THE EFFECTS OF DIETARY LIPIDS ON BONE CHEMICAL, MECHANICAL, AND HISTOLOGICAL PROPERTIES IN JAPANESE QUAIL (*COTURNIX C. JAPONICA*)

DONGMIN LIU

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D. Michael. Denbow, Chairman

Cynthia Denbow

Joseph. H. Herbein

Hugo P. Veit

James H. Wilson

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Blacksburg, Virginia

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DONGMIN LIU

D. M. Denbow, Chairman

Animal and Poultry Sciences

ABSTRACT

Japanese quail were used as animal models in four experiments to evaluate the effects of supplementing diets with different lipids on bone chemical, mechanical, and histological properties. In Exp. 1, laying hens were fed a basal diet containing either 5% soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF), or menhaden fish oil (FO). The addition of SBO in the maternal diet increased the levels of total n-6 fatty acids and arachidonic acid (AA, 20:4n-6) in yolk and tibial bones of newly hatched progeny ($P<0.01$), whereas the maternal FO diet elevated the concentrations of total n-3 fatty acids, eicosapentaenoic acid (EPA, 22:5n-3), docosahexaenoic acid (DHA, 22:6n-3) and total saturated acid, but greatly decreased the amount of AA in both egg and progeny tibiae ($P<0.01$). The maternal HSBO diet resulted in the accumulation of trans-18:1 fatty acid in egg yolks and tibiae at hatch. The addition of FO or HSBO to the maternal diet significantly lowered the *ex vivo* PGE_2 production of tibiae in newly hatched quail compared to those from hens given the SBO or CF diets ($P<0.01$). In Exp. 2, the addition of different lipids in the maternal diets did not affect growth, tibial length, diameter or collagen content of the progeny. However, supplementing the maternal diet with 5 % FO or HSBO increased the percent bone ash , increased bone pyridinium crosslinks of collagen, enlarged the cartilaginous proliferative and hypertrophied zones, increased diaphyseal cortical thickness of the tibiae in embryos ($P<0.05$), and subsequently increased tibial shear force, stiffness ($P<0.05$) and improved cortical thickness, density and trabecular density in early growth and development of progeny compared to those from hens consuming the SBO or CF diets ($P<0.05$). In Exp. 3, male quail at one month of age were fed a basal diet containing either 5% SBO, HSBO, CF or FO for seven months. Long-term supplementation in the diets of different lipids did not affect body weight, food intake, tibial length or diameter, but the FO group had the highest tibial percent ash, and both FO and HSBO increased tibial mineral content in aged quail compared to those fed the SBO or CF diets ($P<0.05$). At 8 months of age, quail fed FO had the highest concentrations of (n-3) fatty acids (20:5n-3, 22:5n-3, 22:6n-3) but the lowest amounts of 20:4n-6 in lipids from tibial cortical bone, whereas the SBO and CF diets greatly elevated (n-6) fatty acids and 20:4n-6 levels. The HSBO diet which contains t18:1 fatty acid resulted in t18:1 accumulation in bone. Long-term supplementation with FO or HSBO increased tibial shear force, stiffness and shear stress, as well as improved cortical thickness and density compared with the SBO or CF diets

(P<.05). In Exp. 4, the addition of SBO or CF to the diet for seven months decreased tibial mineral content compared to the FO diet (P<0.05). Quail fed SBO increased collagen concentration in the tibiae (P<0.05), but the level of collagen crosslinks was higher in quail fed FO or HSBO compared to those given the SBO or CF diets (P<0.05). The PGE_2 production in bone organ culture and marrow was greatly increased in quail maintained on the SBO or CF diets (P<0.05). PGE_2 production in the bone microenvironment was negatively correlated with the tibial percent ash and collagen crosslinks but had a positive correlation with tibial collagen concentration. The results of these studies demonstrate that either supplementing the maternal diets with or long-term exposure to different lipids alters the chemical composition and metabolism of skeletal tissue in both embryos and aged quail. Maternal dietary SBO or CF had an adverse effect on bone growth and development in embryos. Likewise, long-term exposure to SBO or CF diet impaired bone metabolism and remodeling. In contrast, the FO or HSBO diet had beneficial effects on bone modeling in embryos and remodeling in adult quail.

(***Key words*****:** Lipids, PGE_2, fatty acids, mechanical properties, histology, collagen, crosslinks, quail)

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INTRODUCTION

Commercial broilers have been selected for maximal growth rate. Such broilers often have compromised skeletal development and serious leg problems costing the poultry industry millions of dollars annually. Although dietary calcium and vitamin D_3 are traditionally believed to reduce bone mineral loss, calcium intake above normal requirement does not stimulate bone formation (Watkins *et al*., 1997a).

In humans, osteoporosis is one of the major challenges to health care. This disease is quickly becoming more severe in economically developed countries usually afflicting the aging population, particularly post-menopausal women. The incidence of osteoporosis-related fractures in North America is increasing dramatically (Bengner *et al*., 1988). Although this increase is partially attributable to an aging population (Gallagher, 1990; Horowitz, 1993), the increase in fracture incidence is extremely high. It is reported that there are 300,000 new cases of osteoporotic hip fractures annually in the United States alone (Horowitz, 1993), and 1.5 million people around the world suffer from osteoporosis (Das, 1994).

New research has shown that dietary lipids, depending upon the type and amount ingested, modify the fatty acid composition of bone, and this can modulate PGE_2 production and impact bone formation rate and bone modeling in young animals (Alam, *et al*., 1993; Shen *et al*., 1994; Xu *et al*., 1994; Watkins *et al*., 1996,1997b). One proposed mechanism by which dietary lipids influence bone metabolism is by altering prostaglandin biosynthesis (Watkins *et al*, 1996). Bone formation and its resorption are regulated by systemic hormones and locally produced factors by bone cells themselves (Mundy, 1993; Baylink, 1993; Raisz, 1993a). Prostaglandins, especially PGE_2, are produced from 20-carbon polyunsaturated essential fatty acid precursors (arachidonic acid) in osteogenic cells (Sardesai, 1992; Kokkinos *et al*., 1993; Waktins *et al*., 1997a) and have powerful effects on bone metabolism. *In vitro*, although PGE_2 can stimulate bone formation at a low concentration, its main function over a long term is to stimulate bone resorption due to an increase in replication and differentiation of new osteoclasts (Marks and Miller, 1993; Raisz *et al*., 1993b; Fall *et al*., 1994). However, no one has demonstrated with an experimental model whether polyunsaturated fatty acid sources in the diet affect the bone mechanical properties and histopathology, and whether this influence, if it exists, is related to changes in the bone organic matrix.

A few studies have demonstrated that different maternal dietary lipid sources resulted in significant changes in the fatty acid composition of postnatal developmental tissue in chicks (Cherian *et al*., 1991) and sucking mice (Huang *et al*., 1992) which generally reflected the maternal dietary lipid profile. Interestingly, the fatty acid composition of some tissues retain responsiveness to changes in the dietary provision of polyunsaturated fatty acids for about 3 weeks after hatching (Maldjian *et al*., 1996). At this stage, it is still unclear whether maternal dietary fatty acid sources affect the bone metabolism, growth and development of embryos and postnatal progeny. There is a paucity of information on the effects of the maternal diet on embryonic bone development. Previous studies in our laboratory noted that broiler chicks from hens fed chicken fat or soybean oil exhibited larger frame size and greater tibial weight and strength compared to chicks from hens fed menhaden oil (Marks, 1996). However, that study did not look at bone histology and metabolism. Such a study will lead to a better

understanding of the role of n-6 and n-3 fatty acids in bone biology and improving skeletal development of both commercial poultry as well as humans. The present study, therefore, was designed to investigate 1) the effects of different maternal dietary lipids on bone fatty acid composition and *ex vivo* PGE_2 biosynthesis in progeny of Japanese quail; 2) the effects of different maternal dietary lipids on bone chemical composition, mechanical and histological properties in progeny of Japanese quail; 3) long-term effects of dietary lipids on bone fatty acid composition, mechanical and histological properties; and 4) long-term effects of dietary lipids on bone mineral content, collagen, crosslinks and *ex vivo* PGE_2 production in Japanese quail.

REFERENCES

Alam, S. Q., P. P. Kokkinos, and B. S. Alam, 1993. Fatty acid composition and arachidonic acid concentrations in alveolar bone of rat fed diets with different lipids. Calcif. Tissue Int. 53:330-332.

Baylink, D. J., R. D. Kinkelman, S. Mohan, 1993. Growth factors to stimulate bone formation. J. Bone Miner. Res. 8:S565-S572.

Bengner, U., O. Johnell, and I. J. Redlund, 1988. Changes in incidence and prevalence of vertebral fractures during 30 years. Calicif. Tissue Int. 42:293-296.

Cherian, G., and J. S. Sim, 1991. Effects of feeding full fat flax and canola seeds to laying hens on the fatty acid composition of eggs, embryos, and newly hatched chicks. Poultry Sci. 70:917-922.

Das, U. N., 1994. Can essentiala fatty acids prevent osteoporosis? Med. Sci. Res. 22:163-165.

Fall, P. M., D. T. Breault, and L. G. Raisz, 1994. Inhibition of collagen synthesis by prostaglandins in the immortalized rat osteoblastic cell line pyla: structure-activity relations and signal transduction mechanisms. J. Bone Miner. Res. 9:1935-2943.

Gallagher, J. C., 1990. The pathogenesis of osteoporosis. Bone Miner. 9:215-227.

Horowitz, M.C., 1993. Cytokine and estrogen in bone: anti-osteoporotic effects. Science 260:626-627.

Huang, Y. S., P. E. Wainwright, P. R. Redden, D. E. Mills, B. B. Fleming, and D. F. Horrobin, 1992. Effects of maternal dietary fats with variable n-3/n-6 ratios on tissue fatty acid composition in sucking mice. Lipids 27:104:110.

Kalu, D. N., C. C. Liu, E. Salerno, M. Salih, R. Echon, M. Ray, B. W. Hollis, 1991. Insulin-like growth factor-I partially prevents ovariectomy-induced bone loss: A comparative study with human parathroid hormone. J. Bone Miner. Res. 6:548-553.

Kokkinos, P. P, R. Shaye, B. S. Alam, and S. Q. Alam, 1993. Dietary lipids, prostaglandin E_2 levels, and tooth movement in alveolar bone of rats. Calcif. Tissue Int. 53:333-337.

Machwate, M., E. Zerath, X. Holy, P. Pastoureau, 1994. Insulin-like growth factor-I increases trabecular bone formation and osteoblastic cell proliferation in unloaded rats. Endocrinology 134:1031-1038.

Maldjian, A., C. Cristofori, R. C. Noble, and B. K. Speake, 1996. The fatty acid

composition of brain phospholipids from chicken and duck embryos. Comp. Biochem. Physiol. 115B:153-158.

McCarthy, T. L., S. Casinghino, M. Centrella, and E. Canalis, 1994. Complex pattern of insulin-like growth factor binding protein expression in primary rat osteoblast enriched cultures: Regulation by prostaglandin E_2, growth hormone, and insulin-like growth factors. J. Cell. Physiol. 128:2895-2900.

Marks, E. L., 1996. Effect of maternal dietary fats on growth rate and bone development of commercial broilers. MS Thesis. Virginia Polytechnic institute and State University.

Marks, S. C. Jr., and S. C. Miller, 1993. Prostaglandins and skeleton: The legacy and chanllenges of two decades of research. Endocr. J. 1:337-344.

Mundy, G. R., 1993. Cytokines and growth factors in the regulation of bone remodeling. J. Bone Miner. Res. 8: S505-S510.

Raisz, L. G., 1993a. Bone cell biology: New approaches and unanswered questions. J. Bone Miner. Res. 8: S457-S465.

Raisz, L. G., C. C. Pilbeam, and P. M. Fall, 1993b. Prostaglandins: Mechanisms of action and regulation of production in bone. Osteoporosis Int. Suppl. 1:S136-S140.

Raisz, L. G., and P. M. Fall, 1990. Biphasic effects of prostaglandin E2 on bone formation in cultured fetal rat calvariae: Interaction with cortisol. Endocrinology 126:1654-1659.

Sardesai, V. M., 1992. Biochemical and nutritional aspects of eicosanoids. J. Nutr. Biochem. 3:562-579.

Schmid, C., I. Schlapfer, M. Waldvogel, J. Zapf, and E. R. froesch, 1992. Prostaglansin E_2 stimulates synthesis of insulin-like growth factor binding protein-3 in rat bone cells *in vitro*. J. Bone Miner. Res. 7:1157-1163.

Spencer, E. M., C. C. Liu, E. C. C. Si, G. A. Howard, 1991. *In vivo* actions of insulin-like growth factor-I on bone formation and resorption in rats. Bone 12:21-26.

Watkins, B. A, C-L, Shen, K. G. D. Allen, and M .F. Seifert, 1996. Dietary (n-3) and (n-6) polyunsaturates and acetylsalicylic acid alter ex vivo PGE_2 biosynthesis, tissue IGF-I levels, and bone morphometry in chicks. J. bone Miner. Res. 11:1321-1332.

Watkins, B. A., M. F. Seifert, and K. G. D. Allen, 1997a. Importance of dietary fat in modulating PGE_2 responses and influence of vitamin E on bone morphometry. World Rev. Nutr. Diet. 82:250-259.

Watkins, B. A., C. L. Shen, J. P. Mcmurtry, H. Xu, S. D. Bain, 1997b. Dietary lipids modulate bone prostaglandin E2 production, insulin-like growth factor-I concentration and bone formation rate in chicks. J. Nutr. 127:1084-1097.

Xu, H., B. A. Watkins, and H. D. Adkisson, 1994. Dietary lipids modify the fatty acid composition of cartilage, isolated chondrocytes and matrix vesicles. Lipids 29:619-625.

LITERATURE REVIEW

1. *Biochemistry and Physiology of Polyunsaturated Fatty Acids*

Contemporary understanding of the biochemical and physiological significance of essential fatty acids and their metabolism in poultry is still limited. Recent literature documented that some individual fatty acids, and especially their derivatives, are biologically active substances, having a wide range of functions in animals and humans (Nicosia *et al*., 1989; Simopoulos *et al*., 1991; Sardesai *et al*., 1992b; Das *et al*, 1991, 1994; Watkins, 1995). The dietary essential fatty acids are converted to polyunsaturated fatty acids (PUFAs) in poultry and mammals. Some of the PUFAs are substrates for biomembranes and eicosanoid biosynthesis, and the formation of eicosanoid is ubiquitous in the tissues and organs.

1.1. *Essential Fatty Acids*

The n-6 and n-3 series are two families of naturally occurring fatty acids derived from the parent fatty acids linoleic acid (LA, 18:2 n-6) and α-linolenic acid (ALA, n-3), respectively, which can't be synthesized endogenously, and thus are identified as essential fatty acids (EFAs) in animals. LA and ALA contain two and three methylene double bonds in their carbon chains, respectively, and are both 18 carbons in length. The n-3 and n-6 series of PUFAs are characterized by the position of first double bond present. The n-3 series have the terminal double bond at the third carbon from the methyl end of the acyl chain. Likewise, n-6 series of PUFAs have their terminal double bond at the sixth carbon from the methyl end. Seed and vegetable oils such as soybean oil, corn oil, safflower oil, sunflower oil, etc. are rich in LA, whereas linseed oil is a rich source of ALA. Eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) are present in significant amounts in some fish oil, such as menhaden fish oil. ALA is specifically required during embryonic development and early postnatal growth because significant concentrations of some α-linolenic acid derived long-chain fatty acids are found in the lipids of retina and brain (Budowski and Crawford, 1986; Anderson *et al*., 1989). Linoleic acid can affect fluidity, permeability, receptor activity and enzyme function of biomembranes by changing their fatty acid compositions (Murphy, 1990). The linoleic acid requirement for growing chickens, turkeys and quails is suggested to be 1% of the diet (NRC, 1994).

Most of the early work on EFA in either animals or humans focused on establishing the requirements and demonstrating deficiency symptoms. Thus, the important role of EFA on growth, reproduction and health maintenance has been documented extensively in the past years (Watkins, 1991, 1995;Grundy, 1996; Innis, 1996) and will not be further discussed here. However, the role of EFA on calcium metabolism and bone mineralization received much less attention. It was not until the end of the 1950's and early 1960's that a limited number of publications demonstrated that EFA deficiency in animals led to severe osteoporosis which was characterized by loss of normal collagen in bone and other tissues, loss of normal cartilage, bone demineralization and bone fragility (Sinclair, 1957; Biran, 1964). The mechanism by which these effects on bone was totally unclear at that time.

1.2. *Metabolism of Polyunsaturated Fatty Acids*

Animals and humans are able to synthesize saturated, n-7 and n-9 series of unsaturated fatty acids *de novo* from acetyl CoA. However, the n-3 and n-6 series of fatty acids can not be synthesized in mammals because mammalian cells lack both the delta-12 and 15 desaturases necessary for insertion of a double bond at the n-6 and n-3 positions, respectively, of the fatty acid chain (Lnnis, 1991, 1996; Sprecher, 1992). The EFA are metabolized by a number of pathways which include β-oxidation in mitochondria to generate energy, a series of alternating desaturations and chain elongations leading to the long-chain PUFA, and incorporation into glycerolipids. Apart from oxidation for energy and direct acylation for synthesizing tissue triglycerides, cholesteryl esters and phospholipids, the pathways and metabolic fates with emphasis on illustrating the desaturation and chain elongation in liver for EFA and *de novo* synthesis of fatty acids are summarized in Fig. 1.

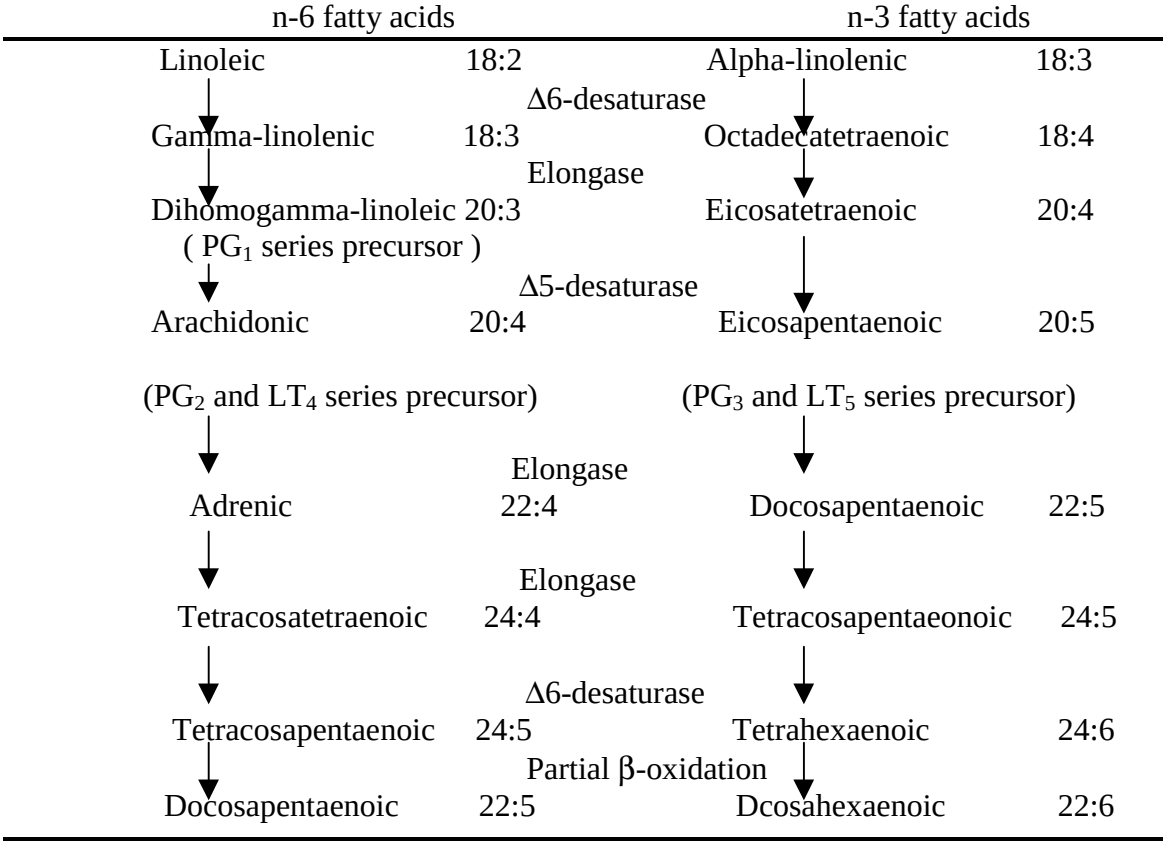

Fig. 1. Potential pathways of n-6 and n-3 fatty acid metabolism. PG_1, PG_2 and PG_3 are 1, 2 and 3 series of prostaglandin, respectively, and LT4 and LT5 are series 4 and series 5 leukotrienes.

In animal cells, ALA can be converted into eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). Similarly, dietary LA is converted via γ-

linolenic (20:3n-6) to arachidonic acid (20:4n-6). The introduction of a single double bond at the carbon 9 or at 7 position is catalyzed by the enzyme Δ^9-desaturase, which is universally present in both plants and animals, and converts stearic acid (18:0) to oleic acid (18:1n-9) or palmitic acid (16:0) to palmitoleic acid (16:1n-7) (Calder, 1997). The n-9, n-6 and n-3 families of PUFAs are not metabolically interconvertiable in mammals. In these series of reactions, it is believed that the same sequential enzymes metabolize both LA and ALA, and the different fatty acids compete for the same enzyme. Therefore, the metabolic utilization of one type of fatty acid will be influenced by the total of dietary fat, type, amounts and fractions of EFA (Holman, 1986; MacDonald and Sprecher, 1991). The rate limiting step is the Δ-6 desaturase which prefers a substrate in an order of ALA>LA>oleic acid.

1.3. *Effect of Fatty Acids on Glycerolipid Synthesis and Biomembrane Compositions*

An important role of EFA, PUFA, MUFA and saturated fatty acids is to synthesize glycerolipids, in which phospholipids are essential for cellular and subcellular membrane formation, as well as normal function (Murphy, 1990). The pathways, enzymes and regulation of glycerolipid synthesis was excellently reviewed elsewhere (Tijburg *et al*., 1989) and described by Watkins (1995). Previous studies have demonstrated that dietary fatty acids and PUFAs are not only incorporated into animal tissue lipids, but also that the fatty acid compositions of phospholipid-rich tissues reflect the dietary fatty acid profiles (Cherian *et al*., 1992; Sardesai, 1992b). In poultry, the lipid compositions and metabolism of yolk (Guenter *et al*., 1971; Cherian *et al*., 1992), embryo, brain (Budowski and Crawford, 1986; Anderson, *et al*., 1989) and various other tissues (Rogel and Watkins, 1987; Phetteplace and Watkins, 1989) can be effectively manipulated by dietary lipid supplementation. Studies also elucidated that the fatty acid composition of membrane phospholipids can be modified by dietary means, and by alteration of cellular desaturation and acylation reactions (Hwang, 1992; Sardesai, 1992a). When animals are fed fat-free or completely saturated fat diets, tissue lipids have only small amounts of n-3 and n-6 fatty acids and accumulate PUFAs of n-7 and n-9 series, which are then rapidly replaced by n-3 and/or n-6 fatty acids if supplemented in the diets. In addition, not only can dietary lipids enriched in n-3 fatty acids or trans-fatty acids be incorporated into animal as well as human tissues, but also concomitantly suppress the formation of arachidonic acid, presumably by competing with linoleic acid for the Δ-6 enzymes (Watkins, 1991). Thus, dietary lipid intake determines to a great extent the fatty acid composition of cell membranes and tissues (Horrobin, 1983), which in turn influence the eicosanoid production. Since cellular function is closely associated with membrane form, any slight changes in phospholipid fatty acid compositions of membrane might modulate membrane function such as fluidity, permeability and even structure.

1.4. *Dietary Fatty Acids and Eicosanoid Biosynthesis*

1.4.1. *Dietary Fatty Acids as Precursors of Eicosanoid Synthesis*

The eicosanoids are derived from 20-carbon polyunsaturated fatty acids (PUFAs), including dihomogamma linolenic (DGA), arachidonic (AA) and eicosapentaenoic (EPA). These precursor PUFAs are released from cell membrane phosphatidylcholine by the action of phospholipase A_2 or from membrane phosphatidylinositol-4,5-bisphosphate by the action of phospholipase C and a diacylglycerol lipase (Calder, 1997). Eicosanoids are generally categorized into two subsets, the cyclooxygenase (CO) and lipoxygenase (LO) products (Wood, 1990). The former include prostaglandins (PGs) and thromboxanes (TXs), which are together termed prostanoids. The latter products include leukotrienes (LTs), lipoxins (LXs), hydroperoxyeicosatetraenoic acids (HPETEs) and hydroxyeicosatetraenoic acids (HETEs) (Fig. 2). The amounts and types of eicosanoids synthesized are determined by the relative availability of precursor fatty acids, by the activities of phospholipase A_2 and phospholipase C as well as by the activities of cyclooxgenase and lipoxygenase. EPA is a poor substrate for cyclooxygenase but a preferred one for lipoxygenase (Hwang, 1992). Therefore, normal tissues synthesize less triene PGs and TXA_3 than diene PGs and TXA_2, but more amounts of LTB_5 than LTB_4. In many cases, the eicosanoids formed from 20:5n-3 have weaker effects than their analogues derived from 20:4n-6. The production of eicosanoids is usually in minute quantities and are inactivated in a very short period of time. Thus, they act locally in an autocrine or paracrine manner via interaction with specific G-protein-linked receptors (Lnnis, 1996).

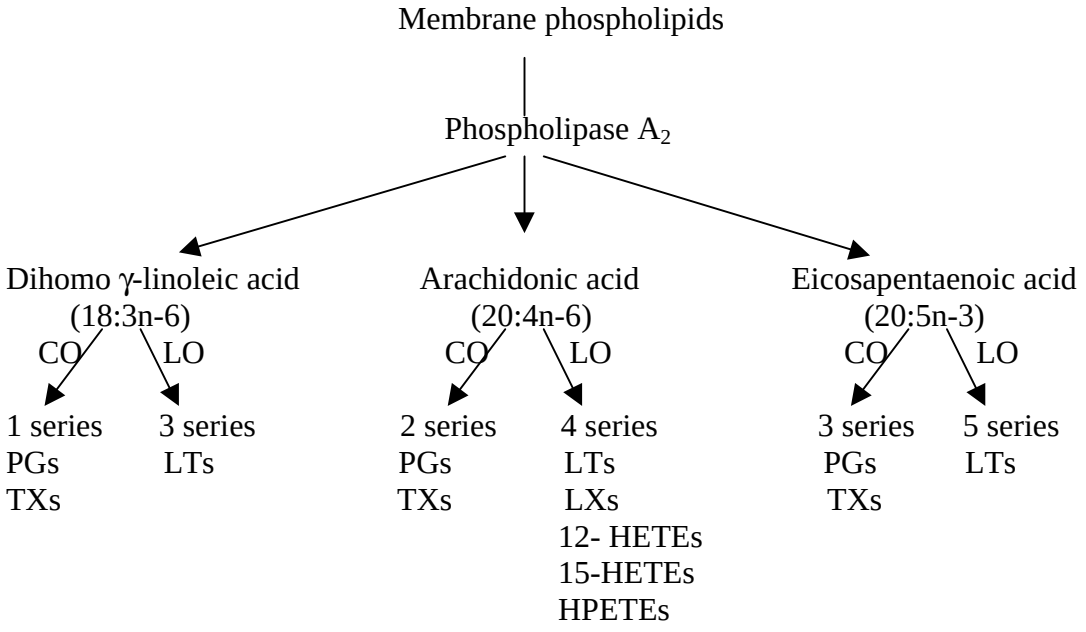

Fig. 2. Fatty acids and eicosanoid biosynthesis.

1.4.2. *Modulation of Eicosanoid Biosynthesis by Different Dietary Fatty Acids*

The modification of eicosanoid biosynthesis is achieved by the following two steps. First, there is competitive inhibition among LA and ALA families for their desaturation and enlongation toward precursor acids of eicosanoids. Another step in which dietary fatty acids can alter the production of eicosanoids is that both EPA and DHA competitively inhibit the oxygenation of AA by cyclooxygenase (Sardesai, 1992a). Since substrate availability is an important limiting factor in the biosynthesis of eicosanoids (Hwang, 1992), the amount of precursor acid released from phospholipids can limit the rate and amount of eicosanoids. As discussed earlier, the amounts and types of precursor fatty acids released from phospholipids depend on the composition of fatty acids in tissue phospholipids, which in turn is influenced by the composition of dietary fatty acids. Therefore, it is predictable that increasing dietary n-3 PUFAs can decrease the conversion of LA to AA and depress the formation of eicosanoids from AA, such as PGE_2 and LTB_4. The nutritional importance of this is that modification of dietary fatty acid compositions can be easily used to modify the biosynthesis of eicosanoids and subsequently alter physiological responses. One study suggested that the ratio of n-3/n-6 fatty acids is more efficient in modulating the amount of eicosanoid production than the absolute amount of fatty acids in the diet (Boudreau *et al*., 1991). However, more research is needed to assess desirable levels of dietary n-3/n-6 fatty acids for any specific purpose.

2. *Bone Tissue and Growth*

Bone is a dynamic, multifunctional tissue that consists of a structural framework of mineralized matrix and living cells, including chondrocytes, osteoblasts, osteocytes, osteoclasts, endothelial cells, monocytes, macrophages, lymphocytes, and hematopoietic cells. Osteoblasts are bone-forming cells that originate locally from mesenchymal stem cells. Functionally, osteoblasts synthesize and secret organic matrix containing type I collagen and other proteins which will be rapidly mineralized with hydroxyapitate of calcium phosphate. In addition to synthesizing the bone matrix, osteoblasts also synthesize various proteins such as alkaline phosphatase, osteocalcin, as well as produce numerous regulatory factors such as prostaglandins, cytokines and growth factors, which are reported to stimulate bone formation as well as bone resorption (Schmid *et al*., 1992; Baylink *et al*., 1993; Canalis *et al*., 1993; Mundy, 1993; Raisz, 1993a). The osteocyte, the most abundant cell type in mature bone, becomes incorporated in the mineralized bone matrix. The role of osteocytes in bone metabolism is still unclear. Recent studies suggest that they may transduct signals to osteoblasts lining the surfaces of trabecular or cortical bone during mechanical loading (Cowin *et al*., 1991).

Osteoclasts are large multinuclear bone-resorbing cells derived from hemopoietic stem cells in bone marrow by vascular infusion. During bone resorption, osteoclasts produce and release lysosomal enzymes, collagenase, hydrogen protons and free radicals which dissolve the mineral and degrade bone organic matrix (Blair *et al*., 1993; Watkins, 1997a).

All bone is originated from mesenchymal cells, but two different histogenetic processes exist for different types of bone formation. Generally, flat bones are formed by intramembranous ossification. Basically, the mesenchymal cells directly differentiate into osteogenic cells, which deposit organic matrix within embryonic connective tissue membrane and then become mineralized. Long bones are formed by endochondral ossification, a process in which cartilage is replaced by bone. The initial chondrocytes originate from mesenchymal cells. Then, bone grows simultaneously in diameter and length. The diameters of bone increase through apposition of bone matrix by osteoblasts located within the periosteum (interstitial ossification). The longitudinal bone growth however, is by endochondral ossification, and is more complicated. The first step in this process involves deposition of cartilage rather than bone. Next, the formed cartilage extracellular matrix mineralizes, and is followed by invasion of bone-forming cells and calcification with capillaries within the cartilage model (Pines and Hurwitz, 1990). The epiphyseal growth plate interposed between the epiphysis and metaphyseal region of a bone follows these sequential steps. There are several distinctive zones passing from the epiphyseal to the diaphyseal side of the growth plate: the resting zone containing stem cells; the proliferative zone with stacks of flattened cells lining in columns parallel to the long axis of the bone; the hypertrophic zone with hypertrophied chondrocytes; and the degenerative zone with a calcifying extracellular matrix and invading capillaries. During early bone growth, diaphyseal, and then epiphyseal centers of ossification develop with the cartilage mineralization and invasion of vasculature. When the bone reaches its ultimate length, the growth plate loses its functionality, becomes bone tissue and disappears.

During the bone growth and development, cell proliferation and hypertrophy in the growth plate are precisely controlled, but affected by growth hormone, insulin-like growth factor-I (IGF-I) and nutrients (reviewed by Pines and Hurwitz, 1990). After a bone becomes mature, it undergoes constant remodeling for structural obligation and metabolic responsibility. The cellular interactions occurring during a remodeling cycle include four main events: activation, resorption, reversal and formation (reviewed by Watkins, 1997a). Briefly, the remodeling cycle begins with recruitment of osteoclasts that resorb bone and form a cavity. Osteoblasts refill the cavity with deposition of new matrix, which subsequently becomes mineralized. In steady states, bone resorption is equal to bone formation and net bone loss is zero (Nilas, 1993). Therefore, bone loss occurs when bone resorption exceeds bone formation.

Anatomically, the skeleton is composed of two types of bone. Cortical bone is the compact outer layer of bone and the predominant bone in the shafts of long bones. Trabecular bone is the spongy internal network of bone that predominates in the vertebrae and metaphysis of long bones. Both types of bone contribute to bone strength (Nilas, 1993).

3. *Collagen, Crosslinks and Bone Strength*

Bone is a complex tissue composed of organic matrix and small ceramic crystals of calcium phosphate, which, as major solid components of bone, are highly organized and deposited into organic matrix. The bone inorganic matrix is well characterized and will not be further discussed here.

3.1. *Collagen and Crosslinks*

The predominant organic matrix in bone is type I collagen, the structural unit of which is tropocollagen (Lawson and Czernuszka, 1998). The tropocollagens are bundled into microfibrils forming larger fibrils, which further form a rope-like structure. The stability of collagen fibrils is achieved by the formation of cross-links, which can be intramolecular or intermolecular. The best characterized and most widely distributed cross-links of bone collagen are hydroxylysylpyridinoline (HP) and its deoxy analog, lysylpyridinoline (LP), also referred to as pyridinoline and deoxypyridinoline, which are derived from lysine and hydroxylysine, respectively. The first step in the cross-linking process is deamination of lysine and hydroxylysine by lysyl oxidase, producing aldehyde (Oxlund *et al*., 1995). Pyridinoline or deoxypyridinoline residues are then formed by a condensation reaction between an aldehyde and a lysyl or hydroxylysyl residues of other collagen molecules, with the resulting structure condensing with other aldehydes, finally forming the pyridinoline and deoxypyridinoline crosslinks (Dennis *et al*., 1996).

In the process of bone resorption, these pyridinium crosslinks will be released and excreted into urine. In recent years, the urinary concentrations of pyridinoline residues HP and LP have been widely accepted as one of the most promising markers of bone metabolism, particularlly of bone resorption and a wide range of bone disorders (Borisova *et al*., 1994; Robins, 1994; Marowska *et al*., 1996; Takahashi *et al*., 1996), since they are specifically located in collagen (Robins, 1994), and bone and dentin appear to be the only appreciable sources of this analogue (Farquharson *et al*., 1989).

3.2. *Bone Strength*

Bone strength is a complex property, which is still incompletely understood. Generally, collagen dominated organic matrix provides bone with its tensile strength and structural scaffolds for the deposition of minerals, while inorganic matrix, primarily hydroxyapatite, provides compressional strength (Rath *et al*., 1999). Bone strength and quality are not only related to the inorganic matrix, but also to the organic matrix. The relative amounts and properties of the mineral and collagen, as well as the bone microarchitecture and anatomy such as collagen fiber orientation, porosity, density and molecular structure of mineral crystals determine biomechanical strength (Currey, 1988; Martin and Ishida, 1989; Martin and Boardman, 1993; Landis, 1995; Shah *et al*., 1995). A decrease in bone collagen content results in a decrease in mechanical strength both in humans (Bailey *et al*., 1999) and poultry (Masse *et al*., 1996). However, past studies of the effects of alterations in bone microarchitecture on mechanical properties have focused more on the mineral content and/or mineral compositions than on collagen matrix (Crenshaw *et al*., 1986; Currey, 1988; Martin and Boardman, 1993; Verhaeghe *et al*.,

1994). Therefore, dietary supplementation of nutrients that affect mineralization is usually used to improve inorganic matrix of bone in both animals and humans, and the nutrients used are always limited to calcium, phosphorous and vitamin D_3, whereas little attention has been paid to improve the organic matrix quality.

In the past several years, it has been shown that not only bone mineral and collagen, but also intermolecular cross-links of collagen molecules contribute to bone strength. Recent studies consistently found that cross-links in both normal and osteoporotic bone have a strong and positive correlation with its mechanical strength (Batge *et al*., 1992; Knott *et al*., 1995; Oxlund *et al*., 1996; Rath *et al*., 1999). Furthermore, factors that affect cross-link formation can adversely affect bone strength. Animals with inhibition of lysyl oxidase or diet induced defects of collagen cross-linking such as vitamin B_6 or copper deficiency resulted in decreases in torsional stiffness and strength of bone, although the mineral content in the bone is normal (Farquharson *et al*., 1989; Wilmarth *et al*., 1992; Jonas *et al*., 1993; Masse *et al*., 1996). These results illustrated that decreased cross-links alone can impair the mechanical performance of bone tissue in the absence of mineral abnormalities.

It is interesting to indicate that not only cross-link content, but also the ratio of pyridinoline and deoxypyridinoline collagen cross-link in bone can be changed by nutritional factors, as observed recently in guinea pigs (Tsuchiya and Bates, 1997, 1998), but it is unclear whether the alteration of this ratio affects the bone strength. Furthermore, there is still a lack of information on how nutritional factors affect bone chemistry and bone strength. Therefore, research characterized to establish the relationships between nutrition, chemistry and bone strength may provide significant insight into the regulatory role of nutrients in bone modeling, and assist to improve bone quality and reduce skeletal problems in poultry by dietary manipulation.

4. Regulation *of Bone Metabolism by Local Factors*

Bone formation and resorption are regulated by locally produced factors. Certain eicosanoids [e.g. prostaglandins (PG), leukotrienes (LT)] exert stimulatory effects on bone formation and resorption. PGE_2 stimulates bone formation at low concentrations but resorption at high concentrations (Raisz and Fall, 1990; Raisz, 1993a). Also, PGE_3 (Raisz *et al*., 1989) and the leukotrienes, particularly LTB_4, LTC_4 and LTD_4 (Meghji *et al*., 1988) are potent stimulatory agents of bone resorption. In addition to eicosanoids, cytokines and local growth factors produced by immunocompetent cells and osteoblasts exert powerful effects on bone.

4.1. *Prostaglandins*

Prostaglandins, especially PGE_2, are produced from 20-carbon polyunsaturated essential fatty acid precursors (arachidonic acid) in osteogenic cells (Sardesai, 1992a; Kokkinos *et al*., 1993; Waktins *et al*., 1997a) and have biphasic effects on bone metabolism. *In vitro*, PGE_2 stimulates bone formation at a low concentration, but it enhances bone resorption at high concentration due to an increase in replication and differentiation of new osteoclasts (Marks and Miller, 1993; Raisz *et al*., 1993b; Fall *et al*., 1994). *In vivo* studies demonstrated that systemic administration of moderate amount of

PGE_2 by either subcutaneous, oral or direct infusions into bone induced an anabolic effect by significantly shifting to a positive bone balance, resulting in increased bone mass, mineral density, bone formation rate in intact rats (Jee. *et al*., 1985, 1987, 1990, 1991; Ito *et al*., 1993; Yang *et al*., 1993; Lin *et al*., 1995; Weinreb *et al*., 1997), ovariectomized rats (Wronski *et al*., 1988; Mori *et al*., 1990, 1992; Ke *et al*., 1992, 1993; Ma *et al*., 1994) and dogs (Li *et al*., 1990). This appeared to be due to stimulation of the replication and differentiation of osteoblast precursors to generate more osteoblasts after administration of PGE_2 (Jee *et al*., 1987; Weinreb *et al*., 1997; Suponitzky and Weinreb, 1998). Recent studies suggest that increased *ex vivo* PGE_2 biosynthesis (Watkins *et al*., 1996a, 1997b) in bone organ culture and *in vivo* PGE_2 production (Kokkinos *et al*., 1993) induced by dietary n-6 fatty acids are associated with decreased bone formation rate. It is therefore presumed that high level of endogenously produced PGE_2 may stimulate bone resorption.

4.2. *Cytokines*

4.2.1. *Definition and Classification*

Cytokines are extracellular signalling proteins, secreted for paracrine or autocrine functions (Endres and Schacky, 1996). They include the families of interleukins (IL), tumor necrosis factors (TNFs), interferons, and the haematopoietic growth factors (Endres, 1996). Among this large group of proteins, a subgroup is identified that has similar biological activities and consists of both IL-α and IL-β, TNFα, transforming growth factors (TGF-α) and TGF-β. The cytokines involved in bone growth and development, as well as health and disease, include epidermal growth factor (EGF), fibroblast growth factor (FGF), interferon-γ, IL-1, IL-6, TNF-α, TGF-α and TGF-β, and insulin like growth factors (IGF-I, IGF-II) (Watkins, and Seifert, 1997a). Whereas most cytokines stimulate bone resorption, only a few enhance bone formation. In osteoporosis and other bone diseases associated with estrogen and androgen deficiency, recent attention has been focused on cytokines generated in the bone marrow and microenvironment that may be responsible for stimulation of bone resorption. The most thoroughly studied cytokines involved in bone resorption are IL-1, IL-6 and TNF-α, which will be discussed in this section.

4.2.2. *Role of Cytokines in Bone*

Interleukin-1, produced primarily by monocytes and macrophages, is the first cytokine identified to be a powerful bone resorption stimulator seen in normal human bone marrow cultures (Devlin *et al*., 1998), ovariectomized mice (Miyaura *et al*., 1995) and postmenopausal osteoporosis (Pacifici *et al*., 1989,1990; Bismar *et al*., 1995). As aforementioned, there are two related molecules, IL-1α and IL-1β, which seem to have identical functions on bone resorption (Mundy, 1993). Tumor necrosis factor-α, which is secreted along with IL-1 from mononuclear cells, also stimulates osteoclast mediated bone resorption when used individually (Bertolini *et al*., 1986; Passeri *et al*., 1994; Rifas, 1999). Interleukin-1 and TNF may act on bone resorption independently since simultaneous inhibition of IL-1 and TNF is required to completely prevent bone resorption in the early postovariectomized mice (Kimble, 1995). Recently, these

cytokines were strongly suggested to regulate estrogen-deficient bone loss, since IL-1, TNF-α and IL-6 levels of human bone marrow cells are significantly increased after menopause (Bismar *et al*., 1995), and bone loss has been prevented in ovariectomized rats treated with IL-1 and TNF-α inhibitors (Pacifici, 1996). Therefore, an increase in bone-resorbing cytokines may be considered as a mechanism for postmenopausal bone loss. IL-1 and TNF have the ability to stimulate their own and each other's synthesis in an autocrine and synergistic fashion (Jilka, 1998).

