growing stage.

In contrast, male and female rabbits fed on the control diet; rich in LA with high dietary ω-6/ω-3 ratio had significantly lower Ca, P and Mg contents; despite their high intake of these minerals as a consequence of high food intake than those fed DHA, DHA/ARA or FO diets with low dietary ω-6/ω-3 ratios. This result clearly showed that the elevation in ω-6/ω-3 ratio as a consequence of the increase in LA had a detrimental effect on bone mineralization thereby bone formation and growth.

In regard to the Zn content in the femur, compared to the control group, the low ω-6/ω-3 ratio found in DHA/ARA group was significantly increased in males while the low ω-6/ω-3 ratio found in FO group was significantly decreased in females. Dietary Zn intake was low in both male and female rabbits fed DHA/ARA or FO diets as a consequence of low food intake. During Zn deficiency, the amount of this mineral in the bone decreases because bone is an endogenous source of Zn when the dietary supply is low (Jackson *et al*., 1982). The mechanism underlying the different response between males and females in femur Zn content to low ω-6/ω-3 ratio found in the present study is unclear and need further investigation; however, it suggests that Zn metabolism is altered in response to LCPUFA particularly to their ratio.

Unlike the current study where the researchers found marked effects of dietary lipid treatments varying in their ω-6/ω-3 ratio on the forearm, humerus, tibia and femur weights, other researchers found no effects on femur (Sirois *et al*., 2003; Green *et al*., 2004; Mollard *et al*., 2005b) or tibia weights (Liu *et al*., 2003; 2004; Lobo *et al*., 2009) of rats or quails. The reduction in the forearm, humerus, tibia and femur weights of male and female rabbits fed the FO, DHA or DHA/ARA diets in the current study is unclear and need further investigations. With respect to bone widths, the result of the current study found that female rabbits but not males fed the fish oil diet had narrower proximal femur width than the control group, however femur diaphysis width did not differ due to diet in females but not in males. This result was in agreement with the result of Sirois *et al.* (2003) that femur diaphysis width was not altered by the fish oil diet in female rats; in contrast it disagrees with the result of Mollard *et al.* (2005b) that diet had a main effect on the femur diaphysis width; male rats fed the fish oil diet had a greater width than the control group, however, proximal femur width did not differ due to diet. Bone diaphysis (shaft) is largely composed of cortical bone whereas bone epiphysis (head) contains mainly trabecular bone (Boskey, 1999; Anderson, 2000). Trabecular bone has higher metabolic activity than cortical bone and thus may respond more rapidly to dietary and/or pharmacological interventions (Malluche and Fraugere, 1986). This may explain why the proximal femur width of female rabbits fed the FO diet in the current study responds to dietary treatment while the femur diaphysis width did not. However, male rabbits fed the DHA algae oil diet in the current study had narrower proximal femur and femur diaphysis widths than the control group.

There are several possible reasons to explain this discrepancy. The differences in animal model (rabbits versus rats) and gender (females versus males) as well as the fat source (fish oil+soy bean oil vs. fish oil+ safflower oil) may account for these discrepancies in results. On the other hand, the negative effect of feeding the fish oil diet on the proximal femur width in the current

study presumably due to long-term feeding, however whether the reduction in the proximal femur width has detrimental effect on bone formation and mass need further investigation to clarify this issue. In addition, the result of the present study showed that humerus, forearm, tibia and femur lengths were similar in all experimental groups, suggesting that dietary lipid treatments had no significant effect on bone length. This result is in accord with observations obtained previously by others where they found that no diet effects on humerus (Li, 1999), femur (Kelly *et al*., 2003; Sirois *et al*., 2003; Green *et al*., 2004; Mollard *et al*., 2005b), or tibia lengths (Liu *et al*., 2003, 2004; Lobo *et al*., 2009) of rats or quails, however it disagrees with the result of Judex *et al*. (2000) where they found that tibia length of male rabbits fed the FO diet was significantly shorter than the control group. The reason for having detrimental effect from fish oil on tibia length in the study of Judex *et al*. (2000) was due to the high fat diet (100 g kg^{-1} diet) versus (70 g kg^{-1}) in the current study. In animal models increased fat intake has typically generated deleterious skeletal effects (Zernicke *et al*., 1995; Sanderson *et al*., 1997).