In contrast to IL-1 and TNF, IL-6 is produced primarily by bone marrow stromal cells and osteoblastic cells (Ishimi *et al*., 1990; Mannolagas, 1995) in response to IL-1 and TNF (Cheleuitte *et al*., 1998; Kim *et al*., 1999). It is also found to be secreted in higher amounts from mononuclear cells isolated from postmenopausal subjects (Ralston *et al*., 1990; Cohen-Solal *et al*., 1993). Interleukin-6 has a similar function as IL-1 and TNF concerning bone resorption. Recent studies have shown that IL-6 stimulates the formation of osteoclasts and enhances osteoclastic bone resorption in murine metatarsal bone cultures (Ishimi *et al*., 1990), in cultures of human marrow mononuclear cells (Kukita *et al*., 1990), and in organ cultures of estrogen deficient mouse long bones (Miyaura *et al*., 1995; Manolagas, 1995, 1998). Furthermore, estrogen deficiency induced increases in osteoclast number and bone loss are effectively prevented in animals that lack the ability to synthesize or respond to IL-1, TNF or TL-6 (Poli *et al*., 1994; Kimble *et al*., 1995; Ammann *et al*., 1997; Lorenzo *et al*., 1997). IL-6 may mediate the effects of IL-1 and TNF on osteoclast-like cell formation in normal human bone marrow because neutralizing antibody to IL-6 also inhibited the stimulatory effects of IL-1 or TNF-α on osteoclast-like multineauclear cell formation (Devlin *et al*., 1998). However, another study has shown that IL-6 stimulates bone resorption cooperatively with IL-1 in estrogen deficient mice (Miyaura, 1995). These data also demonstrated that estrogen is critical for bone health maintenance. The new research indicated that one of the mechanisms by which estrogen acts on bone cells is by decreasing the production of bone resorbing cytokines from osteoblasts, or bone marrow cells (Girasole *et al*., 1992; Passeri *et al*., 1993), and this regulation has been elucidated to be at the gene trasncriptional level (Pottratz *et al*., 1994; Ray *et al*., 1994).

In summary, IL-1, IL-6 and TNF represent an interacting cascade, inducing osteoclastic bone resorption cooperatively or independently, either by inducing the proliferation of osteoclast precusor cells or inducing the activation of the differentiated osteoclasts (Kanatani *et al*., 1994; Pacifici 1995; Suda *et al*., 1995). Excessive production of these cytokines in bone is closely associated with the pathophysiology of bone loss and will eventually lead to several diseases, including hypercalcemia, osteopenia (Jilka *et al*., 1998) and particularly to postmenopausal osteoporosis (Mundy, 1993).

4.2.3. *Role of Dietary Lipids in Modulating Cytokine Production*

The effects of dietary fatty acids or lipids on cytokine productions have been extensively investigated both in human and animals in the past years.

4.2.3.1. *Human Studies*

A large number of studies have been performed to characterize the effects of supplementation of the diet of healthy subjects with fish oil rich in n-3 fatty acids on cytokine synthesis by peripheral blood mononeuclear cells (PBMNCs). Research also has been done on patients with autoimmune and inflammatory diseases. The results of most of these studies consistently report a reduction in bone resorbing cytokine production after supplementation with fish oil or other n-3 fatty acid enriched oil, as compared to n-6 enriched oil. Endres *et al*. (1989) first reported that n-3 polyunsaturated fatty acids (PUFAs) suppressed *ex vivo* production of IL-1 and TNF by 40% and 20%, respectively, and further indicated that the diminished levels of these cytokines remained for 10 weeks after the supplementation had terminated. Meydani *et al*. (1991) supplemented the diet of healthy young (20-33 years old) and older (51-68 years of age) women with 2.4 g n-3 PUFAs per day for 3 months, and examined the *ex vivo* production of cytokines by PMBNCs at 4, 8 and 12 weeks. There were time-dependent and significant reduction of IL-1β (decreased by 48%-90%) and TNF (decreased by 58%-84%) in both young and older women. More recently, Caughey *et al*. (1996) indicated that a high dose of α-linolenic acid supplied via linseed oil has a similar function in suppressing the production of IL-1β and TNF-α as fish oil, and there were inverse relationships between the eicosapentaenoic acid [EPA, 20:5(n-3)] content of mononeuclear cell lipids and the production of IL-1 and TNF. Fatty acids also modulate the cytokine release *in vitro*. When human lymphocytes from heathy human volunteers were incubated with various fatty acids (Purasiri, 1997), both EPA and docosahexaenoic acid (DHA, 22:6(n-3) showed a greater inhibition of IL-1β and TNF-α release compared to other fatty acids. In another *in vitro* study, however, the results showed that both arachidonic acid (AA, 20:4(n-6) and EPA increased the release of IL-1 from human monocytic cell line and isolated PMNCs from healthy subjects after incubation with these fatty acids (Baldie *et al*., 1993). Suppression of IL-1 and TNF-α synthesis by n-3 fatty acids or n-3 fatty acid enriched fish oil has been found in other studies *in vivo* (Kremer, 1990; Cooper, 1993). Although human studies provide more consistent data, the research is mostly limited to circulating monocytes and lymphocytes.

4.2.3.2. *Animal Studies*

Studies with animal models have shown conflicting results. While some investigators report decreased bone resorbing cytokine synthesis similar to those found in human cells (Billiar *et al*., 1988; Renier *et al*., 1993; Grimm *et al*., 1994; Yaqoob *et al*., 1995; Robinson *et al*., 1996), others find increased cytokine synthesis (Lokesh *et al*., 1990; Hardardottir *et al*., 1991; Turek *et al*., 1991, 1998; Blok *et al*., 1992; Chang *et al*., 1995). It is most likely that the discrepancies in the literature result from the differences in the cell types and experimental protocols. Studies differ greatly in the amount of lipid given (50-200g/kg feed), duration of feeding (2-15 weeks), the species of animal used (mouse, rat, pig) and the sources of macrophage used (alveolar, perotoneal, Kupffer or peripheral blood). There is no study in any animal species or humans reporting the effect of dietary fatty acids upon bone cells or bone marrow cytokine synthesis. However, it might be expected that synthesis of bone resorbing cytokines in the bone

microenvironment can be altered by different dietary lipids, that will consequently affect bone modeling and remodeling.

4.2.4. *Insulin Like Growth Factors*

Insulin growth factors (IGF) are polypeptides secreted by cells in a paracrine or autocrine manner. These compounds stimulate growth and synthesis of DNA, RNA and proteins in cells, and subsequently stimulate differentiation of various cell types (Seifert *et al*., 1997). The biosynthesis and secretion of IGF is controlled by growth hormone (Erust *et al*., 1990; Watkins and Seifert, 1997a). IGF-II is generally more abundant than IGF-I in bone tissues of various species including human, neonatal mice and chicks, whereas adult mice and rats have more IGF-I than IGF-II (Bautista *et al*., 1990; Mohan and Baylink, 1991). IGFs, particularly IGF-I, may have an important regulatory role in bone modeling and remodeling, and it may partially mediate the action of other local factors. For example, the stimulatory function of PGE_2 on bone formation is always accompanied by an increase in the production of insulin-like growth factor-I (IGF-I) (Raisz and Fall, 1990; McCarthy *et al*., 1991; Schmid *et al*., 1992; Bichell, *et al*., 1993; Raisz *et al*., 1993b; Watkins *et al*., 1997b). The locally synthesized IGF-I by bone cells has been shown to stimulate replicated and differentiated function of osteoblasts *in vitro*, and consequently enhance the bone formation and prevent bone loss (Baylink *et al*., 1993; Machwate *et al*., 1994). IGF-I also stimulates bone formation and prevents bone loss *in vivo*. Spencer *et al*. (1991) showed that continuous infusion of IGF-I for 14 days increased both cortical and trabecular bone formation in adult female rats. Kalu *et al*. (1991) reported that daily subcutaneous injections of IGF-I for 5 weeks partially prevented loss of trabecular bone. Therefore, IGF-I plays a key role in bone growth and development of animals although most of the evidences was obtained from rats.

5. *Role of Dietary Lipids and Bone Metabolism*

Essential fatty acids (EFAs) have long been suspected as having a role in calcium metabolism and the sites of calcification, but until recently, they have been largely ignored in relation to osteoporosis and other leg problems. An early study indicated that the degree of mineralization in hypertrophic and calcified cartilage was closely related to the amount of acidic phospholipids present in the tissue (Wuthier, 1968, 1975). Essential fatty acid deficiency in weanling rats led to eicosatrienoic acid (C20:3) accumulation and bone partial hypomineralization (Odutuga, 1981). Matrix vesicles (MV) are believed to initiate *de novo* mineralization of calcified tissues (Anderson, 1989; Wuthier, 1993), and phospholipids in the MV membrane are biosynthesized from PUFA (Seifert and Waltkins, 1997). When mineralization is initiated, crystals of hydroxyapatite begin to accumulate and grow within the chondrocyte-derived MV, which eventually rupture and deposite mineral within the matrix (Wuthier, 1988; Anderson, 1989). Further study demonstrated that acidic phospholipids enhance the formation of calcium complexes within matrix vesicles (MV) of growth plate cartilage and facilitate matrix mineralization (Wuthier, 1993). The finding that osteoblasts synthesize and secrete fatty acids as droplets into the extracellular space which formed the pH-dependent

mutilamellar structures in bone suggests that some fatty acids probably are directly involved in bone mineralization (Takahashi, 1994).

5.1. *Dietary Lipids Modify Bone Lipid Profile*

As aforementioned, although the importance of fatty acids in calcium metabolism and bone biology has long been suggested, little attention has been paid in characterizing the relationships between dietary lipids and bone health until in the 1990s. Recent studies with rats and chicks demonstrated that fatty acid compositions of bone and cartilage reflect the lipid composition of diet. Watkins *et al*. (1991) first reported that chickens fed the diet containing hydrogenated soybean oil (HSBO) had lower concentrations of arachidonic acid (AA, 20:4n-6) in growth plate cartilage. In another study, Xu *et al*. (1994) demonstrated that chicks given semi-purified diet containing menhaden fish oil (FO, rich in n-3 fatty acids) had reduced the level of AA but increased eicosapentaeioic acid (EPA, 20:5n-3) concentration in cartilage, matrix vesicles (MV) and epiphyseal chondrocytes, as compared to those consuming soybean oil (SBO), butter +corn oil (BC), or margarine + corn oil (MC). More recently, Watkins *et al*.(1997) further showed that chicks fed menhaden oil + safflower oil also had the highest concentration of n-3 fatty acids and the lowest amount of 20:4(n-6) in lipids of cortical bone. In addition, diets containing greater amount of saturated fatty acids and t-18:1 fatty acids resulted in high accumulation of these fatty acids in bone.

In rat studies, dramatic results were reproduced similar to those obtained in chicks. Male rats fed diet containing 9% ethyl esters of 20:5(n-3) and 20:6(n-3) significantly elevated the concentrations of these fatty acids but greatly decreased the level of 20:4(n-6) in phospholipids of alvelor bone compared to those given corn oil (Alam *et al*., 1993; Kokkinos *et al*., 1993; Alam *et al*., 1994). The conjugated linoleic acid (CLA) isomers were also found to be incorporated in bone tissue of rats supplemented with dietary CLA (Li *et al*., 1999). An important finding is that the production of PGE_2 in bone tissue, a critical bone resorbing agent, is varied according to the levels of the n-3 and n-6 fatty acids in the diets (Kokkinos *et al*., 1993; Watkins *et al*., 1996a, 1997b). It is therefore presumed that dietary lipids may impact bone formation and resorption.

5.2. *Dietary Lipids Alter Bone Growth*

The consequences of altering the fatty acid composition of cartilage and bone with dietary lipids have been recently investigated. Several studies with animal models have indicated that dietary lipids, depending upon the type and amount ingested, may enhance or impair bone formation, as well as modulate bone mineral content and some protein synthesis. Models that have been used include rats (Kokkinos *et al*., 1993; Alam *et al*., 1993; Claassen *et al*., 1995a, 1995b; Kruger *et al*., 1997), chicks (Xu *et al*., 1994; Watkins *et al*., 1996a, 1996b, 1997a, 1997b; Wohl *et al*., 1998), and humans (Gunnes and Lehmann, 1995; van Papendorp *et al*., 1995; Chen *et al*., 1997). Lipid sources that are used include hydrogenated soybean oil (HSBO; high in trans-fatty acids) butter (rich in trans-fatty acids), soybean oil (SBO; enriched with 18:2n-6), mehadan fish oil (FO; high

in 20:5n-3 and 22:6n-3) or saturated fat (SF). In addition, several studies also use different ratios of purified individual n-6: n-3 fatty acids directly.

5.2.1. *Animal Studies*

Kokkinos and associates (1993) supplemented the rat diet with either 10% corn oil or 9% ethyl ester concentrate of n-3 fatty acid plus 1 % corn oil for 5 weeks. The results showed that tooth movement, as evaluated using computerized image analysis, was significantly reduced in the n-3 group, which was coupled with a large decrease in arachidonic acid (AA; 20:4n-6) and PGE_2 levels. This observation suggests that AA concentration in phospholipids of alveolar bone may be an important factor that can influence tooth movement which is probably mediated by changes in PGE_2 production in bone.

Claassen and coworkers (1995a) studied the effect of different ratios of n-6/n-3 fatty acids on calcium balance and bone in rats. The results showed that rats administrated gamma-linoleic acid (GLA, 18:3n-6) and EPA (n-3) in a ratio of 1:3 decreased the urinary calcium excretion compared to groups given the ratios of 1:1 and 3:1. However, the calcium balance and bone calcium increased significantly in the 3:1 group compared with the control which was supplemented with LA (sunflower oil) and ALA (linseed oil) in a ration of 3:1. Claassen *et al*. (1995b) further reported that, concomitant with a bone calcium increase, higher GLA: EPA ratios (1:1 and 3:1) also significantly decreased urinary excretion of bone collagen pyridinium crosslinks in rats, which currently have been shown to be sensitive markers of bone status and indicators of bone matrix breakdown. These results suggest that dietary supplementation with relative high GLA: EPA ratios are more effective in inhibiting bone resorption than their parent fatty acids (LA and ALA).

Sakaguchi *et al*. (1994) reported for the first time the interaction between estrogen deficiency, PUFAs and bone metabolism. When ovariectomized rats were given a diet rich in EPA and low in calcium for 5 weeks, the results showed that EPA administration prevented the bone weight loss as well as bone strength decrease caused by estrogen deficiency. This result is supported by the findings that administration of EPA (20:5n-3) or DHA (22:6n-3) prevented the development of osteopenia-related fragility in diabetic animals (Yamada *et al*., 1995). Recently, Kruger *et al*. (1997) described that DGLA, DHA and EPA levels in red blood cell membranes were positively correlated with femur calcium ($r=0.54$, 0.65 and 0.59, respectively, $P<0.01$), and DGLA was negatively correlated with deoxypyridinoline excretion ($r= -0.61$, $P<0.002$) in ovariectomized rats, indicating an inhibiting effect of these fatty acids on bone resorption induced by estrogen deficiency.

Although Atteh *et al*. (1984) suggested that dietary fat type and level may significantly alter bone ash mass and bone calcium content in broiler chicks, studies with chicks have received little attention until recently. The pioneering work done by Watkins and coworkers (1996a) with chicks demonstrated that supplementation with LA (50μM) impaired collagen synthesis and caused celluar injury partially by lipid peroxidation in primary cultures of epiphyseal chondrocytes. Epiphyseal chondrocytes may be sensitive to excessive amounts of 18:2n-6 and have a lower tolerance to oxdative stress. *In vivo* studies revealed that bone formation rate, as determined by histomorphometry, was

significantly greater in chicks fed a blended fat of mehaden oil + safflower oil compared to those given soybean oil (Watkins *et al*., 1996b). Watkins *et al*. (1997b) further found that saturated fat supplemented as butter + corn oil (BC) also significantly increased tibial periosteal and total new bone formation rates (158% and 168% of the SBO group, respectively), and resulted in a higher level of circulating hexosamines compared with those in chicks given SBO. These results reflected that dietary BC supplementation increased the bone modeling of growing chicks compared to those consuming SBO. No difference in tibial mineral content between treatments was found in these studies, which is consistent with similar studies with rats. However, it is unclear whether increases in bone formation rate in chicks and calcium content of bone in rats induced by certain dietary fatty acids can lead to greater biomechanical strength. Studies have shown that a diet high in saturated fat, particularly palmitic acid (16:0), impairs bone mineralization in growing chicks (Atteh *et al*., 1984) and adversely affects bone mechanical properties both in rats (Zernicke *et al*., 1995) and adult broiler chicks (Wohl *et al*., 1998).

5.2.2. *Human Studies*

Studies in this area with humans are limited. A possible role of EFA in osteoporosis was suggested in 1993 by Das, with particular emphasis on their modulating effects on several bone-resorbing cytokines involved in the process of osteoporosis, which has been reviewed earlier. Since then, some investigators have observed an association of fat types and amounts of intake with bone formation. van Papendorp *et al*. (1995) first studied the effects of dietary lipids on osteoporotic patients. After patients were supplemented with evening primrose oil (PO, a source of GLA), FO, olive oil (rich in monounsaturated fatty acids) and a mixture of PO and FO, their serum calcium, creatine, alkaline phosphatse, osteocalcin and procollagen were measured. Alkaline phosphatase was greatly decreased while osteocalcin and procollagen increased in the patients with FO and FO+PO mixture supplementation. These changes in bone remodeling markers may indicate an increase in bone formation in these groups. A study in children showed that bone mineral density in the forearm was closely associated with dietary saturated fat intake (Gunnes and Lehmann, 1995).

6. *Interactions between Dietary Lipids, Local Regulatory Factors and Bone Metabolisms*

Bone modeling and remodeling are closely regulated by both systemic hormones and local factors. Recent investigations implied that the mechanisms by which dietary lipids or fatty acids modulate the bone formation are at least partially mediated by local produced factors in bone, such as prostaglandins, cytokines and IGF-I.

Kokkinos and associates (1993) reported that PGE_2 production in alveolar bone was much higher in rats fed corn oil compared to those given ethyl ester of n-3 fatty acids. Consistently, Watkins *et al*. (1996a and 1997b) indicated that the levels of *ex vivo* PGE_2 production in tibial bone organ cultures were significantly elevated (3.5 fold) in chicks fed SBO than those from chicks given either FO or saturated fat. These results were in agreement with the higher concentration of AA (20:4n-6) in bone of animals given SBO in the diets. In chicks, static and kinetic analysis of bone modeling by

histomorphometric measurements revealed that an increase in bone resorptive activity, as conformed by a reduction in bone volume, decreases in trabecular volume and bone formation rate were coupled with elevated PGE_2 production.

Presumably, PGE_3 should be the principle PG in bone of animals fed FO, since the concentration of EPA was 10.8 times higher, while AA concentration was about 50% lower than that in bone of chicks fed SBO. Despite the fact that PGE_3 also stimulated bone resorption in cultured fetal rat long bone, and its potency was nearly equal to that of PGE_2 (Raisz *et al*., 1989), it may be produced in significantly less amounts than that of PGE_2 because it is a poor substrate for cyclooxygenase. It is therefore presumed that dietary lipids impact bone formation and resorption activities, at least in part, by modulating PGE_2 biosynthesis (Watkins, 1998). PGE_2 may mediate part of its effect on bone by regulating IGF-I synthesis. The anabolic effects of PGE_2 are always accompanied by an increase in production of IGF-I by osteoblasts, or by increasing bone cell responsiveness to IGF-I (Raisz and Fall, 1990; McCarthy *et al*., 1991; Schmid *et al*., 1992; Raisz *et al*., 1993b). As aforementioned, IGF-I stimulates bone formation *in vitro* and *in vivo*. Watkins *et al*. (1997b) recently reported that chicks consuming FO had a higher level of IGF-I in epiphyseal cartilage, cortical bone and liver compared to those fed SBO. This result demonstrated that different dietary lipids, dependent on their fatty acid compositions and amount of intake, may alter IGF-I production in bone via their ability to modulate the local biosythesis of PGE_2. However, when chicks were supplemented with asprin in addition to the SBO or FO diet (Watkins *et al*., 1996a), *ex vivo* PGE_2 production was abolished in both groups, independent of tissue AA concentration. It is surprising to notice that bone formation rate was still significantly higher in chicks fed FO compared to those given SBO regardless of whether asprin was in the diets. In addition, feeding asprin failed to induce a change of IGF-I concentration commensurate with a reduction of PGE_2. These results suggest that other metabolites from PUFAs, possibly leukotrienes, also play an important role in regulating bone formation and IGF-I activity.

It is evident from the preceding discussion that IL-1, IL-6 and TNF mediate the bone resorption activity and that dietary n-3 fatty acids or lipids higher in these fatty acids inhibit the production of these bone resorbing cytokines. Thus, there seems to be a close interaction between dietary lipids, cytokines, PGs and bone metabolism, which is summarized in Figure 4.

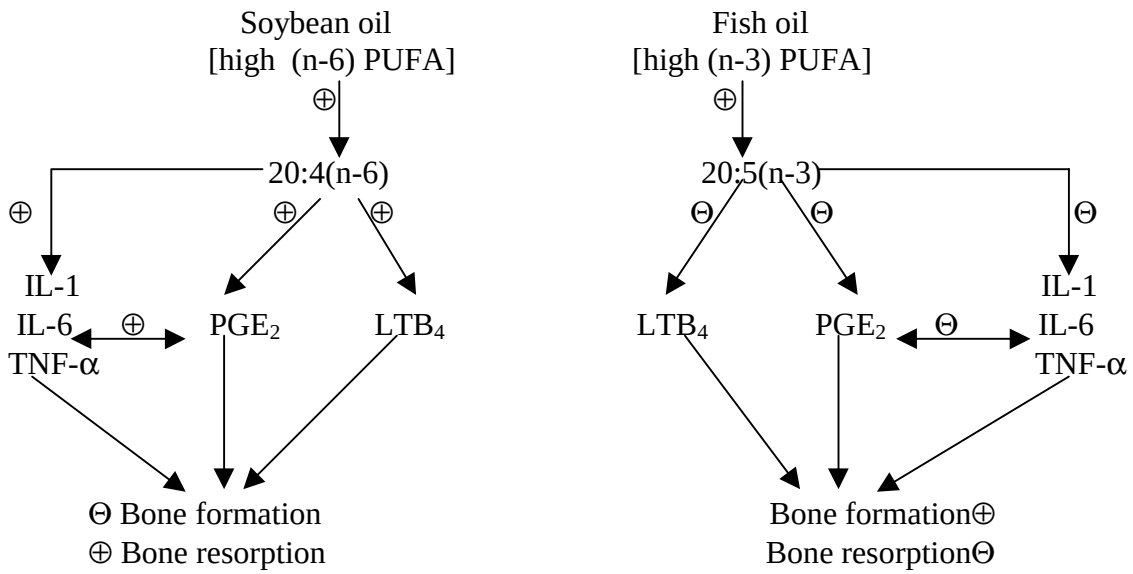

Figure 4. Scheme showing the possible interactions between cytokines, PUFAs, eicosanoids and bone metabolism. ⊕ indicates stimulation, synthesis or formation; Θ indicates inhibition or resorption.

Obviously, there are several pathways by which dietary lipids act on bone metabolism. They influence bone formation synergistically or independently. LTB_4 has a similar function as PGE_2 and is more active than LTB_5. Exogenous IL-1 and TNF not only stimulate PGE_2 production by bone cells *in vitro (*Sato *et al*., 1987; Harrison *et al*., 1994), but also activate phospholipase A_2, which in turn induces the release of AA (Das, 1991), and may further elevate PGE_2 biosynthesis. Similarly, the secretion of IL-1, TNF-α, IL-6 and PGE_2 are all significantly increased in bone marrow after menopause or discontinuation of estrogen replacement (Bismar *et al*., 1995). Reversibly, the increased activities of these endogenous bone-resorbing cytokines in estrogen deficiency-induced bone loss were significantly diminished by indomethacin *in vitro*, which is an inhibitor of cyclooxygenase and therefore blocks the PGE_2 synthesis. These results imply that the production of cytokines and PGE_2 are mutually activated and cytokines may stimulate bone resorption cooperatively with or in response to PGE_2, which in turn, is controlled by its precursor fatty acid. More investigations are needed to characterize the relationships and their interactions between dietary lipids, cytokines and PGE_2 in various conditions.

7. Conclusion

Current research indicates that dietary lipids play an important role in skeletal metabolism and bone health. The fatty acid composition of bone and cartilage has been shown to reflect the fatty acid profile in the diet. Moderate dietary n-3 and saturated fat depressed the concentration of 20:4(n-6), decreased PGE_2 production, elevated IGF-I production in bone and increased bone formation rate more than diet enriched with n-6 fatty acids in young animals. Therefore, dietary lipids appear to modulate bone metabolism partially by controlling local regulatory factors. Osteoporosis may be

partially due to excessive production of cytokines and PGs locally. Supplementation of a certain ratio of n-3/n-6 fatty acids in the diet may reduce or prevent the occurrence of osteoporosis affilicting the aging population. Investigations are needed to characterize the effects of dietary fats on bone metabolism and mechanical properties with regard to their possible impact on osteoporosis. Clearly, further research designed to investigate the relationships between lipids, eicosanoids, growth factors and cytokines in normal bone modeling and remodeling will improve the knowledge of bone biology in both animals and humans.

REFERENCES

Ailhaud, G. P., N. Abumrad, Ez-Z. Amri, and P. A. Grimaldi, 1994. A new look at fatty acids as signal-transducting molecules. World Rev. Nutr. Diet. 75:35-45.

Alam, S. Q., M. Henderson, and B. S. Alam, 1994. Effect of essential fatty acid deficiency on the fatty acid composition and aarachidonic acid levels in rat marillae and mandibles. Calcif. Tiss. Int. 55:169-172.

Alam, S. Q., P. P. Kokkinos, and B. S. Alam, 1993. Fatty acid composition and arachidonic acid concentrations in alveolar bone of rat fed diets with different lipids. Calcif. Tissue Int. 53:330-332.

Alam, S. Q., P. P. Kokkinos, and B. S. Alam, 1993. Fatty acid composition and arachidonic acid concentration in alveolar bone of rats fed diets with different lipids. Clacif. Tissue Int. 53:330-332.

Ammann, P., R. Rissoli, J. P. Bonjour, S, Bourrin, J. M. Meyer, P. Vassalli, andd I. Garcia, 1997. Transgenic mice expressing soluble tumor necrosis factor-receptor are protected against bone loss caused by estrogen deficiency. J. Clin. Invest. 1699:1699-1703.

Amnolagas, S. C., 1998. The role of IL-6 type cytokines and their receptors in bone. Ann. N. Y. Acad. Sci. 840:194-204.

Anderson, H. C., 1989. Biology of disease: mechanisms of mineral formation in bone. Lab Invest. 60:320-330.

Anderson, G. J., W. E. Connor, J. D. Corliss, and D. S. Lin, 1989. Rapid modulation of the(n-3) docosahexaenoic acid levels in the brain and retina of the newly hatched chick. J. Lipid Res. 30:443-441.

Atteh, J. O., and S. Leeson, 1984. Effects of dietary saturated or unsaturated fatty acids and calcium levels on performance and mineral metabolism of broiler chicks. Poultry Sci. 63:2252-2260.

Badie, G., D. Kaimakamis, and D. Rotondo, 1993. Fatty acid modulation of cytokine release fromn human monocytic cells. Biochim. Biophys. Acta. 1179:125-133.

Bailey, A. J., T. J. Sims, E. N. Ebbesen, J. P. Mansell, J. S. Thomsen, and L. Mosekilde, 1999. Age-related changes in the biochemical properties of human cancellous bone collagen: relationship to bone strength. Calcif. Tissue Int. 65:203-210.

Batge, B., J. Diebold, H. Stein, M. Bodo, and P. K. Muller, 1992. Compositional analysis of the collagenous bone matrix. A study on adult normal and osteopenic bone tissue. Eur. J. Clin. Invest. 22:805-812.

Bautista, C. M., S. Mohan, and D. J. Baylink, 1990. Insulin-like growth factors I and II are present in the skeletal tissues of ten vertebrates. Metabolism 39:96-100.

Baylink, D. J., R. D. Finkelman, and S. Mohan. 1993. Growth factors to stimulate bone formation. J. Bone Miner. Res. 8 (Suppl. 2):S565-S572.

Bertolini D. R., G. E. Nedwin, T. S. Bringman, D. D. Smith, and G. R. Mundy, 1986. Stimulation of bone resorption and inhibition of bone formation in vitro by human tumour necrosis factors. Nature 319:516-518.

Bichell, D. P., p. Rotwein, and T. L. McCarthy. 1993. Prostaglandin E_2 rapidly stimulates insulin-like growth factor-I gene expression in primary rat osteoblast cultures: evidence for transcriptional control. Endocrinology 133:1020-1028.

Billiar, T., P. Bankey, and B. Svingen, 1988. Fatty acid intake and Kupffer cell function: fish oil alters eicosanoid and monokine production to endotoxin stimulation. Surgery 104:343-349.

Biran, L. A., W. Bartley, C. W. Carter, and A. Renshaw, 1964. Studies on essential fatty acid deficiency. Effect of the deficiency on the lipids in various rat tissues and the influence of dietary supplementation with essential fatty acids on deficient rats. Biochem. J. 93:492-498.

Bismar, H., I. Diel, R. Ziegler, and J. Pfeilschifter, 1995. Increased cytokine secretion by human marrow cells after menopause or discontinuation of estrogen replacement. J. Clin. Endocr. Metab. 80:3351-3355.

Blair, H. C., P. H. Schlesinger, F. P. Ross, and S. L. Teitelbaum, 1993. Recent advances toward understanding osteoclast physiology. Clin. Orthop. 294:7-22.

Blok, W. L., M .T. Vogels, J. H. Curfs, W. M. Eling, W. A. Buurman, and J. W. vab der Meer, 1992. Dietary fish oil supplementation in experimental gram-negative infection and in cerebral malaria in mice. J. Infec. Dis. 165:898-903.

Borisova, N. V., A. Ya. Pokrovskaya, E. Yu. Zakharova, and K.D. Krasnopol'Skaya, 1994. Analysis of collagen hydroxypyridinium crosslinks in samples of tissue and urine of patients with inheritedd connective tissue disorders. Connective Tissue. Res. 30:177-190.

Boudreau, M. D., P .S .Chanmugam, S. B. Hart, S. H. Lee, and D. H. Hwang, 1991. Lack of dose response by different levels of dietary n-3 fatty acids at a constant n-3/n-6 fatty acid ratio in suppressing eicosanoid biosynthesis from arachidonic acid. Am. J. Clin. Nutr. 54:111-116.

Budowski, P, and M. A. Crawford, 1986. Effect of dietary linoleic acid and α-linolenic acids on the fatty acid composition of brain lipidss in the young chick. Prog. Lipid Res. 25:615-618.

Calder, P. C., 1997. N-3 polyunsaturated fatty acids and cytokine production in health and diseases. Ann. Nutr. Metab. 41:203-234.

Calder, P. C., 1999. Dietary fatty acid and the immune system. Lipids 34:S137-S140.

Canalis, E., J. Pash, B. Gabbitas, S. Rydziel, and S. Varghese, 1993. Growth factors regulate the synthesis of insulin-like growth factor-I in bone cell cultures. Endocrinology 133:33-38.

Caughey, G.E., E.Mantzioris, R. A. Gibson, L. G. Cleland, and M. James, 1996. The effect on human tumor necrosis factorα and interleukin 1β production of diets enriched in n-3 fatty acids from vegetable oil or fish oil. Am. J. Clin. Nutr. 63:116-22.

Chang, H. R., D. Arsenijevis, I. R. Vladoianu, L. Girardier, and A. G. Dullo, 1995. Fish oil enhances macrophage tumor necrosis factor-alpha mRNA expression at the transcriptional level. Metabolism 44:800-805.

Chen, Z., T. G. Lohman, W. A. Stini, C. Riitenbaugh, and M. Aickin, 1997. Fat or lean tissue mass: which one is the major determinant of bone mineral mass in healthy postmenopausal women? J. Bone Miner. Res. 12:144-151.

Cheleuitte, D., S. Mizuno, and J. Glowacki, 1998. In Vitro Secretion of cytokines by humna bone marrow effects of age and estrogen status. J. Clin. Endorinol. Metab. 83:2043-2051.

Cherian, G., and J. S. Sim, 1991. Effects of feeding full fat flax and canola seeds to laying hens on the fatty acid composition of eggs, embryos, and newly hatched chicks. Poultry. Sci. 70:917-922.

Cherian, G., and J. S. Sim, 1992. Omega-3 fatty acid and cholesterol content of newly hatched chicks from α-linolenic acid enriched eggs. Lipids 27:706-710.

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

Claassen, N., H. C. Potgieter, M. Seppa, W. J. H. Vermaak, H. Coetzer, 1995b. Supplemented gamma-linolenic acid and eicosapentaeonic acid influence bone status in young male rats: effects on free urinary collagen crosslinks, total urinary hydroxyprolinee, and bone calcium content. Bone 16 (suppl):385-392.

Cohen-Solal, M. E., A. M. Graulet, M. A. Denne, J. Gueris, D. Baylink, and M. C. de Vernejoul, 1993. Peripheral monocyte culture supernatants of menopausal women can induce bone resportion: involvement of cytokines. J. Clin. Endocrinol. Metab. 77:1648-1653.

Cooper, A. L., L. Gibbon, M. A. Horan, R. A. Little, and N. J. Rothwell, 1993. Effect of dietary fish oil supplementation on fever and cytokine production in human volunteers. Clin. Nutr. 12:321-328.

Cowin, S. C., L. S. Moss, and M. L. Moss, 1991. Candidates for the mechanosensory system in bone. J. Biomech. Eng. 113:191-197.

Crebshaw, T. D., 1986. Reliability of dietary calcium and phosphorous levels and bone mineral content as predictors of bone mechanical properties at various time of periods in growing swine. J. Nutr. 116:2155-2170.

Cumming, R. G., and M. C. Nevitt, 1997. Calcium for prevention of osteoporotic fractures in post-menopausal women. J. Bone Miner. Res. 12:1321-1329.

Currey, J. D., 1988. The effect of porosity and mineral content on the Young's modulus of elasticity of compact bone. J. Biomech. 21:131-139.

Das, U. N., 1991. Interaction(s) between essential fatty acids, eiosanoids, cytokines, growth factors and free radicals: Relevance to new therapeutic strategies in rheumatoid arthritis and other collagen vascular diseases. Prostaglandins Leukot. Essent. Fatty Acids. 44:201-210.

Das, U. N., 1994. Can essential fatty acids prevent osteoporosis? Med. Sci. Res. 22:163-165.

Dawson-Hughes, B., G. E. Dallal, and E. A. Krall, 1990. A controlled trial of the effect of calcium supplementation on bone density in post-menopausal women. New Eng. J. Med. 323:878-883.

Devlin, R. D., S. V. Reddy, R. Savino, G. Ciliberto, and G. D. Roodman, 1998. IL-6 mediates the effects of IL-1 or TNF, but not PTHrP or 1,25$(OH)_2D_3$ on osteoclast-like cell formation in normal human bone marrow cultures. J. Bone Miner. Res. 13:393-399.

Endres, S., 1996. N-3 fatty polyunsaturated fatty acids and human cytokine synthesis. Lipids 31:S239-S242.

Endres S, R. Ghorbani, V. E. Kelley, and K. Georgilis, 1989. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. N. Engl. J. Med. 320:265-271.

Endres, S., and C.V. Schacky, 1996. N-3 polyunsaturated fatty acids and human cytokine syntehsis. Current Opinion Lipido. 7:48-52.

Ernst, M., and G. A. Rodan. 1990. Increased activity of insulin-like growth factor (IGF) in osteoblastic cells in the presence of growth hormone (GH): Positive correlation with the presence of the GH-induce IFG-binding protein BP-3. Endocrinology 127:807-814.

Fall, P. M., D. T. Breault, and L. G. Raisz, 1994. Inhibition of collagen synthesis by prostaglandins in the immortalized rat osteoblastic cell line pyla: structure-activity relations and signal transduction mechanisms. J. Bone Miner. Res. 9:1935-2943.

Farquharson, C., A. Duncan, and s. Robins, 1989. The effects of copper deficiency on the pyridinium crosslinks of mature collagen in the rat skeleton and cardiovascular system. P. S. E. B.M. 192:166-171.

Girasole, G., R. L. Jilka, and G. Passeri, 1992. 17β-estrodiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro:a potential mechanisn for the antiosteoporotic effect of estrogens. J. Clin. Invest. 89:883-891.

Grimm, H., A. Tibell, B. Norrlind, C. Blecher, S. Wilker, and K. Schwemmle, 1994. Immunoregulation by parenteral lipids: Impact of the n-3:n-6 fatty acid ratio. J. Parent. Enter. Nutr. 18:417-421.

Grundy, S. M., 1996. Dietary fat. Recent Knowledge in Nutrition (seventh edition). Edited by Ekhard E. Ziegler and L. J. Filer Jr. ILSI Press, DC.

Guenter W, D. B. Bragg, and P. A. Kondra, 1971. Effect of dietary linoleic acid on fatty acid composition of egg yolk, liver and adipose tissue. Poultry Sci. 50:845-50.

Gunnes, M., and E. H. Lehmann, 1995. Dietary calcium, saturated fat, fiber and vitamin C as predictors of forearm cottical and trabecular bone mineral density in healthy children and adolescents. Acta Paediatr. 84:388-392.

Hanson, D. A., and Eyre, D. A., 1996. Molecular site specificity of pyridinoline and pyrrole cross-links in type I collagen of human bone. J. Biol. Chem. 271:26508-26516.

Hardaardottir, I., and J. E. Kinsella, 1991. Tumor necrosis factor production by murine resident peritoneal macrophages is enhanced by dietary n-3 fatty polyunsaturated fatty acids. Biochim. Biophys. Acta. 1095:187-195.

Harrison, J. R., J. A. Lorenzo, H. Kawaguchi, L. G. Raisz, C. C. Pilbeam, 1994. Stimulation of prostaglandin E_2 production by interleukin-Iα and trasnsforming growth factor α in osteoblast MC 3T3-E1 cells. J. Bone Miner. Res. 9:817-823.

Heaney, R. P., 1992. Calcium in the prevention and treatmemnt of osteoporosis. J. Intern. Med. 231:169-180.

Holman, R. T., 1986. Nutritional and biochemical evidences of acyl interaction with respect to essential polyunsaturated fatty acids. Prog. Lipids Res. 25:29.

Horrobin, D. F. 1983, The regulation of prostaglandin biosynthesis by the manipulation of essential fatty acid metabolism. Rev. Pure Applied Pharmacol. Sci. 4:339-383.

Hosking, D. J., P. D. Ross, D .E. Thompson, R. D. Wasnich, M. McClung, N. H. Bjaarnason, P. Ravn, G. Cizza, M. Daley, and J. Yates, 1998. Evidence that increased calcium intake does not prevent early postmenopausal bone loss. Clin. Therap. 20:933-944.

Hou, J. C. H., R. F. Zernicke, and R. J. Barnard, 1990. High-fat sucrose diet effects on femoral neck geometry and biomechanics. Clin. Biomech. 5:162-168.

Huang, Y. S., P. E. Wainwright, P. R. Redden, D. E. Mills, B. B. Fleming, and D. F. Horrobin, 1992. Effects of maternal dietary fats with variable n-3/n-6 ratios on tissue fatty acid composition in sucking mice. Lipids 27:104-110.

Hwang, D., 1992. Dietary fatty acids and eicosanoid. Fatty Acids in Food and Their Health Implications. Edited by C. K. Chow. M. Dekker, InC. NY.

Innis, S. M., 1996. Essential dietary lipids. Recent Knowledge in Nutrition (seventh edition). Edited by Ekhard E. Ziegler and L. J. Filer Jr. ILSI Press, DC.

Ishimi, Y., C. Miyaura, C. H. Jin, T. Akatsu, T. Abe, Y. Nakamura, A. Yamaguchi, S. Yoshiki, T. Hirano, T. Kishimoto, and T. Suda, 1990. IL-6 is produced by osteoblasts and induce bone resorption. J. Immunol. 145:3297-3303.

Ito, H., H. Z. Ke, W. S. S. Jee, and T. Sakou. 1993. Anabolic responses of an adult cancellous bone site to prostaglandin E_2 in the rat. Bone Mineral. 21:219-236.

Jee, W. S. S., H. Z. Ke, and X. J. Li, 1991. Long-term anabolic effects of prostaglandin E_2 on tibial diaphyseal bone in male rats. Bone Mineral. 15:33-55.

Jee. W. S. S., S. Mori, X. J. Li, and S. Chan, 1990. Prostaglandin E_2 enhances cortical bone mass and activates intracortical bone remodeling in intact and ovariectomized female rats. Bone 11:253-266.

Jee, W.S.S., K. Ueno, Y. P. Deng, and D. M. Woodbury, 1985. The effect of prostaglandin E_2 in growing rats: increased metaphyseal hard tissue and cortico-endosteal bone formation. Calcif. Tissue Int. 37:148-157.

Jee, W. S. S., K. Ueno, D. B. Kimmel, D. M. Woodbury, P. Price, and L. A. Woodbury, 1987. The role bone cells in increasing metaphyseal hard tissue in rapidly growing rats treated with prostaglandin E_2. Bone 8:171-178.

Jilka, R. L., 1998. Cytokines, bone remodeling, and estrogen deficiency: a 1998 update. Bone 23:75-81.

Jonas, J., J. Burns, E. W. Abel, M. J. Cresswell, J. J. Strain, and C. R. Paterson, 1993. Impaired mechanical strength of bone in experimental copper deficiency. Ann. Nutr. Metab. 37:245-252.

Kalu, D. N., C. C. Liu, E. Salerno, M. Salih, R. Echon, M. Ray, B. W. Hollis, 1991. Insulin-like growth factor-I partially prevents ovariectomy-induced bone loss: A comparative study with human parathroid hormone. J. Bone Miner. Res. 6:548-553.

Kanatani, M., T. Sugimoto, M. Fukase, and K. Chihara, 1994. Role of interleukin-6 and postglandins in the effect of monocyte conditioed medium on osteoclast formation. Am. J. Physiol. 267:E868-E876.

Ke, H. Z., W. S. S. Jee, Q. Q. Zeng, M. Li, and B. Y. Lin, 1993. Prostaglandin E_2 increased rat cortical bone mass when administered immediately following ovariectomy. Bone Mineral. 21:189-201.

Ke, H. Z., M. Li, and W. S. S. Jee, 1992. Prostaglandin E_2 prevents ovariectomy-induced cancellous bone loss in rats. Bone Mineral. 19:45-62.

Kim, C. H., H. K. Kim, Y. K. Shong, K. U. Lee, and G. S. Kim, 1999. Thyroid hormone stimulates basal and interleukin (IL)-1-induced IL-6 production in human bone marrow stromal cells: a possible mediator of thyroid hormone-induced bone loss. J. Endocrinol. 160:97-102.

Kimble, R. B., A. B. Matayoshi, J. L. Vaanice, V. T. Kung, C. Williams, and R. Pacific, 1995. Simultaneous block of interleukin-1 and tumor necrosis factor is required to completely prevent bone loss in the early postvariectomy period. Endocrinology 136:3054-3061.

Knott, L., C. C. Whitehead, R. H. Fleming, and Aaa. J. Bailey, 1995. Biochemical changes in the collagenous matrix of osteoporotic avian bone. Biochem. J. 310:1045-1051.

Kokkinos P. P., R. Shaye, B. S. Alam, and S. Q. Alam, 1993. Dietary lipids, prostaglandin E_2 levels, and tooth movement in alveolar bone of rats. Calcif. Tissue Int. 53:333-337.

Kremer, J., D. Lawrence, W. Jubiz, R. Di Giacomo, R. Rynes, L. E. Bartholomew, and M. Sherman, 1990. Dietary fish oil and oilve oil supplementation in patients with rheumatoid arthritis, Clinical and Immunological effects. Arthritis Rheum. 33:810-820.

Kruger, M. C., N. Claassen, C. M. Smuts, and H. C. Potgieter, 1997. Correlation between essential fatty acids and parameters of bone formation and degradation. Asia Pacific J. Clin. Nutr. 6:235-238.

Kukita, A., L. Bonewald, D. Rosen, S. Seyedin, G. R. Mundy, G. D. Roodman, 1990. Osteinductive factor inhibits formation of human osteoclast-like cells. Proc. Natl. Acad. Sci. 87:3023-3026.

Landis, W. J., 1995. The strength of a calcified tissue depends in part on the molecular structure and organization of its constituent mineral crystals in their organic matrix. Bone 16:533-544.

Li, X. J., W. S. S. Jee, Y. L. Li, and P. P. Buckendahl, 1990. Transient effects of subcutaneously administered prostaglandin E_2 on cancellous and cortical bone in young adult dogs. Bone 11:353-364.

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

Li, K. C., R. F. Zernicke, R .J. Barnard, and A. F. Y. Li, 1990. Effects of a high-fat sucrose diet on cortical bone morphology and biomechanics. Calcif. Tissue Int. 47:308-313.

Lin, C. H., W. S. S. Jee, Y. F. Ma, and R. B. Setterberg, 1995. Early effects of prostaglandin E_2 on bone formation and resorption in different bone sites of rats. Bone 17(suppl.4):255S-259S.

Lnnis, S. M., 1991. Essential fatty acids in growth and development. Prog. Lipids Res. 30:39-103.

Lnnis, S. M., 1996. Essential dietary lipids. Recent Knowledge in Nutrition (Seventh edition). Edited by Ekhard E. Ziegler and L. J. Filer, Jr. ILSI Press. Whashington, DC.

Lawson, A. C., and T. J. Czernuszka, 1998. Collagen-calcium phosphate composites. Proc. Instn. Mech. Engrs. 212:413-425.

Lokesh, B. R., T. J. Sayers, and J.E. Kinsella, 1990. Interleukin-1 and tumor necrosis factor synthesis by mouse peritoneaal macrophages is enhanced by dietary n-3 polyunsaturated fatty acids. Immunol. Lett. 23:281-285.

Lorenzo, J. A. Naprta, Y. Rao, C. Alander, M. Glaccum, G. Kalinowski, and C. Pilbeam, 1997. Mice deficienct in the functional interleukin-1 receptor (IL-1R1) do not loss bone mass after ovariectomy. J. Bone Miner. Res. 12(suppl.):S126-S130.

Ma, Y. F., H. Z. Ke, and W. S. S. Jee, 1994. Prostaglandin E_2 adds bone to a cancellous bone site with a closed growth plate and low bone turnover in ovariectomized rats. Bone 15:137-146.

MacDonald, J. I. S., and H. Sprecher, 1991. Phospholipid fatty acid remodeling in mammalian cells. Biochim. Biophys. Acta. 1084:105.

Machwate, M., E. Zerath, X. Holy, P. Pastoureau, 1994. Insulin-like growth factor-I increases trabecular bone formation and osteoblastic cell proliferation in unloaded rats. Endocrinology 134:1031-1038.

Maldjian, A., C. Cristofori, R. C. Noble, and B. K. Speake, 1996. The fatty acid composition of brain phospholipids from chicken and duck embryos. Comp. Biochem. Physiol. 115B:153-158.

Manolagas, S. C., 1995. Role of cytokines in bone resorption. Bone 17 (suppl. 2):63S-67S.

Marks, S. C. Jr., and S. C. Miller, 1993. Prostaglandins and skeleton: The legacy and chanllenges of two decades of research. Endocrine. J. 1:337-344.

Marowska, J., M. Kobylinska, J. Lukassszewicz, A. Talajko, B. Rymkiewicz-Kluczynska, and R. S. Lorenc, 1996. Pyridinium crosslinks of collagen as a marker of bone resorption rates in children and adolescents: normal values and linical application. Bone 19:669-677.

Martin, R. B., and D. L. Boardman, 1993. The effects of collagen fiber orientation, porosity, density, and mineralization on bovine cortical bone bending properties. J. Biomech. 26:1047-1054.

Martin, R. B., and J. Ishida, 1989. The relative effects of collagen fiber orientation, porosity, and mineralization on bone strength. J. Biomech. 22:419-426.

Masse, P. G., C. M. Rimnac, M. Yamauchi, S. P. Coburn, R. B. Rucker, D. S. Howell, and A. L. Boskey, 1996. Pyridoxine deficiency affects biomechanical properties of chick tibial bone. Bone 18:567-574.

McCarthy, T. L., S. Casinghino, M. Centrella, and E. Canalis, 1994. Complex pattern of insulin-like growth factor binding protein expression in primary rat osteoblast enriched cultures: Regulation by prostaglandin E2, growth hormone, and insulin-like growth factors. J. Cell. Physiol. 128:2895-2900.

McCarthy, T. L., M. Centrella, L. G. Raisz, and E. Canalis. 1991. Prostaglandin E_2 stimulates insulin-like growth factor-I synthesis in osteoblast-enriched cultures from fetal rat bone. Endocrinology 128:2895-2900.

Meghji, S., J. R. Sandy, A. M. Scutt, W. Harvey, and M. Harris, 1988. Stimulation of bone resorption by lipoxygenase metabolites of arachidonic acid. Prostaglandins 36:139-149.

Meydani, S.N., M. W.Margo, A. D.Barry, and L.G.Sherwood, 1991. Oral (n-3) fatty acid Supplementation Suppresses Cytokine Production and Lymphocyte Proliferation: Comparision Between Young and Older Women. J. Nutr. 121:547-55.

Miyaura, C., K. Kusano, T. Masuzawa, O. Chaki, Y. Onoe, M. Aoyagi, and T. Sasaki, 1995. Endogenous bone-resobing factors in estrogen deficiency: cooperative effects of IL-1 and IL-6. J. Bone Miner. Res. 10:1365-1373.

Mohan, S., and D. J. Baylink, 1991. Bone growth factors. Clin. Orthop. 263:30-48

Mori, S., W. S. S. Jee, and X. J. Li, 1992. Production of new trabecular bone in osteopenic ovariectomized rats by prostaglandin E_2. Calcif Tissue Int. 50:80-87.

Mori. S., W. S. S. Jee., X. J. Li, S. Chan, and D .B. Kimmel, 1990. Effects of prostaglandin E_2 on production of new cancellous bone in the axial skeleton of ovariectomized rats. Bone 11:103-113.

Mundy, G. R., 1993. Cytokines and growth factors in the regulation of bone remodeling. J. Bone Miner. Res. 8: S505-S510.

Murphy, M. G., 1990. Dietary fatty acids and membrane protein function. J. Nutr. Biochem. 1:68-73.

National Research Council, 1994. Nutrient Requirements of Poultry, 9^{th} Rev. Ed. National Academy Press, Washington DC.

Nicosia, S., and C. Patrono, 1995. Eicosanoid biosythesis and action: novel opportunities for pharmacological intervention. FASEB J. 3:1941.

Nilas, L., 1993. Clacium intake and osteoporosis. World Rev. Nutr. Diet. 73:1-26.

Odutuga, A. A., 1981. Effects of low-zinc status and essential fatty acid deficiency on bone development and mineralization. Comp. Biochem. Physiol. 71A:383-388.

Oxlund, H., Li. Mmosekilde, and G. Ørtoft, 1996. Reduced concentration of collagen reducible cross-links in human trabecular bone with respect to age and osteoporosis. Bone 19:479-484.

Oxlund, H., M. Barckman, G. Ørtoft, and T. T. Andreassen, 1995. Reduced concentrations of collagen cross-links are associated with reduced strength of bone. Bone 17 (suppl):365S-371S.

Pacifici, R., 1995. Cytokines and osteoclast activity. Calcif. Tissue Int. 56 (Suppl.1):S27-S28.

Pacifici, R, 1996. Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis. J. Bone. Miner. Res. 11:1043-1051.

Pacific, R., L. Rifas, R. McCracken, and L. V. Avioli, 1990. The role of interleukin-1 in postmenopausal bone loss. Exp. Gerontol. 25:309-316.

Pacific, R., L. Rifas, R. McCracken, I. Vered, C. McMurthy, L. V. Avioli, and W. A. Peck, 1989. Ovarian steroid treatment blocks a postmenopausal increase in blood monocyte interleukin-1 release. Proc. Natl. Acda. Sci. 86:2398-2402.

Passeri, G., G. Girasole, R. L. Jilka, and S. C. Manolagas, 1993. Increased interleukin-6 production by murine bone marrow and bone cells after estrogen withdrawal. Endocrinology 133:822-828.

Phetteplace, H. W., and B. A. Watkins, 1989. Effects of various (n-3) lipid sources on fatty acid compositions in chicken tissues. J. Food Anal. 2:104-117.

Pines, M., and S. Hurwitz, 1990. The role of growth plate in longitudinal bone growth. Poultry Sci. 70:1806-1814.

Poli, V., R. Balena, E. Fattori, A. Markatos, A. Yamamoto, H. Tanaka, G. Ciliberto, G. A. Rodan, andd F. Costantini, 1994. Interleukin-6 deficient mice are protected from bone loss caused by estrogen depletion. Eur. Mol. Org. J. 13:1189-1196.

Pottratz, S. T., T. Bellido, H. Mocharla, D. Crabb, and S. C. Manolagas, 1994. 17β-estradiol inhibits expression of humand interleukin-6 promoter-reporter constructs by a receptor-dependent mechanism. J. Clin. Invest. 93:944-950.

Purasiri, P., S. D.Mckechnie, and O. E.Heys, 1997. Modulation in vitro of human natural cytotoxicity, lymphocyte proliferative response to mitogens and cytokine production by essential fatty acids. Immunology 92:166-72.

Raisz, L. G., 1993a. Bone cell biology: New approaches and unanswered questions. J. Bone Miner. Res. 8: S457-S465.

Raisz, L. G., C. B. Alander, and H. A. Simmons, 1989. Effects of prostaglandin E_3 and eicosapentaenoic acid on rat bone in organ culture. Prostaglandins 37:615-625.

Raisz, L. G., and P. M. Fall. 1990. Biphasic effects of prostaglandin E_2 on bone formation in cultured fetal rat calvariae: Interaction with cortisol. Endocrinology 126:1654-1659.

Raisz, L. G., C. C. Pilbeam, and P. M. Fall, 1993b. Prostaglandins: Mechanisms of action and regulation of production in bone. Osteoporosis Int. 3 (Suppl.1):S136-S140.

Ralston, S. H., R. G. Russell, M. Gowen, 1990. Estrogen inhibits release of tumor necrosis factor from peropheral blood mononuclear cells in postmenopausal women. J. Bone Miner. Res. 5:983-988.

Ray, A., K. E. Prefontaine, and P. Ray, 1994. Down-modulation of interleukin-6 gene expression by 17 beta-estradiol in the absebce of high affinity DNA binding by the estrogen receptor. J. Biol. Chem. 269:12940-12946.

Renier, G., E. Skamene, J. de Sanctis, and D. Radzioch, 1993. Dietary n-3 polyunsaturated fatty acids prevent the development of atherosclerotic lesions in mice: Modulation of macrophage secretory activities. Arterioscler. Thomb. 13:1515-1524.

Rifas, L., 1999. Bone and cytokines: Beyond IL-1, IL-6 and TNF-α. Calcif. Tissue Int. 64:1-7.

Riis, B., K. Thomsen, and C. Christiansen, 1987. Does calcium supplementation prevent post-menopausal osteoporosis? New Eng. J. Med. 316:173-177.

Robins, S. P., 1994. Biochemical markers for assessing skeletal growth. Eur. J. Clin. Nutr. 48(Suppl.1):S199-S209.

Robinson, D. R., M. Urakaze, R. Huang, H. Taki, E. Sugiyama, C. T. Knoell, L. Xu, E. T. Yeh, and P. E. Auron, 1996. Dietary marine lipids suppress continuous expression of interleukin-1β gene transcription. Lipids 31:23S-31S.

Rogel, A. M., and B. A. Watkins, 1987. Liver subcellular fatty acid profiles of chicks fed diets containing hydrogenated fats and varying linoleate levels. Lipids 22:637-642.

Sakaguchi, K., I. Mortita, and S. Murota, 1994. Eicosapentaenoic acid inhibits bone loss due to ovariectomy in rats. Prostaglandins Leukot. Essent. Fatty Acids. 50:81-84.

Sardesai, V. W., 1992a. Biochemical and nutritional aspects of eicosanoids. J. Nutr. Biochem. 3:562-579.

Sardesai, V. W., 1992b. Nutritional role of polyunsaturated fatty acids. J. Nutr. Biochem. 3:154-166.

Sato, K., K. Kasono, Y. Fujii, 1987. Tumor necrosis factor α stimulates mousee osteoblast-like cells to produce macrophage-colony stimulating activity and prostaglandin E_2. Biochem. Biophys. Res. Commun. 145:323-329.

Schmid, C., I. Schlapfer, M. Waldvogel, J. Zapf, and E. R. Froesch. 1992. Prostaglandin E_2 stimulates synthesis of insulin-like growth factor binding protein-3 in rat bone cells in vitro. J. Bone Miner. Res. 7:1157-1163.

Seifert, M. F., and B. A. Waltkins, 1997. Role of dietary lipid and antioxidants in bone metabolism. Nutr. Res. 17:1209-1228.

Shah, K. M., J. C. Goh, R. Karunanithy, S. L. Low, D .S. Das, and K. Bose, 1995. Effect of decalcification on bone mineral content and bending strength of feline femur. Calcif. Tissue Int. 56:78-82.

Simopoulos, A. P., 1991. Omega-3 fatty acids in health and disease and in growth and development. Am. J. Clin. Nutr. 54:438.

Sinclair, H. M, 1957. Essential fatty acids. 249-256. Butterworths. London.

Spencer, E. M., C. C. Liu, E. C. C. Si, G. A. Howard. 1991. *In vivo* actions of insulin-like growth factor-I on bone formation and resorption in rats. Bone 12:21-26.

Sprecher, H., 1992. Interconversions between 20- and 22- carbon ω-3 and ω-6 fatty acids via 4-desaturase independent pathways. Essential fatty acids and Eicosanoids. American Oil Chemists' Society. Campaign, IL. 18-22.

Suda, T., N. Udagawa, I. Nakamura, C. Miyaura, and N. Takahashi, 1995. Modulation of osteoclast differentiation by local factors. Bone 17:87S-91S.

Suponitzky, I., and M. Weinreb, 1998. Differential effects of systemic prostaglandin E_2 on bone mass in rat long bones and calvariae. J. Endocrinol. 156:51-57.

Takada, M, I. Y., I. Yu, and Y. Kigami, 1995. Chronic intramedullary infusion of prostaglandin E_2 stimulates bone formation both in the bone marrow and in the periosteum. Endocrinol. J. 42:323-330.

Takahashi, M., K. Kushida, H. Hoshino, M. Suzuki, M. Sano, and S. Miyamoto,1996. Concentrations of pyridinoline and deoxypyridinoline in joint tissues from patients

with osteoarthritis or rheumatoid arthritis. Ann. Rheum. Dis. 55:324-327.

Takahashi, T., S. Ueda, K. Takahashi, and R. O. Scow, 1994. pH-dependent multilamellar structures in fetal mouse bone: possible involvement of fatty acids in bone mineralization. Am. J. Physiol. 266:C590-C600.

Tijburg, L. B. M., M. J. H. Geelen, and L. M. G. Gilde, 1989. Regulation of the biosynthesis of triacylglycerol, phosphatidylcholine andd phosphatidylethanolamine in the liver. Biochim. Biophys. Acta. 1004:1-19.

Triyuwanta, C. L., and Y. Nys, 1992. Dietary phosphorus and food allowance of dwarf breeders affect reproductive performance of hens and bone development of their progeny. Br. Poult. Sci. 33:363-379.

Tsuchiya, H., and C. J. Bates, 1997. Vitamin C and copper interactions in quinea-pigs and a study of collagen cross-links. Br. J. Nutr. 77:315-325.

Tsuchiya, H., and C. J. Bates, 1998. Changes in collagen cross-link ratios in bone and urine of guinea pigs fed graded dietary vitamin C: a functional index of vitamin C status. J. Nutr. Biochem. 9:402-407.

Turek, J. J., Y. Li, I. A. Schoenlein, K. D. G. Allen, 1998. Modulation of macrophage cytokine production by conjugated linoleic acids is influenced by the dietary n-6:n-3 fatty acid ratio. J. Nutr. Biochem. 9:258-266.

Turke, J. J., A. Schoenlein, and G. D. Bottoms, 1991. The effect of dietary n-3 and n-6 fatty acids on tumor necrosis factor-α production and leucine aminopeptidase levels in rat peritoneal macrophages. Prostaglandins. Leuk. Essent. Fatty Acids. 43:141-149.

Urist, M.R., K. Behnam, F. Kerendi, D. Bagga, H. D. Adkisson, J. Nevenzel, and T. Malinin, 1997. Neutral lipids facilitate transfer of bone morphogenetic proteins and other noncollagenous proteins. Med. Hypothesis. 49:465-475.

Van Papendorp, D. H., H. Coetzer, and M. C. Kruger, 1995. Biochemical profile of osteoporotic on essential fatty acid supplementation. Nutr. Res. 15:325-334.

Verhaeghe, J., A. M. H. Suiker, T. A. Einhorn, P. Geusens, W. J. Visser, E. Van Herck, R. Van Bree, S. Magitsky, and R. Bouillon, 1994. Brittle bones in spontaneously diabetic female rats cannot be predicted by bone mineral measurements: studies in diabetic and ovariectomized rats. J. Bone Miner. Res. 9:1657-1667.

Watkins, B. A., 1988. Influences of biotin deficiency and dietary trans-fatty acids on tissue lipids in chickens. Br. J. Nutr. 61:99-111.

Watkins, B. A., 1991. Importance of essential fatty acids and their derivatives in poultry. J. Nutr. 121:1475-1485.

Watkins, B. A., 1995. Biochemical and physiological aspects of polyunsaturates. Poult Avian Biol. Rev. 6:1-18.

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

Watkins, B. A., and M. F. Seifert, 1997a. Food lipids and bone health. Food Lipids and Bone Health. Edited by McDonald, R. E., and B. D. Min. 71-116. Marcel Dekker, Inc. NY.

Watkins, B. A., C. L. Shen, K. J. D. Allen, and M. F. Seifert, 1996a. Dietary (n-3) and (n-6) polyunsaturates and acetylsalicylic acid alter ex vivo PGE_2 biosynthesis, tissue IGF-I levels, and bone morphometry in chicks. J. Bone Miner. Res. 11:1321-1332.

Watkins, B. A., H. Xu, and J. J. Turek, 1996b. Linoleate impairs collagen synthesis in primary cultures of avian chondrocytes. P. S. E. B. M. 212:153-159.

Watkins, B. A., C. L. Shen, J. P. Memurtry, H. Xu, and S. D. Bain, 1997b. Dietary lipids modulate bone prostaglandin E_2 production, insulin-like growth factor-I concentration and formation rate in chicks. J. Nutr. 127:1084-1091.

Watkins, B. A, M. F.Seifert, and K. G. D. Allen, 1997c. Importance of dietary fat in modulating PGE_2 responses and influence of vitamin E on bone morphometry. World Rev. Nutr. Diet. 82:250-259.

Watkins, B. A., C. L, Shen, M. F. Seifert, and D. C. McFarland, 1994. Dietary lipid modulates fatty acid composition, PGE_2 biosynthesis and IGF-I in chick tibia. J. Bone Miner. Res. 9:B104, S256.

Watkins, B. A., C. C. Whitehead, S. R. Duff, 1991. Hydrogenated oil decreases tissue concentrations of n-6 polyunsaturated fatty acids and may contribute to dyschondroplasia in broilers. Br. Poult. Sci. 32:1109-1119.

Watkins, S. M., and J. B. German, 1998. Omega fatty acids. Food Lipids. 463-493.

Weinreb, M, I. Suponitzky, and S. Keila, 1997. Systemic administration of an anabolic dose of PGE_2 in young rats increases the osteogenic capacity of bone marrow. Bone 20:521-526.

Wilmarth, K. R., and J. R. Froines, 1992. In vitro and in vivo inhibition of lysyl oxidase by aminopropionitriles. J. Toxicol. Envrion. Health. 37:411-423.

Wohl, G. R., L Loehrke, B. A. Watkins, and R. F. Zernicke, 1998. Effects of high-fat diet on mature bone mineral content, structure, and mechanical properties. Calcif. Tissue Int. 63:74-79.

Wood, J. N., 1990. Essential fatty acids and their metabolites in signal transduction. Biochem. Soc. Trans. 18:785-786.

Wronski, T. J., M. Clintron, and L. M. Dann, 1988. Temporal relationship between bone loss and increase bone turnover in ovariectomized rats. Calcif. Tissue Int. 43:179-183 .

Wuthier, R. E., 1968. Lipids of mineralizing epiphyseal tissues in the bovine fetus. J. Lipids Res. 9:68-78.

Wuthier, R.E., 1975. Lipids composition of isolated epiphyseal cartilage cells, membrane and matrix vesicles. Biochim. Biophys. Acta. 409-128.

Wuthier, R. E., 1988. Mechanism of matrix vesicle-mediated mineralization of cartilage. ISI Atlas Sci. Biochem. 1:231-241.

Wuthier, R. E., 1993. Involvement of cellular metabolism of calcium and phosphate in calcification of avian growth plate cartilage. J. Nutr. 123:301.

Xu, H., B. A. Watkins, and H. D. Adkisson, 1994. Dietary lipids modify the fatty acid composition of cartilage, isolated chondrocytes and matrix vesicles. Lipids 29:619-625.

Yamada, Y., H. Fushimi, T. House, Y. Matsuyama, M. Kameyama, T. Minami, Y. Okasaki, Y. Noguchi, and T. Kasama, 1995. Effect of eicosapentaenoic acid and docosahexaenoic acid on diabetic osteopenia. Diabetes Res. Clin. Pract. 30:37-42.

Yang, R. S., T. K. Liu, and S. Y. L. Shiau, 1993. Increased bone growth by local prostaglandin E_2 in rats. Calcif tissue Int. 52:57-61.

Yaqoob, P., and P. C. Calder, 1995. Effects of dietary lipid manipulation upon inflammatory mediator production by murine macrophages. Cell Immunol. 163:120-128.

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.

CHAPTER ONE

Maternal Dietary Lipids Modify Composition of bone Lipids and *ex vivo* PGE_2 Production in Early Postnatal Japanese Quail

ABSTRACT: This study examined the effects of maternal dietary lipids on fatty acid composition and *ex vivo* PGE_2 biosynthesis of bone tissues in progeny of Japanese quail. Laying hens were fed a basal diet containing either soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) or menhaden fish oil (FO) at 50g/kg of the diet. Fertilized eggs were incubated and newly hatched quail were used either for tibial fatty acid analysis and PGE_2 measurement, or were fed an identical diet until 2 weeks of age. Egg yolks and tibial bones of newly hatched quail from hens fed SBO diet contained significantly higher levels of total n-6 fatty acids and arachidonic acid [AA, 20:4(n-6)] ($P<0.01$), whereas those from hens consuming the FO diet had increased concentrations of total n-3 fatty acids, eicosapentaenoic acid [EPA, 20:5(n-3)], docosapentaenoic acid [22:5(n-3)], docosahexaenoic acid [DHA, 22:6(n-3)] and total saturated fatty acids ($P<0.01$), but greatly reduced amounts of AA in both egg yolks and tibiae ($P<0.01$). The maternal diet containing t18:1 resulted in the accumulation of this fatty acid in egg yolks and tibiae of newly hatched quails. At one week of age, the concentrations of EPA, DHA and t18:1 in tibiae still reflected the maternal dietary fatty acid profile. At two weeks of age, differences in fatty acid composition between treatments found in the early age had disappeared. Addition of FO or HSBO to the maternal diet significantly lowered the *ex vivo* PGE_2 production of tibiae in newly hatched quail compared to those from hens fed SBO or CF diet ($P<0.01$). These results suggest that maternal dietary lipids may have the potential to influence bone development and growth of embryos by modifying the fatty acid composition of this tissue.

(***Key words***: maternal lipids, fatty acids, tibia, quail, prostaglandin E_2)

INTRODUCTION

The fatty acid composition of the yolk varies according to the dietary fatty acid profile, and either n-3 or n-6 fatty acids can be incorporated and enriched in yolk lipids by appropriate maternal dietary manipulations (Caston and Lesson, 1990; Cherian and Sim, 1991; Hargis, *et al*., 1991; Baucells *et al*., 2000). Likewise, a number of studies have consistently indicated that alterations in the fatty acid composition of yolk lipids, brought about by maternal dietary lipid sources, result in significant changes in the fatty acid composition of phospholipids in brain tissues of the embryos, which generally reflected the maternal dietary fatty acid profile (Anderson *et al*., 1990; Cherian and Sim, 1991; Enslen *et al*., 1991; Maldjian *et al*., 1996). Although lipids in the yolk are extensively utilized during incubation (Noble and Cocchi, 1990), a large proportion remain unused at hatch and are sequestered into the body cavity and utilized within 5 days after hatch (Latour *et al*., 1994). Studies indicate that the fatty acid composition in certain tissues still reflect the maternal dietary lipid profile for about 3 weeks after hatching (Anderson *et al*., 1989; Cherian and Sim, 1991).

Lipids are believed to play an important role in skeletal metabolism and bone health. Essential fatty acid deficiency of weanling rats led to eicosatrienoic acid (20:3n-9) accumulation and bone partial hypomineralization (Odutuga, 1981). Acidic phospholipids enhance the formation of calcium complexes within matrix vesicles of the growth plate cartilage and facilitate matrix mineralization (Wuthier, 1993). Current findings indicate that neutral lipids may also play a role in bone development induced by bone morphogenetic proteins (BMPs) because lipids freed of BMPs did not induce bone formation (Urist *et al*., 1997). The finding that osteoblasts synthesize and secrete fatty acids as droplets into the extracellular space, which form the pH-dependent multilamellar structures in bone, suggests that some fatty acids probably are directly involved in bone mineralization (Takahashi, 1994). In addition, polyunsaturated fatty acids (PUFAs) serve as substrates for prostaglandin (PG) biosynthesis (Xu *et al*., 1994). PGE_2, produced in bone tissue from arachidonic acid (AA; 20:4n-6) (Sardesai, 1992), acts as a potent bone resorbing agent (Marks and Miller, 1993; Raisz *et al*., 1993; Fall *et al*., 1994). Chick embryonic long bones undergo mineral resorption in the presence of PGE_2 concentration ranging from 10^{-9} to 10^{-5} M (Watkins 1995). Therefore, PUFAs are physiologically important for bone mineralization and modeling. However, the impacts of maternal dietary lipids on bone metabolism of progeny are still unknown. The present study, therefore, was designed to investigate effects of maternal dietary lipids, varying in amounts of n-6 and n-3 fatty acids, as well as saturated and trans-fatty acids, on the fatty acid composition of bone tissue in newly hatched and postnatal quail of different ages. Newly hatched quail were used to determine if maternal dietary lipids modulate PGE_2 production in bone tissue.

MATERIALS AND METHODS

Animals and Diets

One hundred and sixty newly hatched Japanese quail were placed in temperature-controlled battery brooders with 24 h of light and raised with the same starter diet (Table1) until 3 weeks of age. At 4 weeks of age, the birds were individually wing-banded, weighed and equally assigned to 4 dietary treatments. Each treatment group was fed a basal diet supplemented with either 5% soybean oil [SBO; rich in linoleic acid (18:2n-6)], hydrogenated soybean oil [HSBO; high in trans oleic acid (t18:1)], chicken fat [CF; blend fat; high in 18:2n-6] or menhaden fish oil [FO; rich in eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA;22:6n-3)] (Table 2). The fatty acid composition of the diets is shown in Table 3. The diets were formulated to meet or exceed the nutrient requirements of laying quail (NRC, 1994). Water and feed were provided for *ad libitum* consumption throughout the whole experimental period.

At 6 weeks of age, the birds (20 males and 20 females per treatment) were housed individually in cages. From 7 weeks of age, hens were naturally mated 3 times a week and eggs were collected daily according to treatments beginning at 8 weeks of age. The eggs, collected for one week, were incubated. Newly hatched quail were killed by cervical dislocation. Another set of eggs, collected continuously for 10 days, were incubated and the hatched quail were weighed and placed into the temperature-controlled battery brooders according to maternal diet. All quail were fed an identical starter diet (Table 1) and raised under the same management conditions for 2 weeks.

Sample Collection

Tibial bones were collected from progeny at day old, one week and two weeks of age. After quail were killed by cervical dislocation, tibial bones were excised, and the surrounding soft tissue removed. Tibial bones from 4 birds at one day of age were pooled to obtain one sample, and tibiae from two birds at one week and two weeks of age were pooled as one sample. Six samples in each treatment at each age were obtained, placed on ice and frozen at -20^{o}C for fatty acid analysis. In addition, 15 eggs were collected from each group and the yolks were separated from the rest of the egg. Three yolks were pooled as a single sample to get 5 samples for each treatment group and frozen immediately at -20^{o}C for fatty acid analysis. Additional 10 newly hatched birds from each treatment were scarified, and the tibiae, after removal of marrow, were collected for measurement of *ex vivo* PGE_2 biosynthesis.

Fatty Acid Analysis

The lipids in the diet, yolk and tibial cortical bone were extracted with chloroform/methanol (2:1,v/v). The shaft portion of the tibia was used for fatty acid analysis. After removal of the periosteum and marrow, cortical bone was frozen in liquid nitrogen and pulverized to powder with a mortar and pestle, and then weighed and placed in 7 ml of methanol and 14 ml of chloroform for 24 h prior to the extraction of lipids. Fatty acid methyl esters (FAME) from lipid fractions were prepared by transesterification using 14% Boron Trifluoride (BF_3) in methanol (Watkins *et al*., 1997). The FAME were extracted in hexane and analyzed using a gas-liquid chromatograph (HP 5890A, flame ionization detector, autosampler, Hewlett Packard, Sunnyvale, CA) equipped with a silica capillary 88 column (100 m×0.25 mm i.d., 0.2 μm film thickness, Middelburg,

Netherland) with hydrogen as the carrier gas. The initial oven temperature of 175^{o}C was held for 10 min and increased at a rate of 10^{o}C/min to a final 220^{o}C and held for 20 min. The duration of gas chromatographic analysis was 110.5 min. The temperatures of the injector and detector were 250^{o}C and 255^{o}C, respectively. A known amount of internal free 11:1 fatty acid standard in hexane was used to develop the calibration table, and 18:0 fatty acid was used as a reference compound for uncalibrated peaks. Fatty acid composition of the diets and bone samples was expressed as μg/100 μg of FAME in the lipids. The amounts of total saturated fatty acids (SF), monounsaturated fatty acids (MONUs), polyunsaturated fatty acids (PUFAs), total (n-3) and (n-6) PUFA, as well as the (n-6)/(n-3) ratios were calculated from the GLC analysis.

Ex vivo PGE_2 analysis

Tibia bones from newly hatched quail were used for PGE_2 measurement. Bone cultures for *ex vivo* PGE_2 production were prepared as described by Dekel *et al*. (1981) and Watkins *et al*. (1996). Briefly, the tibia from each bird were split in half longitudinally, bone marrow cells removed, weighed, and immediately immersed in 1ml of Hank's balanced salt solution (sigma Chemical Co., St. Louis, MO) and incubated for 2 h at 40^{o}C with shaking (Watkins *et al*., 1996). Following incubation, the bone culture medium was collected and stored at –80^{o}C for future analysis of *ex vivo* PGE_2 production of bone. PGE_2 biosynthesis in bone organ cultures was measured by radioimmunoassay using EIA kits (Cayman Chemical Co., CA). Each sample and standard were measured in duplicate and PGE_2 levels were expressed as ng of per gram bone wet weight. The anti-PGE_2 antibody had the following cross-reactivities: PGE_2, 100%; PGE_3, 43%, PGE_1, 18.7%; $PGF_{2\alpha}$; <0.01.

Data Analysis

All data were subjected to a one-way ANOVA analysis, and significant differences ($P<0.05$) between treatment means were performed by Duncan's multiple range test (SAS Institute Inc., 1998, Cary, NC). Variation within treatment was expressed as pooled standard error of the treatment mean (SEM).

RESULTS

Maternal Diet Fatty Acid Composition

The fatty acid composition of the four diets shown in Table 3 demonstrated that the SBO diet provided the highest amount of 18:2(n-6) and 18:3(n-3), whereas the FO diet had the greatest levels of 14:0, 16:1(n-7), 20:5(n-3), 22:5(n-3) and 22:6(n-3). The HSBO diet contained the largest amount of 18:0 and trans-18:1, and the CF diet was the highest in 18:1 and the second highest in the amount of 18:2(n-6). The SBO diet had the highest levels of PUFAs and (n-6) fatty acids, but the FO diet provided the largest amount of (n-3) fatty acids, total SAT and the lowest ratio of (n-6)/(n-3). The HSBO diet contained the greatest level of total MONU. The CF diet, however, also contained 2 fold the amount of MONU compared to the FO diet, and much higher levels of total (n-6)

fatty acids compared to both the HSBO and FO diets. Based on the GLC analysis and fat content calculation, all the diets contained 1.3-4.0 % of 18:2(n-6) which was well above the recommended requirement for laying quail (NRC, 1994).

Yolk Fatty Acid Composition

The fatty acid composition of yolks collected from laying hens fed different dietary lipids is presented in Table 4. The yolk fatty acid composition reflected the fatty acid profile of the diet. Specifically, yolks from hens fed SBO contained the highest amount of 18:2(n-6), 18:3(n-3) and 20:4(n-6) fatty acids ($P<0.01$), but significantly higher ($P<0.01$) incorporation of 14:0, 20:5(n-3), 22:5(n-3) and 22:6(n-3) fatty acids was obtained in the egg yolks from hens given the FO diet. Concurrently, the concentration of 20:4(n-6) fatty acid was significantly decreased in the FO group ($P<0.01$). Yolks from hens fed HSBO diet had the highest levels of t18:1. Consistently, yolks of hens fed SBO contained the highest amount of total (n-6) fatty acids and PUFAs ($P<0.01$), whereas those from hens fed FO were the greatest in the levels of, 20:5n-3, 22:6n-3, total (n-3) fatty acids and the lowest in the ratio of (n-6)/(n-3) ($P<0.01$). Yolks collected from hens consuming the HSBO diet had the highest MONU. Furthermore, yolks from hens maintained on the CF diet had significantly greater levels of (n-6) fatty acids than those collected from HSBO or FO fed hens ($P<0.01$), and also exhibited higher amount of MONU than yolks obtained from those fed the SBO diet ($P<0.01$).

Fatty Acid Composition of Progeny Bone Tissue

The egg yolk lipid composition had a significant effect upon the bone fatty acid profile of the newly hatched chicks, which was largely reflected in the yolk lipid profile (Table 5). Significantly higher concentrations of 18:2(n-6), 18:3(n-3) and 20:4(n-6) fatty acids were detected in the tibiae of progeny from hens fed SBO diet in comparison with the other groups ($P<0.01$), and the most pronounced increases in 20:5(n-3) and 22:6(n-3) fatty acids were observed in the FO group ($P<0.01$). Concurrent with the increase in (n-3) fatty acids, there was a significant decrease in the concentration of 20:4(n-6) fatty acids in FO group. Likewise, tibiae of progeny from hens given the HSBO diet still had a significantly higher level of t18:1 compared to other groups ($P<0.01$). The CF group was significantly higher in the amount of 18:2(n-6) fatty acid than FO group ($P<0.01$), and also markedly greater in the level of 20:4(n-6) fatty acid than both HSBO and FO groups ($P<0.01$). Totally, tibiae from progeny of hens fed SBO were the highest in total (n-6) fatty acids and PUFA, whereas those from the FO group contained the highest levels of total (n-3) fatty acids, total saturated acids, but lowest in the amounts of total (n-6) fatty acids, PUFA and the ratio of (n-6) to (n-3) ($P<0.01$). The HSBO groups had the greatest level of MONU ($P<0.01$).

The fatty acid analysis of bone tissues from one-week old progeny is shown in Table 6. It revealed that most of the fatty acids incorporated from yolk or maternal diet into tibial lipids were replaced by the quail starter diet fed to all groups. However, some of the fatty acids in tibia still reflected the effects of the yolk fatty acid composition. Particularly, quail whose maternal diet contained FO had significantly higher levels of 20:3(n-3), 20:5(n-3), and 22:6(n-3) fatty acids and a decreased ratio of n-6/n-3 ($P<0.05$)

at one week of age. Those hatched from HSBO enriched eggs still contained a significantly higher amount of t18:1 in the bone tissues than CF and FO groups ($P<0.01$). There were no significant differences in the concentrations of total SAT, PUFA, (n-6) or (n-3) fatty acids between treatment groups at this stage. At two weeks of age, all progeny showed similar fatty acid composition of lipids in the tibial bones regardless of maternal dietary lipids (Table 7).

Bone Ex vivo PGE_2 Production

The productions of *ex vivo* PGE_2 in bone organ cultures of day old quail paralleled the corresponding amounts of bone arachidonic acid (AA), the precursor of PGE_2 (Fig. 1). Newly hatched quail from hens consuming SBO or CF enriched diets had notably higher biosynthesis of *ex vivo* PGE_2 in bone organ cultures (28.65 ± 2.71, and 20.78 ± 1.78, respectively) compared with those whose maternal diets were FO or HSBO (15.06 ± 2.57, and 8.09 ± 1.34) ($P<0.01$). In addition, SBO group had significantly higher production of PGE_2 compared to CF ($P<0.01$). Likewise, the HSBO group showed a significantly higher biosynthesis of PGE_2 than FO group in bone organ cultures ($P<0.01$).

DISCUSSION

In the present study, maternal dietary lipids had a profound effect on egg yolk fatty acid composition and subsequent bone fatty acid profiles of progeny. Quail fed a diet higher in either n-6 or n-3 PUFAs led to the production of eggs with significant increases in the levels of these fatty acids. A maternal diet high in tran-18:1 also led to a greater deposition of this fatty acid in the yolks. These results are in accordance with those obtained by others (Caston and Lesson, 1990; Cherian and Sim, 1991; Hargis *et al*., 1991; Baucells *et al*., 2000). Linoleic acid [LA, 18:2(n-6)] and α-linolenic acid [LNA, 18:3(n-3)], which serve as precursors of AA, and EPA and DHA respectively, were the almost the only n-6 and n-3 fatty acids in the SBO, HSBO and CF diets. However, the egg yolks from hens fed these diets showed higher increases in the amounts of AA, EPA and DHA than those in the diets, indicating that laying quails can convert LA and LNA to longer chain n-3 and n-6 fatty acids by desaturation and elongation pathways (Sardesai, 1992). Egg yolks from FO or HSBO diet fed hens contained significantly lower levels of AA compared to those from the SBO or CF groups. This may be partially attributable to the relatively lower concentrations of the precursor fatty acids in the FO and HSBO diets. On the other hand, diets enriched in EPA and DHA have been shown to inhibit the Δ6 and Δ5 desaturases, reducing synthesis of AA from LA, and to compete between AA and EPA for acylation into phospholipids (Sardesai, 1992; Innis, 1996). Trans-18:1 fatty acids was also shown to compete with linoleic acid (18:2n-6) for the Δ^6 desaturase (Emken, 1984), inhibit this enzyme activity (Kirstein, 1983), and suppressed the AA synthesis in various animal tissues (Watkins, 1991; Bysted, 1998).

During incubation, egg yolk lipids are the only source of lipids for developing embryos (Maldjian, 1996). The fatty acid composition of bone tissues in newly hatched quail displayed a similar pattern of yolk fatty acid composition. The n-6 fatty acid enriched eggs from the SBO group resulted in much higher levels of both total n-6 and AA in the tibia, and eggs higher in EPA and DHA efficiently elevated these fatty acids

and greatly decreased the AA formation in bones of newly hatched quail. In addition, the concentrations of other fatty acids and the n-6/n-3 ratio in bone tissues still largely reflected the yolk lipid profiles. The results of this study indicated that during embryonic development, yolk fatty acids are efficiently converted, delivered and extensively utilized by developing bone tissues of embryos. Although substantial information is available regarding the fatty acid composition of lipids in the yolk and in some of the developing tissues of the avian embryo (Anerdson *et al*., 1990; Cherian and Sim, 1991; Enslen *et al*., 1991; Vilchez *et al*., 1990, 1992; Maldjian *et al*., 1996; Speake *et al*., 1996), there is no report describing the relationship between yolk fatty acid composition and that in the skeleton. The present data, in many ways, are consistent with those studies analyzing other tissues.