CONCLUSION

The present study demonstrated that in growing rabbits, long-term supplementation of various dietary ω-6/ω-3 ratios in the diets altered bone marrow fatty acids level and consequently modulated *Ex vivo* PGE_2 release in bone thereby reducing bone resorption and improving bone mass during growth. The reduction in the ω-6/ω-3 ratio resulted in significantly increased femur Ca, P and Mg contents in both genders, although low dietary intake of these minerals; suggesting an important role of LCPUFA in mineral metabolism and bone mineralization. Furthermore, the reduction in the ω-6/ω-3 ratio using fish oil or marine algae oils as sources of ARA, EPA and DHA supports femur Ca, P, Mg and Zn contents depending upon the dietary amount of these fatty acids and more important their ratio; suggesting that ARA, EPA and DHA may indirectly influence bone mass by making more minerals available for calcification.

In addition, the significant elevation in mineral content and the maintenance of optimal Ca/P ratio in bone of DHA/ARA and DHA fed groups proved that algae oils may be promising dietary sources for promoting bone mineralization and formation thus improving bone mass during the growth stage. Thus, it is critical to study more about the interactions among ω-6 (ARA) and ω-3 (EPA and DHA) LCPUFAs and mineral and bone metabolism during growth and early adulthood because these are the periods in which peak bone mass is set.

ACKNOWLEDGEMENTS

This study was funded by King Abdulaziz City for Sciences and Technology KACST (Research No. AT-17-45) as well as by Research Center of the Center for Female Scientific and Medical Colleges CFSMC, King Saud University KSU.

REFERENCES

AOAC, 1962. AOAC official method 962.11 phosphorus in wines colorimetric method first action 1962 final action 1963. JAOAC, 45: 624-624.

AOAC., 2002. AOAC Official Method 965.33, Peroxide Value of Oils and Fats. 17th Edn., AOAC International, Gaithersburg, MD., USA.

Anderson, J.B., 2000. Nutrition in Bone Health. In: Krause's Food, Nutrition and Diet Therapy, Mahan, L.K.S. and S. Escott-Stump (Eds.). W.B. Saunders Co., Philadelphia, USA., pp: 567-593.

Baron, R., L. Neff, D. Louvard and P.J. Courtoy, 1985. Cell-mediated extracellular acidification and bone resorption: Evidence for a low pH in resorbing lacunae and localization of a 100-kD lysosomal membrane protein at the osteoclast ruffled border. J. Cell Biol., 101: 2210-2222.

Baron, R., J.H. Ravesloot, L. Neff, M. Chakraborty, D. Chatterjee, A. Lomri and W. Horne, 1993. Cellular and Molecular Biology of the Osteoclast. In: Cellular and Molecular Biology of Bone, Noda, M. (Ed.). Academic Press Inc., San Diego, USA., pp: 445-495.

Baron, R., 1999. Anatomy and Ultrastructure of Bone. In: Primer on the Metabolic Diseases and Disorders of Mineral Metabolism, Flavus, M.J. (Ed.). Lippincott Williams and Wilkins, Philadelphia, USA., pp: 3-10.

Blanaru, J.L., J.R. Kohut, S.C. Fitzpatrick-Wong and H.A. Weiler, 2004. Dose response of bone mass to dietary arachidonic acid in piglets fed cow milk-based formula. Am. J. Clin. Nutr., 79: 139-147.

Bligh, E.G. and W.J. Dyer, 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol., 37: 911-917.

Boskey, A.L., 1999. Mineralization, Structure and Function of Bone. In: Dynamics of Bone and Cartilage Metabolism, Seibel, M.J., S.P. Robins and J.P. Bilezikian (Eds.). Academic Press, New York, USA., pp: 153-164.

Boyle, F.G., R.J. Yuhas, K. Goldberg and E.L. Lien, 1998. Interaction of n-3 long-chain polyunsaturated fatty acids with n-6 fatty acids in suckled rat pups. Lipids, 33: 243-250.

Broadus, A.E., 1999. Mineral Balance and Homeostasis. In: Primer on the Metabolic Diseases and Disorders of Mineral Metabolism, Flavus, M.J. (Ed.). Lippincott Williams and Wilkins, Philadelphia, USA., pp: 74-79.

Burdge, G.C., 2006. Metabolism of á-linolenic acid in humans. Prostaglandins Leukot. Essent. Fatty Acids, 75: 161-168.