The fatty acid analysis of tibial bones in quail at one and two weeks of age revealed that the effect of maternal dietary lipids on bone fatty acid profile of the progeny diminished with age and completely disappeared at two weeks of age. During the two weeks posthatch, all quail were fed an identical diet regardless of their maternal treatments. However, at one week of age, bones from the FO group still contained significantly higher amounts of EPA and DHA, and those from the HSBO group had a greater level of tran-18:1 fatty acids. These results indicated that skeletal tissue continued to reflect the changes in the maternal dietary supplementation of these fatty acids for about one week, which is analogous with other studies on brain tissue in chicks (Anderson *et al*., 1989; Cherian and Sim, 1991).

In the present study, *ex vivo* PGE_2 production in the bone organ culture of newly hatched quails from hens fed SBO or CF were 3.5 and 2.5 fold of that in quail fed FO, respectively, and 1.9 and 1.4 times of that in quail given HSBO. These substantial decreases in PGE_2 biosynthesis in tibia of FO and HSBO groups were consistent with the relative concentrations of AA [20:4(n-6)], the precursor of PGE_2, which was the highest in the tissue of quail whose maternal dietary lipid was SBO, and was the lowest in the FO group. This result demonstrated that newly hatched quail had already developed the highly active enzyme system, capable of catalyzing the PG formation. Since the level of EPA [20:5(n-3)], the precursor of PGE_3, was significantly higher in tissue of FO group compared to those of other groups, and the antibodies used to measure PGE_2 had 43% cross-reactivity against PGE_3, the value of PGE_2 for quail in this group may represent a mixture of PGE_2 and PGE_3. However, EPA is a poor substrate for cyclooxygenase (Hwang, 1992), an enzyme catalyzing the formation of PGE_2 and PGE_3. Raisz *et al*. (1989) reported that EPA was only one-tenth as effective for PGE_3 formation as AA for PGE_2 production. Therefore, the relative proportion of PGE_3 is believed to be very low, and the decrease in PGE_2 production in the FO group was mainly attributable to depressed PGE_2 biosynthesis.

To our knowledge, we are the first to investigate the effects of different lipids, supplemented in the maternal diets, on the bone PGE_2 production in the progeny in avian species. Recent research consistently indicated that growing animals fed a diet enriched with n-3 fatty acids depressed the bone PGE_2 production, whereas those consuming a diet high in n-6 fatty acids significantly elevated the production of PGE_2 in bone tissues (Kokkinos *et al*., 1993;Watkins *et al*., 1996, 1997; Li and Watkins, 1998). Our results are in accordance with these observations.

The importance of PGE_2 in bone biology has been well studied since the first discovery that this AA metabolite stimulates bone resorption in fetal rat long bone organ culture (Klein and Raisz, 1970). It is currently believed that PGE_2 is a powerful agent regulating the bone modeling and remodeling, but its effect in bone is biphasic. At lower concentration, PGE_2 can stimulate bone formation (Raisz and Fall, 1990). At higher concentration, however, its main function is to stimulate bone resorption (Raisz and Fall, 1990; Marks and Miller, 1993; Raisz *et al*., 1993b; Fall *et al*., 1994). Therefore, excessive production of PGE_2 may adversely affect bone modeling as observed in chicks fed a diet enriched with n-6 fatty acids (Watkins *et al*., 1996, 1997). At this stage however, it is unclear whether maternal diet-induced changes in AA level in the bone tissue of progeny, and subsequent modification of PGE_2 can influence the bone development of embryos.

In summary, the data from this experiment confirms previous reports that supplementation of diets with different lipids to laying hens alters egg yolk fatty acid composition. We further demonstrated that egg yolk fatty acid composition had a significant effect on the fatty acid composition of bone tissue in embryos. Bone fatty acid concentrations in embryos highly reflected the lipid profile of egg yolks. This effect, however, diminished with age and had disappeared by two weeks of age. It was also observed that maternal dietary lipid induced large changes in bone AA concentrations in newly hatched quail, and the AA concentrations had a tremendous influence on PGE_2 production in bone organ cultures. Research is needed to characterize the effects of maternal dietary lipids on the bone growth and development of progeny.

Table 1. Dietary ingredients of quail starter diet[1]

Ingredients	(%)
Yellow corn	42.2
Soybean meal	38.0
Blend fat	3.5
Fish meal	7.0
Meat+bone scrap	5.0
Dehydrated alfalfa meal	1.25
Defluorinated phosphate	1.0
Limestone	0.5
Vitamin premix[2]	1.0
Trace mineral premix[3]	0.05
Salt	0.5

[1] The diet was formulated to contain 29.6% crude protein, 2970 kcal/kg metabolizable energy, 5.5% crude fat, 2.70% crude fiber, 1.40% calcium, and 0.70% phosphorous.

[2] Provided per kilogram of diet: vitamin A, 880 IU; vitamin D_3, 330 IU; vitamin E, 5.5 IU; menadione sodium bisulfite, 3.52 mg; thiamine HCL, 1.1 mg; riboflavin, 4.4 mg; calcium D-pantothenate, 8.8 mg; niacin, 44 mg; choline chloride, 374 mg; vitamin B_{12}, 0.011 mg; folic acid, 1.1 mg; pyridoxine HCL, 1.1mg; biotin, 0.11 mg; DL-methionine, 990 mg; Bacitracin,5.5 mg; selenium, 0.20 mg; ethoxyquin, 0.124 mg.

[3] Provided per kilogram of diet: manganese, 22 mg; zinc, 23.8 mg; iron, 25.0 mg; copper, 3.13 mg; iodine, 1.0 mg; and selenium, 0.15 mg.

Table 2. Dietary ingredient composition of the basal diets given to laying hens[1]

	Dietary lipid treatment[2]			
Ingredients (%)	SBO	HSBO	CF	FO
Yellow corn	53.55	53.55	53.55	53.55
Soybean meal	32.00	32.00	32.00	32.00
Soybean oil	5.00	—	—	—
Hydrogenated soybean oil	—	5.00	—	—
Chicken fat	—	—	5.00	—
Menhaden oil	—	—	—	5.00
Limestone	6.50	6.50	6.50	6.50
Dicalcium phosphate	1.50	1.50	1.50	1.50
Vitamin Premix[3]	1.00	1.00	1.00	1.00
Trace mineral premix[4]	0.10	0.10	0.10	0.10
salt	0.35	0.35	0.35	0.35

[1] The diet was formulated to contain 21.0% crude protein, 2997.2-3007.2 kcal/kg metabolizable energy, 7.36% crude fat, 2.43% crude fiber, 2.81% calcium, and 0.67% phosphorous.

[2] Diets include soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) or menhaden fish oil (FO) at 50g/kg of diet.

[3] Provided per kilogram of diet: vitamin A, 1100 IU; vitamin D_3, 330 IU; vitamin E, 33 IU; menadione sodium bisulfite, 3.52mg; Thiamine HCL, 1.1mg; riboflavin, 6.6mg; calcium D-pantothenate, 16.5mg; niacin, 44mg; choline chloride, 374mg; vitamin B_{12}, 0.0165mg; folic acid, 1.1mg; pyridoxine HCL, 1.1mg; biotin, 0.055mg; Bacitracin,22.0 mg; selenium, 0.20mg; ethoxyquin, 0.124mg.

[4] Provided per kilogram of diet: manganese, 44mg; zinc, 47.5 mg; iron, 50.0 mg; copper, 6.25mg; iodine, 2.0 mg; and selenium, 0.3 mg.

Table 3. Fatty acid composition (μg/100 μg) of maternal basal diet

	Dietary lipid treatment[1]			
Fatty acids	SBO	HSBO	CF	FO
14:0	0.06	0.19	0.57	5.29
15:0	0.02	0.04	0.09	0.42
16:0	9.95	11.26	15.01	15.45
16:1(n-7)	0.08	0.15	2.30	6.95
18:0	3.54	11.11	6.53	3.26
t18:1	0.05	25.49	2.57	1.45
18:1	25.82	30.76	39.50	15.07
18:2(n-6)	54.79	18.11	30.00	19.16
18:3(n-3)	4.33	0.98	1.54	2.32
20:0	0.30	0.34	0.23	0.20
20:3(n-3)	ND[5]	ND	0.05	0.11
20:4(n-6)	0.01	0.01	0.16	0.51
20:5(n-3)	0.01	ND	0.02	18.49
22:1	ND	ND	0.03	0.24
22:4(n-6)	0.01	0.01	0.03	0.09
22:5(n-3)	0.02	0.03	0.02	1.47
22:6(n-3)	ND	ND	0.01	7.47
SAT[2]	13.87	22.94	22.42	24.62
MONU[3]	25.95	56.42	44.43	23.91
PUFA[4]	59.18	19.15	31.82	49.61
(n-6) PUFA	54.81	18.13	30.19	19.75
(n-3)PUFA	4.37	1.02	1.63	29.86
(n-6)/(n-3)	12.55	17.85	18.47	0.66

[1] Maternal dietary treatments include soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat, and menhaden fish oil (FO) at 50 g/kg of diet.

[2] SAT, total saturated fatty acids.

[3] MONU, total monounsaturated fatty acids.

[4] PUFA, total polyunsaturated fatty acids.

[5] ND, not detected.

Table 4. Fatty acid composition (μg/100 μg) of the egg yolks collected from laying hens fed different lipids[1]

	Dietary lipid treatment[2]					
Fatty acids	SBO	HSBO	CF	FO	Pooled SEM	P values
14:0	0.45[b]	0.50[b]	0.49[b]	0.91[a]	0.05	0.0002
15:0	0.12	0.16	0.17	0.19	0.04	0.6940
16:0	28.17	31.15	31.63	30.35	1.92	0.6109
16:1(n-7)	3.01	3.90	4.23	4.98	0.44	0.0763
18:0	13.10	12.18	12.89	13.61	0.91	0.7529
t18:1	0.21[b]	3.68[a]	0.56[b]	0.22[b]	0.11	0.0001
18:1	29.85	34.60	33.40	29.49	4.61	0.8206
18:2(n-6)	19.86[a]	9.73[b]	12.22[b]	10.56[b]	1.03	0.0001
18:3(n-3)	0.79[a]	0.19[c]	0.23[c]	0.51[b]	0.04	0.0001
20:0	0.04	0.09	0.17	0.10	0.03	0.0522
20:3(n-3)	0.15[a]	0.10[b]	0.12[ab]	0.07[c]	0.01	0.0015
20:4(n-6)	2.36[a]	0.56[b]	2.35[a]	0.61[b]	0.12	0.0001
20:5(n-3)	0.12[b]	0.04[b]	0.05[b]	3.19[a]	0.08	0.0001
22:4(n-6)	0.10[a]	0.07[b]	0.12[a]	0.03[c]	0.01	0.0001
22:5(n-3)	0.23[b]	0.07[c]	0.12[bc]	0.68[a]	0.03	0.0001
22:6(n-3)	1.08[b]	0.39[c]	0.60[bc]	3.79[a]	0.14	0.0001
SAT[2]	41.85	44.08	45.36	45.16	2.84	0.8134
MONU[3]	33.06[c]	42.18[a]	39.19[ab]	34.68[bc]	1.17	0.0019
PUFA[4]	24.67[a]	12.15[c]	15.80[bc]	19.41[b]	1.38	0.0003
(n-6) PUFA	22.31[a]	11.36[c]	14.69[b]	11.19[c]	1.11	0.0001
(n-3)PUFA	2.36[b]	0.80[c]	1.11[c]	8.22[a]	0.26	0.0001
(n-6)/(n-3)	9.46[c]	14.30[a]	13.31[b]	1.37[d]	0.21	0.0001

[1] Mean values for yolk fatty acid composition (n=5) within a row having different superscripts are significantly different (P<0.05).

[2] Maternal dietary treatments include soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat, and menhaden fish oil (FO) at 50 g/kg of diet.

[3] SAT, total saturated fatty acids.

[4] MONU, total monounsaturated fatty acids.

[5] PUFA, total polyunsaturated fatty acids.

Table 5. Effects of feeding different lipids to laying hens on the fatty acid composition (μg/100 μg) of lipids isolated from tibial bones in newly hatched quail[1]

	Dietary lipid treatment[2]				Pooled	
Fatty acids	SBO	HSBO	CF	FO	SEM	P values
14:0	0.59[b]	0.57[b]	0.62[b]	0.95[a]	0.04	0.0058
15:0	0.07[c]	0.08[bc]	0.11[b]	0.17[a]	0.01	0.0089
16:0	22.35[c]	24.39[bc]	24.82[ab]	26.28[a]	0.5	0.0461
16:1(n-7)	1.81	2.43	2.18	2.39	0.11	0.0637
18:0	11.82[b]	10.07[c]	13.0[b]	15.13[a]	0.5	0.0025
t18:1	0.33c	2.20[a]	0.67[b]	0.39[c]	0.03	0.0001
18:1	26.90[b]	38.58[a]	36.92[a]	36.39[a]	0.73	0.0001
18:2(n-6)	18.92[a]	11.73[b]	11.72[b]	9.74[b]	0.69	0.0001
18:3(n-3)	0.32[a]	0.12[b]	0.08[b]	0.11[b]	0.02	0.0005
20:0	0.26	0.21	0.23	0.28	0.02	0.4215
20:1(n-9)	0.15[c]	0.35[b]	0.49[a]	0.56[a]	0.04	0.0104
20:3(n-3)	0.46[a]	0.37[b]	0.29[c]	0.34[bc]	0.03	0.0123
20:4(n-6)	10.92[a]	5.74[c]	7.92[b]	1.94[d]	0.57	0.0003
22:1	0.17[c]	0.21[bc]	0.27[b]	0.37[a]	0.03	0.0378
20:5(n-3)	0.69[b]	0.21[c]	0.14[c]	2.28[a]	0.16	0.0108
22:4(n-6)	1.10[a]	0.69[b]	0.68[b]	0.16[c]	0.07	0.0034
22:5(n-3)	0.48[a]	0.21[b]	0.17[b]	0.54[a]	0.04	0.0143
22:6(n-3)	1.00[b]	0.65[c]	0.65[c]	2.00[a]	0.09	0.0001
SAT[3]	35.45[c]	35.69[c]	37.29[b]	43.54[a]	0.96	0.0136
MONU[4]	29.35[c]	43.82[a]	40.53[ab]	40.11[b]	0.71	0.0001
PUFA[5]	34.88[a]	20.16[b]	21.69[bc]	15.76[c]	1.45	0.0006
(n-6) PUFA	30.94[a]	18.62[b]	20.37[b]	10.93[c]	1.18	0.0001
(n-3)PUFA	3.35[b]	1.55[c]	1.32[c]	4.84[a]	0.32	0.0035
(n-6)/(n-3)	7.89[b]	12.70[a]	15.43[a]	2.30[c]	0.71	0.0001

[1] Mean values for bone fatty acid composition (n=8) within a row having different superscripts are significantly different (P<0.05).

[2] Maternal dietary treatments include soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat, and menhaden fish oil (FO) at 50 g/kg of diet.

[3] SAT, total saturated fatty acids.

[4] MONU, total monounsaturated fatty acids.

[5] PUFA, total polyunsaturated fatty acids.

Table 6. Effects of feeding different lipids to laying hens on the fatty acid composition (μg/100 μg) of lipids isolated from tibial bones in one week old posthatched quail[1]

	Dietary lipid treatment[2]					
Fatty acids	SBO	HSBO	CF	FO	Pooled SEM	P values
14:0	0.94[a]	0.88[b]	0.86[bc]	0.84[c]	0.02	0.0014
15:0	0.87	0.78	0.84	0.72	0.06	0.4444
16:0	24.27	23.78	23.93	23.53	0.45	0.7441
16:1(n-7)	2.56[c]	2.91b[c]	3.54[a]	2.98[b]	0.13	0.0003
18:0	9.72	8.91	8.41	9.27	0.50	0.3000
t18:1	1.44[ab]	1.67[a]	1.32[b]	1.32[b]	0.09	0.0021
18:1	28.00	28.32	30.31	27.38	0.73	0.0843
18:2(n-6)	21.68	22.24	21.53	21.86	0.79	0.9513
18:3(n-3)	0.87	0.96	0.81	0.83	0.08	0.6034
20:0	0.16	0.14	0.13	0.16	0.01	0.0600
20:1(n-9)	0.32	0.29	0.28	0.30	0.01	0.4541
20:3(n-3)	0.32[b]	0.35[b]	0.30[b]	0.43[a]	0.02	0.0015
20:4(n-6)	3.22	3.72	3.16	4.09	0.26	0.0854
22:1	0.11[b]	0.10[b]	0.09[b]	0.20[a]	0.01	0.0001
20:5(n-3)	0.48[b]	0.51[b]	0.46[b]	0.83[a]	0.09	0.044
22:4(n-6)	0.45	0.53	0.44	0.49	0.03	0.1981
22:5(n-3)	0.32	0.39	0.32	0.48	0.05	0.1067
22:6(n-3)	0.53[b]	0.55[b]	0.45[b]	0.96[a]	0.08	0.0015
SAT[3]	35.80	34.49	34.17	34.52	0.65	0.1597
MONU[4]	32.73	33.29	35.54	32.18	0.70	0.0624
PUFA[5]	28.07	29.25	27.47	29.97	1.11	0.0815
(n-6) PUFA	25.55	26.49	25.13	26.44	0.88	0.0844
(n-3)PUFA	2.51	2.75	2.35	3.53	0.31	0.0678
(n-6)/(n-3)	10.18[a]	9.63[ab]	10.69[a]	7.49[b]	0.86	0.0395

[1] Mean values for bone fatty acid composition (n=8) within a row having different superscripts are significantly different (P<0.05).

[2] Maternal dietary treatments include soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat, and menhaden fish oil (FO) at 50 g/kg of diet.

[3] SAT, total saturated fatty acids.

[4] MONU, total monounsaturated fatty acids.

[5] PUFA, total polyunsaturated fatty acids.

Table 7. Effects of feeding different lipids to laying hens on the fatty acid composition (μg/100 μg) of lipids isolated from tibial bones in two weeks old posthatched quail[1]

	Dietary lipid treatment[2]					
Fatty acids	SBO	HSBO	CF	FO	Pooled SEM	P values
14:0	0.92	0.92	0.90	0.92	0.03	0.9805
15:0	0.13	0.13	0.12	0.18	0.01	0.0976
16:0	23.37	24.10	23.19	24.04	0.2	0.5433
16:1(n-7)	2.98	3.40	3.68	3.81	0.22	0.0787
18:0	8.84	8.78	8.70	8.36	0.18	0.3442
t18:1	1.45	1.45	1.27	1.35	0.12	0.6365
18:1	31.77	32.85	33.63	32.76	0.70	0.3574
18:2(n-6)	23.94	22.15	21.69	22.91	0.64	0.7356
18:3(n-3)	1.01	0.89	0.90	0.98	0.05	0.3451
20:0	0.11	0.11	0.11	0.10	0.01	0.2732
20:1(n-9)	0.31	0.32	0.31	0.29	0.01	0.3668
20:3(n-3)	0.19	0.18	0.21	0.20	0.01	0.2660
20:4(n-6)	1.89	1.81	2.01	1.65	0.11	0.2010
22:1	0.05	0.07	0.08	0.05	0.01	0.0650
20:5(n-3)	0.28	0.24	0.23	0.27	0.06	0.9119
22:4(n-6)	0.30	0.30	0.36	0.29	0.02	0.0677
22:5(n-3)	0.22	0.20	0.20	0.23	0.03	0.8600
22:6(n-3)	0.34	0.26	0.29	0.34	0.07	0.7671
SAT[3]	34.91	35.54	35.65	34.94	0.30	0.1615
MONU[4]	36.62	38.13	38.15	38.18	0.81	0.2219
PUFA[5]	28.17	26.03	25.89	26.87	4.28	0.9805
(n-6) PUFA	26.13	24.26	24.06	24.85	4.37	0.9748
(n-3)PUFA	2.04	1.77	1.83	2.02	0.21	0.6239
(n-6)/(n-3)	12.81	13.71	13.23	12.13	1.09	0.7793

[1] Mean values for bone fatty acid composition (n=8) within a row having different superscripts are significantly different (P<0.05).

[2] Maternal dietary treatments include soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat, and menhaden fish oil (FO) at 50 g/kg of diet.

[3] SAT, total saturated fatty acids.

[4] MONU, total monounsaturated fatty acids.

[5] PUFA, total polyunsaturated fatty acids.

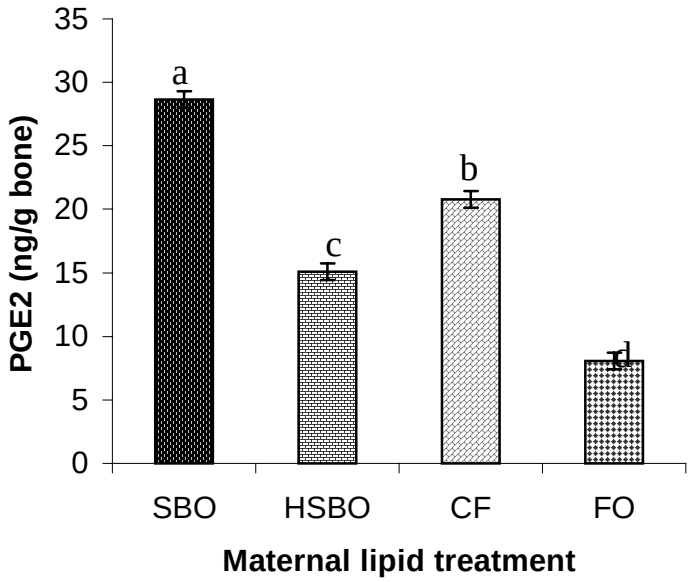

Figure 1. PGE_2 production in bone organ culture in day old quail from hens fed soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) or menhaden fish oil (FO) at 50g/kg of the diet. Data are expressed as mean ± SEM; Bars having different superscripts are significantly different ($P<0.001$).

REFERENCES

Anderson, G. J., W. E. Connor, J. D. Corliss, and D. S. Lin, 1989. Rapid modulation of the n-3 docosahexaenoic acid levels in the brain and retina of the newly hatched chick. J. Lipid Res. 30:433-441.

Anderson, G. J., W.E. Connor, and J. D. Corliss, 1990. Docosahexaenoic acid is the preferred dietary n-3 fatty acid for the development of the brain and retina. Pediatr. Res. 27:89-97.

Baucells, M. D., N. Crespo, A. C. Barroeta, S. Lopez-Ferre, and M. A. Crashorn, 2000. Incorporation of different polyunsaturated fatty acids into eggs. Poultry Sci. 79:51-59.

Bysted, A., G. H∅lmer, and P. Lund, 1998. Influence of moderate amounts of trans fatty acids on the formation of polyunsaturated fatty acids. JAOCS. 75:225-234.

Caston, L., and S. Leeson, 1990. Research note: dietary flax and egg composition. Poultry Sci. 69:1617-1620.

Cherian, G., and J. S. Sim, 1991. Effect of feeding full fat flax and canola seeds to laying hens on the fatty acid composition of eggs, embryos and newly hatched chicks. Poultry Sci. 70:917-922.

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

Emken, E. A., 1984. Nutrition and biochemistry of trans and positional fatty acid isomers in hydrogenated oils. Ann. Rev. Nutr. 4:339-376.

Enslen, M., H. Milon, and A. Malnoe, 1991. Effect of low intake of n-3 fatty acids during development on brain phospholipid fatty acid composition and exploratory behavior in the rat. Lipids 26:203-208.

Fall, P. M., D. T. Breault, and L. G. Raisz, 1994. Inhibition of collagen synthesis by prostaglandins in the immortalized rat osteoblastic cell line pyla: structure-activity relations and signal transduction mechanisms. J. Bone Miner. Res. 9:1935-2943.

Hargis, P. S., M. E. Van Elswyk, and B. M. Hargis, 1991. Dietary modification of yolk lipid with menhaden oil. Poultry Sci. 70:873-883.

Hwang, Y. S. P. E. Wainwright, P. R. Redden, D. E. Mills, B. B. Fleming, and D. F. Horrobin, 1992. Effect of maternal dietary fats with variable n-3/n-6 ratios on tissue fatty acid composition in sucking mice. Lipids 27:104-110.

Innis, S. M., 1996. Essential dietary lipids. Recent Knowledge in Nutrition. 7th ed. ILSI Press. Washington D. C.

Kirstein, D., C. E. HΦy, and G. HΦlmer, 1983. Effects of dietary fats on the Δ6- and Δ5-desaturation of fatty acids in rat liver microsomes. Br. J. Nutr. 50:749-756.

Klein, D. C., and L .G. Raisz, 1970. Prostaglandins: stimulation of bone resorption in tissue culture. Endocrinology 86:1436-1440.

Kokkinos P. P., R. Shaye, B. S. Alam, and S. Q. Alam, 1993. Dietary lipids prostaglandin E_2 levels, and tooth movement in alveolar bone of rats. Calcif. Tissue Int. 53:333-337.

Latour, M. A., E. D. Peebles, C. R. Boyle, and J .E. Brake, 1994. The effects of dietary fat on growth performance, carcass composition, and feed efficiency in the broiler chick. Poultry Sci. 73:1362-1369.

Li, Y., and B. A. Watkins, 1998. Conjugated linoleic acids alter bone fatty acid composition and reduce *ex vivo* prostaglandin E_2 biosynthesis in rats fed n-6 or n-3 fatty acids. Lipids 33:417-425.

Maldjian, A., Cristofori, C, R. C. Noble, and B. K. Speake, 1996. The fatty acid composition of brain phospholipids from chicken and duck embryos. Comp. Biochem. Physiol. 115B:153-158.

Marks, S. C. Jr., and S. C. Miller, 1993. Prostaglandins and skeleton: The legacy and challenges of two decades of research. Endocrine. J. 1:337-344.

Miyaura, C., K. Kusano, T. Masuzawa, O. Chaki, Y. Onoe, M. Aoyag, T. Sasaki, T. Tamura, Y. Koishihara, Y. Ohsugi, and T. Suda, 1995. Endogenous bone-resorbing factors in estrogen deficiency: cooperative effects of IL-1 and IL-6. J. Bone Miner. Res. 10:1365-1373.

Noble, R. C., and M. Cocchi, 1990. Lipid metabolism and the neonatal chicken. Prog. Lipid Res. 29:107-140.

National Reseach Council, 1994. Nutrient requirements of poultry. 9th rev. ed. National Academic Press, Washington DC.

Odutuga, A. A., 1981. Effects of low-zinc status and essential fatty acid deficiency on bone development and mineralization. Comp. Biochem. Physiol. 71A:383-388.

Raisz, L. G., C. B. Alander, and H. A. Simmons, 1989. Effects of prostaglandin E_3 and eicosapentaenoic acid on rat bone in organ culture. Prostaglandins 37:615-625.

Raisz, L. G., and P. M. Fall, 1990. Biphasic effects of prostaglandin E_2 on bone

formation in cultural fetal rat calvariae: Interaction with cortisol. Endocrinology 126:1654-1659.

Raisz, L. G., C. C. Pilbeam, and P. M. Fall, 1993. Prostaglandins: Mechanisms of action and regulation of production in bone. Osteoporosis Int. 3 (Suppl.1):S136-S140.

Sardesai, V. M., 1992. Biochemical and nutritional aspects of eicosanoids. J. Nutr. Biochem. 3:562-579.

Speak, B. K., C. Cristoforic, R. J. McCartney, and R. C. Noble, 1996. The relationship between the fatty acid composition of the lipids of the york and the brain of the duck embryos. Biochem. Soc. Trans. 24:181S.

Takahashi, T., S. Ueda, K. Takahashi, and R. O. Scow, 1994. PH-dependent multilamellar structures in fetal mouse bone: possible involvement of fatty acids in bone mineralization. Am. J. Physiol. 266:C590.

Urist, M.R., K. Behnam, F. Kerendi, D. Bagga, H. D. Adkisson, J. Nevenzel, and T. Malinin, 1997. Neutral lipids facilitate transfer of bone morphogenetic proteins and other noncollagenous proteins. Med. Hypothesis. 49:465.

Vilchez, C., S. P. Touchburn, E. R. Chavez, and C. W. Chan, 1990. Effect of feeding palmitic, oleic, and linoleic acids to Japanese quail hens (*Coturnix coturnix japonica*). 1. Reproductive performance and tissue fatty acids. Poultry Sci. 70:2484-2493.

Vilchez, C., S. P. Touchburn, E. R. Chavez, and C. W. Chan, 1992. Effect of feeding palmitic, oleic, and linoleic acid to Japanese quail hens (*Coturnix coturnix japonica*). 2. Maternal diets and stage of incubation on the lipid metabolism of quail embryos. Poultry Sci. 71:1032-1042.

Watkins, B. A., 1995. Biochemical and physiological aspects of polyunsaturates. Poultry Avian Biol. Rev. 1-14.

Watkins, B. A., C-L. Chen, J. P. McMurthy, H. Xu, and S. D. Bain, 1997. Dietary lipids modulate bone prostaglandin E_2 production, insulin-like growth factor-I concentration and formation rate in chicks. J. Nutr. 127:1084-1091.

Watkins, B. A, C-L, Shen, K. G. D. Allen, and M .F. Seifert, 1996. Dietary (n-3) and (n-6) polyunsaturates and acetylsalicylic acid alter *ex vivo* PGE_2 biosynthesis, tissue IGF-I levels, and bone morphometry in chicks. J. Bone Miner. Res. 11:1321-1332.

Watkins, B. A., C. C. Whitehead, and R. L. Duff, 1991. Hydrogenated oil decreases tissue concentrations of n-6 polyunsaturated fatty acids and may contribute to dyschondroplasia in broilers. Br. Poultry Sci. 32:1109-1119.

Wuthier, R. E., 1993. Involvement of cellular metabolism of calcium and phosphate in calcification of avian growth plate cartilage. J. Nutr. 123:301.

Xu, H., B. A. Watkins, and H. D. Adkisson, 1994. Dietary lipids modify the fatty acid composition of cartilage, isolated chondrocytes and matrix vesicles. Lipids 29:619-625.

CHAPTER TWO

Effects of Maternal Dietary Lipids on Bone Chemical Composition, Mechanical Properties and Histological Characteristics in Progeny of Japanese Quail

ABSTRACT: Maternal dietary lipids can modify the bone fatty acid composition and *ex vivo* PGE_2 biosynthesis in bone organ cultures of newly hatched Japanese quail. This study evaluated the effects of maternal dietary lipids on chemical components, mechanical and histological properties of tibia in progeny of hens fed different dietary lipids. Laying hens were fed a basal diet containing either soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) or menhaden fish oil (FO) at 50g/kg of the diet. Fertilized eggs were incubated and newly hatched quail were either sacrified for bone composition and histological analysis or raised using the identical diet for later measurements. The various maternal dietary lipid treatments did not affect growth of progeny at any developmental stage. There were also no significant differences in tibial length, diameter and collagen content. Tibial percent ash was significantly higher in newly hatched quail from hens fed the FO and HSBO diets, and it remained higher in the FO groups than in the CF group at 14 days of age ($P<0.05$). The levels of tibial deoxypyridinoline (DP) and total pyridinium crosslinks were markedly higher in the FO and HSBO groups at hatch ($P<0.05$). At 7 days of age, the tibial DP links remained higher in the FO group compared to the CF and SBO groups ($P<0.05$). Likewise, quail from hens consuming the FO or HSBO diet had higher tibial shear force and stiffness at 7 and 14 days of age. There were no pronounced differences in tibial fracture energy and deflection among treatments. Furthermore, supplementing the maternal diet with FO or HSBO enlarged the cartilaginous proliferative and hypertrophic zones (HZ) of the tibial proximal end in newly hatched quails ($P<0.05$), which was accompanied with a thicker cortical bone in the diaphysis in these groups ($P<0.05$). However, the width of the HZ tended to be smaller in these two groups coupled with improvement in trabecular density and cortical thickness in the proximal end and cortical density in the diaphysis at three weeks of age. These results suggest that maternal dietary lipids altered bone development by influencing organic matrix quality and mineralization in embyros, which consequently modified bone mechanical properties in early postnatal growth.

(***Key words*:** Maternal fatty acids, collagen, crosslinks, bone, quail)

INTRODUCTION

Commercial broilers have been selected for enhanced growth rate which concurrently has increased the incidence and severity of leg disorders, bone deformities and breakage by compromising bone strength and quality (Skinner *et al*., 1990; Rath *et al*., 1999). Bone strength is related to both the organic and inorganic matrix composing bone tissue. The inorganic matrix provides the bone with compressional strength, whereas the collagen dominated organic matrix provides bone with its tensile strength and structural scaffolds for the deposition of minerals (Lawson and Czernuszka, 1998; Rath *et al*., 1999). Therefore, a decrease in bone collagen content also results in a decrease in mechanical strength both in humans (Bailey *et al*., 1999) and animals (Masse *et al*., 1996). New research indicates that not only collagen, but also its intermolecular crosslinks (e.g. pyridinoline and deoxypyridinoline) contribute to bone mechanical strength (Batge *et al*., 1992; Bailey *et al*., 1993; Knott *et al*., 1995; Oxlund *et al*., 1995 and 1996; Rath *et al*., 1999).

Traditionally, dietary calcium, phosphorous and vitamin D_3 are often used to improve the inorganic matrix, but calcium intake above normal requirement does not stimulate bone formation (Watkins and Seifert, 1997). New research suggests that dietary lipids play an important role in the development, growth and modeling of long bones. Atteh and Leeson (1983, 1989) indicated that dietary sources and levels of fat influence mineral metabolism and bone mineralization of broiler chicks. More recently, several studies reported that dietary lipids, varying in the amounts of n-3, n-6 and saturated fatty acids, modulate bone fatty acid composition, the production of local regulatory factors and bone formation rate in growing animals (Alam *et al*., 1993; Kokkinos *et al*., 1993; Watkins 1996a, 1997a). In addition, changes in bone calcium and pyridinium crosslinks in response to dietary essential fatty acid manipulation were found (Claassen *et al*,. 1995; Kruger *et al*., 1997), which imply modulation in bone organic matrix. Furthermore, high fat diets decrease bone mineralization and negatively affect the bone strength in both young and adult animals (Li *et al*., 1990; Zernicke *et al*., 1995; Wohl *et al*., 1998).

The effects of lipid supplementation in the maternal diet on tissue fatty acid composition in chick embryos have been well established. In an earlier study with quail (Chapter 1), it was found that different maternal dietary lipids dramatically modify the concentrations of n-3, n-6 and trans fatty acids in tibia, as well as *ex vivo* PGE_2 production in bone organ culture of newly hatched quail. The objectives of this study were to investigate if maternal dietary lipids, varying in the amounts of n-3, n-6, saturated and trans fatty acids, modulate the bone growth and development in progeny through the application of biochemical, histological and mechanical techniques.

MATETRALS AND METHODS

Animals and Diets

One hundred and sixty newly hatched Japanese quail were placed in temperature-controlled battery brooders with 24 h of light and fed with the same standard diet (Table

1) until 3 weeks of age. At 4 weeks of age, birds were individually wing-banded, weighed and equally assigned to 4 dietary treatments. Each treatment group was fed a basal diet supplemented with either 5% soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) or menhaden fish oil (FO) (Table 2). The fatty acid composition of the diets is shown in Table 3. The diets were formulated to meet or exceed the nutrient requirements of laying quail (NRC. 1994). Birds were given free access to feed and water throughout the whole experimental period. At 6 weeks of age, the birds were housed individually in cages with 20 females and 20 males in each treatment. From 7 weeks of age, hens were naturally mated 3 times a week and eggs were collected daily according to treatments from the 8^{th} week. Two sets of fertilized eggs, with each collected for 10 consecutive days, were set by maternal dietary treatments, incubated, and hatched.

In study one, 10 newly hatched quail in each treatment from the first set of eggs were sacrificed for collecting samples at day old age. The remaining birds (about 50/ treatment) were weighed and placed in temperature-controlled battery brooders, separated according to maternal diet, and raised for histological measurements. In study two, 11 newly hatched quail in each treatment from the second set of eggs were sacrified for the first sampling. The rest of birds (55/ treatment) were raised for 2 weeks and then used for determining bone chemical composition and biomechanical properties. Quail from all maternal dietary treatments were fed an identical starter diet (Table 1) and raised under the same management conditions with continuous light. Feed and water were provided for *ad libitum* consumption. Individual body weight and feed consumption on a pen basis were recorded weekly.

Sample Collections

In study one, at one day old, 3 and 6 weeks of age, 10 quail from each treatment were killed for histological examination. The birds were weighed, and the tibiae from both legs removed, cleaned of adhering tissue, and the length measured with calipers. The tibiae were fixed in 10% phosphate-buffered formalin solution for two days and then, those from 3- and 6- week-old birds were decalcified with formic acid solution for histological analysis. In study two, 15 newly hatched quail were killed, and the tibiae were collected as described above. After the length and diameter of the diaphysis were measured, the right tibiae were kept on ice and frozen at -20^{o}C for collagen and its crosslink analysis, and the left tibiae were collected for ash measurement. At 1 and 2 weeks of age, 25 quail at each age in each maternal dietary treatment were sacrificed by cervical dislocation. Both tibial bones from each bird were excised, cleaned of surrounding soft tissue and weighed. The length of the right tibiae of each bird was measured and the diameter at the center of diaphysis was determined by measuring the widest and narrowest sites using calipers. Then right tibiae were sealed in plastic bags and immediately frozen (-20^{o}C) until the day of mechanical testing. Fifteen left tibiae in each maternal dietary treatment were collected for ash and mineral measurements, and the remaining 10/treatment were kept on ice and frozen at -20^{o}C for collagen and crosslink determination.

Histological Measurements

The bone samples were split longitudinally in halves and then cut into two parts at the center of diaphysis. Both the proximal and distal parts of tibiae were embedded in paraffin, and later cut into approximately 5 micron sections. Randomly selected sections were stained with hematoxylin and eosin. The specimens were identified so as to blind treatment group, examined under a microscope and photographed. Widths of the proliferative zone, hypertrophic zone, mineralized zone and cortical thickness were evaluated and measured as units of the visual field of microscopy. Cortical and trabecular density was scored in a range of 1-3 from low to high density.

Bone Mechanical Testing

On the day of testing, tibial bones were thawed at room temperature. All mechanical tests were performed on an Material Testing System (MTS System Co, Cary, NC). A shear test was used to determine the mechanical properties of the tibial bone using a double shear block apparatus (Wilson and Ruszler, 1997). Tests were conducted at room temperature (23 ^{o}C ± 2 ^{o}C) and specimens were kept moist during testing. Loaded at the midpoint of the shaft, the tibia were subjected to a shear test at a constant loading rate of 5 mm/min, using a 1000 N load cell, which was connected to a microcomputer via an amplifier. The ultimate shear force, shear fracture energy (area under the load-deflection curve up to peak load), maximal deflection before fracture and stiffness (tangent to the angle α) were read directly or calculated from the computer recordings (Fig. 1).

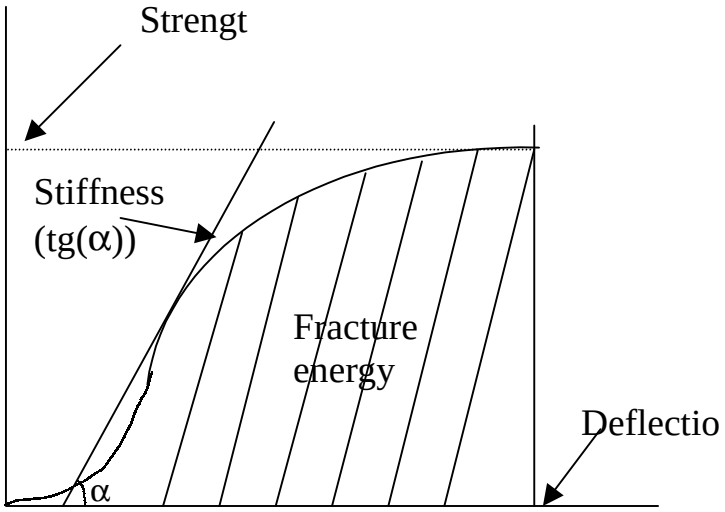

Fig. 1. Typical load-deflection relationships and the parameters of deflection at ultimate load, ultimate stiffness and fracture energy.

Collagen and Pyridinum Crosslink Determination

Tibial bones of newly hatched and one week old quail, following bone marrow removal, were demineralized in several changes of 0.5 M EDTA (pH 7.2) at 4^{o}C for one week and then washed with distilled water (Farquharson *et al*., 1989; Tsuchiya and Bates, 1997, 1998). The non-diffusible residues of decalcified bone samples were hydrolyzed in 5 ml hydrochloric acid (6 mol/L) by heating at 110^{o}C for 24 hr in screw capped glass tubes. The hydrolysates of bone samples were then evaporated to dryness at 37^{o}C under a stream of nitrogen to remove hydrochloric acid (Tsuchiya and Bates, 1998). They were then reconstituted in 5 ml of distilled water, adjusted to a neutral pH with 1 N sodium hydroxide and kept at –4^{o}C for measurement of hydroxyproline, pyridinoline and deoxypyridinoline.

The hydroxyproline concentration of the acid hydrolysates of bones, which is an indicator of collagen content, was measured by the procedure of Logan (1950) with modification (Monnier et al., 1986). Each sample was analyzed in duplicate for hydroxyproline concentration at 540 nm wawelength using a spectrophotometer (Bechman, Fullerton, CA). The values, automatically calculated by a connected computer, were used to calculate the molar proportions of pyridinium crosslinks in collagen. The collagen crosslinks (pyridinoline and deoxypyridinoline, separately) were measured in acid hydrolysates of decalcified right tibial bone by antibody-based ELISA kit assays (Metra Biosystems Co., CA). These are competitive enzyme immunoassays with high sensitivity for deoxypyridinoline and pyridinoline, respectively, which have been proved to be highly comparable with the results from HPLC (Delmas *et al*., 1993; Robins *et al*., 1994). Duplicate measurements were performed for each sample, the optical density at 405 nm was measured with a microplate reader (Biotek Instruments, Inc. VT), and the data were expressed as nmol/μmol of hydroxyproline.

Ash and Mineral Content

Tibia samples were oven-dried at 105^{o}C for 48 h, weighed, and ashed in a muffle furnace at 600^{o}C for 14 h in porcelain crucibles. Tibial ash was expressed as a percentage of dry weight, and mineral content was represented by ash weight (mg) per unit of tibial length.

Statistical Analysis

Data were analyzed by GLM procedure (one-way ANOVA) of the SAS® (SAS Institute Inc., 1998, Cary, NC) with individual birds as the experimental unit for all measurements, and significant differences of means between treatments were tested using studentized tukey test at the 5% probability level. Variation within treatment was expressed as the standard error of the treatment mean (SEM).

RESULTS

Body Weight and Bone Measurements

The maternal dietary treatments had no significant effect on body weight of progeny at any age throughout the duration of the feeding experiments both in study one and study two. Similarly, maternal dietary lipid supplementation had no influence on tibia bone length or diameter of progeny in either trial (Tables 4-10). However, the percentage of ash in the tibia bone of day old quail from hens fed FO and HSBO was significantly higher than those from hens fed the SBO diet ($P<0.05$) (Table 4). Day old quail from hens given HSBO had a significantly higher mineral content in the tibiae than those whose maternal diet was SBO ($P<0.05$). Quail from hens fed CF, although they were lower in tibial ash and mineral content level than those from hens fed the FO or HSBO diet, had no differences in these parameters with quail from other treatment groups. There were no significant differences among treatments in bone percent ash and bone mineral content in progeny quail at 7 days of age (Table 5), whereas the FO group continued to have the highest percent ash in tibia at 14 days of age, which was significantly higher compared to that in CF group ($P<0.05$) (Table 6). The comparison of ash contents and changes with ages were shown in Figure 2.

Collagen and Pyridinium Crosslinks

The effect of maternal dietary lipids on bone collagen as measured by hydroxyproline is shown in Tables 4-6. There was no significant effects of maternal dietary lipids on bone total hydroxyproline (hence total collagen) content per unit of bone weight in progeny. Supplementation of different lipids in the maternal diets also had no significant effects on bone pyridinoline crosslinks in quail at day old of age or one week of age. Notably, however, at day old, quail from hens fed the diet supplemented with either 5 % FO or 5 % HSBO had higher deoxypyridinoline and total pyridinium crosslinks in tibia compared to those from hens fed the same amount of either SBO or CF ($P<0.05$). At one week of age, the levels of deoxypyridinoline in tibia of FO and HSBO groups were significantly higher than those whose maternal diets was SBO, and the amount of deoxypyridinoline in FO group, but not in HSBO group, was also significantly higher than that from the CF group ($P<0.05$). There was no significant difference of total bone pyridinium crosslinks at one week of age.

Bone Mechanical Properties

The results of the tibial bone mechanical test, presented in Tables 5 and 6, and Figures 3 and 4, showed that maternal dietary lipids had a significant effect on the biomechancial properties of tibial bones in the progeny. At 7 days of age, birds whose maternal diets were supplemented with SBO or CF had significantly lower values in shear force (required to break tibia) than those from hens consuming FO or HSBO ($P<0.05$), while tibial fracture energy was not effected by maternal lipid difference at this stage. The tibial fracture deflection was the highest in quail from hens fed CF, but was only significantly greater than that in quail whose maternal diet was HSBO ($P<0.05$). In

addition, stiffness analysis revealed that the tibia in HSBO group was significant higher in this value than that in the CF group ($P<0.05$), but was comparable to those from the FO and SBO groups. At 14 days of age, higher tibial shear force was still found in birds from the FO and HSBO maternal diets, but significant differences were observed only between the FO and CF groups ($P<0.05$). There were no significant differences in fracture energy or fracture deflection of tibiae between treatments at this age, whereas birds from FO and HSBO maternal diets still showed markedly higher values in tibial stiffness compared to that of CF group, but no significant difference with the value from SBO group.

Anatomic and Histologic Parameteres

There were no differences in the length of the tibiae subjected to histological analysis in day old, 3 and 6 weeks old progeny quail between maternal dietary lipid treatments (Tables 7-10). Day old quail from the FO and HSBO maternal diets had a significantly greater cartilaginous proliferative zone compared to those from hens fed the CF diet, and also were statistically larger in the hypertrophic zone width compared to those from hens fed SBO or CF in the proximal end of tibia ($P<0.05$) (Table 7). In addition, diaphyseal cortical thickness was increased in quail from hens given FO and HSBO compared to those from SBO and CF groups ($P<0.05$). Furthermore, cortical thickness in both the proximal and distal sides of the tibiae in quail from the FO and HSBO groups tended to be larger than those from the SBO and CF diets. In the distal end of the tibiae, newly hatched quail from the FO maternal diet also had the widest hypertrophic zone. However, there were no statistical differences in other parameters including the width of cartilaginous mineralized zone, cortical density and bone width in all examined areas, and cortical thickness in the ends of proximal and distal tibiae between maternal diets at this age.

At 21 days of age, progeny from hens fed FO and HSBO had a significantly higher cortical thickness in the proximal end of the tibiae compared to those from hens given the SBO and CF diets ($P<0.05$). Quail from the FO group also showed higher trabecular density in the proximal end and greater cortical density in the diaphysis than those from the SBO and CF groups ($P<0.05$). Additionally, the tibiae in the distal ends from the FO group were the lowest in the hypertrophic zone width than those from other groups at this developmental stage ($P<0.05$). The effect of maternal lipids on the cartilaginous proliferative zone width, as observed in newly hatched quail, disappeared at this time, and neither bone width nor cortical density was measurably effected by maternal dietary lipids. At 42 days of age, there were no detectable differences in cortical density, cortical thickness and bone width in either males or females between maternal dietary treatments (Table 9, 10).

DISCUSSION

The previous study showed dramatic effects of maternal dietary lipids, varying in the amounts of n-3, n-6, saturated and trans fatty acids, on bone fatty acid composition and bone *ex vivo* prostaglandin E_2 (PGE_2) production in progeny of quail (Chapter one). This study was performed in order to investigate whether maternal dietary lipids

influence body and bone growth, bone histology and physical strength in embryos and early postnatal stage, in response to the changes in fatty acid composition and local PGE_2 biosynthesis manipulated by maternal dietary lipid supplementation. The results demonstrated that the body weights, tibial lengths, weight and diameters in progeny of all experimental groups were similar, suggesting that maternal dietary lipids had no significant effect on early growth of body or bones in birds. However, the percentage of tibial ash was significantly higher in newly hatched quail from hens fed the FO and HSBO diets and FO group continued to be higher in this parameter than that in the CF group at 14 days of age. This result may suggest that supplementation of FO or HSBO in the maternal diet improves mineral deposition and leads to better mineralization of bone in the early development compared to the SBO and CF diets.

Although it has been reported that the type of fat in the diet influences the mineral metabolism in chicks (Attech *et al*., 1983; Attech and Leeson, 1983, 1984), there were no published data describing a relationship between lipid supplementation in the diet and bone mineralization until recently. Attech *et al*. (1989) indicated that SBO in the diet significantly decreased bone ash of broiler chicks. More recently, Watkins et al. (1996a, 1997a) reported that n-6 enriched diet such as SBO retarded dynamic bone formation rate as shown by histomorphometric measurements in growing chicks. Our observations were in accordance with these findings. As far as we aware, there are no reported studies characterizing the relationships between different maternal dietary lipid supplementation and bone growth and development of the subsequent progeny.

Tibial bone matrix analysis revealed that maternal lipid intakes did not alter tibial hydroxyproline concentration (used as a measure of collagen content) in subsequent embryos and progeny. However, significantly less amounts of pyridinium crosslinks in the tibiae of quail from hens fed either the SBO or CF diet were formed compared to those from hens given the FO or HSBO diet at the early development of the bone. Approximately 90% of the organic matrix of bone is type I collagen (Seyedin and Rosen, 1990; Bailey *et al*., 1993). Collagen dominated organic matrix provides bone with its tensile strength and structural scaffolds for the deposition of minerals (Lawson and Czernuszka, 1998; Rath *et al*., 1999). Quantitatively, a decrease in bone collagen content results in a decrease in mechanical strength both in humans (Bailey *et al*., 1999) and animals (Masse *et al*., 1996). Mature type I collagen in bone is cross-linked by pyridinoline and deoxypyridinoline, specific molecules which have a strong and positive correlation with bone mechanical strength in both normal and osteoporotic bones (Batge *et al*., 1992; Oxlund *et al*., 1995, 1996; Knott *et al*., 1995; Rath *et al*., 1999). Therefore, not only quantity, but more importantly, a change in the collagen quality, particularly in the crosslinks also could lead to loss of mechanical strength (Bailey *et al*., 1993).

As mineral content and organic matrix properties are positively correlated with bone strength (Crenshaw *et al*., 1986; Currey, 1988; Martin and Boardman, 1993), it may be expected that maternal dietary FO and HSBO would also improve the strength of the tibiae, which was demonstrated in this study. Although there was no significant difference in fracture energy between treatments which represents bone brittleness (Wilson and Ruszler, 1998; Rath *et al*., 1999), supplementing FO or HSBO in the maternal diet markedly improved tibia breaking strength of the progeny. The much higher fracture deflection and relatively lower fracture energy in bones from the CF group suggest that the tibia in this group were the weakest and softest. Additionally, the

stiffness of bones was increased in quail from hens consuming the FO or HSBO diet in the early developmental stage.

Bone strength is related to its structural properties and material properties (Grynpas *et al*., 1992; Aerssens *et al*.; Zernicke *et al*., 1995;Wilson and Ruszler, 1998; Bailey *et al*., 1999; Wohl *et al*., 1998; Rath *et al*., 1999; Yoshitake *et al*., 1999). The structural properties of a bone depend in part on its geometry (Zernicke *et al*., 1995). This study indicated that birds in all treatments had similar bone geometry, suggesting that the modification of mechanical properties by maternal dietary lipids be mainly related to changes in material properties of the bones in progeny. It may be concluded from these results that bones from the FO and HSBO groups were mineralized better and stronger than those in the SBO and CF groups during the early two weeks of growth and development.

The present study further demonstrated that collagen crosslinks play an important role in bone strength, and impairment of crosslink formation lead to the reduction of mechanical strength even if collagen level is constant. To our knowledge, no study has been published that shows the effects of maternal dietary lipid intakes on the concentration of the collagen crosslinks, pyridinoline and deoxypyridinoline in bone.

The histological features of the tibiae from newly hatched and 21 day-old quail were distinctive. Passing from the physeal to metaphyseal areas several zones are apparently identified: 1) the basal zone, the thin layer of progenitor cartilage cells; 2) the proliferative zone, the zone containing proliferating, flattened chondrocytes arranged in rows or columns parallel to the long axis of the bone; 3) the hypertrophic zone, in which chondrocytes enlarge and produce much basophilic extracellular matrix; 4) the mineralized zone or degenerative zone, consisting mainly of mineralizing extracellular matrix and invading capillaries from the metaphyseal tissue. The observations from the histological study demonstrated that proliferative and hypertrophic zones were narrowed in the proximal end, and cortical bone was thinner at the diaphyseal area in newly hatched quail from hens fed the SBO or CF diets.

Longitudinal bone growth occurs as a consequence of chondrocyte proliferation and hypertrophy to form cartilage within the physis (Pines and Hurwitz, 1990). The generated cartilage extracellular matrix is then mineralized, degraded and replaced by osseous tissue. Therefore, the maternal SBO or CF diet influenced the proliferation and hypertrophy of chondrocytes and their subsequent extracellular matrix within the physis, which consequently modifies the conversion of cartilage to mineralized bone in the tibiae of embryos, as confirmed by ash measurements.

During the postnatal growth and development, degenerative and hypertrophic zones in tibia become narrowed gradually and disappear eventually because rapid mineralization takes place. At 21 days of age, the degenerative zone disappeared by ossification in the tibiae of all groups and the hypertrophic zone was relatively much shorter compared to that in newly hatched quail. The hypertrophic zone diminished slower in quail of the SBO and CF groups from day old to 21 days of age when compared to the FO and HSBO groups, at these ages. In addition, the cortical thickness, density and trabecular density were notably decreased in these groups. These findings, which agree with those in phosphorous deficiency animals (Long *et al*., 1984; Edwards, 1988; Qian *et al*., 1996), support and further explain the other results in this study in which

maternal dietary supplementation of SBO or CF influences the bone matrix chemistry and decreases the bone strength in the early growth and development of quail progeny.

The mechanisms by which dietary fat impacts on cartilage and bone metabolism and function are not completely clear. However, it is well established that dietary lipids can modulate the fatty acid profile in cartilage and bone tissues (Alam *et al*., 1993; Shen *et al*., 1994; Xu *et al*., 1994; Watkins *et al*., 1996a, 1997a). Specifically, the concentrations of n-3 and n-6 fatty acids in bone could be dramatically increased by their corresponding supplementation in the diet. Recent studies showed that some fatty acids and lipids have a direct and important role in facilitating the mineralization of bone tissues (Wuthier, 1993;Takahashi, 1994; Urist *et al*., 1997). In addition, polyunsaturated fatty acids (PUFAs) serve as substrate for prostaglandin (PG) biosynthesis. PGE_2 which is synthesized in osteogenic cells by the cyclooxygenase pathway from arachidonic acid [AA, 20:4(n-6)], acts as a potent bone resorbing agent in high concentration (Watkins *et al*., 1997b). The research of others show that dietary saturated fatty acids or FO depressed the PGE_2 production to increase bone formation rate compared to dietary SBO (Shen *et al*. 1994; Watkins *et al*., 1996a, 1997a).

In addition, enrichment of cartilage with linoleate (18:2(n-6)) may be detrimental to normal chondrocyte function (Watkins, *et al*., 1996b). Furthermore, PGE_2 at 100 nM notably suppressed the lysyl oxidase synthesis (Roy *et al*., 1996), an enzyme that catalyzes deamination of lysine and hydroxylysine to form aldehydes that eventually form mature pyridinium crosslinks (Seibel, *et al*., 1992; Oxlund *et al*., 1995; Knott and Bailey, 1998). In a previous study (Chapter one), it was showed that maternal dietary SBO and CF dramatically increased n-6 fatty acid concentration and *ex vivo* PGE_2 production in tibia of newly hatched quail. Thus, it is speculated that the adverse effects of supplementation of SBO or CF in the maternal diet on bone development of progeny may be at least partially attributable to a relatively higher local production of PGE_2. The cartilage and bone in the embryos may be sensitive to excessive amounts of PGE_2.

In summary, the data from this study showed that supplementing the maternal diet with either SBO or CF decreased the bone percent ash in progeny, which was accompanied by decreases in bone strength and collagen crosslinks. Consistent with these findings, we further observed that maternal dietary SBO and CF narrowed physeal hypertrophic zone width in embryonic tibia and variably decreased the cortical density and thickness in various areas in embryos and early postnatal quail. Coupled with the findings of the previous study (Chapter one), our current results suggest that maternal dietary lipids affect bone modeling of progeny perhaps by modulating the production of local regulatory factor such as PGE_2.

Table 1. Dietary ingredients of quail starter diet[1]

Ingredients	(%)
Yellow corn	42.2
Soybean meal	38.0
Stabilized fat	3.5
Fish meal	7.0
Meat+bone scrap	5.0
Dehydrated alfalfa meal	1.25
Deflurorinated phosphate	1.0
Limestone	0.5
Vitamin premix[3]	1.0
Trace mineral premix[4]	0.05
Salt	0.5

[1] All quail were fed the same diet through whole experimental period.

[2] The diet was formulated to contain 29.6% crude protein, 2970 kcal/kg metabolizable energy, 5.5% crude fat, 2.70% crude fiber, 1.40% calcium, and 0.70% phosphorous.

[3] Provided per kilogram of diet: vitamin A, 880 IU; vitamin D_3, 330 IU; vitamin E, 5.5 IU; menadione sodium bisulfite, 3.52 mg; thiamine HCL, 1.1 mg; riboflavin, 4.4 mg; calcium D-pantothenate, 8.8 mg; niacin, 44 mg; choline chloride, 374 mg; vitamin B_{12}, 0.011 mg; folic acid, 1.1mg; pyridoxine HCL, 1.1 mg; biotin, 0.11 mg; DL-methionine, 990 mg; Bacitracin,5.5 mg; selenium, 0.20 mg; ethoxyquin, 0.124 mg.

[4] Provided per kilogram of diet: manganese, 22 mg; zinc, 23.8 mg; iron, 25.0 mg; copper, 3.13 mg; iodine, 1.0 mg; and selenium, 0.15 mg.

Table 2. Dietary ingredient composition of the basal diets given to laying hens[1]

	Dietary lipid treatment[2]			
Ingredients (%)	SBO	HSBO	CF	FO
Yellow corn	53.55	53.55	53.55	53.55
Soybean meal	32.00	32.00	32.00	32.00
Soybean oil	5.00	—	—	—
Hydrogenated soybean oil	—	5.00	—	—
Chicken fat	—	—	5.00	—
Menhaden oil	—	—	—	5.00
Limestone	6.50	6.50	6.50	6.50
Dicalcium phosphate	1.50	1.50	1.50	1.50
Vitamin Premix[3]	1.00	1.00	1.00	1.00
Trace mineral premix[4]	0.10	0.10	0.10	0.10
salt	0.35	0.35	0.35	0.35

[1] The diet was formulated to contain 21.0% crude protein, 2997.2-3007.2 kcal/kg metabolizable energy, 7.36% crude fat, 2.43% crude fiber, 2.81% calcium, and 0.67% phosphorous.

[2] Diets include soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) or menhaden fish oil (FO) at 50g/kg of diet.

[3] Provided per kilogram of diet: vitamin A, 1100 IU; vitamin D_3, 330 IU; vitamin E, 33 IU; menadione sodium bisulfite, 3.52mg; thiamine HCL, 1.1mg; riboflavin, 6.6mg; calcium D-pantothenate, 16.5mg; niacin, 44mg; choline chloride, 374mg; vitamin B_{12}, 0.0165mg; folic acid, 1.1mg; pyridoxine HCL, 1.1mg; biotin, 0.055mg; Bacitracin, 22.0 mg; selenium, 0.20mg; ethoxyquin, 0.124mg.

Table 3. Fatty acid composition (μg/100 μg) of maternal basal diet

	Dietary lipid treatment[1]			
Fatty acids	SBO	HSBO	CF	FO
14:0	0.06	0.19	0.57	5.29
15:0	0.02	0.04	0.09	0.42
16:0	9.95	11.26	15.01	15.45
16:1(n-7)	0.08	0.15	2.30	6.95
18:0	3.54	11.11	6.53	3.26
t18:1	0.05	25.49	2.57	1.45
18:1	25.82	30.76	39.50	15.07
18:2(n-6)	54.79	18.11	30.00	19.16
18:3(n-3)	4.33	0.98	1.54	2.32
20:0	0.30	0.34	0.23	0.20
20:3(n-3)	ND[5]	ND	0.05	0.11
20:4(n-6)	0.01	0.01	0.16	0.51
20:5(n-3)	0.01	ND	0.02	18.49
22:1	ND	ND	0.03	0.24
22:4(n-6)	0.01	0.01	0.03	0.09
22:5(n-3)	0.02	0.03	0.02	1.47
22:6(n-3)	ND	ND	0.01	7.47
SAT[2]	13.87	22.94	22.42	24.62
MONU[3]	25.95	56.42	44.43	23.91
PUFA[4]	59.18	19.15	31.82	49.61
(n-6) PUFA	54.81	18.13	30.19	19.75
(n-3)PUFA	4.37	1.02	1.63	29.86
(n-6)/(n-3)	12.55	17.85	18.47	0.66

[1] Maternal dietary treatments include soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat, and menhaden fish oil (FO) at 50 g/kg of diet.

[2] SAT, total saturated fatty acids.

[3] MONU, total monounsaturated fatty acids.

[4] PUFA, total polyunsaturated fatty acids.

[5] ND, not detected.

Table 4. Body weight, collagen content, crosslinks and other measurements of tibiae in day old quail from hens fed different lipids[1]

Measurements	Maternal dietary lipid treatment[2]				Pooled	
	SBO	HSBO	CF	FO	SEM	P values
Body weight (g)	5.52	5.45	5.71	5.53	0.13	0.5022
Tibial length (mm)	17.21	17.14	17.40	17.08	0.14	0.4362
Tibial Diameter(mm)	0.63	0.60	0.61	0.64	0.02	0.1649
Dry weight of tibia(mg)	10.86	11.56	11.74	11.14	0.30	0.1793
Tibia ash (%)	25.57^{b}	27.63^{a}	26.54^{ab}	27.87^{a}	0.32	0.0001
Mineral Content(mg/mm)	0.16^{b}	0.19^{a}	0.18^{ab}	0.17^{ab}	0.01	0.0014
Hydroxyproline (g/mg, bone$^{\eta}$)	15.6	15.5	15.5	16.1	0.5	0.8004
Collagen Crosslinks:						
Pyridinoline (nmol/μmol HP)	0.212	0.308	0.220	0.314	0.050	0.1798
Deoxypyridinoline (nmol/μmol HP$^{\xi}$)	0.266^{b}	0.450^{a}	0.217^{b}	0.371^{a}	0.033	0.0002
Total Crosslinks (nmol/μmol)	0.469^{b}	0.708^{a}	0.434^{b}	0.673^{a}	0.058	0.0069

[1] n=25 for body weight, tibial length and diameter, n=15 for ash measurement, all other measurement n=10, mean values within rows having different superscripts are significantly different (P<0.05).

[2] Maternal dietary treatments included soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF), and menhaden fish oil (FO) at 5 % of the diet. All progeny for the experiment were fed the same diet until the end of this study.

$^{\xi}$. Denotes hydroxyproline.

$^{\eta}$. Dry weight of bone.

Table 5. Body weight, mechanical properties, collagen content, crosslinks and other measurements of tibiae in one week old quail from hens fed different lipids[1]

Measurements	Maternal dietary lipid treatment[2]				Pooled	
	SBO	HSBO	CF	FO	SEM	P values
Body weight (g)	15.2	16.0	14.6	14.6	0.5	0.0889
Tibial length (mm)	25.1	25.4	25.0	24.5	0.3	0.1453
Tibial diameter (mm)	1.13	1.12	1.10	1.10	0.03	0.7709
Dry weight of tibia (mg)	37.2	40.1	38.0	35.6	1.8	0.3329
Tibial ash (%)	36.5	37.1	35.1	36.5	1.3	0.6819
Mineral content (mg/mm)	0.54	0.58	0.54	0.54	0.04	0.7916
Hydroxyproline (μg/mg)	24.4	25.9	25.4	25.6	0.7	0.5548
Tibial force (Newton)	19.8^{b}	25.0^{a}	20.4^{b}	23.7^{a}	0.8	0.0001
Fracture deflection (mm)	0.72^{ab}	0.70^{b}	0.86^{a}	0.72^{ab}	0.04	0.0213
Tibial energy (N-mm)	12.5	12.3	14.5	12.6	0.8	0.2426
Stiffness (N/mm)	30.4^{ab}	37.8^{a}	25.2^{b}	33.6^{ab}	3.0	0.0097
Collagen crosslinks:						
Pyridinoline (nmol/μmol HP^{ξ})	0.216	0.180	0.187	0.174	0.045	0.2685
Deoxypyridinoline (nmol/μmol HP)	0.114^{c}	0.171^{ab}	0.147^{bc}	0.217^{a}	0.051	0.0040
Total Crosslinks (nmol/μmol)	0.330	0.350	0.335	0.391	0.074	0.3698

[1] n=25 for body weight, bone mechanical properties, n=15 for ash measurement, all other measurement n=10, mean values within rows having different superscripts are significantly different ($P<0.05$).

[2] Maternal dietary treatments included soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF), and menhaden fish oil (FO) provided at 5 % of the diet. All progeny for the experiment were fed the same diet until the end of this study.

$^{\xi}$ Denotes hydroxyproline.

Table 6. Body weight, mechanical properties, collagen content and other measurements of tibiae in two weeks old quail from hens fed different lipids[1]

Measurements	Maternal dietary lipid treatment[2]				Pooled	
	SBO	HSBO	CF	FO	SEM	P values
Body weight (g)	30.5	29.6	28.2	30.1	1.1	0.5085
Tibial length (mm)	30.9	30.9	31.0	31.4	0.5	0.9061
Tibial diameter (mm)	1.44	1.50	1.34	1.44	0.03	0.0928
Dry weight of tibia (mg)	92.7	85.4	81.4	89.7	5.8	0.5441
Tibial ash (%)	39.9^{ab}	38.5^{ab}	36.3^{b}	41.2^{a}	1.0	0.0089
Mineral content (mg/mm)	1.20	1.05	0.97	1.17	0.07	0.1033
Hydroxyproline (μg/mg bone)	26.4	27.3	24.2	25.6	0.8	0.1088
Tibial force (Newton)	28.5^{ab}	32.1^{ab}	27.4^{b}	32.8^{a}	1.6	0.0398
Fracture deflection (mm)	0.36	0.39	0.45	0.43	0.03	0.1971
Tibial energy (N-mm)	7.5	8.3	8.5	9.2	0.8	0.5319
Stiffness (N/mm)	82.4^{ab}	91.0^{a}	66.5^{b}	90.3^{a}	6.1	0.0250

[1] n=20 for body weight, bone mechanical properties, n=15 for ash measurement, all other measurement n=11, mean values within rows having different superscripts are significantly different ($P<0.05$).

[2] Maternal dietary treatments included soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF), and menhaden fish oil (FO) provided at 5 % of the diet. All progenies for experiment were fed the same diet until the end of this study.

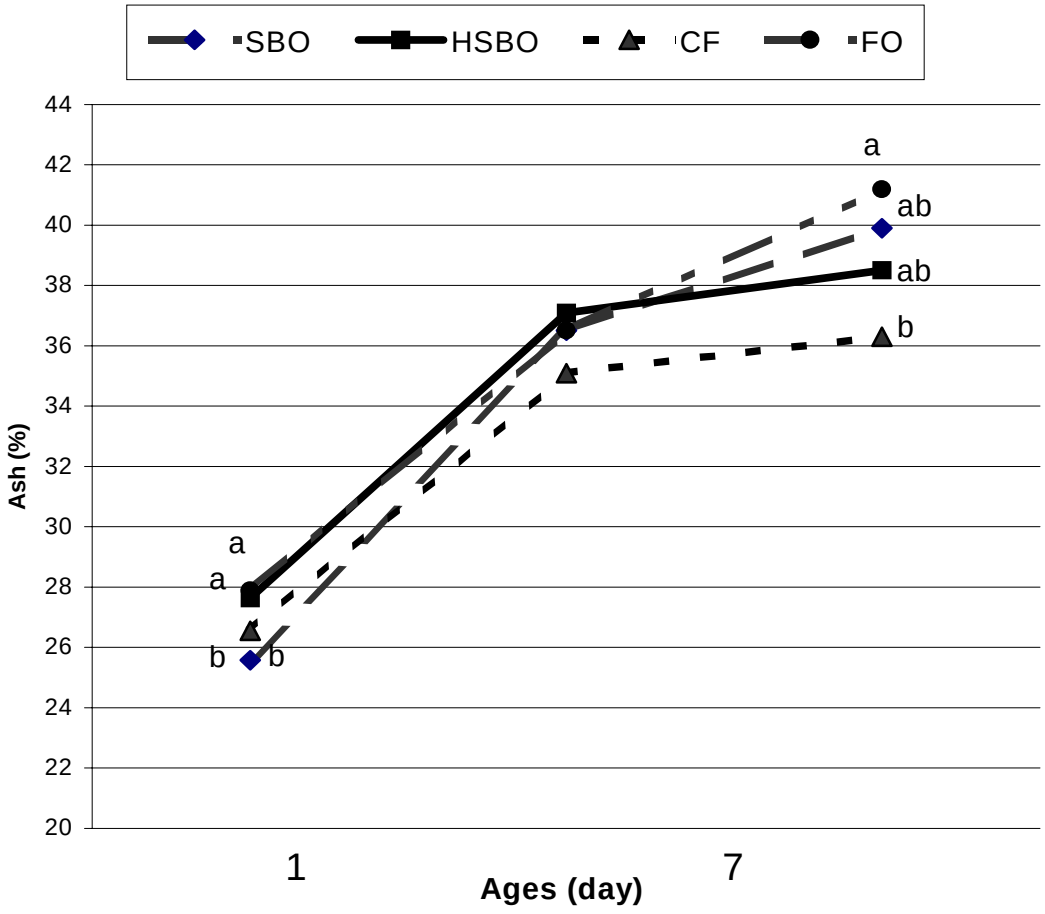

Fig. 2. Effect of maternal dietary lipids on percent bone ash in progeny from quail fed maternal diets which included either soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) or menhaden fish oil (FO) at 50g/kg of the diet. Different letters on the graph indicate significant differences at that age.

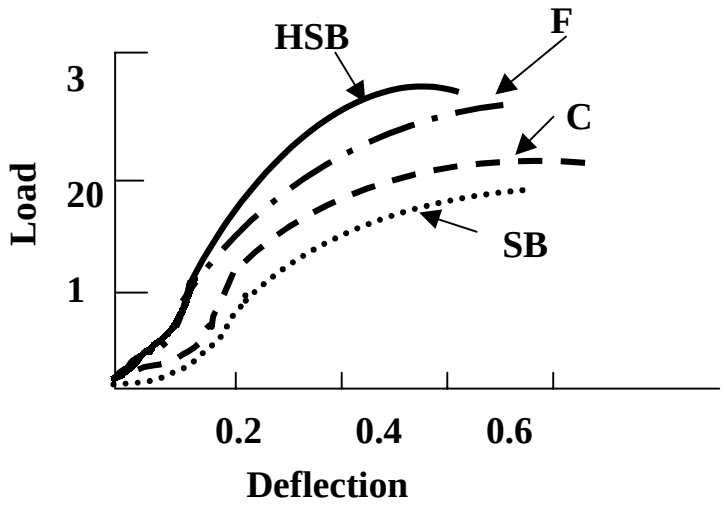

Fig. 3. Comparison of load-deformation curves of tibial bones in one-week-old quail from hens fed diets containing either soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) or menhaden fish oil (FO) at 50g/kg of the diet. The tibiae from the HSBO and FO groups had a higher maximum load compared to the SBO and CF groups, whereas tibiae from the CF group were greater in fracture deflection compared to the HSBO group.

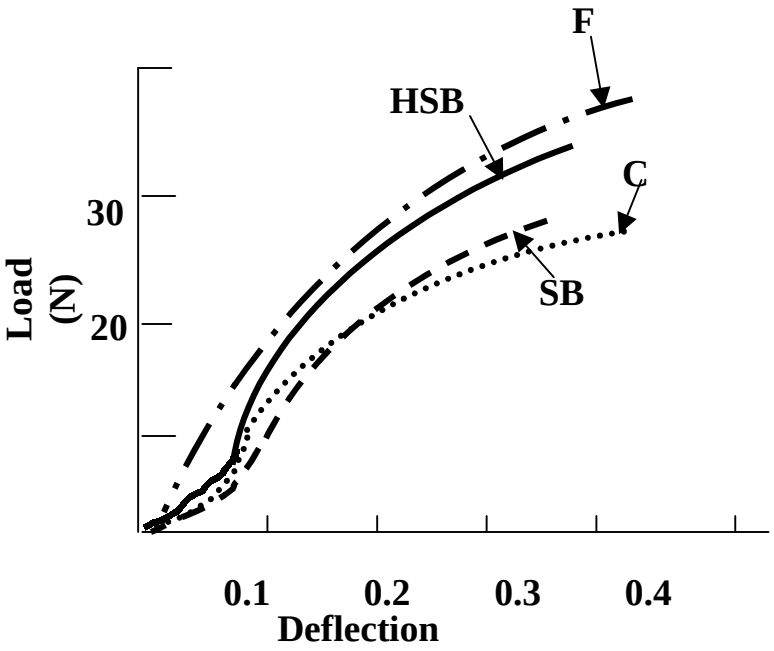

Fig. 4. Comparison of load-deformation curves of tibial bones in two-week-old quail from hens fed diets containing either soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) or menhaden fish oil (FO) at 50g/kg of the diet. The tibiae from the FO group were higher in maximum load compared to the CF group. There were no differences in fracture energy or deflection between four groups.

Table 7. Body weight, tibia length and histological measurements in the tibiae of day old quail from hens fed different lipids[1]

	Maternal Dietary lipid treatment[2]				Pooled	
Measurements	SBO	HSBO	CF	FO	SEM	P values
Body weight (g)	5.9	5.8	6.0	5.7	0.1	0.1886
Tibia length (mm)	17.5	17.7	18.0	17.8	0.2	0.5793
<u>Proximal tibia</u>						
Proliferative Zone (20×)	1.6^{ab}	1.7^{a}	1.5	1.7^{a}	0.03	0.0006
Hypertrophic zone (20×)	4.8^{b}	5.6^{a}	4.5^{b}	5.4^{a}	0.06	0.0002
Mineralized zone (20×)	11.8	12.2	13.5	12.2	0.22	0.1188
Cortical thickness (20×)	0.91	1.00	0.90	1.01	0.02	0.1597
Cortical density (20×)	2.2	2.2	2.2	2.3	0.03	0.8610
Bone width (20×)	9.2	9.7	8.9	9.0	0.3	0.1975
<u>Diaphysis</u>						
Cortical thickness (20×)	1.3^{b}	1.6^{a}	1.2^{b}	1.6^{a}	0.1	0.0006
Cortical density (20×)	2.3	2.1	2.2	2.2	0.06	0.5598
<u>Distal tibia</u>						
Proliferative zone (20×)	1.6	1.5	1.5	1.5	0.03	0.2104
Hypertrophic zone (20×)	3.9^{b}	4.0^{b}	4.3^{ab}	4.6^{a}	0.04	0.0002
Mineralized zone (20×)	10.7	11.2	11.9	10.6	0.53	0.4469
Cortical thickness (20×)	0.84	1.01	0.88	0.93	0.04	0.1751
Cortical density (20×)	1.9	1.7	1.8	1.9	0.06	0.1812
Bone width (20×)	7.9	8.6	7.4	7.9	0.3	0.1516

[1] n=16 for body weight, bone length and all histological measurements, mean values within rows having different superscripts are significantly different ($P<0.05$).

[2] Maternal dietary treatments included soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF), and menhaden oil at 5% of the diet. All progeny for the experiment were fed the same diet until the end of this study.

Table 8. Body weight, tibia length and histological meassurements in the tibiae of three weeks old quail from hens fed different lipids[1]

	Maternal Dietary lipid treatment[2]				Pooled	
Measurements	SBO	HSBO	CF	FO	SEM	P values
Body weight (g)	61.0	60.4	58.9	61.6	1.6	0.4531
Tibia length (mm)	37.1	37.1	37.3	37.2	0.3	0.8234
<u>Proximal tibia</u>						
Proliferative zone (10×)	1.0	1.0	1.0	1.0	0.03	0.8693
Hypertrophic zone (10×)	4.9	4.6	4.9	4.4	0.3	0.0857
Cortical thickness (10×)	1.4[b]	1.9[a]	1.5[b]	1.8[a]	0.1	0.0001
Cortical density (10×)	2.5	2.3	2.6	2.4	0.1	0.1203
Trabecular density (10×)	1.9[b]	2.2[ab]	2.1[b]	2.5[a]	0.1	0.0038
Bone width (10×)	7.6	7.4	7.6	7.5	0.03	0.5643
<u>Diaphysis</u>						
Cortical thickness (10×)	1.7	1.6	1.8	1.8	0.1	0.1524
Cortical density (10×)	2.9[b]	3.1[ab]	2.9[b]	3.6[a]	0.1	0.0041
Bone width (10×)	4.9	4.8	4.8	4.9	0.1	0.7740
<u>Distal tibia</u>						
Proliferative zone (10×)	0.5	0.5	0.5	0.5	0.03	0.9746
Hypertrophic zone (10×)	1.5[a]	1.4[ab]	1.6[a]	1.1[b]	0.1	0.0002
Cortical thickness (10×)	1.2	1.2	1.5	1.3	0.1	0.0800
Cortical density (10×)	2.3	2.4	2.6	2.6	0.1	0.4050
Trabecular density (10×)	2.6	2.5	2.8	2.9	0.1	0.0662
Bone width (10×)	4.4	4.5	4.6	4.5	0.2	0.6325

[1] n=20 for body weight and bone length, all histological measurements n=16, mean values within rows having different superscripts are significantly different (P<0.05).

[2] Maternal dietary treatments included soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF), and menhaden oil at 5% of the diet. All progeny for the experiment were fed the same diet until the end of this study.

Table 9. Body weight, tibia length and histological measurements in the tibiae of six weeks old quail (male) from hens fed different lipids[1]

	Maternal Dietary lipid treatment[2]				Pooled	
Measurements	SBO	HSBO	CF	FO	SEM	P values
Body weight (g)	110.0	112.4	113.6	109.9	1.6	0.3391
Tibia length (mm)	44.0	43.8	44.6	44.0	0.4	0.1387
Proximal tibia						
Cortical thickness (10×)	1.6	1.7	1.6	1.7	0.1	0.3825
Cortical density (10×)	2.8	2.9	2.7	2.9	0.2	0.8354
Bone width (10×)	7.6	7.6	7.7	7.5	0.3	0.1928
Distal tibia						
Cortical width (10×)	1.2	1.2	1.5	1.3	0.1	0.8250
Cortical density (10×)	3.9	4.0	4.0	3.9	0.2	0.9321
Bone width (10×)	4.5	4.4	4.4	4.3	0.2	0.7441

[1] n=10 for body weight, bone length, and n=8 for all histological measurements, mean values within rows having different superscripts are significantly different (P<0.05).

[2] Maternal dietary treatments included soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF), and menhaden oil at 5% of the diet. All progeny for the experiment were fed the same diet until the end of this study.

Table 10. Body weight, tibia length and histological measurements in the tibiae of six weeks old quail (female) from hens fed different lipids[1]

	Maternal Dietary lipid treatment[2]				Pooled	
Measurements	SBO	HSBO	CF	FO	SEM	P values
Body weight (g)	134.9	142.1	135.8	132.1	4.3	0.4631
Tibia length (mm)	46.1	46.5	46.3	46.3	0.4	0.7030
			Proximal tibia			
Cortical thickness (10×)	1.7	1.6	1.6	1.5	0.1	0.3634
Bone width (10×)	8.9	8.8	8.8	8.1	0.3	0.2093
			Distal tibia			
Cortical thickness (10×)	1.1	1.2	1.2	1.1	0.1	0.8322
Bone width (10×)	5.0	5.3	4.9	4.9	0.2	0.2596

[1] n=10 for body weight and bone length, and n=8 for all histological measurements, mean values within rows having different superscripts are significantly different ($P<0.05$).

[2] Maternal dietary treatments included soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF), and menhaden oil at 5% of the diet. All progeny for the experiment were fed the same diet until the end of this study.

REFERENCES

Aerssens, J., R. V. Audekercke, P. Geusens, L. P. C. Schot, A. A-H. Osman, 1993. Mechanical properties, bone mineral content, and bone composition(collagen, osteocalcin, IGF-I) of the rat femur: influence of ovariectomy and namdrolone decanoate (anabolic steroid) treatment. Calcif. Tissue Int. 53:269-277.

Alam, S. Q., P. P. Petros, and B. S. Alam, 1993. Fatty acid composition and arachidonic acid concentrations in alveolar bone of rats fed diets with different lipids. Calcif. Tissue Int. 53:330-332.

Atteh, J. O., and S. Leeson, 1983. Effects of dietary fatty acids and calcium levels on performance and mineral metabolism of broiler chicks. Poultry Sci. 62:2412-2419.

Atteh, J. O., and S. Leeson, 1984. Effects of dietary saturated and unsaturated fatty acids and calcium levels on performance and mineral metabolism of broiler chicks. Poultry Sci. 63:2252-2260.

Atteh, J. O., S. Leeson, and R. J. Julian, 1983. Effects of dietary levels and types of fat on performance and mineral metabolism of broiler chicks. Poultry Sci. 62:2403-2411.

Attech, J. O., S. Lesson, and J. D. Summers, 1989. Effects of dietary sources and levels of fat on performance, nutrient retention and bone mineralization of broiler chicks fed two levels of calcium. Can. J. Anim. Sci. 69:459-467.

Bailey, A. J., T. J. Sims, E. N. Ebbesen, J. P. Mansell, J. S. Thomsen, and L. Mosekilde, 1999. Age-related changes in the biochemical properties of human cancellous bone collagen: relationship to bone strength. Calcif. Tissue Int. 65:203-210.

Bailey, A. J., S .F. Wotton, T. J. Sims and P.W. Thompson, 1993. Biochemical changes in the collagen of human osteoporotic bone matrix. Connective Tissue Res. 29:119-132.

Batge, B., J. Diebold, H. Stein, M. Bodo, and P. K. Muller, 1992. Compositional analysis of the collagenous bone matrix. A study on adult normal and osteopenic bone tissue. Eur. J. Clin. Invest. 22:805-812.

Claassen, N., H. C. Potgieter, M. Seppa, W. J. Vermaak, H. Coetzer, D. H. van Papendorp, and M. C. Kruger, 1995. Supplementated gamma-linoleic acid and eicosapentaenoic acid influence bone status in young male rats: effects on free urinaru collagen crosslinks, total urinary hydroxyproline and bone calcium content. Bone 16:385S-392S.

Crenshaw, T. D., 1986. Reliability of dietary calcium and phosphorous levels and bone mineral content as predictors of bone mechanical properties at various time of periods in growing swine. J. Nutr. 116:2155-2170.

Currey, J. D., 1988. The effect of porosity and mineral content on the Young's modulus of elasticity of compact bone. J. Biomech. 21:131-139.

Delmas, P. D., E. Gineyts, A. Bertholin, P. Garnero, and F. Marchand, 1993. Immunoassay of pyridinoline crosslink excretion in normal adults and in paget's disease. J. Bone Miner. Res. 8:643-648.

Edwards, H. M., 1988. Effects of dietary calcium, phosphorous, chloride on the development of tibial dyschondroplasia. Poultry Sci. 67:1436-1446.

Farquharson, C., A. Duncan, and S. Robins, 1989. The effects of copper deficiency on the pyridinium crosslinks of mature collagen in the rat skeleton and cardiovascular system. P. S. E. B.M. 192:166-171.

Grynpas, M. D., A. Acito, M. Dimitriu, B. P. Mertz, and J. M. Very, 1992. Changes in bone mineralization, architeture and mechanical properties due to long-term (1 year) administration of pamidronate (APD) to adult dogs. Osteoporosis Int. 2:74-81.

Knott, L., and A. J. Bailey, 1998. Collagen cross-links in mineralizing tissues: a review of their chemistry, function, and clinical relevance. Bone 22:181-187.

Knott, L., C. C. Whitehead, R. H. Fleming, and Aaa. J. Bailey, 1995. Biochemical changes in the collagenous matrix of osteoporotic avian bone. Biochem. J. 310:1045-1051.

Kokkinos, P. P., R. Shaye, B. S. Alam, and S. Alam, 1993. Dietary Lipids, prostaglandin E_2 levels, and tooth movement in alveolar bone rats. Calcif. Tissue Int. 53:333-337.

Kruger, M. C., N. Claassen, C. M. Smuts, and H. C. Potgieter, 1997. Correlation between essential fatty acids and parameters of bone formation and degradation. Asia Pacific J. Clin. Nutr. 6:235-238.

Lawson, A. C., and T. J. Czernuszka, 1998. Collagen-calcium phosphate composites. Proc. Instn. Mech. Engrs. 212:413-425.