Carlson, S.E., 1997. Functional effects of increasing omega-3 fatty acid intake. J. Pediatr., 131: 173-175.

Claassen, N., H. Coetzer, C.M. Steinmann and M.C. Kruger, 1995. The effect of different n-6/n-3 essential fatty acid ratios on calcium balance and bone in rats. Prostaglandins Leukot. Essent. Fatty Acids, 53: 13-19.

Coetzee, M., M. Haag and M.C. Kruger, 2007. Effects of arachidonic acid, docosahexaenoic acid, prostaglandin E_2 and parathyroid hormone on osteoprotegerin and RANKL secretion by MC3T3-E1 osteoblast-like cells. J. Nutr. Biochem., 18: 54-63.

Connor, T.B., P. Toskes, J. Mahaffey, L.G. Martin, J.B. Williams and M. Walser, 1972. Parathyroid function during chronic magnesium deficiency. John Hopkins Med. J., 131: 100-117.

Crawford, M.A., 2000. Placental delivery of arachidonic and docosahexaenoic acids: Implications for the lipid nutrition of preterm infants. Am. J. Clin. Nutr., 71: 275S-284S.

Croft, K.D., J.P. Codde, A. Barden, R. Vandongen and L.J. Beilin, 1988. Effect of dietary fish oils on the formation of leukotriene B_4 and B_5, thromboxane and platelet activating factor by rat leukocytes. Clin. Exp. Pharmacol. Physiol., 15: 517-525.

Czajka-Narins, D.M., 1996. Minerals. In: Krause's Food, Nutrition and Diet Therapy, Mahan, L.K. and S. Escott-Stump (Eds.). 9th Edn. W.B. Saunders Co., Philadelphia, USA., pp: 123-166.

Das, U.N., 2000. Essential fatty acids and osteoporosis. Nutrition, 16: 386-390.

Decsi, T. and B. Koletzko, 1994. Polyunsaturated fatty acids in infant nutrition. Acta Paediatr. 395: 31-37.

Dekel, S., G. Lenthall and M.J.O. Francis, 1981. Release of prostaglandins from bone and muscle after tibial fracture: An experimental study in rabbits. J. Bone Joint Surg. Br., 63: 185-189.

Fernandes, G., R. Lawrence and D. Sun, 2003. Protective role of n-3 lipids and soy protein in osteoporosis. Fatty Acids, 68: 361-372.

Fernstrom, J.D., 2000. Can nutrient supplements modify brain function. Am. J. Clin. Nutr., 71: 1669S-1673S.

Folch, J., M. Lees and G.H. Sloane Stanley, 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226: 497-509.

Green, K.H., S.C. Wong and H.A. Weiler, 2004. The effect of dietary n-3 long-chain polyunsaturated fatty acids on femur mineral density and biomarkers of bone metabolism in healthy, diabetic and dietary restricted growing rats. Prost. Leukotr. Ess. Fatty Acids, 71: 121-130.

Hattersley, G., J.A. Kerby and T.J. Chambers, 1991. Identification of osteoclast precursors in multilineage hemopoietic colonies. Endocrinology, 128: 259-262.

Heinegard, D. and A. Oldberg, 1989. Structure and biology of cartilage and bone matrix noncollagenous macromolecules. FASEB J., 3: 2042-2051.

Innis, S.M., 2000. Essential fatty acids in infant nutrition: Lessons and limitations from animal studies in relation to studies on infant fatty acid requirements. Am. J. Clin. Nutr., 71: S238-S244.

Jackson, W.J., D.A. Jones and R.H.T. Edwards, 1982. Tissue zinc levels as an index of body zinc status. Clin. Physiol., 2: 333-343.

James, M.J., R.A. Gibson and L.G. Cleland, 2000. Dietary polyunsaturated fatty acids and inflammatory mediator production. Am. J. Clin. Nutr., 71: 343S-348S.

Judex, S., G.R. Wohl, R.B. Wolff, W. Leng, A.M. Gillis and R.F Zernicke, 2000. Dietary fish oil supplementation adversely affects cortical bone morphology and biomechanics in growing rabbits. Calcified Tissue Int., 66: 443-448.