Li, K-C., R. F. Zernicke, R. J. Barnard, and A-F. Y. Li, 1990. Effects of high fat-sucrose diet on cortical bone morphology and biomechanics. Calcif. Tissue Int. 47:308-313.

Logan, N., 1950. Determination of hydroxyproline. J. Bio. Chem. 184:299-306.Marks, S. C. Jr., and S. C. Miller, 1993. Prostaglandins and skeleton: The legacy and challenges of two decades of research. Endocrine. J. 1:337-344.

Long, P. H., S .R. Lee., G. N. Rowland, and W. M. Britton, 1984. Experimental rickets in broilers: gross, microscopic, andd radiographic lesions. I. Phosphorous deficiency and calcium excess. Avian Dis. 28:460-474.

Martin, R. B., and D. L. Boardman, 1993. The effects of collagen fiber orientation, porosity, density, and mineralization on bovine cortical bone bending properties. J. Biomech. 26:1047-1054.

Masse, P. G., C. M. Rimnac, M. Yamauchi, S. P. Coburn, R. B. Rucker, D. S. Howell, and A. L. Boskey, 1996. Pyridoxine deficiency affects biomechanical properties of chick tibial bone. Bone 18:567-574.

Monnier, V. M., V. Vishwanath, K .E. Frank, C. A. Elmets, P. Dauchot, and R. R. Kohn, 1986. Relation beteen complications of type I diabetes mellitus and collagen-linked fluoresence. N. Eng. J. Med. 314:403-408.

National Reseach Council, 1994. Nutrient requirements of poultry. 9th rev. ed. National Academic Press, Washington DC.

Oxlund, H., Li. Mmosekilde, and G. Ørtoft, 1996. Reduced concentration of collagen reducible cross-links in human trabecular bone with respect to age and osteoporosis. Bone 19:479-484.

Oxlund, H., M. Barckman, G. Ørtoft, and T. T. Andreassen, 1995. Reduced concentrations of collagen cross-links are associated with reduced strength of bone. Bone 17(Suppl):365S-371S.

Pines, M., and S. Hurwitz, 1990. The role of growth plate in longitudinal growth. Poultry Sci. 70:1806-1814.

Qian, H., H. P. Veit, E. T. Kornegay, V. Ravindran, and D. M. Denbow, 1996. Effects of supplemental phytase and phosphorous on histological and other tibial characteristics and performances of broilers fed semi-purified diets. Poultry Sci. 75:618-626.

Rath, N. C., J. M. Balog, W. E. Huff, G. R. Huff, G. B. Kukarni, and J. F. Tierce, 1999. Comparative differences in the composition and biomechanical properties of tibiae of deven- and seventy-two-week-old male and female broiler breeder chickens. Poultry Sci. 78:1232-1239.

Robins, S. P., 1994. Biochemical markers for assessing skeletal growth. Eur. J. Clin. Nutr. 48(Suppl.1):S199-S209.

Roy, R., P. Polgar, Y. Wang, R. H. Goldstein, L. Taylor, and H. M. Kagan, 1996. Regulation of lysyl oxidase and cyclooxygenase expression in human lung fibroblasts: interactions among TGF-beta, IL-I beta, and prostaglandin E. J. Cell Biochem. 62:411-417.

Seibel, M. J., S. P. Robins, and J. P. Bilezikian, 1992. Urinary crosslinks of collagen: specific markers of bone resorption in metabolic bone disease. Trends Endo. Metab. 3:263-270.

Seyedin, S. M., and D. M. Rosen, 1990. Matrix proteins of the skeleton. Curr. Opin. Cell Biol. 2:914-919.

Shen, C-L., B. A. Watkins, S. S. Lim, and D. C. McFarland, 1994. Dietary lipid modulation of fatty acid composition of tibiotarsal bone and IGF-I responses in chicks. FASEB J. 8:A929.

Skinner, J. T., J. N. Beasley, and P. W. Waldroup, 1990. Effects of dietary amino acid levels on bone development in broiler chickens. Poultry Sci. 70:941-946.

Takahashi, T., S. Ueda, K. Takahashi, and R. O. Scow, 1994. PH-dependent multilamellar structures in fetal mouse bone: possible involvement of fatty acids in bone mineralization. Am. J. Physiol. 266:C590-C600.

Tsuchiya, H., and C. J. Bates, 1997. Vitamin C and copper interactions in guinea-pigs and a study of collagen cross-links. Br. J. Nutr. 77:315-325.

Tsuchiya, H., and C. J. Bates, 1998. Changes in collagen cross-link ratios in bone and urine of guinea pigs fed graded dietary vitamin C: a functional index of vitamin C status. J. Nutr. Biochem. 9:402-407.

Urist, M.R., K. Behnam, F. Kerendi, D. Bagga, H. D. Adkisson, J. Nevenzel, and T. Malinin, 1997. Neutral lipids facilitate transfer of bone morphogenetic proteins and other noncollagenous proteins. Med. Hypothesis. 49:465-475.

Watkins, B. A., C. Shen, K. G. D. Allen, and M. F. Seifert, 1996a. Dietary (n-3) and (n-6) polyunsaturates and acetylsalicylic acid alter Ex Vivo PGE_2 biosynthesis, tissue IGF-I levels and bone morphometry in chicks. J. Bone Miner. Res. 11:1321-1332.

Watkins, B., and M. F. Seifert, 1997. Food lipids and bone health. Food Lipids and Health. Edited by McDonald, R. E. and B. D. Min. Marcel Dekker, InC., NY. 71-116.

Watkins, B. A., M . F. Seifert, K. G. D. Allen, 1997b. Importance of dietary fat in modulating PGE_2 responses and influence of vitamin E on bone morphometry. World Rev. Nutr. Diet. 82:250-259.

Watkins, B. A., C. L. Shen, J. P. Mcmurtry, H. Xu, S. D. Bain, 1997a. Dietary lipids modulate bone prostaglandin E2 production, insulin-like growth factor-I concentration and bone formation rate in chicks. J. Nutr. 127:1084-1097.

Watkins, B. A., H. Xu, and J. J. Turek, 1996b. Linoleate impairs collagen synthesis in primary cultures of avian chondrocytes. P. S. E. B. M. 212:153-159.

Wilson, J. H., and P. L. Ruszler, 1998. Long term effects of boron on layer bone strength and production parameters. Br. Poultry Sci. 39:11-15.

Wohl, G. R., L. Loehhrke, B. A. Watkins, and R. F. Zernicke, 1998. Effects of high-fat diet on mature bone mineral content, structure, and mechanical properties. Calcif. Tissue. Int. 63:74-79.

Wuthier, R. E., 1993. Involvement of cellular metabolism of calcium and phosphate in calcification of avian growth plate cartilage. J. Nutr. 123:301.

Xu, H., B. A. Watkins, and H. D. Adkisson, 1994. Dietary lipids modify the fatty acid composition of cartilage isolated chondrocytes and matrix vesicles. Lipids 29:619-625.

Yoshitake, K., K. Yokota, Y. Kasugai, M. Kagawa, T. Sukamoto, and T. Nakamura, 1993. Effects of 16 weeks of treatment with tibolone on bone mass and bone mechanical and histomorphometric indices in mature ovariectomized rats with established osteopenia on a low-calcium diet. Bone 25:311-319.

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

CHAPTER THREE

Long-Term Effects of Dietary lipids on Mature Bone Mineral Contents, Mechanical Properties and Histological Characteristics of Japanese Quail

ABSTRACT: The purpose of this study was to investigate the effects of long-term supplementation of fat in the diets on the fatty acid composition, chemical, mechanical and histological properties of mature tibial bone. Month-old male quail were fed a basal diet containing either soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) or menhaden fish oil (FO) at 50g/kg of the diet and maintained on these diets for 7 months. Lipid treatments did not affect body weight, food intake, tibial length or diameter. The FO diet group had the highest percentage of tibial ash and both the FO and HSBO significantly increased tibial mineral content compared to those given SBO or CF. The type and amount of fatty acids in the diets had a profound influence on fatty acid composition of lipids in tibial cortical bones. Quail fed FO had the highest concentration of n-3 fatty acids and those fed SBO were the hightest in n-6 fatty acids. The HSBO diet, containing high level of trans-fatty acids, led to the accumulation of these fatty acids in bone. In quail, long-term supplementation of FO or HSBO compared to those given SBO or CF increased tibial shear force, stiffness and shear stress and improved histological cortical thickness and density. These results suggest that long-term exposure to FO or HSBO diet has a significant beneficial effect on mature bone metabolism.

(***Key words:*** Lipids, bone, mechanical properties, histology, quail)

INTRODUCTION

Osteoporosis is a major health care problem which is quickly becoming more severe in economically developed countries. Usually afflicting the aging population, It is estimated to cost $10 billion a year in the U. S. to treat such patients (Seifert and Watkins, 1997). It is reported that there are 300,000 new cases of osteoporotic hip fractures annually in the United States alone, and 1.5 million people around the world suffer from osteoporosis (Das, 1994). Many nutrients influence the growth, development, modeling and remodeling of bones. Among them, the effects of calcium, phosphorous and 1,25-dihydroxyvitamin D_3 on bone growth and health are well studied. Dietary supplementation of calcium and vitamin D_3 are traditionally believed to be effective in prevention of bone loss. Therefore, an increase in dietary calcium intake is often recommended for reducing postmenopausal bone loss (Heaney, 1992; Cumming *et al*., 1997). However, new evidence indicates that increased calcium intake above normal does not prevent bone loss in post-menopausal women (Riis *et al*., 1987; Dawson *et al*., 1990; Hosking *et al*., 1998).

The relationships between dietary fat, calcium metabolism and bone development have only recently been studied. An early study indicated that the degree of mineralization in hypertrophic and calcified cartilage was closely related to the amounts of acidic phospholipids present in the tissue (Wuthier, 1968, 1975). A diet high in saturated fat adversely affected bone mineralization and consequently compromised structural and mechanical properties of bones in growing rats (Hou *et al*., 1990; Li *et al*., 1990; Salem *et al*., 1992; Zernicke *et al*., 1995) and chicks (Atteh *et al*., 1983; Wohl *et al*., 1998). In other studies, the results indicated that dietary lipids, depending upon the type and amount ingested, may enhance or impair bone growth and development, and also modulate bone mineral content. Models that have been employed include rats (Alam et al., 1993; Claassen *et al*., 1995) and chicks (Xu *et al*., 1994; Watkins *et al*., 1996a, 1996b, 1997a, 1997b). Lipid sources that are mostly often used include hydrogenated soybean oil (HSBO), soybean oil (SBO), menhaden fish oil (FO) and saturated fat (SF), which are enriched with trans fatty acids, n-6 fatty acids, n-3 fatty acids, and saturated fatty acids, respectively. Briefly, the results of these studies indicated that dietary lipids modify the fatty acid composition of cartilage and bone, which reflects the dietary lipid profile. Supplementation of the diet with n-3 sources of lipids elevate 20:5(n-3) and 20:6(n-3) fatty acids in phospholipids of bone, while decreasing the concentration of 20:4(n-6) fatty acids compared to those fed either the corn oil or SBO. Consequently, growing animals fed either n-3 fatty acids or saturated fat enriched diets showed significantly greater bone formation rate compared to those given soybean oil as determined by histomorphometry (Watkins *et al*., 1996a, 1997b).

High fat diets are pervasive in western countries and dietary fat constitutes 30-40% of the food calories (Waktins, 1997a). Thus, the type of fat consumed may have a significant impact on the bone metabolism and growth in the young. There are no studies on how different dietary lipids affect adult bone following long term supplementation. Furthermore, the effects of dietary lipids on bone mechanical and histological properties have not been previously documented. Therefore, the purpose of this study was to investigate the long-term effects of different dietary lipids, varying in the amounts of n-3 and n-6 fatty acids, saturated and trans-fatty acids on the fatty acid

composition of old bone tissue in Japanese quail. Bone biomechanical properties and histological charactersitics were performed to determine the effects of lipids on these bone parameters.

MATERINALS AND METHODS

Animals and Diets

Two hundred and eighty day-old quail were placed in temperature-controlled battery brooders on continuous light and raised at the same dietary and managerial conditions until 4 weeks old. Birds were then sexually identified and the males were individually wing-banded, weighed and randomly divided into four groups with 30 birds each. The quail were fed a basal diet containing either 5 % (50g/kg diet) soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) or menhaden fish oil (FO). The basal diet (Table 1) was formulated to meet or exceed the nutrient requirements of the breeding quail (NRC, 1994). At six weeks of age, quail were placed into individual cages where they remained throughout the rest of the experiment. Diets were mixed twice each month and stored in cool conditions. The quail were given free access to feed and water throughout the 7-month experimental period. Body weight and feed consumption were recorded monthly.

Sample Collections

Twenty quail per dietary treatment were used for bone histological, mechanical and mineral measurements. At eight months of age, quail were sacrified by cervical dislocation. Both tibial bones from each bird were excised and cleaned of surrounding soft tissue. The length of the right tibia of each bird was measured and the diameter at the center of diaphysis was determined by measuring the widest and narrowest sites using a caliper. The right tibiae were then sealed in plastic bags immediately and frozen (-20^{o}C) until the day of mechanical testing. An average of bone wall thickness was derived following the mechanical test. After recording the weight, ten left tibiae were fixed in 10% phosphate-buffered formalin solution for two days and then decalcified with formic acid solution for histological analysis. The rest of the left tibiae were collected and frozen at –20^{o}C for ash and mineral measurements. The remaining eight birds per treatment were sacrificed at the same age and the tibiae were collected and kept in –20^{o}C for fatty acid analysis.

Bone Mechanical Testing

On the day of testing, tibial bones were thawed at room temperature. All mechanical tests were performed on an Material Testing Machine (MTS Systems Co., Cary, NC). A shear test was used to determine the mechanical properties of the tibial bone with a double shear block apparatus (Wilson and Ruszler, 1998). Tests were conducted at room temperature and specimens were kept moist during testing. Loaded at the midpoint of the shaft, the tibia was subjected to shear test to failure at a constant loading rate of 2mm/min, using a 1000 N load cell, which was connected to a

microcomputer via an amplifier. The ultimate shear force, shear fracture energy (area under the load-deflection curve), maximal deflection before fracture and stiffness (tangent to the angle α) were read directly or calculated from the computer recordings. Because the strength values are influenced by differences in bone size, the ultimate stress was calculated using the following formula: $\text{Stress}=\text{Force}/2\pi(D*BW-BW^2)$ where D is the diameter of tibia, and BW is the thickness of bone wall at the failure.

Histological Measurements

The decalcified bone samples were split longitudinally and then cut into two parts at the center of the diaphysis. Both the proximal and distal ends of the tibia were embedded in paraffin, and later cut into about 5 micron thick sections. Randomly selected sections were stained with hematoxylin and eosin. The specimens were examined in a blind fashion under a microscope and photographed. Cortical bone thickness at the proximal end and distal end, and in the middle of the diaphysis was evaluated and measured microscopically. Cortical density at the same sites was scored in a range of 1-4 from low to high density.

Lipid Analysis

Lipids in the diet, and tibial cortical bone were extracted with chloroform/methanol (2:1,v/v). The shaft portion of tibia was used for fatty acid analysis. After being freed of the periosteum and marrow, cortical bone was cooled with liquid nitrogen and pulverized to powder with a mortar and pestle, and then weighed and placed in 7 ml methanol and 14 ml chloroform for 24 h prior to the extraction of lipids. Fatty acid methyl esters (FAME) from lipid fractions were prepared by transesterification using 14% BF_3 in methanol (Watkins *et al*., 1997b). The FAME were extracted in hexane and analyzed using a gas-liquid chromatograph (HP 5890GC, flame ionization detector, autosampler, Sunnyvale, CA) equipped with a silica capillary 88 column (100 m×0.25 mm i.d., 0.2 μm film thickness, Middelburg, Netherlands) with hydrogen as the carrier gas. The initial oven temperature of 175^{o}C was held for 10 min and increased at a rate of 10^{o}C/min to a final 220^{o}C and held for 20 min. The duration of the gas chromatographic analysis was 110.5 min. The temperatures of the injector and detector were 250^{o}C and 255^{o}C, respectively. Known amounts of the internal free 11:1 acid standard in hexane was used to develop the calibration table. The 18:0 fatty acid was used as reference compound for uncalibrated peaks. The fatty acid composition of the diets and bone samples were expressed as μg/100 μg of FAME in the lipids. The amounts of total saturated fatty acids (SF), monounsaturated fatty acids (MONU), polyunsaturated fatty acids (PUFA), total n-3 and n-6 PUFA, as well as the n-6/n-3 ratios, were calculated from the GLC analysis. The amount of conjugated linoleic acid represented the collection of all detected isomers.

Ash, Calcium and Phosphorous Analysis

Tibia samples were oven-dried at 105^{o}C for 48 h, weighed, and ashed in a muffle furnace at 600^{o}C for 14h in porcelain crucibles. Tibial ash was expressed as a percentage

of dry weight, and mineral content was represented by ash weight (mg) per unit of tibial length. Afterwards, the ash was digested with nitric acid:perchloric acid mixture (5:3 v/v). The Ca and P contents of tibial ash were determined with an atomic absorption spectrophotometer (Perkin Elmer, 5100 PC, Norwalk, CT), and expressed as a percentage of dry weight of the tibiae.

Statistic Analysis

Data were analyzed by a one-way ANOVA of the SAS® (SAS Institute Inc., 1998, Cary, NC), and significant differences of means between treatments were tested using studentized tukey test at the 5% probability level. Variation within treatment was expressed as the standard error of the treatment mean (SEM).

RESULTS

Fatty Acid Composition of the Diets and Bone Lipids

The fatty acid composition of the four diets shown in Table 2 demonstrated that the SBO diet provided the highest amount of 18:2(n-6) and 18:3(n-3), whereas the FO diet had the greatest levels of 14:0, 16:1(n-7), 20:5(n-3), 22:5(n-3) and 22:6(n-3). The HSBO diet contained the largest amount of 18:0 and trans-18:1, and the CF diet was the highest in 18:1. Consequently, the SBO diet had the highest levels of total polyunsaturates and n-6 fatty acids, but the FO diet provided the largest amount of n-3 fatty acids, total saturates and the lowest ratio of n-6/n-3. The HSBO diet contained the greatest level of total monounsaturates. The CF diet, however, also contained a one fold increase in the amount of monounsaturates compared to the FO diet, and much higher levels of total n-6 fatty acids compared to both the HSBO and FO diets. All above diets contained 1.3 %-4.0 % of 18:2(n-6) which was well above the recommended requirement (1.0%) by laying quail (NRC, 1994).

Significant differences in bone fatty acid composition were observed between the dietary treatment groups. Quail given SBO had the highest concentrations of 18:2(n-6), 18:3(n-3), 20:4(n-6) and total polyunsaturates in the tibiae, whereas those consuming FO were the highest in 14:0, 16:1(n-7), 20:5(n-3), 22:6(n-3) and total saturates but were lowest in the level of 20:4(n-6) and the ratio of n-6:n-3 (Table 3). Likewise, the tibiae from birds given the HSBO had higher trans-18:1 and total monounsaturates. The CF group was significantly higher in 18:2(n-6), 20:4(n-6) and total n-6 fatty acids than those in the FO and HSBO groups, and also had higher levels of total monounsaturates than those in the SBO and FO groups. The effect of lipid supplementation in the diet on the concentrations of t18:1 and major polyunsaturates in bone are summarized in Fig. 1.

Body Weight, Feed Intakes and Bone Measurements

No significant differences among the lipid treatment groups were observed for body weight and feed intake across the dietary treatments of quail during the whole experimental period (Table 4). Accordingly, long-term dietary lipid supplementation had no pronounced effect on tibial bone length, diameter or weight. However, the percentage

of ash in the tibiae was significantly higher from quail fed FO than from the other treatment groups. Mineral content was significantly higher both in the FO and HSBO groups than that in the SBO or CF group. In addition, birds from the FO treatment group also showed the highest Ca and P contents in the tibiae. There were significant differences between the other groups (Table 4).

Biomechanical Properties

There were significant differences in the biomechancial properties of tibial bones between dietary lipid treatment groups. Birds fed SBO had significantly lower values in shear force, stress and stiffness than those fed FO or HSBO. Similarly, the CF group was also significantly lower in these parameters except the value of shear force, which was still lower, but not statistically significant, compared to the FO or HSBO groups (Table 5). There were no differences in these parameters between the SBO and CF groups, or between the FO and HSBO groups. The shear fracture energy was significantly higher for birds fed SBO than those given HSBO, but not different compared to those consuming the FO or CF diet (Table 5). Mean load-deformation curves for all groups are shown in Fig. 2.

Anatomic and Histologic Parameters

There were no differences in the diameter and length of the tibiae subjected to histological analysis between dietary lipid treatments (Table 6). In the proximal end of the tibia, cortical bone thickness was not different between treatments, but birds fed either FO or HSBO showed a significantly higher cortical density compared to those given CF or SBO diet. In the diaphysis, both cortical thickness and density in birds fed FO, and cortical density in birds fed HSBO were significantly higher compared to those consuming the SBO or CF diet. In the distal end of the tibiae, significantly higher cortical thickness was found in quail provided the FO or HSBO diet than those fed SBO, but there was no differences in cortical density in this area between treatments.

DISCUSSION

This study was performed in order to investigate the long-term effect of different lipid supplementation in the diet on fatty acid composition, mechanical and histological properties in aged tibial bone, using the quail as an animal model. The results demonstrated that the body weights, tibial lengths, weights and diameters in all experimental groups were similar, suggesting that dietary lipids had no significant effect on these parameters in birds. However, the percentages of tibial ash, Ca and P were significantly higher in quail fed FO, compared to those of the other groups. Mineral content was significantly higher in both FO and HSBO treatment groups. Although Watkins *et al*. (1996a, 1997b) reported that (n-6) enriched diet such as SBO impaired bone formation rate as measured by histomorphometric measurements in growing chicks, there were no difference in percent bone ash and mineral content between chicks fed dietary SBO and those given the other dietary lipids either at 21 or 42 days of age. Our previous study (data not shown) also demonstrated that different dietary lipids did not

lead to a significant change in bone mineralization in young quail when supplemented for a relatively short-term, but a significant difference was observed when dietary lipids were provided for 12 weeks. The current results suggest that the effect of lipids on bone mineralization and metabolism is a cumulative process, and long-term dietary treatment exacerbates the effects of lipids on bone turnover. Thus, in this dietary condition, SBO and CF diets may eventually lead to negative bone turnover and mineral loss in aged quail compared to those fed FO diet. This hypothesis, however, needs to be confirmed by a more detailed study.

The fatty acid composition of lipids in bone was significantly altered by the dietary lipids. Specifically, the concentrations of 18:2(n-6) and 20:4(n-6) were higher in tibial cortical bone of quail fed SBO, whereas dietary enrichment with n-3 fatty acids greatly increased the levels of 20:5(n-3) and 22:6(n-3) but depressed the concentration of 20:4(n-6), the precursor of PGE_2, compared with other groups. Alam *et al*. (1993) and Kokkinos *et al*. (1993) reported that feeding ethyl esters of (n-3) polyunsaturates for over 5 weeks elevated the concentrations of 20:5(n-3) and 22:6(n-3) but lowered the level of 20:4(n-6) in alveolar bone of rats. Watkins *et al*. (1996a, 1997b) demonstrated that dietary SBO increased the concentrations of 20:4(n-6), whereas menhaden fish oil (rich in n-3 fatty acids) decreased the level of this fatty acid but increased the concentration of (n-3) fatty acids in the tibiae of growing chicks. In addition, trans-fatty acids (mainly t18:1) were incorporated into bone lipids in chicks fed HSBO (Watkins, *et al*., 1991). The results of the present study are in agreement with these findings.

The study of mechanical properties revealed that, in quail fed FO or HSBO compared to those consuming CF and SBO, bone shear force was increased by 16.6%-29.3%, shear stress, determining the internal resistance of the molecular structure of a material to deformity (Wilson and Ruszler, 1998; Rath *et al*., 1999), was increased by 26.2%-37.2%, and stiffness, measuring the bone rigidity, was increased by 21.9%-55.7%. Fracture energy, representing brittleness, was comparable between treatments except the SBO group had higher fracture energy than quail fed HSBO. Overall, these measurements showed that bones from quail fed FO or HSBO were stronger than those given SBO or CF.

The biomechanics of bone has been extensively investigated in both animals and humans (Grynpas *et al*., 1992; Aerssens *et al*., 1993; Barengolts *et al*., 1993; Zernicke *et al*., 1995; Wilson and Ruszler, 1998; Bailey *et al*., 1999; Wohl *et al*., 1998; Path *et al*., 1999; Yoshitake *et al*., 1999). Bone strength is related to its structural properties (e.g. shear force, energy, stiffness) and material properties (e.g. stress, matrix chemistry). The structural properties of bones depend in part on bone geometry (Zernicke *et al*., 1995). This study indicated that birds in all treatments had similar gross geometry and body weight, suggesting that the deleterious effects of the SBO and CF diets were mainly related to changes in material properties of the cortical bones. Further, histological observations from this study demonstrated that both cortical bone density and thickness were markedly improved in quail fed FO or HSBO compared to those given SBO or CF at eight months of age. Based on these data and mineral measurements, it is suggested that the SBO and CF diet-related changes in mechanical properties of cortical bones resulted from both reduced cortical bone quality and quantity.

Several studies indicated that short-term supplementation of a high level of SBO in the diet decreased bone formation rate compared to FO or saturated fat as determined

by histomorphometry in rapidly growing animals (Watkins *et al*., 1996a, 1997b), but they failed to assess the bone mechanical properties. In this study, mature quail were used as animal model, and the results therefore largely reflect the influence of lipids on bone remodeling (formation and resorption), which is the primary mechanism of mature bone turnover (Wohl *et al*., 1998). Thus, it can be assumed that long-term supplementation of SBO or CF in the diets caused a negative bone balance by inducing bone resorption, which resulted in decreased bone mass, mineral density, and impaired mechanical properties.

Mature bone remodeling is regulated partially by locally produced factors within the skeleton (Baylink *et al*., 1993; Marks and Miller, 1993; Mundy, 1993; Raisz, 1993a). Prostaglandins (PGs) have been extensively investigated and have been found to play an important role in bone metabolism. Certain fatty acids are precursors of PGs, and therefore, one mechanism by which dietary lipid influences bone formation and resorption is by altering prostaglandin production. Prostaglandin E_2 (PGE_2), which is produced from the 20-carbon polyunsaturated essential fatty acid (arachidonic acid) in osteogenic cells (Sardesai, 1992; Kokkinos *et al*., 1993; Watkins *et al*., 1997), has powerful effects on bone metabolism. *In vitro*, although PGE_2 can stimulate bone formation at a low concentration, its main function over the long term is to stimulate bone resorption (Marks and Miller, 1993; Raisz *et al*., 1993b; Fall *et al*., 1994). Recent studies indicate that diets enriched with n-6 PUFA increased the concentration of arachidonic acid [20:4(n-6)] in bone and elevated bone PGE_2 production in chicks (Watkins *et al*., 1996a, 1997b) and rats (Kokkinos *et al*., 1993), but depressed bone formation rate. In this study, quail fed SBO or CF not only had a significantly higher level of arachidonic acid, but also maintained a higher n-6/n-3 ratio in the tibiae than those consuming FO or HSBO. Unfortunately, this study was conducted without performing PGE_2 measurement simultaneously. However, it may be reasonably presumed that quail given the SBO or CF diet had a higher level of endogenous PGE_2 production in bone, which may stimulate the bone resorption more than bone formation, and eventually decrease the bone mechanical properties.

In summary, our current study demonstrated that in mature quail, long-term supplementation of lipids in the diets altered fatty acid composition of bone lipids, which reflected the fatty acid profile of the diet. Specifically, quail fed SBO or CF had elevated bone arachidonic acid concentrations, a precursor of PGE_2. Precious study in quail showed a direct relationship between AA concentration and PGE_2 production. The beneficial effects of FO and HSBO on bone mineral content, mechanical and histological properties in this study might be, at least in part, attributable to their decreased PGE_2 production-induced bone resorption. Further study on the effects of dietary lipids on bone should include biochemical assays and histomorphometrical measurements to determine the effects of lipids on bone extracellular matrix chemistry and both trabecular and cortical architecture.

Table 1. Dietary ingredient composition of the basal diets given to quail[1]

	Dietary lipid treatment[2]			
Ingredients (%)	SBO	HSBO	CF	FO
Yellow corn	53.55	53.55	53.55	53.55
Soybean meal	32.00	32.00	32.00	32.00
Soybean oil	5.00	—	—	—
Hydrogenated soybean oil	—	5.00	—	—
Chicken fat	—	—	5.00	—
Menhaden oil	—	—	—	5.00
Limestone	6.50	6.50	6.50	6.50
Dicalcium phosphate	1.50	1.50	1.50	1.50
Vitamin Premix[3]	1.00	1.00	1.00	1.00
Trace mineral premix[4]	0.10	0.10	0.10	0.10
salt	0.35	0.35	0.35	0.35

[1] The diet was formulated to contain 21.0% crude protein, 2997.2-3007.2 kcal/kg metabolizable energy, 7.36% crude fat, 2.43% crude fiber, 2.81% calcium, and 0.67% phosphorous.

[2] Diets include soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) or menhaden fish oil (FO) at 50g/kg of the diet.

[3] Provided per kilogram of diet: vitamin A, 1100 IU; vitamin D_3, 330 IU; vitamin E, 33 IU; menadione sodium bisulfite, 3.52 mg; thiamine HCL, 1.1 mg; riboflavin, 6.6 mg; calcium D-pantothenate, 16.5 mg; niacin, 44 mg; choline chloride, 374 mg; vitamin B_{12}, 0.0165 mg; folic acid, 1.1 mg; pyridoxine HCL, 1.1 mg; biotin, 0.055 mg; Bacitracin, 22.0 mg; selenium, 0.20 mg; ethoxyquin, 0.124 mg.

[4] Provided per kilogram of diet: manganese, 44mg; zinc, 47.5 mg; iron, 50.0 mg; copper, 6.25mg; iodine, 2.0 mg; and selenium, 0.3 mg.

Table 2. Fatty acid composition (μg/100 μg) of the quail basal diet

	Dietary lipid treatment[1]			
Fatty acids	SBO	HSBO	CF	FO
14:0	0.06	0.19	0.57	5.29
15:0	0.02	0.04	0.09	0.42
16:0	9.95	11.26	15.01	15.45
16:1(n-7)	0.08	0.15	2.30	6.95
18:0	3.54	11.11	6.53	3.26
t18:1	0.05	25.49	2.57	1.45
18:1	25.82	30.76	39.50	15.07
18:2(n-6)	54.79	18.11	30.00	19.16
18:3(n-3)	4.33	0.98	1.54	2.32
20:0	0.30	0.34	0.23	0.20
20:3(n-3)	ND[5]	ND	0.05	0.11
20:4(n-6)	0.01	0.01	0.16	0.51
20:5(n-3)	0.01	ND	0.02	18.49
22:1	ND	ND	0.03	0.24
22:4(n-6)	0.01	0.01	0.03	0.09
22:5(n-3)	0.02	0.03	0.02	1.47
22:6(n-3)	ND	ND	0.01	7.47
SAT[2]	13.87	22.94	22.42	24.62
MONU[3]	25.95	56.42	44.43	23.91
PUFA[4]	59.18	19.15	31.82	49.61
(n-6) PUFA	54.81	18.13	30.19	19.75
(n-3)PUFA	4.37	1.02	1.63	29.86
(n-6)/(n-3)	12.55	17.85	18.47	0.66

[1] Dietary treatments include soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat, and menhaden fish oil (FO) at 50 g/kg of the diet.

[2] SAT, total saturated fatty acids.

[3] MONU, total monounsaturated fatty acids.

[4] PUFA, total polyunsaturated fatty acids.

[5] ND, not detected.

Table 3. Fatty acid composition (μg/100 μg) of tibial cortical bone from mature quails fed different lipids[1]

	Dietary lipid treatment[2]					
Fatty acids	SBO	HSBO	CF	FO	Pooled SEM	P values
14:0	0.43^{c}	0.57^{b}	0.69^{b}	4.43^{a}	0.06	0.0001
15:0	0.08^{b}	0.08^{b}	0.11^{b}	0.38^{a}	0.01	0.0001
16:0	15.74^{b}	18.25^{ab}	19.19^{a}	19.65^{a}	0.72	0.0155
16:1(n-7)	3.09^{c}	4.78^{b}	4.61^{b}	8.79^{a}	0.33	0.0001
18:0	5.49^{b}	6.92^{a}	5.61^{b}	5.58^{b}	0.36	0.0135
t18:1	0.32^{c}	7.05^{a}	1.47^{b}	0.41^{c}	0.19	0.0001
18:1	28.14^{b}	41.26^{a}	41.40^{a}	27.91^{b}	0.76	0.0001
18:2(n-6)	29.95^{a}	14.21^{b}	15.61^{b}	14.38^{b}	1.05	0.0001
CLA[3]	0.02^{c}	0.56^{a}	0.14^{b}	ND[4]	0.02	0.0001
18:3(n-3)	2.22^{a}	0.54^{c}	0.70^{c}	1.19^{b}	0.08	0.0001
20:0	0.11	0.12	0.08	0.12	0.02	0.6643
20:1(n-9)	0.20^{c}	0.24^{c}	0.30^{b}	0.71^{a}	0.01	0.0001
20:3(n-3)	0.11	0.40	0.09	0.14	0.02	0.4236
20:4(n-6)	12.10^{a}	4.10^{c}	8.75^{b}	3.71^{c}	0.48	0.0001
20:5(n-3)	0.10^{b}	0.08^{b}	0.06^{b}	6.14^{a}	0.27	0.0001
22:4(n-6)	0.11	0.14	0.11	0.07	0.03	0.6432
22:5(n-3)	0.07^{b}	0.05^{b}	0.04^{b}	1.14^{a}	0.07	0.0001
22:6(n-3)	0.16^{b}	0.12^{b}	0.14^{b}	3.83^{a}	0.15	0.0001
SAT[5]	22.26^{c}	26.42^{b}	26.23^{b}	30.93^{a}	0.92	0.0006
MONU[6]	31.82^{d}	53.46^{a}	47.87^{b}	37.94^{c}	0.72	0.0001
PUFA[7]	45.75^{a}	19.89^{d}	25.64^{c}	30.58^{b}	1.25	0.0001
(n-6) PUFA	43.10^{a}	19.11^{c}	24.61^{b}	18.16^{c}	1.25	0.0001
(n-3)PUFA	2.65^{b}	0.88^{b}	1.03^{b}	12.43^{a}	0.52	0.0001
(n-6)/(n-3)	16.29^{b}	22.22^{a}	23.98^{a}	1.49^{c}	0.88	0.0001

[1] Mean values for bone fatty acid composition (n=6) within a row having different superscripts are significantly different by Duncan multiple comparison test (P<0.05).

[2] Dietary lipids includes soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) and menhaden fish oil (FO) at 50g/kg of the diet.

[3] CLA, total conjugated linoleic acids.

[4] ND, not detected.

[5] SAT, total saturated fatty acids.

[6] MONU, total monounsaturated fatty acids.

[7] PUFA, total polyunsaturated fatty acids.

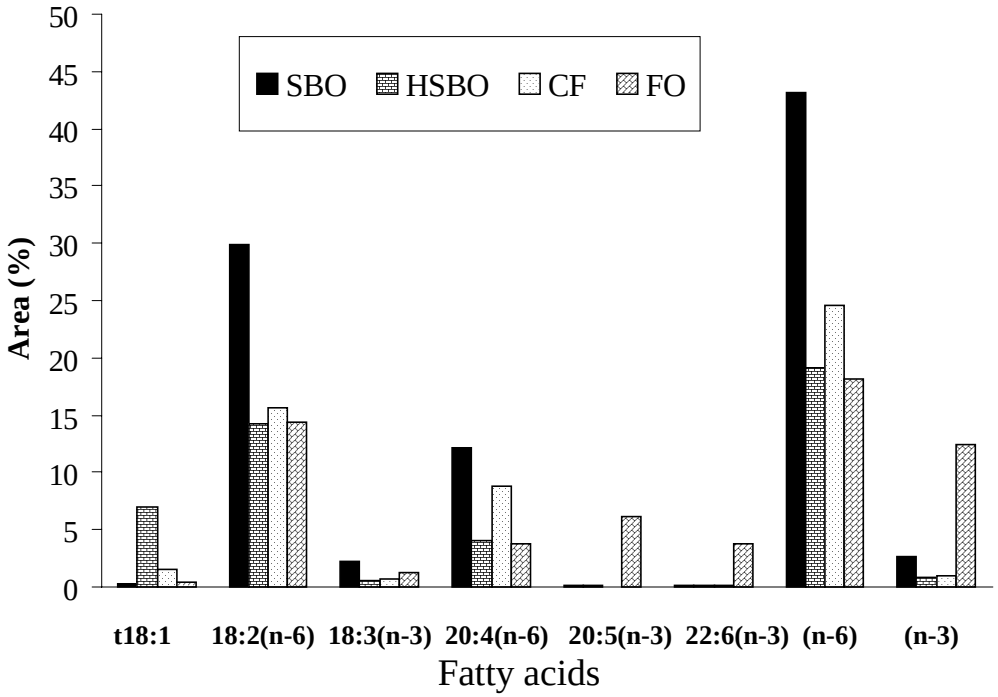

Fig 1. Major fatty acid composition (μg/100 μg) of lipids extracted from tibial cortical bone in aged quail fed the basal diet containing soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) and menhaden fish oil (FO) at 50 g/kg of the diet. The fatty acid composition of bone lipids reflected the dietary lipid profile.

Table 4. Body weight, tibial bone measurements and mineral contents of mature quail (male) fed different lipids[1]

	Dietary lipid treatment[2]				
Measurements	SBO	HSBO	CF	FO	P values
Body weight (g)	114.5±2.3	116.0±2.1	115.6±2.3	114.2±1.9	0.9244
Feed Intakes (g/d)	11.9±0.2	12.1±0.3	12.0±0.3	11.9±0.3	0.9211
Tibia length (mm)	46.8±0.4	46.6±0.4	46.4±0.4	46.3±0.3	0.7062
Tibia diameter (mm)	2.25±0.02	2.22±0.03	2.22±0.04	2.22±0.03	0.8420
Tibia weight (g)	0.34±0.01	0.37±0.01	0.34±0.01	0.36±0.01	0.5102
Ash (%)	39.0±1.0[b]	39.0±0.6[b]	39.7±0.6[b]	43.7±1.2[a]	0.0023
Mineral Content (mg/mm)	2.89±0.06[b]	3.10±0.05[a]	2.88±0.09[b]	3.27±0.09[a]	0.0011
Calcium (%)	14.0±0.3[b]	13.9±0.3[b]	14.1±0.3[b]	16.2±0.5[a]	0.0004
Phosphorus (%)	6.5±0.2[b]	6.6±0.1[b]	6.8±0.1[b]	7.5±0.3[a]	0.0059

[1] n=20 for body weight and bone measurements, n=10 for ash and mineral measurements. Mean values (means±SEM) within rows having different superscripts are significantly different ($P<0.05$).

[2] Dietary lipids treatments included soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF), and menhaden oil provided at 50g/kg of the diet. All day old quail were fed standard diets for 4 weeks and then randomly divided into 4 experimental groups and fed above experimental diets for 7 months before killed.

Table 5. Mechanical properties of tibial bones in mature quail (male) fed different dietary lipids[1]

	Dietary lipid treatment[2]				
Measurements	SBO	HSBO	CF	FO	P values
Shear force (N)	52.6±3.1[b]	68.0±3.2[a]	57.8±3.8[ab]	67.4±3.6[a]	0.0098
Fracture energy (N-mm)	17.2±4.1	15.1±2.6	15.7±4.6	14.6±4.4	0.0819
Stiffness (N/mm)	3.43±0.23[b]	5.34±0.20[a]	4.20±0.31[b]	5.12±0.30[a]	0.0001
Shear stress (N/mm^2)	19.6±1.2[b]	26.9±1.4[a]	21.0±1.1[b]	26.5±1.6[a]	0.0003

[1] n=20 for mechanical measurements. Mean values (means±SEM) within rows having different superscripts are significantly different ($P<0.05$).

[2] Dietary lipids treatments included soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF), and menhaden oil at 50 g/kg of the diet.

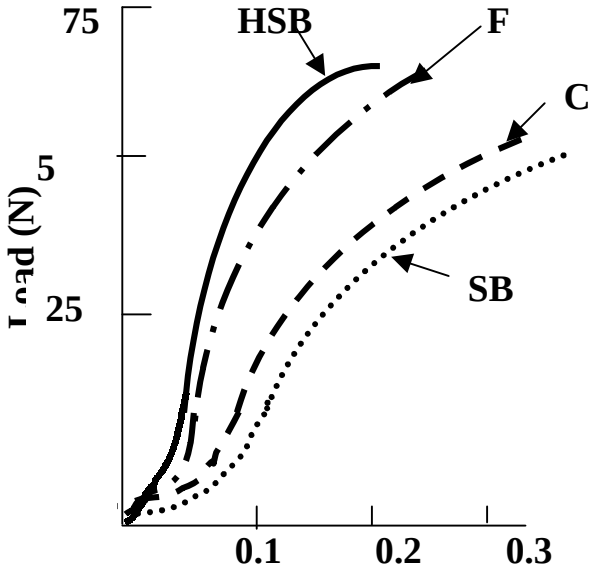

Fig. 2. Load-deformation curves of the tibial bones from four groups of quail fed soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) or menhaden fish oil (FO) at 50g/kg of the diet. The tibiae from the HSBO and FO groups had higher maximum load but lower fracture deflection compared to the CF and SBO groups.

Table 6. Histological measurements of tibial bones from mature quail fed different dietary lipids[1]

	Dietary lipid treatment[2]				
Measurements	SBO	HSBO	CF	FO	P values
Diameter (mm)	2.18±0.03	2.20±0.04	2.25±0.04	2.24±0.04	0.9230
Length (mm)	46.2±0.5	46.3±0.4	46.5±0.5	46.2±0.5	0.7662
			Proximal end of tibia		
Cortical thickness (10×)	1.3±0.2	1.6±0.2	1.4±0.2	1.9±0.2	0.3721
Cortical density (10×)	1.2±0.2^{b}	2.7±0.3^{a}	1.7±0.3^{b}	3.0±0.3^{a}	0.0026
			Diaphysis of tibia		
Cortical thickness (10×)	1.7±0.2^{b}	2.3±0.2^{ab}	1.6±0.3^{b}	2.7±0.2^{a}	0.0125
Cortical density (10×)	3.0±0.4^{b}	4.1±0.2^{a}	3.3±0.2^{b}	4.4±0.2^{a}	0.0078
			Distal end of tibia		
Cortical thickness (10×)	0.5±0.02^{b}	0.8±0.09^{a}	0.6±0.07^{ab}	0.9±0.08^{a}	0.0163
Cortical density (10×)	3.1±0.2	4.1±0.3	4.1±0.4	4.2±0.4	0.2050

[1] n=8 for histological measurements. Mean values (means±SEM) within rows having different superscripts are significantly different ($P<0.05$).

[2] Dietary lipids treatments included soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF), and menhaden oil at 50 g/kg of the diet. All day old quail were fed standard diets for 4 weeks and then randomly divided into 4 experimental groups and fed above experimental diets for 7 months before killed.

REFERENCES

Aerssens, J., R. V. Audekercke, P. Geusens, L. P. C. Schot, A. A-H. Osman, 1993. Mechanical properties, bone mineral content, and bone composition(collagen, osteocalcin, IGF-I) of the rat femur: influence of ovariectomy and namdrolone decanoate (anabolic steroid) treatment. Calcif. Tissue Int. 53:269-277.

Alam, S. Q., P. P Kokkinos, and B. S. alam, 1993. Fatty acid composition and arachidonic acid concentrations in alveolar bone of rats fed diets with different lipids. Clacif. Tissue Int. 53:330-332.

ASAE Physical Properties of Agricultural Products Committee, 1998. Shear and three-point bending test of animal bone. ASAE Standards. 568-570.

Atteh, J. O., S. Leeson, and R. J. Julian, 1983. Effects of dietary levels and types of fat on performance and mineral metabolism of broiler chicks. Poultry Sci. 62:2403-2411.

Bailey, A. J., T. J. Sims, E. N. Ebbesen, J. P. Mansell, J. S. Thomsen, and Li. Moskilde, 1999. Age-related changes in the biochemical properties of human cancellous bone collagen: relationship to bone strength. Calcif. Tissue Int. 65:203-210.

Barengolts, E.I., D. J. Curry, M. S. Bapna, S. C. Kukreja, 1993. Effects of endurance exercise on bone mass and mechanical properties in intact and ovariectomized rats.J. Bone Miner. Res. 8:937-942.

Baylink, D. J., R. D. Finkelman, and S. Mohan, 1993. Growth factors to stimulate bone formation. J. Bone Miner. Res. 8:S565-S572.

Claassen, N., H. Coetzer, C. M. L. 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 Leuk. Essent. Fatty Acids. 53:13-19.

Cumming, R. G., and M. C. Nevitt, 1997. Calcium for prevention of osteoporotic fractures in post-menopausal women. J. Bone Miner. Res. 12:1321-1329.

Das, U. N., 1994. Can essentiala fatty acids prevent osteoporosis? Med. Sci. Res. 22:163-165.

Dawson-H., B., G. E. Dallal, and E. A. Krall, 1990. A controlled trial of the effect of calcium supplementation on bone density in post-menopausal women. New Eng. J. Med. 323:878-883.

Fall, P. M., D. T. Breault, and L. G. Raisz, 1994. Inhibition of collagen synthesis by prostaglandins in the immortalized rat osteoblastic cell line pyla: structure-activity relations and signal transduction mechanisms. J. Bone Miner. Res. 9:1935-2943.

Grynpas, M. D., A. Acito, M. Dimitriu, B. P. Mertz, and J. M. Very, 1992. Changes in bone mineralization, architeture and mechanical properties due to long-term (1 year) administration of pamidronate (APD) to adult dogs. Osteoporosis Int. 2:74-81.

Heaney, R. P., 1992. Clacium in the prevention and treatmemnt of osteoporosis. J. Intern. Med. 231:169-180.

Hosking, D. J., P. D. Ross, D .E. Thompson, R. D. Wasnich, M. McClung, N. H. Bjaarnason, P. Ravn, G. Cizza, M. Daley, and J. Yates, 1998. Evidence that increased calcium intake does not prevent early postmenopausal bone loss. Clin. Therap. 20:933-944.

Hou, J. C-H.,, R. F. Zernicke, and R. J. Barbard, 1990. High-fat sucrose diet effetcs on femoral neck geometry and biomechanics. Clin. Biomech. 5:162-168.

Kokkinos, P. P., R. Shaye, B. S. Alam, and S. Q. Alam, 1993. Dietary lipids, prostaglandin E_2 levels and tooth movement in alveolar bone of rats. Calcif. Tissue Int. 53:333-337.

Li, K-C., R.F. Zernicke, R. J. Barnard, and AF-Y. Li, 1990. Effects of a high-fat sucrose diet on cortical bone morphology and biomechanics. Calcif. Tissue Int. 47:308-313.

Marks, S. C. Jr., and S. C. Miller, 1993. Prstaglandins and the skeleton: the legacy and challenges of two decades of research. Endocr. J. 1:337-344.

Mundy, G. R., 1993. Cytokines and growth factors in the regulation of bone growth. J. Bone Miner. Res. 8(Suppl. 2):S505-S510.

National Reseach Council, 1994. Nutrient requirements of poultry. 9th rev. ed. National Academic Press, Washington DC.

Raisz, L. G., 1993a. Bone cell biology: New approches and unanswered questions. J. Bone Miner. Res. 8:S457-S465.

Raisz, L. G., C. C. Pilbeam, and P. M. Fall, 1993b. Prostaglandins: Mechanisms of action and regulation of production in bone. Osteoporosis Int. 3 (Suppl. 1):S136-S140.

Rath, N. C., J. M. Balog, W. E. Huff, G. R. Huff, G. B. Kukarni, and J. F. Tierce, 1999. Comparative differences in the composition and biomechanical properties of tibiae of seven- and seventy-two-week-old male and female broiler breeder chickens. Poultry Sci. 78:1232-1239.

Riis, B., K. Thomsen, and C. Christiansen, 1987. Does calcium supplementation prevent

post-menopausal osteoporosis? New Eng. J. Med. 316:173-177.

Salem, G. J., R. F. Zernicke, and R. J. Barnard, 1992. Diet-related changes in mechanical properties of rat vertebrae. Am. J. Physiol. 262:R318-R321.

Sardesai, V. M., 1992. Biochemical and nutritional aspects of eicosanoids. J. Nutr. Biochem. 3:562-579.

Seifert, M. F., and B. A. Watkins, 1997. Role of dietary lipids and antioxidants in bone metabolism. Nutr. Res. 17:1209-1228.

Watkins, B. A., M. F. Seifert, and K. G. D. Allen, 1997a. Importance of dietary fat in modulating PGE_2 responses and influence of vitamin E on bone morphometry. World Rev. Nutr. Diet. 82:250-259.

Watkins, B. A., C. Shen, K. G. D. Allen, and M. F. Seifert, 1996a. Dietary 9n-3) and (n-6) polyunsaturates and acetylsalicylic acid alter Ex Vivo PGE_2 biosynthesis, tissue IGF-I levels and bone morphometry in chicks. J. Bone Miner. Res. 11:1321-1332.

Watkins, B. A., C. L. Shen, J. P. Mcmurtry, H. Xu, S. D. Bain, 1997b. Dietary lipids modulate bone prostaglandin E_2 production, insulin-like growth factor-I concentration and bone formation rate in chicks. J. Nutr. 127:1084-1097.

Watkins, B. A., C. C. Whitehead, and S. R. I. Duff, 1991. Hydrogenated oil decreases tissue concentration of n-6 polyunsaturated fatty acids and may contribute to dyschondroplasia in broilers. Br. Poult. Sci. 32:1109-1119.

Watkins, B. A., H. Xu, and J. J. Turek, 1996b. Linoleate impairs collagen synthesis in primary cultures of avian chondrocytes. P. S. E. B. M. 212:153-159.

Wilson, J. H., and P. L. Ruszler, 1998. Long term effects of boron on layer bone strength and production parameters. Br. Poultry Sci. 39:11-15.

Wohl, G. R., L. Loehhrke, B. A. Watkins, and R. F. Zernicke, 1998. Effects of high-fat diet on mature bone mineral content, structure, and mechanical properties. Calcif. Tissue. Int. 63:74-79.

Wuthier, R. E., 1968. Lipids of mineralizing epiphyseal tissues in the bovine fetus. J. Lipids Res. 9:68-78.

Wuthier, R.E., 1975. Lipids composition of isolated epiphyseal cartilage cells, membrane and matrix vesicles. Biochim. Biophys. Acta. 409-128.

Xu, H., B. A. Watkins, and H. D. Adkisson, 1994. Dietary lipids modify the fatty acid composition of cartilage, isolated chondrocytes and matrix vesicles. Lipids 29:619-625.

Yoshitake, K., K. Yokota, Y. Kasugai, M. Kagawa, T. Sukamoto, and T. Nakamura, 1993. Effects of 16 weeks of treatment with tibolone on bone mass and bone mechanical histomorphometric indices in mature ovariectomized rats with established osteopenia on a low-calcium diet. Bone 25:311-319.

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

CHAPTER FOUR

Effects of Long-Term Dietary Lipids on Mature Bone Mineral Content, Collagen, Cross-links and PGE_2 Production in Japanese Quail

ABSTRACT: This study investigated the effects of long-term dietary lipids on aged bone mineral content, collagen concentration, crosslink levels, bone marrow and *ex vivo* PGE_2 biosyntheiss, as well as the relationship of PGE_2 production to these bone formation parameters. One-month old male Japanese quail were given a basal diet containing one of four lipid sources: soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) or menhaden fish oil (FO) at 50 g/kg of the diet. At eight months of age, lipid treatments did not affect bone length, diameter or weight in quail. Quail fed SBO or CF had significantly lower levels of mineral content in tibial bones compared to those given FO. Bone collagen level was significantly higher in quail consuming SBO than those given HSBO or CF. Collagen crosslink concentration was markedly increased in birds provided FO or HSBO compared with those fed the SBO or CF. PGE_2 biosynthesis in bone organ culture and marrow were greatly increased in quail maintained on SBO or CF diet compared to those given the FO or HSBO diet. PGE_2 production in the bone microenvironment was negatively correlated with tibial ash and collagen crosslinks but had a positive correlation with tibial collagen levels. These results supported our previous findings that long-term exposure to diets high in SBO or CF impaired mature bone mechanical properties and histological characteristics. Further, the results suggest that long-term supplementation of SBO or CF in the diet had a significant adverse effect on mature bone metabolism, and that dietary lipids altered bone metabolism perhaps partially by controlling the production of local regulatory factor in bone.

(***Key words**:* Lipids, bone, prostaglandin E_2, collagen, crosslinks, quail)

INTRODUCTION

Bone strength is a complex property which is still not completely understood. Generally, collagen dominated organic matrix provides bone with its tensile strength and structural scaffolds for the deposition of minerals (Rath, 1999), while inorganic matrix provides compressional strength. Bone strength and quality are, therefore, not only related to the inorganic matrix, but also to the organic matrix. The relative amounts and properties of the mineral and collagen, as well as the bone microarchitecture and anatomy such as collagen fiber orientation, porosity, density and molecular structure of mineral crystals determine biomechanical strength (Currey, 1988; Martin and Ishida, 1989; Martin and Boardman, 1993; Landis, 1995; Shah *et al*., 1995). A decrease in bone collagen content results in a decrease in mechanical strength both in humans (Bailey *et al*., 1999) and animals (Masse *et al*., 1996). Moreover, it has been shown that not only bone mineral and collagen, but also intermolecular cross-links of collagen molecules contribute to bone strength.

Recent studies consistently found that pyridinium cross-links (pyridinoline and deoxypyridinoline) in both normal and osteoporotic bone have a strong and positive correlation with its mechanical strength (Batge *et al*., 1992; Knott *et al*., 1995; Oxlund *et al*., 1996; Rath *et al*., 1999). Furthermore, factors that affect cross-link formation can adversely affect bone strength. Animals with inhibition of lysyl oxidase or diet induced defects of collagen cross-linking such as vitamin B_6 or copper deficiency resulted in decreases in torsional stiffness and strength of bone even if bone mineral content is not altered (Farquharson *et al*., 1989; Wilmarth *et al*., 1992; Jonas and Froines, *et al*., 1993; Masse, *et al.*, 1996). However, past studies concerning the effects of alterations in bone microarchitecture on mechanical properties have focused more on the mineral content and/or mineral composition than on collagen matrix (Crenshaw *et al*., 1986; Currey, 1988; Martin and Boardman, 1993; Verhaeghe *et al*., 1994). Therefore, dietary supplementation of nutrients that affect mineralization is usually used to improve inorganic matrix of bone in both animals and humans, whereas little attention has been paid to improve the organic matrix quality.

Recent papers reported that administration of eicosapentaenoic acid (EPA; 20:5n-3) increased bone calcium and strength in ovarectomized rats (Sakaguchi *et al*., 1994). Administration of EPA in combination with dihomogamma-linoleic acid (GLA; 20:3n-6) significantly decreased collagen degradation as measured by urinary hydroxyproline and pyridinium cross-links both in growing and ovarectomized rats (Claassen *et al.*, 1995; Kruger *et al*., 1997). Watkins *et al*. (1996, 1997) demonstrated that diets enriched with n-6 PUFA elevated concentration of eicosanoid precursor arachidonic acid (AA; 20:4n-6), increased *ex vivo* PGE_2 production in bone organ culture, and depressed bone formation rate in rapidly growing animals. Prostaglandin E_2 is produced from AA in osteogenic cells (Sardesai, 1992; Kokkinos *et al*., 1993; Watkins *et al*., 1997) and has powerful effects on bone metabolism. *In vitro*, although PGE_2 can stimulate bone formation at a low concentration, its main function over a long term is to stimulate bone resorption and inhibit collagen synthesis due to an increase in replication and differentiation of new osteoclasts (Marks and Miller, 1993; Raisz *et al*., 1993; Fall *et al*., 1994). Our previous study demonstrated that long-term supplementation of different lipids in the diets altered the mature bone fatty acid composition, and changed bone biomechanical properties and

histological parameters in adult Japanese quail (Chapter three). Specifically, bone fatty acid concentration varied according to the amounts and types of PUFA in the diet. Diets supplemented with menhaden fish oil [FO; rich in EPA and DHA (22:6n-3)] or partially hydrogenated soybean oil (HSBO; rich in saturated fatty acids and a source of trans- fatty acids) greatly lowered the concentration of AA [20:4(n-6)] in bone. Accordingly, bone mineral content in birds fed a FO enriched diet and tibial bone strength in birds fed either the FO or HSBO diets were significantly increased compared to those fed diets supplemented with either soybean oil (SBO; rich in n-6 PUFA) or chicken fat (rich in monounsaturated and n-6 PUFA).

Prior to this study, it is unclear as to how dietary lipids influence the bone mechanical and histological properties, it may be a result of impairment of the organic matrix such as collagen and its cross-links. In addition, it has not been demonstrated with an experimental model how long-term dietary lipids affect endogenous PGE_2 production in aged bone microenvironment and how it is related to the parameters of bone formation and degradation. Therefore, the present study was designed to evaluate the long-term effects of dietary lipids, varying in amounts of (n-3) and (n-6) PUFA, saturated and trans- fatty acids, on adult bone collagen concentration and cross-link levels. Endogenous PGE_2 production both in bone marrow and bone tissue was performed to investigate the relationship between PGE_2 and the parameters of bone formation and degradation.

MATERIALS AND METHODS

Animals and Diets

Two hundred and forty day-old quail were placed in temperature-controlled battery brooders on continuous light and raised at the same dietary and managerial conditions until 4 weeks old. Then birds were sexually identified and males were individually wing-banded, weighed and randomly divided into four groups with 20 birds each. The quail were fed a basal diet containing either 5 % (50g/kg diet) soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) or menhaden fish oil (FO). The basal diet (Table 1) was formulated to meet or exceed the nutrient requirements of the breeding quails (NRC, 1994). The fatty acid composition of the diets is presented in Table 2. At six weeks of age, quail were placed into individual cages where they remained throughout the rest of experiment. Diets were mixed twice each month and stored in cool condition. Quail were given free access to feed and water throughout the 7-month experimental period. Body weight and feed consumption were recorded monthly.

Sample Collections

Eleven birds per treatment were killed by cervical dislocation at eight months of age. Both tibial, and one femur bones from each bird were excised, deprived of surrounding soft tissue and their weights recorded. Tibial length and its diaphyseal diameter were measured with a caliper. The femur bones were sealed in plastic bags and

frozen at –20 ^{o}C for ash measurement. The bone marrow was removed from the left tibial bones before analysis for collagen and cross-links.

Analytical Procedures

To measure of collagen (as determined by hydroxyproline) and its cross-links (deoxypyridinoline and pyridinoline), after removal of the bone marrow, bones were demineralized in several changes of 0.5 M EDTA (pH 7.2) at 4^{o}C for two weeks and then washed with distilled water (Farquharson *et al*., 1989; Tsuchiya and Bates, 1997, 1998). The non-diffusible residues of decalcified bone samples were hydrolyzed in 5 ml hydrochloric acid (6 mol/L) by heating at 110^{o}C for 24 hr in screw capped glass tubes. The hydrolysates of bone samples were then evaporated to dryness at 37^{o}C under a stream of nitrogen to remove hydrochloric acid (Tsuchiya and Bates, 1998). They were then reconstituted in 5 ml of distilled water, adjusted to neutral pH with 1 N sodium hydroxide and were kept at –4^{o}C for measurement of hydroxyproline, pyridinoline and deoxypyridinoline.

The hydroxyproline concentrations (an indicator of collagen content) of the acid hydrolysates of bones were measured by the procedure of Logan (1950) with modification (Monnier *et al*., 1986). Each sample was analyzed in duplicate for hydroxyproline concentration at 540 nm wavelength using a spectrophotometer (Beckman InC, CA). The values were automatically calculated by a connected computer, and were used to calculate the molar proportions of pyridinium crosslinks in collagen. The collagen crosslinks (pyridinoline and deoxypyridinoline, separately) were measured in acid hydrolysates of decalcified right tibia bone by antibody-based ELISA kit assays (Metra Biosystems Co., CA). These are specifically competitive enzyme immunoassays with high sensitivity for deoxypyridinoline and pyridinoline, which have been proved to be highly comparable with the results from HPLC (Delmas, *et al*., 1993; Robins *et al*., 1994) and were previously validated for measuring quail bone in our laboratory. Duplicate measurements were performed for each sample, the optional density at 405 nm was measured with a microplate reader (Biotek Instruments, Inc. VT), and the data were expressed as nmol/μg hydroxyproline.

The right tibia bones were used for PGE_2 measurement. Tibia shafts (20 mm in length) were cut, the weight recorded, and bone marrow cells were collected by carefully flushing out the bone marrow with 1 ml of phenol red-free minimum essential medium (α-MEM) as described (Miyaura *et al*., 1995). The cell suspension was centrifuged at 10,000g for 3 minutes and the bone marrow supernatant was harvested and stored at -80^{o}C for analysis for PGE_2. Tibia bone cultures for *ex vivo* PGE_2 production were prepared as described by Dekel *et al*. (1981) and Watkins *et al*. (1996). The tibial bones from which the bone marrow was removed were further flushed with 0.9 % NaCl to remove any remained marrow cells. Shafts were then immersed in 2 ml of Hank's balanced salt solution (sigma Chemical Co.) and incubated for 2 h at 40^{o}C with shaking (Watkins *et al*., 1996). Following incubation, the bone culture medium was collected and stored at –80^{o}C for analysis of *ex vivo* PGE_2 production of bone. PGE_2 biosyntheis in bone organ cultures and bone marrow supernatants was measured by radioimmunoassay using RIA kits (Cayman Chemical Co., CA). Each sample and standard were measured in duplicate and PGE_2 levels were expressed as ng per gram of bone wet weight. The

anti-PGE_2 antibody had the following cross-reactivities: PGE_2, 100%; PGE_3, 43%, PGE_1, 18.7%; $PGF_{2\alpha}$; <0.01.

For bone ash measurement, samples of femur bones were dried for 24 hr, weighed, and ashed at 600 ^oC in a muffled furnace for 14 hr. Bone ash content was expressed as a percentage of dry weight of a bone.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) using the General Linear Model procedure of SAS® (SAS Institute Inc., 1998, Cary, NC), and treatment differences were subjected to a Tukey's studentized range test or Duncan's multiple comparison test at the 5% probability. Variation within treatments was expressed as the standard error of the mean (SEM). The Pearson correlation coefficients and linear association between variables were determined using SAS REG model. A significant linear relationship between two variables was determined at $P<0.05$ level.

RESULTS

Body Weight, Feed Intake and Bone Measurements

The dietary treatment did not affect the body weight and feed consumption (data not shown) during the whole feeding experimental period (Table 3). Likewise, long-term dietary lipid supplementation had no influence on tibial bone length, diameter or weight. However, the percentage of ash in the femur was significantly higher from quail fed the FO diet than those from other treatment groups. In addition, birds from the HSBO treatment group also showed greater ash levels compared with those fed the CF diet.

Collagen and Pyridinium Crosslinks

There were significant effects of dietary lipids on tibia collagen as indicated by the content of tibial hydroxyproline ($P=0.0018$). Quails fed a diet supplemented with 5 % SBO had a hgher content of tibial hydroxyproline than those from quail fed the HSBO or CF diet, but there was no difference from quail fed FO (Table 4).

Long –term supplementation of either FO or HSBO in the diet significantly increased the tibial concentrations of both pyridinoline and deoxypyridinoline ($P<0.05$). The tibia from birds fed the SBO diet had the lowest content of pyridinoline and those fed CF had the lowest level of deoxypyridinoline. For total crosslinks, there were no differences between birds fed SBO and CF, or between those from birds fed FO and HSBO.

Bone Marrow Supernatants and Tissue ex vivo Prostaglandin E_2 Production

Quail consuming the SBO or CF enriched diets had dramatically higher concentration of PGE_2 in bone marrow supernatant and *ex vivo* PGE_2 production in bone organ cultures compared with those given FO and HSBO at eight months of age (Table 5). In addition, birds given SBO had significantly higher levels of PGE_2 in both

measurements compared with birds fed CF, and similarly, birds consuming HSBO showed a significantly higher concentration of PGE_2 in the bone marrow supernatant, but no difference in bone organ cultures, compared to those fed the FO diet.

Correlations between PGE_2 and Bone Matrix Parameters

Correlations were calculated between PGE_2 and parameters of bone matrix, specifically ash content, pyridinoline, deoxypyridinoline, total pyridinium and bone collagen (Table 6). These results showed that both bone marrow and bone tissue PGE_2 were negatively correlated with these bone parameters, except for collagen. Particularly, PGE_2 was negatively correlated with ash, pyridinoline and total crosslinks (r= -0.38– -0.50; P=0.015-0.001) (Table 6, Fig. 1-4). The data also shows that bone PGE_2 had a positive correlation with collagen (r=0.34; P=0.03) (Table 6, Fig. 5-6). In addition, bone ash was positively correlated with all other matrix parameters. The order of correlation of ash with other matrix parameters were total pyridinium > deoxypyridinoline > pyridinoline > hydroxyproline.

DISCUSSION

The conclusions of the present study have confirmed and extended those of our previous study (Chapter three). Our previous findings demonstrated that long-term supplementation of dietary SBO or CF significantly modified fatty acid composition, decreased the percentage of bone ash and bone strength of mature bones in Japanese quail compared to those given either FO or HSBO diets. The objectives of this study were to further investigate the biochemical and molecular basis related to the alterations in biomechanical properties of tibial bones as a result of long-term dietary lipid supplementation.

The results of this study demonstrated that different dietary lipids significantly influenced the bone prostaglandin production and bone metabolism. The assessment of correlations between PGE_2 production and parameters of bone formation and degradation strongly suggested that this prostaglandin, derived from 20:4(n-6) fatty acid, is involved in bone metabolism. There were not any treatment differences in body weight, feed intake, tibial weight, length or diameter during the feeding experimental period. These results were consistent with our previous study (Chapter 3), and in agreement with those of others using similar dietary treatments in rats (Kokkinos *et al*., 1993) and chicks (Watkins *et al*., 1996, 1997). However, the percentage of tibial ash was the highest in quails fed FO, and it is the lowest in quails fed CF. This observation is consistent with our previous study (Chapter three) and those obtained by Claassen *et al*,. (1995) and Kruger *et al*. (1997). Watkins et al. (1996, 1997) reported that there were no difference in percent bone ash between chicks fed dietary SBO and those given other dietary lipids either at 21 or 42 days of age. The current result, therefore, seems to be that the period of dietary lipid treatment needs to be prolonged to cause any significant effect on percent bone ash.

Tibial bone matrix analysis revealed that long-term dietary lipid intake caused changes in bone formation and resorption activity. The collagen concentration, as represented by hydroxyproline level, was significantly higher in quail fed SBO compared

with that from quail given HSBO or CF, but was not different from birds consuming FO. However, pyridinium crosslinks in tibiae were greatly decreased in quail fed either SBO or CF, compared to those given FO or HSBO. Approximately 90% of the organic matrix of bone is type I collagen (Seyedin and Rosen, 1990). Mature type I collagen in bone is cross-linked by pyridinoline and deoxypyridinoline, specific molecules which have a strong and positive correlation with mechanical strength in both normal and osteoporotic bones (Batge *et al*., 1992; Oxlund *et al*., 1995, 1996; Knott *et al*., 1995; Rath *et al*., 1999). Similarly, bone collagen also contributes to bone strength, and a decrease in bone collagen content results in a decrease in mechanical strength (Masse *et al*., 1996; Bailey *et al*., 1999).

Mature bone is constantly undergoing formation and resorption, a process called remodeling, which is required for body calcium homeostasis and overall bone health. Pyridinium crosslinks are released into circulation during the bone resorption process and excreted in the urine (Delmas *et al*., 1991). Therefore, the urinary concentrations of pyridinium crosslinks have been considered as suitable markers of bone resorption and a wide range of bone disorders (Borisova *et al*., 1994; Robins, 1994; Marowska *et al*., 1996; Takahashi et al., 1996). Watkins *et al*. (1996, 1997) reported that SBO significantly decreased the bone formation rate in various ages of growing chicks compared with those in chicks given either FO or saturated fat. Other studies have demonstrated that varying dietary ratios of essential fatty acids influenced urinary collagen crosslink excretion (Claassen *et al*., 1995; Kruger *et al*., 1997). In this study, although hydroxyproline content in quail fed SBO was increased by 6.5%-10.3% compared to other groups, the total crosslink levels in this group, and in CF group as well, was decreased by 24%-28% in average compared to those in the FO and HSBO groups. Therefore, the greater hydroxyproline concentration, lower crosslinks, along with the decreased mineral mass observed in these groups is indicative of a mineralization defect (Peterson *et al*., 1995). These findings are consistent with and support our previous observations that long-term supplementation of the diets with either SBO or CF remarkably decreased the tibial strength in quail compared to those fed the FO or HSBO diets. The results of this study further demonstrated that collagen crosslinks play an important role in bone strength, and impairment of crosslink formation lead to the reduction of mechanical strength even if collagen level is similar, as seen in quail fed SBO and CF. As far as we are aware, no study has been published showing the effects of dietary lipid intake on the concentration of the collagen crosslinks pyridinoline and deoxypyridinoline in bone.

The difference in crosslinks may be explained by changes in bone resorptive activities under various dietary conditions. In the present study, *ex vivo* PGE_2 production in the tibial bones of quail fed SBO or CF were 1.0 and 1.6 times higher than in quail fed FO, respectively, and 1.6 and 2.2 times that in quail given HSBO. EPA (20:5n-3) is a very poor substrate for cyclooxygenase (Hwang, *et al*., 1992), and both EPA and docosahexaenoic acid (DHA; 22:6n-3) competitively inhibit the oxygenation of AA by cyclooxygenase for PGE_2 formation (Sardesai, 1992). The concentration of AA (20:4n-6) was much higher in the tibial bones of quail given either SBO or CF, whereas the levels of EPA and DHA were much higher in quail fed FO (Chapter three). Trans-18:1 fatty acids was shown to compete with linoleic acid (18:2n-6) for the Δ^6 desaturase (Emken, 1984) and inhibit this enzyme activity(Kirstein, 1983), which catalyzes the

initial rate limiting desaturation step for conversion of linoleic acid to the longer chain n-6 fatty acids. Studies indicated that feeding of trans-fatty acids to animals suppressed the AA synthesis in various tissues (Watkins, 1991; Bysted, 1998). Therefore, although anti-PGE_2 antibody has 43% cross-reactivity against PGE_3, the decreases in PGE_2 productions in the groups fed FO and HSBO diets are mainly attributable to depressed PGE_2 biosynthesis. This result was in agreement with the observations obtained by others (Kokkinos *et al*., 1993; Watkins *et al*., 1996, 1997). *In vitro*, PGE_2 shows biphasic effects on bone formation, stimulating bone formation at low concentrations, and enhancing resorption at high concentrations (Marks and Miller, 1993; Raisz *et al*., 1993). Therefore, long-term supplementation of SBO or CF in the diets may elevate PGE_2 production to a level that could have stimulated an increase in bone resorptive activities, which leads to degradation of bone collagen crosslinks.

The correlation analysis further demonstrated that endogenously produced PGE_2, as measured in bone marrow, and *ex vivo* PGE_2 biosynthesis of bone tissues, had negative correlations with bone pyridinium crosslinks and ash content. However, they were positively correlated to bone collagen content. To our knowledge, this is the first study that the relationships between endogenous PGE_2 production in the bone microenvironment and bone metabolism have been demonstrated. We also found that lower levels of crosslinks were concomitant with a higher content of collagen in the SBO fed group. In addition, it seems that bone ash content in aged bones was independent of its collagen concentration but highly dependent on its crosslinks under this dietary condition. These results further confirmed the above conclusions that a specific high level of local PGE_2 production in a long-term may stimulate the bone resorption activities, which can eventually result in bone loss and osteoporosis. Furthermore, the results suggest that bone crosslink status more accurately reflected the bone metabolism changes than collagen under this dietary condition. Other studies, however, reported that bone resorption is an event in which both collagen and its crosslinks are degraded (Aderssens *et al*., 1993; Claassen *et al*., 1995). Therefore, the changes in bone crosslinks other than collagen in quail fed SBO and CF may also involve a resorption-independent mechanism. Possibly, dietary lipids may also influence the crosslink formation.

A recent study showed that PGE_2 at 100 nM notably suppressed the lysyl oxidase synthesis and this downregulation was at the transcriptional level (Roy *et al*., 1996). Briefly, crosslinks are formed by the enzymatic action of lysyl oxidase, which catalyzes deamination of amino acid lysine and hydroxylysine to form aldehydes that eventually form mature pyridinium links (Seibel *et al*., 1992; Oxlund *et al*., 1995; Knott and Bailey, 1998). Any inhibition of lysyl oxidase leads to the impairment of crosslink formation and decrease in bone strength (Farquharson *et al*., 1989; Wilmarth *et al*., 1992; Jonas and Froines *et al*., 1993; Masse, *et al*., 1996). Therefore, influence of dietary SBO and CF on collagen crosslink concentration may be partially mediated by enzyme-dependent pathways involving PGE_2. From this point of view, high levels of PGE_2 decrease the collagen pyridinium cosslinks in bone, probably by stimulating bone resorption and inhibiting the corsslink formation simultaneously. This hypothesis needs to be confirmed by more detailed investigations.

In conclusion, the present investigation demonstrated that long-term dietary lipids altered the aged bone ash content, collagen, and pyridinium crosslink levels. Significant decreases in the amounts of bone mineral and collagen crosslinks in quail fed SBO or CF

were observed compared to those consuming FO or HSBO. Consequently, PGE_2 levels in bone microenvironment were greatly elevated in the SBO or CF fed group. Notably, the percent ash and crosslinks were negatively correlated to either bone or marrow PGE_2, suggesting that high local PGE_2 production was associated with increased bone resorption. In addition, it appears from these results and from others that dietary lipids may influence lysyl oxidase synthesis indirectly by PGE_2 mediated pathway, and thus affect collagen crosslink formation. Studies designed to characterize the mechanism by which dietary lipids influence bone organic matrix will establish important relationships between dietary lipids and bone metabolism.

Table 1. Dietary ingredient composition of the quail basal diets[1]

	Dietary lipid treatment[2]			
Ingredients (%)	SBO	HSBO	CF	FO
Yellow corn	53.55	53.55	53.55	53.55
Soybean meal	32.00	32.00	32.00	32.00
Soybean oil	5.00	—	—	—
Hydrogenated soybean oil	—	5.00	—	—
Chicken fat	—	—	5.00	—
Menhaden oil	—	—	—	5.00
Limestone	6.50	6.50	6.50	6.50
Dicalcium phosphate	1.50	1.50	1.50	1.50
Vitamin Premix[3]	1.00	1.00	1.00	1.00
Trace mineral premix[4]	0.10	0.10	0.10	0.10
salt	0.35	0.35	0.35	0.35

[1] The diet was formulated to contain 21.0% crude protein, 2997.2-3007.2 kcal/kg metabolizable energy, 7.36% crude fat, 2.43% crude fiber, 2.81% calcium, and 0.67% phosphorous.

[2] Diets include soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF) or menhaden fish oil (FO) at 50g/kg of diet.

[3] Provided per kilogram of diet: vitamin A, 1100 IU; vitamin D_3, 330 IU; vitamin E, 33 IU; menadione sodium bisulfite, 3.52 mg; thiamine HCL, 1.1 mg; riboflavin, 6.6 mg; calcium D-pantothenate, 16.5 mg; niacin, 44 mg; choline chloride, 374 mg; vitamin B_{12}, 0.0165 mg; folic acid, 1.1 mg; pyridoxine HCL, 1.1 mg; biotin, 0.055 mg; Bacitracin, 22.0 mg; selenium, 0.20 mg; ethoxyquin, 0.124 mg.

[4] Provided per kilogram of diet: manganese, 44mg; zinc, 47.5 mg; iron, 50.0 mg; copper, 6.25mg; iodine, 2.0 mg; and selenium, 0.3 mg.

Table 2. Fatty acid composition (μg/100 μg) of the quail basal diet

	Dietary lipid treatment[1]			
Fatty acids	SBO	HSBO	CF	FO
14:0	0.06	0.19	0.57	5.29
15:0	0.02	0.04	0.09	0.42
16:0	9.95	11.26	15.01	15.45
16:1(n-7)	0.08	0.15	2.30	6.95
18:0	3.54	11.11	6.53	3.26
t18:1	0.05	25.49	2.57	1.45
18:1	25.82	30.76	39.50	15.07
18:2(n-6)	54.79	18.11	30.00	19.16
18:3(n-3)	4.33	0.98	1.54	2.32
20:0	0.30	0.34	0.23	0.20
20:3(n-3)	ND[5]	ND	0.05	0.11
20:4(n-6)	0.01	0.01	0.16	0.51
20:5(n-3)	0.01	ND	0.02	18.49
22:1	ND	ND	0.03	0.24
22:4(n-6)	0.01	0.01	0.03	0.09
22:5(n-3)	0.02	0.03	0.02	1.47
22:6(n-3)	ND	ND	0.01	7.47
SAT[2]	13.87	22.94	22.42	24.62
MONU[3]	25.95	56.42	44.43	23.91
PUFA[4]	59.18	19.15	31.82	49.61
(n-6) PUFA	54.81	18.13	30.19	19.75
(n-3)PUFA	4.37	1.02	1.63	29.86
(n-6)/(n-3)	12.55	17.85	18.47	0.66

[1] Dietary treatments include soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat, and menhaden fish oil (FO) at 50 g/kg of diet.

[2] SAT, total saturated fatty acids.

[3] MONU, total monounsaturated fatty acids.

[4] PUFA, total polyunsaturated fatty acids.

[5] ND, not detected.

Table 3. Body weight and tibial measurements of Japanese quail fed different lipids[1]

	Dietary lipid treatment[2]				
Measurements	SBO	HSBO	CF	FO	P Values
Body weight (g)	107.0±2.5	109.7±2.2	105.6±1.8	106.0±1.8	0.5024
Tibial length (mm)	45.3±0.7	44.4±0.5	44.0±0.5	44.0±0.2	0.2544
Tibial diameter(mm)	2.18±0.04	2.27±0.08	2.14±0.04	2.18±0.05	0.3366
Tibial weight (mg)	420.0±12.6	415.5±11.3	403.6±12.0	414.5±6.8	0.7507
Tibia ash (%)	41.78±0.54^{bc}	42.83±0.71^{b}	40.70±0.50^{c}	44.73±0.69^{a}	0.0003

[1] n=11 for all measurements. Mean values (means±SEM) within rows having different superscripts are significantly different ($P<0.05$).

[2] Dietary lipids treatments included soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF), and menhaden oil at 50 g/kg of the diet. All day old quail were fed standard diets for 4 weeks and then randomly divided into 4 experimental groups and fed above experimental diets for 7 months before being sacrified.

Table 4. Tibial collagen and crosslinks in eight-month-old quail fed different dietary lipids[1]

Measurements	Dietary lipid treatment[2]				
	SBO	HSBO	CF	FO	P Values
Hydroxyproline (μg/mg tibia)[*]	13.83±0.28[a]	12.64±0.28[b]	12.54±0.19[b]	12.98±0.20[ab]	0.0018
Collagen Crosslinks:					
Pyridinoline (nmol/mg HP)[ξ]	1.024±0.138[b]	1.495±0.050[a]	1.345±0.107[ab]	1.509±0.071[a]	0.0035
Deoxypyridinoline (nmol/mg HP)	1.637±0.125[ab]	1.805±0.142[a]	1.235±0.081[b]	1.776±0.199[a]	0.0277
Total Crosslinks: (nmol/mg HP)	2.658±0.146[b]	3.298±0.153[a]	2.577±0.140[b]	3.285±0.217[a]	0.0030

[1] n=11 for all measurement, mean values (means±SEM) within rows having different superscripts are significantly different (P<0.05).

[2] Dietary lipids treatments included soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF), and menhaden oil at 50 g/kg of the diet. All day old quail were fed standard diets for 4 weeks and then randomly divided into 4 experimental groups and fed above experimental diets for 7 months before being sacrified.

[*] Tibia weight is based on the wet weight of bone.

[ξ] HP denotes hydroxyproline.

Table 5. Tibial marrow supernatant and bone *ex vivo* PGE_2 production in eight-month-old quail fed different lipids[1]

Measurements	Dietary lipid treatment[2]				
	SBO	HSBO	CF	FO	P values
Bone marrow PGE_2 (ng/ml)	1.73 ± 0.05^{a}	1.06 ± 0.04^{c}	1.43 ± 0.05^{b}	0.87 ± 0.07^{d}	0.0001
Tibial Bone PGE_2 (ng/g bone)*	69.27 ± 3.16^{a}	31.30 ± 2.06^{c}	50.56 ± 1.84^{b}	26.14 ± 2.12^{c}	0.0001

[1] n=11 for all measurement, mean values (means±SEM) within rows having different superscripts are significantly different ($P<0.05$).

[2] Dietary lipids treatments included soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF), and menhaden oil at 50 g/kg of the diet. All day old quail were fed standard diets for 4 weeks and then randomly divided into 4 experimental groups and fed above experimental diets for 7 months before being sacrified.

* Tibia weight is based on the wet weight of bone.

Table 6. Correlation coefficiencts among bone ash, collagen, cross-links and PGE_2 in eight-month-old quail fed different dietary lipids [1]

	Ash	P values	D-links[2]	P values	P-links[2]	P values	T-pyr[2]	P values	HP	P values
Marrow PGE_2	-0.44	0.004	-0.15	0.356	-0.50	0.001	-0.49	0.002	0.28	0.078
Bone PGE_2	-0.38	0.015	-0.18	0.279	-0.43	0.006	-0.45	0.003	0.34	0.033
Ash	1.000		0.37	0.015	0.19	0.223	0.41	0.006	0.02	0.930

[1] Dietary lipids treatments included soybean oil (SBO), hydrogenated soybean oil (HSBO), chicken fat (CF), and menhaden oil at 50 g/kg of the diet. All day old quail were fed standard diets for 4 weeks and then randomly divided into 4 experimental groups and fed above experimental diets for 7 months before being sacrified.

[2] D-links: Deoxypyridinoline; P-links: Pyridinoline; T-pyr: Total pyridinium; HP: hydroxyproline.

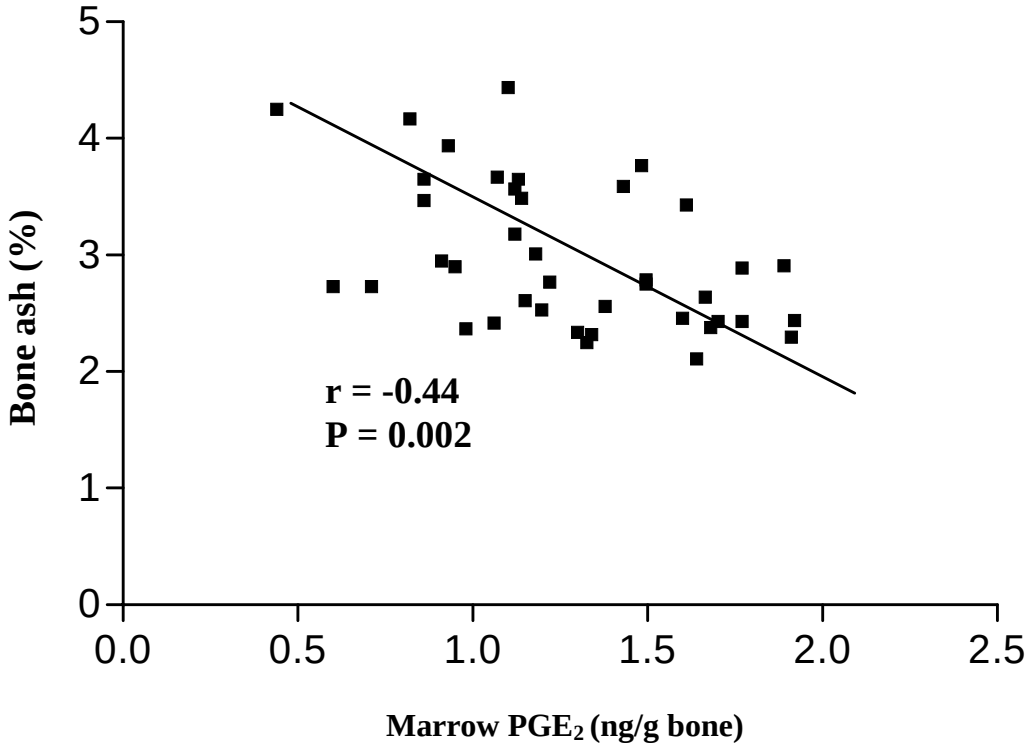

Fig. 1. Correlation between marrow PGE_2 and percent bone ash.