Kelly, O., S. Cusack, C. Jewell and K.D. Cashman, 2003. The effect of polyunsaturated fatty acids, including conjugated linoleic acid, on calcium absorption and bone metabolism and composition in young growing rats. Br. J. Nutr., 90: 743-750.

Kettler, D.B., 2001. Can manipulation of the ratios of essential fatty acids slow the rapid rate of postmenopausal bone loss. Alternative Med. Rev., 6: 61-77.

Lands, W.E., 1992. Biochemistry and physiology of n-3 fatty acids. FASEB J., 6: 2530-2536.

Lau, Y.Y., W.E. Ward, J.X. Kang and D.W.L. Ma, 2009. Femur EPA and DHA are correlated with femur biomechanical strength in young fat-1 mice. J. Nutr. Biochem., 20: 453-461.

Li, Y., 1999. Dietary conjugated linoleic acid reduces bone formation rate and alters biomarkers for bone metabolism in rats. Ph.D. Thesis, Purdue University, West Lafayette, IN., USA.

Li, Y., M.F. Seifert, D.M. Ney, M. Grahn, A.L. Grant, K.G. Allen and B.A. Watkins, 1999. Dietary conjugated linoleic acids alter serum IGF-I and IGF binding protein concentrations and reduce bone formation in rats fed (n-6) or (n-3) fatty acids. J. Bone Miner. Res., 14: 1153-1162.

Lian, J.B. and G.W. Stein, 1999. The Cells of Bone. In: Dynamics of Bone and Cartilage Metabolism, Seibel, M.J., S.P. Robins and J.P. Bilezikian (Eds.). Academic Press Inc., New York, pp: 153-164.

Liu, D., H.P. Veit, J.H. Wilson and D.M. Denbow, 2003. Long-term supplementation of various dietary lipids alter bone mineral content, mechanical properties and histological characteristics of Japanese quail. Poult. Sci., 82: 831-839.

Liu, D., H.P. Viet and D.M. Denbow, 2004. Effects of long-term dietary lipids on mature bone mineral content, collagen, crosslinks and prostaglandin E_2 production in Japanese quail. Poult. Sci., 83: 1876-1883.

Lobo, A.R., J.M. Filho, E.P. Alvares, M.L. Cocato and C. Colli, 2009. Effects of dietary lipid composition and inulin-type fructans on mineral bioavailability in growing rats. Nutrition, 25: 216-225.

Malaval, L., D. Modrowski, A.K. Gupta and J.E. Aubin, 1994. Cellular expression of bone related proteins during *in vitro* osteogenesis in rat bone marrow stromal cell cultures. J. Cell Physiol., 158: 555-572.

Malluche, H.H. and M.C. Fraugere, 1986. Atlas of Mineralized Bone Histology. Krager Publishing, New York, ISBN-13: 978-3805542012, pp: 136.

Mollard, R.C., M.E. Gillam, T.M. Wood, C.G. Taylor and H.A. Weiler, 2005a. (n-3) fatty acids reduce the release of prostaglandin E_2 from bone but do not affect bone mass in obese (fa/fa) and Lean Zucker rats. J. Nutr., 135: 499-504.

Mollard, R.C., H.R. Kovacs, S.C. Fitzpatrick-Wong and H.A. Weiler, 2005b. Low levels of dietary arachidonic and docosahexaenoic acids improve bone mass in neonatal piglets, but higher levels provide no benefit. J. Nutr., 135: 505-512.

Presa-Owens, S.D.L., S.M. Innis and F.M. Rioux, 1998. Addition of triglycerides with arachidonic or docosahexaenoic acid to infant formula has tissue- and lipid class-specific effects on fatty acids and hepatic desaturase activities in formula-fed piglets. J. Nutr., 128: 1376-1384.

Price, C. and P. Thompson, 1995. The role of biochemical tests in the screening and monitoring of osteoporosis. Ann. Clin. Biochem., 32: 244-260.

Reeves, P.G., 1997. Components of the AIN-93 diets as improvements in the AIN-76A diet. J. Nutr., 127: 838S-841S.

Reichling, T.D. and R.Z. German, 2000. Bones, muscles and visceral organs of protein-malnourished rats (*Rattus norvegicus*) grow more slowly but for longer durations to reach normal final size. J. Nutr., 130: 2326-2332.

Rude, R.K., H.E. Gruber, L.Y. Wei, A. Frausto and B.G. Mills, 2003. Magnesium deficiency: Effect on bone and mineral metabolism in the mouse. Calcified Tissue Int., 72: 32-41.

Saltman, P.D. and L.G. Strause, 1993. The role of trace minerals in osteoporosis. J. Am. Coll. Nutr., 12: 384-389.

Sanderson, J.P., N. Binkley, E.B. Roecker, J.E. Champ, T.D. Pugh, L. Aspnes and R. Weindruch, 1997. Influence of fat intake and caloric restriction on bone in aging male rats. J. Gerontol. A Biol. Sci. Med. Sci., 52: B20-B25.

Sauerwald, T.U., D.L. Hachey, C.L. Jensen, H. Chen, R.E. Anderson and W.C. Heird, 1997. Intermidiates in endogenous synthesis of C22:6 ù-3 and C20:4 ù-6 by term and preterm infants. Pediatr. Res., 41: 183-187.

Sherwood, L., 2004. The Peripheral Endocrine Glands. In: Human Physiology: From Cells to Systems, Sherwood, L. (Ed.). 5th Edn., Brooks Cole, Thomson Learning Inc., USA., pp: 701-747.

Sirois, I., A.M. Cheung and W.E. Ward, 2003. Biomechanical bone strength and bone mass in young male and female rats fed a fish oil diet. Prostaglandins Leukot. Essent. Fatty Acids, 68: 415-421.

Sun, D., A. Krishnan, K. Zaman, R. Lawrence, A. Bhattacharya and G. Fernandes, 2003. Dietary n-3 fatty acids decrease osteoclastogenesis and loss of bone mass in ovariectomized mice. J. Bone. Mine. Res., 18: 1206-1216.

Sun, L., H. Tamaki, T. Ishimaru, T. Teruya, Y. Ohta, N. Katsuyama and I. Chinen, 2004. Inhibition of osteoporosis due to restricted food intake by the fish oils DHA and EPA and perilla oil in the rat. Biosci. Biotechnol. Biochem., 68: 2613-2615.

Wallach, S., 1990. Effects of magnesium on skeletal metabolism. Magnes Trace Elem., 9: 1-14.

Ward, G.R., Y.S. Huang, E. Bobik, H.C. Xiang and L. Mutsaers *et al*., 1998. Long-chain polyunsaturated fatty acid levels in formulae influence deposition of docosahexaenoic and arachidonic acid in brain and red blood cells of artificially reared neonatal rats. J. Nutr., 128: 2473-2487.

Watkins, B.A., 1998. Regulatory effects of polyunsaturates on bone modeling and cartilage function. World Rev. Nutr. Diet, 83: 38-51.

Watkins, B.A., Y. Li, H.E. Lippman and S. Feng, 2003. Modulatory effect of omega-3 polyunsaturated fatty acids on osteoblast functions and bone metabolism. Prostaglandins Leukot. Essent. Fatty Acids, 68: 387-398.

Watkins, B.A., S.L.Y. Reinwald and M.F. Seifert, 2005. Protective actions of soy isoflavones and n-3 PUFAs on bone mass in ovariectomized rats. J. Nutr. Biochem., 16: 479-488.

Watkins, B.A., Y. Li and M.F. Seifert, 2006. Dietary ratio of n-6/n-3 PUFAs and docosahexaenoic acid: actions on bone mineral and serum biomarkers in ovariectomized rats. J. Nutr. Biochem., 17: 282-289.

Weiler, H., 2000. Dietary supplementation of arachidonic acid is associated with higher whole body weight and bone mineral density in growing pigs. Pediatr. Res., 47: 692-697.

Weiler, H.A. and S. Fitzpatrick-Wong, 2002. Dietary long-chain polyunsaturated fatty acids minimize dexamethasone-induced reductions in arachidonic acid status but not bone mineral content in piglets. Pediatr. Res., 51: 282-289.

Whelan, J., 1996. Antagonistic effects of dietary arachidonic acid and n-3 polyunsaturated fatty acids. J. Nutr., 126: S1086-S1091.

Zernicke, R.F., G.J. Salem, R.J. Barnard and E. Schramm, 1995. Long-term, high-fat-sucrose diet alters rat femoral neck and vertebral morphology, bone mineral content and mechanical properties. Bone, 16: 25-31.