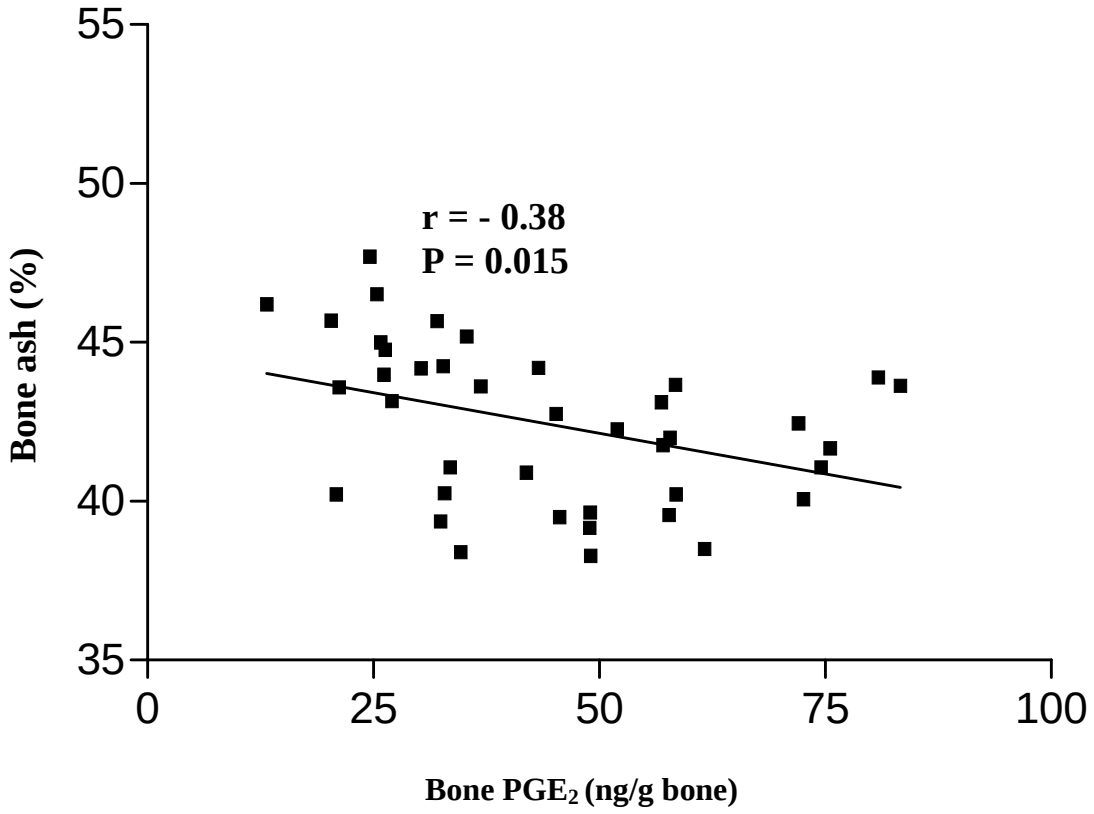

Fig. 2. Correlation between bone PGE_2 and percent bone ash.

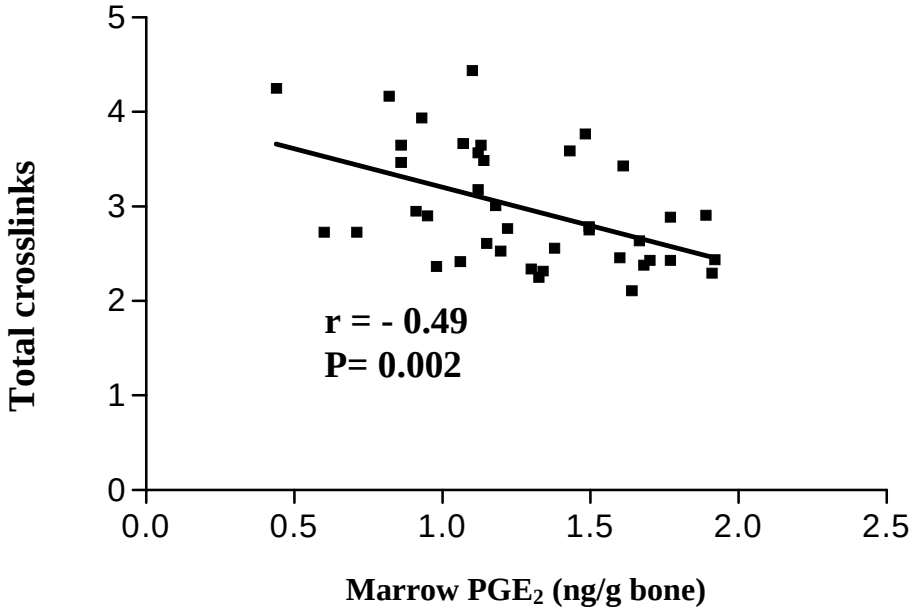

Fig. 3. Correlation between marrow PGE_2 and total crosslinks.

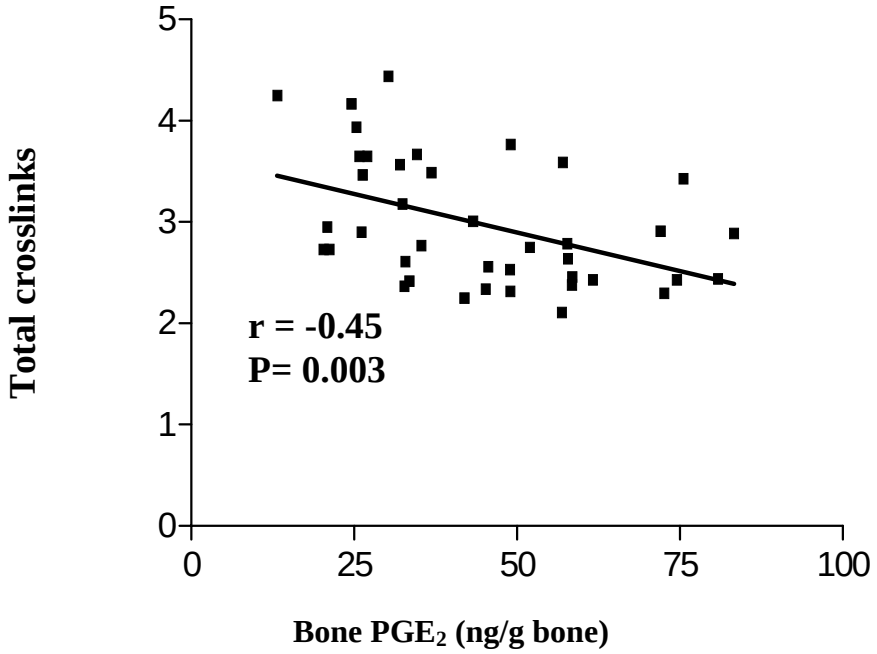

Fig. 4. Correlation between bone PGE_2 and total crosslinks.

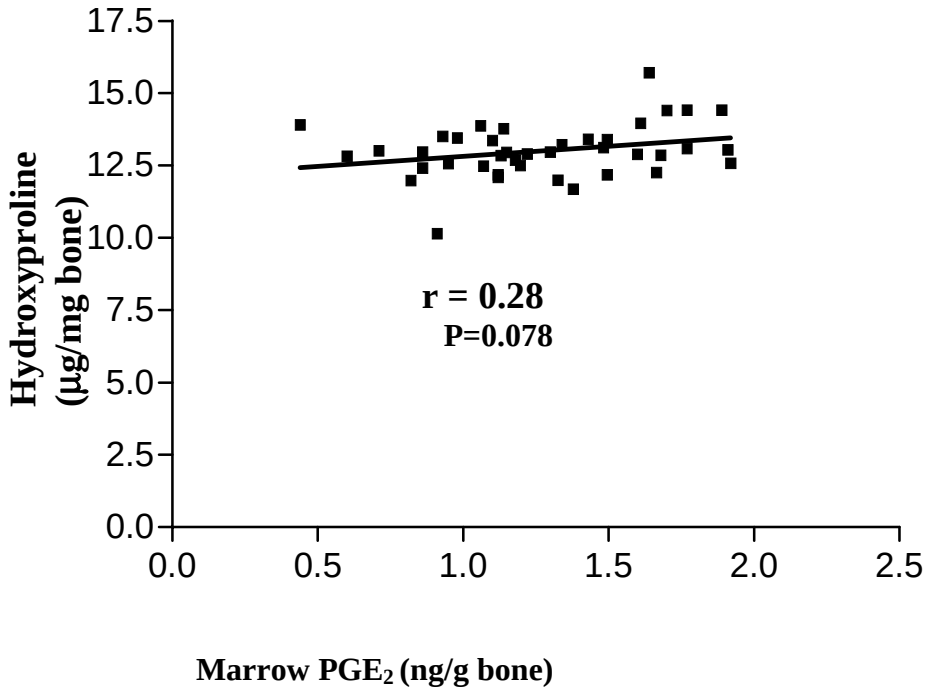

Fig. 5. Correlation between marrow PGE_2 and tibial hydroxyproline.

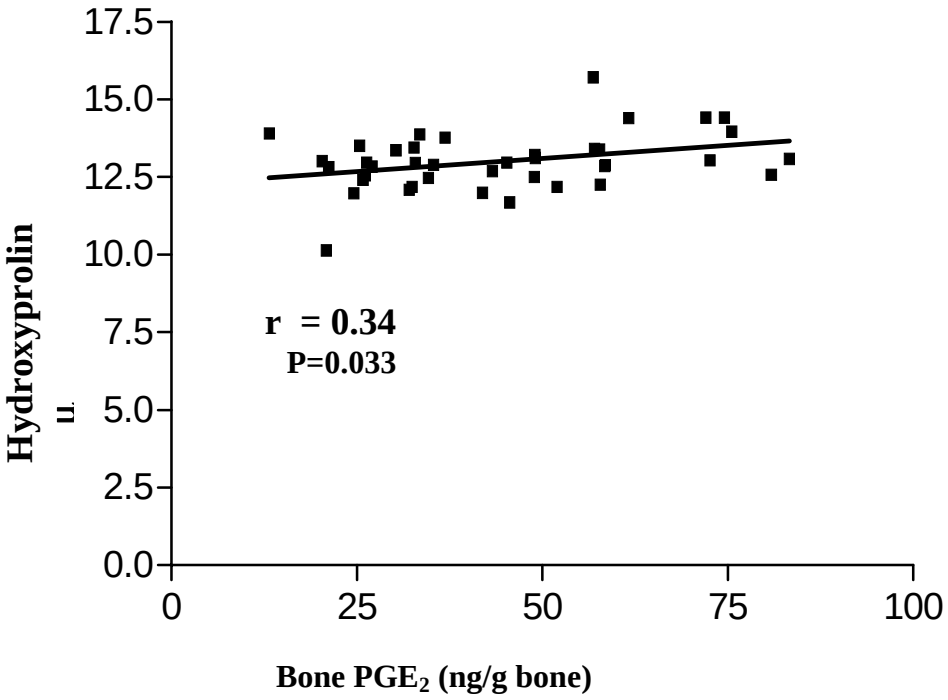

Fig. 6. Correlation between bone PGE_2 and tibial hydroxyproline.

REFERENCES

Aerssens, J. R. Van Audekercke, P. Geusens, L. P. C. Schot, A. A-H. Osman, 1993. Mechanical properties, bone mineral content, and bone composition (collagen, osteocalcin, IGF-I) of the rat femur: influence of ovariectomy and namdrolone decanoate (anabolic steroid) treatment. Calcif. Tisuse Int. 53:269-277.

Bailey, A. J., T. J. Sims, E. N. Ebbesen, J. P. Mansell, J. S. Thomsen, and L. Mosekilde, 1999. Age-related changes in the biochemical properties of human cancellous bone collagen: relationship to bone strength. Calcif. Tissue Int. 65:203-210.

Batge, B., J. Diebold, H. Stein, M. Bodo, and P. K. Muller, 1992. Compositional analysis of the collagenous bone matrix. A study on adult normal and osteopenic bone tissue. Eur. J. Clin. Invest. 22:805-812.

Borisova, N. V., A. Ya. Pokrovskaya, E. Yu. Zakharova, and K.D. Krasnopol'Skaya, 1994. Analysis of collagen hydroxypyridinium crosslinks in samples of tissue and urine of patients with inheritedd connective tissue disorders. Connective Tissue. Res. 30:177-190.

Boskey, A. L., T. M. Wright, and R. D. Blank, 1999. Collagen and bone strength. J. Bone Miner. Res. 14:330-335.

Bysted, A., G. HΦlmer, and P. Lund, 1998. Influence of moderate amounts of trans fatty acids on the formation of polyunsaturated fatty acids. JAOCS 75:225-234.

Claassen, N., H. C. Potgieter, M. Seppa, W. J. H. Vermaak, H. Coetzer, D. H. Van Papendorp, and M. C. Kruger, 1995. Supplemented gamma-linoleic acid and eicosapentaenoic acid influnence bone status in young male rats: effects on free urinary collagen crosslinks, total urinary hydroxyproline, and bone calcium content. Bone 16 (Suppl. 4):385S-392S.

Crenshaw, T. D., 1986. Reliability of dietary calcium and phosphorous levels and bone mineral content as predictors of bone mechanical properties at various time of periods in growing swine. J. Nutr. 116:2155-2170.

Currey, J. D., 1988. The effect of porosity and mineral content on the Young's modulus of elasticity of compact bone. J. Biomech. 21:131-139.

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

Delmas, P. D., E. Gineyts, A. Bertholin, P. Garnero, and F. Marchand, 1993. Immunoassay of pyridinoline crosslink excretion in normal adults and in paget's disease. J. Bone Miner. Res. 8:643-648.

Delmas, P. D., A. Schlemmer, E. Gineyts, B. Riis, and C. Christiansen, 1991. Urinary excretion of pyridinoline crosslinks correlate with bone turnover measured on iliac crest biopsy in patients with vertebral osteoporosis. J. Bone Miner. Res. 6:639-644.

Emken, E. A., 1984. Nutrition and biochemistry of trans and positional fatty acid isomers in hydrogenated oils. Ann. Rev. Nutr. 4:339-376.

Fall, P. M., D. T. Breault, and L. G. Raisz, 1994. Inhibition of collagen synthesis by prostaglandins in the immortalized rat osteoblastic cell line pyla: structure-activity relations and signal transduction mechanisms. J. Bone Miner. Res. 9:1935-2943.

Farquharson, C., A. Duncan, and S. Robins, 1989. The effects of copper deficiency on the pyridinium crosslinks of mature collagen in the rat skeleton and cardiovascular system. P. S. E. B.M. 192:166-171.

Hwang, Y. S., P. E. Wainwright, P. R. Redden, D. E. Mills, B. B. Fleming, and D. F. Horrobin, 1992. Effects of maternal dietary fats with variable n-3/n-6 ratios on tissue fatty acid composition in sucking mice. Lipids 27:104:110.

Jonas, J., J. Burns, E. W. Abel, M. J. Cresswell, J. J. Strain, and C. R. Paterson, 1993. Impaired mechanical strength of bone in experimental copper deficiency. Ann. Nutr. Metab. 37:245-252.

Kirstein, D., C. E. HΦy, and G. HΦlmer, 1983. Effects of dietary fats on the Δ6- and Δ5-desaturation of fatty acids in rat liver microsomes. Br. J. Nutr. 50:749-756.

Knott, L., and A. J. Bailey, 1998. Collagen cross-links in mineralizing tissues: a review of their chemistry, function, and clinical relevance. Bone 22:181-187.

Knott, L., C. C. Whitehead, R. H. Fleming, and Aaa. J. Bailey, 1995. Biochemical changes in the collagenous matrix of osteoporotic avian bone. Biochem. J. 310:1045-1051.

Kokkinos P. P., R. Shaye, B. S. Alam, and S. Q. Alam, 1993. Dietary lipids prostaglandin E_2 levels, and tooth movement in alveolar bone of rats. Calcif. Tissue Int. 53:333-337.

Kruger, M. C., N. Claassen, C. M. Smuts, and H. C. Potgieter, 1997. Correlation between essential fatty acids and parameters of bone formation and degradation. Asia Pacific J. Clin. Nutr. 6:235-238.

Landis, W. J., 1995. The strength of a calcified tissue depends in part on the molecular structure and organization of its constituent mineral crystals in their organic matrix. Bone 16:533-544.

Logan, N., 1950. Determination of hydroxyproline. J. Bio. Chem. 184:299-306.

Marks, S. C. Jr., and S. C. Miller, 1993. Prostaglandins and skeleton: The legacy and challenges of two decades of research. Endocrine. J. 1:337-344.

Marowska, J., M. Kobylinska, J. Lukassszіewicz, A. Talajko, B. Rymkiewicz-Kluczynska, and R. S. Lorenc, 1996. Pyridinium crosslinks of collagen as a marker of bone resorption rates in children and adolescents: normal values and clinical application. Bone 19:669-677.

Martin, R. B., and D. L. Boardman, 1993. The effects of collagen fiber orientation, porosity, density, and mineralization on bovine cortical bone bending properties. J. Biomech. 26:1047-1054.

Martin, R. B., and J. Ishida, 1989. The relative effects of collagen fiber orientation, porosity, and mineralization on bone strength. J. Biomech. 22:419-426.

Masse, P. G., C. M. Rimnac, M. Yamauchi, S. P. Coburn, R. B. Rucker, D. S. Howell, and A. L. Boskey, 1996. Pyridoxine deficiency affects biomechanical properties of chick tibial bone. Bone 18:567-574.

Miyaura, C., K. Kusano, T. Masuzawa, O. Chaki, Y. Onoe, M. Aoyag, T. Sasaki, T. Tamura, Y. Koishihara, Y. Ohsugi, and T. Suda, 1995. Endogenous bone-resorbing factors in estrogen deficiency: cooperative effects of IL-1 and IL-6. J. Bone Miner. Res. 10:1365-1373.

Monnier, V. M., V. Vishwanath, K .E. Frank, C. A. Elmets, P. Dauchot, and R. R. Kohn, 1986. Relation beteen complications of type I diabetes mellitus and collagen-linked fluoresence. N. Eng. J. Med. 314:403-408.

National Research Council, 1994. Nutrient requirements of poultry. 9th rev. ed. National Academic Press, Washington DC.

Oxlund, H., Li. Mmosekilde, and G. Ørtoft, 1996. Reduced concentration of collagen reducible cross-links in human trabecular bone with respect to age and osteoporosis. Bone 19:479-484.

Oxlund, H., M. Barckman, G. Ørtoft, and T. T. Andreassen, 1995. Reduced concentrations of collagen cross-links are associated with reduced strength of bone. Bone 17 (Suppl):365S-371S.

Peterson, C. A, A. C. Eurell, W. K. Keith, and J. W. Erdman, 1995. Bone composition and histological analysis of young and aged rats fed diets of varieed calcium bioavailability. J. Am. Coll. Nutr. 14:278-285.

Raisz, L. G., C. C. Pilbeam, and P. M. Fall, 1993b. Prostaglandins: Mechanisms of action and regulation of production in bone. Osteoporosis Int. 3 (Suppl.1):S136-S140.

Rath, N. C., J. M. Balog, W. E. Huff, G. R. Huff, G. B. Kukarni, and J. F. Tierce, 1999. Comparative differences in the composition and biomechanical properties of tibiae of seven- and seventy-two-week-old male and female broiler breeder chickens. Poultry Sci. 78:1232-1239.

Robins, S. P., 1994. Biochemical markers for assessing skeletal growth. Eur. J. Clin. Nutr. 48(Suppl.1):S199-S209.

Roy, R., P. Polgar, Y. Wang, R. H. Goldstein, L. Taylor, and H. M. Kagan, 1996. Regulation of lysyl oxidase and cyclooxygenase expression in human lung fibroblasts: interactions among TGF-beta, IL-I beta, and prostaglandin E. J. Cell Biochem. 62:411-417.

Sakaguchi, K., I. Morita, and S. Murota, 1994. Eicosapentaenoic acid inhibits bone loss due to ovariectomy in rats. Prostaglandin Leukot. Essent. Fatty Acids. 50:81-84.

Sardesai, V. W., 1992. Biochemical and nutritional aspects of eicosanoids. J. Nutr. Biochem. 3:562-579.

Seibel, M. J., S. P. Robins, and J. P. Bilezikian, 1992. Urinary crosslinks of collagen: specific markers of bone resorption in metabolic bone disease. Trends Endo. Metab. 3:263-270.

Seyedin, S. M., and D. M. Rosen, 1990. Matrix proteins of the skeleton. Curr. Opin. Cell Biol. 2:914-919.

Shah, K. M., J. C. Goh, R. Karunanithy, S. L. Low, D .S. Das, and K. Bose, 1995. Effect of decalcification on bone mineral content and bending strength of feline femur. Calcif. Tissue Int. 56:78-82.

Takahashi, M., K. Kushida, H. Hoshino, M. Suzuki, M. Sano, S. Miyamoto, and T. Inoue, 1996. Concentrations of pyridinoline and deoxypyridinoline in joint tissues from patients with osteoarthritis or rheumatoid arthritis. Ann. Rheum. Dis. 55:324-327.

Tsuchiya, H., and C. J. Bates, 1997. Vitamin C and copper interactions in quinea-pigs and a study of collagen cross-links. Br. J. Nutr. 77:315-325.

Tsuchiya, H., and C. J. Bates, 1998. Changes in collagen cross-link ratios in bone and urine of guinea pigs fed graded dietary vitamin C: a functional index of vitamin C status. J. Nutr. Biochem. 9:402-407.

Verhaeghe, J., A. M. H. Suiker, T. A. Einhorn, P. Geusens, W. J. Visser, E. Van Herck, R. Van Bree, S. Magitsky, and R. Bouillon, 1994. Brittle bones in spontaneously diabetic female rats cannot be predicted by bone mineral measurements: studies in diabetic and ovariectomized rats. J. Bone Miner. Res. 9:1657-1667.

Watkins, B. A, C-L, Shen, K. G. D. Allen, and M .F. Seifert, 1996. Dietary (n-3) and (n-6) polyunsaturates and acetylsalicylic acid alter ex vivo PGE_2 biosynthesis, tissue IGF-I levels, and bone morphometry in chicks. J. Bone Miner. Res. 11:1321-1332.

Watkins, B. A., C-L. Chen, J. P. McMurthy, H. Xu, and S. D. Bain, 1997. Dietary lipids modulate bone prostaglandin E_2 production, insulin-like growth factor-I concentration and formation rate in chicks. J. Nutr. 127:1084-1091.

Watkins, B. A., C. C. Whitehead, and R. L. Duff, 1991. Hydrogenated oil decreases tissue concentrations of n-6 polyunsaturated fatty acids and may contribute to dyschondroplasia in broilers. Br. Poultry Sci. 32:1109-1119.

Wilmarth, K. R., and J. R. Froines, 1992. In vitro and in vivo inhibition of lysyl oxidase by aminopropionitriles. J. Toxicol. Environ. Health. 37:411-423.

SUMMARY

Japanese quail were used as animal models in four experiments to evaluate the effects of supplementing diets with different lipids on bone chemical, mechanical and histological properties. In the first and second study (Chapters one and two), the effects of maternal dietary lipids on bone fatty acid composition, *ex vivo* PGE_2 production, bone collagen, crosslinks, as well as mechanical and histological properties were investigated. In the other two studies (Chapters three and four), long-term effects of supplementing different lipids in the diets on mature bone metabolism and mechanical properties were determined.

The data from these studies demonstrated that dietary lipids significantly modified the fatty acid composition of lipids in the bone tissue. The addition of soybean oil (SBO) in the maternal diet increased the levels of total n-6 fatty acids and arachidonic acid (AA, 20:4n-6) in tibial bones of newly hatched progeny, whereas the maternal menhaden fish oil (FO) diet elevated the concentrations of total n-3 fatty acids, eicosapentaenoic acid (EPA, 22:5n-3), docosahexaenoic acid (DHA, 22:6n-3) and total saturated acid, but greatly decreased the amount of AA in progeny tibiae. The maternal HSBO diet resulted in the accumulation of t18:1 in the tibiae at hatch. This effect, however, diminished with age and had disappeared by two weeks of age. In mature quail with long-term supplementing of different lipids in the diet, dramatic results were reproduced similar to those obtained in progeny from hens fed the different dietary lipids. It was also observed that dietary SBO or chicken fat (CF) elevated the prostaglandin E_2 (PGE_2) production in bone organ cultures in day old progeny, and both in bone and marrow in aged quail.

The mechanical and histological studies demonstrated that both maternal dietary lipids and long-term supplementation in the diet of mature quail had the similar consequences on bone properties. In general, addition of FO or HSBO in the diets had beneficial effects on bone by increasing mineral content and strength in progeny and aged quail compared to SBO and CF. Consistent with these findings, It was further observed that maternal dietary SBO and CF narrowed cartilage growth plate and hypertrophic zone in embryonic tibiae and variably decreased the cortical density and thickness in various areas in embryos and early postnatal quail. When supplemented in mature quail diet in a long term, SBO and CF decreased the cortical bone density and thickness compared to FO and HSBO.

The present investigation further demonstrated that different lipids, either in the maternal diets or supplemented in mature quail diets, altered the collagen pyridinium crosslink levels in the tibiae of progeny and aged quail. Significant decreases in the amounts of bone collagen crosslinks in quail fed SBO or CF were observed compared to those consuming FO or HSBO. The percent ash and collagen crosslinks were negatively correlated to PGE_2 production in bone microenvironment. Taken all together, the studies indicate that supplementing FO or HSBO in maternal diets or in mature quail diets at a moderate level has beneficial effects on bone growth and development in embryos, and on bone metabolism and health in mature quail. Furthermore, these results suggest that one of the mechanisms by which dietary lipids affect bone modeling of progeny and metabolism in mature quail, at least in part, by modulating the production of local regulatory factor such as PGE_2. It is very likely that excessive local PGE_2 production was associated with increased bone resorption. However, more investigations are

needed concerning the pathways by which dietary lipids act on bone and their practical application in poultry industry or human health.

It has been observed that bone crosslink concentration was significantly decreased without concomitant decrease in collagen level in the SBO and CF groups, and that pyridinium crosslink levels had a negative correlation with bone and marrow PGE_2. A recent study showed that PGE_2 at 100 nM suppressed lysyl oxidase (enzyme catalyzing bone pyridinium crosslink formation) synthesis and gene expression in human lung fibroblasts (Roy *et al*., 1996). It is therefore hypothesized that dietary lipids may also regulate the lysyl oxidase synthesis by a PGE_2 mediated pathway. If this is the case, it may open a new avenue of inquiry.

During the last few years, progress has been made toward the understanding of local regulation of bone remodeling, especially in relation to osteoporosis. Cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor (TNF) are powerful bone resorbing agents (Kimble *et al*., 1995; Miyaura *et al*., 1995). The secretions of these cytokines may be activated by PGE_2 and the levels of them are significantly increased in the bone microenvironment after menopause (Bismar *et al*., 1995). Investigations have demonstrated that excessive production of these cytokines in bone is closely associated with the pathophysiology of bone loss and can eventually lead to osteoporosis (Poli *et al*., 1994; Kimble *et al*., 1995; Pacifici, 1996; Ammann *et al*., 1997; Lorenzo *et al*., 1997). A large number of studies have indicated that dietary FO or n-3 fatty acids suppress these cytokine production in blood mononuclear cells of humans (Endres *et al*., 1989; Meydani *et al*., 1991; Caughey *et al*., 1996; Purasiri, 1997). In view of these pieces of evidence, it is suggested that there is a close interaction between n-3 fatty acids, PGE_2 cytokines and bone metabolism, and that the anabolic effect of n-3 fatty acid on bone may be explained by their suppressing the production of these bone resorbing cytokines through PGE_2 mediated pathway in skeletal tissue. This hypothesis, however, must be confirmed by further investigation.

This dissertation only focused on comparing the effects of different lipid sources on skeletal metabolism and health. Because n-3 fatty acids competitively inhibit the availability of n-6 fatty acids for metabolism and utilization (Sardesai, 1992), supplementation of FO alone may increase the requirement for n-6 fatty acid particularly in growing animals (Kruger *et al*., 1997). Although dietary FO alone stimulates bone formation in embryos and is beneficial for bone remodeling in mature quail, our experiment with growing quail showed that dietary supplementation of SBO is better on bone growth and development compared to FO. Therefore, an appropriate ratio of n-3/n-6 fatty acids may be more important regarding to the bone metabolism and health. One study suggested that the ratio of n-63/n-3 fatty acids is more efficient in modulating the amount of eicosanoid production than the absolute amount of fatty acids in the diet (Boudreau *et al*., 1991). Thus, more research is needed to assess the desirable ratio of n-6/n-3 fatty acids that has maximum beneficial effect on bone. In addition, for poultry producers to use these lipids in their diets, it must be cost effective for them to add these products. Therefore, efforts are needed to investigate the acceptable level of these lipids in the diets for meeting both the economic benefit and animal welfare concern.

In conclusion, the present studies are focused on a recently developed area of nutrition and bone metabolism, namely the effects of various dietary lipids on bone growth, metabolism and health. These results indicate that maternal dietary lipids

influence bone growth and development of progeny. Long-term supplementation in the diet of mature quail with different lipids modulates the bone mechanical properties and bone matrix chemistry. Taken together, these data suggest that lipids play functional roles in the biology of bone. The beneficial effects of dietary FO and HSBO on bone modeling and remodeling deserve much more investigation.

REFERENCES

Ammann, P., R. Rissoli, J. P. Bonjour, S, Bourrin, J. M. Meyer, P. Vassalli, andd I. Garcia, 1997. Transgenic mice expressing soluble tumor necrosis factor-receptor are protected against bone loss caused by estrogen deficiency. J. Clin. Invest. 1699:1699-1703

Bismar, H., I. Diel, R. Ziegler, and J. Pfeilschifter, 1995. Increased cytokine secretion by human marrow cells after menopause or discontinuation of estrogen replacement. J. Clin. Endocr. Metab. 80:3351-3355.

Boudreau, M. D., P .S .Chanmugam, S. B. Hart, S. H. Lee, and D. H. Hwang, 1991. Lack of dose response by different levels of dietary n-3 fatty acids at a constant n-3/n-6 fatty acid ratio in suppressing eicosanoid biosynthesis from arachidonic acid. Am. J. Clin. Nutr. 54:111-116.

Caughey, G.E., E.Mantzioris, R. A. Gibson, L. G. Cleland, and M. James, 1996. The effect on human tumor necrosis factorα and interleukin 1β production of diets enriched in n-3 fatty acids from vegetable oil or fish oil. Am. J. Clin. Nutr. 63:116-22.

Endres S, R. Ghorbani, V. E. Kelley, and K. Georgilis, 1989. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. N. Engl. J. Med. 320:265-271.

Kimble, R. B., A. B. Matayoshi, J. L. Vaanice, V. T. Kung, C. Williams, and R. Pacific, 1995. Simultaneous block of interleukin-1 and tumor necrosis factor is required to completely prevent bone loss in the early postvariectomy period. Endocrinology 136:3054-3061.

Kruger, M. C., N. Claassen, C. M. Smuts, and H. C. Potgieter, 1997. Correlation between essential fatty acids and parameters of bone formation and degradation. Asia Pacific J. Clin. Nutr. 6:235-238.

Lorenzo, J. A. Naprta, Y. Rao, C. Alander, M. Glaccum, G. Kalinowski, and C. Pilbeam, 1997. Mice deficient in the functional interleukin-1 receptor (IL-1R1) do not loss bone mass after ovariectomy. J. Bone Miner. Res. 12(Suppl.):S126-S130.

Meydani, S.N., M. W.Margo, A. D.Barry, and L.G.Sherwood, 1991. Oral (n-3) fatty acid supplementation suppresses cytokine production and lymphocyte proliferation: Comparision between young and older women. J. Nutr. 121:547-55.

Miyaura, C., K. Kusano, T. Masuzawa, O. Chaki, Y. Onoe, M. Aoyagi, and T. Sasaki, 1995. Endogenous bone-resobing factors in estrogen deficiency: cooperative effects of IL-1 and IL-6. J. Bone Miner. Res. 10:1365-1373.

Pacifici, R, 1996. Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis. J. Bone. Miner. Res. 11:1043-1051.

Poli, V., R. Balena, E. Fattori, A. Markatos, A. Yamamoto, H. Tanaka, G. Ciliberto, G. A. Rodan, andd F. Costantini, 1994. Interleukin-6 deficient mice are protected from bone loss caused by estrogen depletion. Eur. Mol. Org. J. 13:1189-1196.

Purasiri, P., S. D.Mckechnie, and O. E.Heys, 1997. Modulation in vitro of human natural cytotoxicity, lymphocyte proliferative response to mitogens and cytokine production by essential fatty acids. Immunology 92:166-72.

Roy, R., P. Polgar, Y. Wang, R. H. Goldstein, L. Taylor, and H. M. Kagan, 1996. Regulation of lysyl oxidase and cyclooxygenase expression in human lung fibroblasts: interactions among TGF-beta, IL-I beta, and prostaglandin E. J. Cell Biochem. 62:411-417.

Sardesai, V. W., 1992. Biochemical and nutritional aspects of eicosanoids. J. Nutr. Biochem. 3:562-579.

APPENDIX A

Assays for Pyridinium crosslinks of Collagen in Animal Bone Tissues

Procedure for Tibial Decalcification

1. Bone samples will be freed of soft tissue and bone marrow.
2. The mid-shift segments (1cm) are to be isolated and weight recorded.
3. Samples then will be decalcified in several changes of 0.5 M EDTA (pH 7.2) at 4^{o}C for two weeks and then washed with distilled water.

Preparation of sample hydrolysates

1. The non-diffusible residues of decalcified bone samples will be hydrolyzed for 24 h at 100 ^{o}C in 6 M HCL in a screw capped glass tube (10 mg wet weight/ml).
2. Neutralize the hydrolyzed samples by diluting 1:5 with 1N NaOH (0.25 ml:1.0ml).
3. The neutralized samples then will be evaporated to dryness under a stream of N_2, redissolved and re-evaporated three times from distilled water.
4. Samples will then be dissolved in water for analysis (2.5ml).

Pyrilinks-D (deoxypyridinoline crosslinks) Assay Procedure

Reagent Preparation and Storage

1. Wash buffer-Prepare required amount of 1×wash buffer by diluting 10×wash buffer concentration 1:10 with deionized water. Store at room temperature (20-28^{o}C). Use 1×wash buffer within 24 hours of preparation.
   Special Washing Instructions: The pyrlinks-D assay is sensitive to washing conditions. The entire wash step should be completed within 2 minutes. ***If the wash step CANONOT be completed within 2 minutes, follow the special washing instructions located in the substrate incubation section.***

2. Enzyme Conjugate- Prepare enzyme conjugate within 2 hours of use. Reconstitute each required vial of enzyme conjugate with 7 ml of assay buffer. Store reconstituted conjugate at 2-8^{o}C until use.

3. Working Substrate Solution- The substrate buffer must be brought to room temperature (20-28^{o}C) before beginning the assay. (Two hours to overnight recommended). Prepare working substrate solution within 1 hour of use. Put one substrate tablet into each required bottle of room temperature substrate buffer. Allow 30-60 minutes for tablet(s) to dissolve. Vigorously shake bottle(s) to completely mix.

Sample/Enzyme Conjugate Incubation

1. Dilute samples, standard and controls 1:10 with assay buffer (e.g. 50 µl sample + 450 µl assay buffer).

2. Remove Stripwell Frame and the required number of coated strips from the pouch. Ensure that the pouch containing any unused strips in completed resealed.
3. Add 50 ul diluted standard, control or sample to each well of the coated strips. This step should be completed within 30 minutes.

4. Prepare enzyme conjugate within 2 hours of use. Reconstitute each required vial of enzyme conjugate with 7 ml of assay buffer. Store reconstituted enzyme conjugate at 2-8^{o}C until use.

5. Add 100µl of reconstituted enzyme conjugate to each well. Cover strips with tape cover provided. Incubate for 2 hours (±5 minutes) at 2-8^{o}C. This incubation should be carried out in dark.

6. Prepare working substrate solution within 1 hour of use. Put one substrate tablet into each required bottle of room temperature substrate buffer. Allow 30-60 minutes for tablet(s) to dissolve. Vigorously shake bottle(s) to completely mix.

Substrate Incubation

1. Prepare required amount of 1×wash buffer by diluting 10× wash buffer 1:10 with deionized water. Manually invert/empty strips. Add at east 250 µl of 1×wash buffer to each well and manually invert/empty strips. Repeat two more times for a total of three washes. Vigorously blot the strips dry on paper towels after the last wash. While strips are inverted, carefully wipe bottom of strips with a lint-free paper towel t oensure that the bottom of the strips are clean.

   <u>Special Washing Instructions:</u> Perform wash step as above, using cold (2-8^{o}C) 1×wash buffer. After last wash, allow strips t odrain for 5-10 minutes on paper towels before adding substrate.

2. Add 150µl of working substrate solution to each well.

3. Incubate for 60 minutes (5±minutes) at room temperature (20-28^{o}C).

Stop/Read

1. Add 100µl of stop solution to each well. Add stop solution in the same pattern and time intervals as the substrate solution addition.

2. Read the Optical Density (OD) at 405nm. Assure that no large bubbles are present in the wells and that the bottom of the strip is clean. Strips should be read within **<u>15 minutes</u>** of stop solution addition.

3. Quantitation software with a 4-paramete calibration curve fitting equation must be used to analyze the Pyrlinks-D assay results.

4. Determine concentration of samples and controls from the standard curve.

5. Control values should be within the range specified in the certificate of analysis supplied with the kit.

REFERENCES

Takahashi, M., Kushida, K., Hoshino, H., Suzuki, M., Sano, M., Miyamoto, S., and Inoue, T. 1996. Concentration of pyridinoline and deoxypyridinoline in joint tissues from patients with osteoarthritis or rheumatoid arthritis. Ann. Rheum. Dis. 55:324-327.

Tsuchiya, H., and C. J. Bates. 1997. Vitamin C and copper interactions in guinea-pigs and a study of collagen cross-links. Br. J. Nutr. 77:315-325.

Tsuchiya, H., and C. J. Bates. 1998. Changes in collagen cross-link rations in bone and urine of guinea pigs fed graded dietary vitamin C: a functional index of vitamin C status. J. Nutr. Biochem. 9:402-407.

Metra Biosystems, Inc. 1999. User Manual.

Farquharson, C., A. A. Duncan, and S. P. Robins. 1989. The effects of copper deficiency on the pyridinium crosslinks of mature collagen in the rat skeleton and cardiovascular system. P.S.E.B.M. 192:166-171.

Borisova, N. V., Ya. Pokrovskaya, E. Y. Zakharova, and K. D. Krasnopol'skaya. 1994. Analysis of collagen hydroxypyridinium crosslinks in samples of tissues and urine of patients with inherited connective tissue disoders. Connec. Tissue Res. 33:177-190.

APPENDIX B

Determination of Hydroxyproline in Bone Samples

Procedure

1. Bone samples will be freed of soft tissue and bone marrow.
2. The mid-shift segments (50-300 cm) are to be isolated and weight recorded.
3. Samples then will be decalcified in several changes of 0.5 M EDTA (pH 7.2) at 4^{o}C for two weeks and then washed with distilled water.
4. Put in cap tubes.
5. Add 5 ml HCL (6N) and cap the tubes.
6. Put in oven, set temperature at 110 ^{o}C for 24 hr.
7. At the end, cool the tubes.
8. Neutralize: take the aliquot 1ml, add 2 ml 3N NaOH and vortex.
9. Take aliquot 0.5 ml, add 4.5 ml distilled water and vortex.

Reagents

1. 0.01 M copper sulfate solution (anhydrous or dH_2O, calculate the water).
2. 2.5 N sodium hydroxide.
3. 6% hydrogen peroxide.
4. 3N sulfuric acid.
5. 5% α-dimethylamonobenzaldehyde in c.p. n-propanol.

Assay

1. Add 0.5 ml unknown sample and standard in duplicate.
2. Into each test tube pipette in succession 0.25 ml each of: 0.01 M copper sulfate, 2.5 N sodium hydroxide, 6% H_2O_2. Then vortex for 1min.
3. Place in water bath at 80 ^{o}C for 5 min with frequent vigorous shaking.
4. After chill in ice and water bath, add 1 ml 3N sulfuric acid followed by adding 0.5 1ml α-dimethylamonobenzaldehyde. Vortex.
5. Place tubes in water bath at 70 ^{o}C for 16 min and shaking slightly.
6. Cool in tap water and transfer contents to selected absorption tubes and read at 540 μm. Run black before read standards and samples.

Standard Curve

1. Take 50 mg, dissolve in 50 ml 0.1 N HCl (=1mg/1ml).
2. Dilute 1 to 10 (in 0.1 N HCl).
3. Standard curves: 0.025 ml, 0.05 ml, 0.10 ml, 0.15 ml, 0.20 ml, 0.25 ml. All bring to 0.5 ml.

REFRENCES

Logan, N., 1950. Determination of hydroxyproline. J. Biol. Chem. 184:299-306.

Tsuchiya, H., and C. J. Bates. 1997. Vitamin C and copper interactions in guinea-pigs and a study of collagen cross-links. Br. J. Nutr. 77:315-325.

Tsuchiya, H., and C. J. Bates. 1998. Changes in collagen cross-link rations in bone and urine of guinea pigs fed graded dietary vitamin C: a functional index of vitamin C status. J. Nutr. Biochem. 9:402-407.

APPENDIX C

Assay for *ex vivo* PGE_2 Production in Bone Organ Cultures

Bone and Marrow Supernatant Preparation

1. Tibia shafts (20 mm in length), weight recorded. Then bone marrow cells will be flushed out and collected into tubes with 1 ml of phenol red-free minimum essential medium (Sigma Chemical Co.).
2. The cell suspension will be centrifuged at 10,000g for 3 min and the bone marrow supernatant harvested into tubes, then stored at –80°C.
3. Bone will be further flushed with 0.9% NaCl to remove remained marrow cells.
4. Shafts will be immersed in 2 ml of Hank's balanced salt solution (Sigma Chemical Co.) and incubated for 2 hr at 40°C with moderate shaking.
5. The bone culture medium will be collected and stored at –80°C.

Reconstitution of Reagents

1. **Prostaglandin E_2 Standard:** Dilute the PGE_2 standard with 900 µl UltraPure water. Obtain 8 clean tubes and number them from 1 to 8. Aliquot 900 µl EIA Buffer to tube 1 and 500 µl EIA buffer to tube 2-8. Transfer 100 µl of the bulk standard (ng/ml) to tube 1 and mix thoroughly. Serially dilute the standard by removing 500 µl from tube 1 and placing in tube 2, mix thoroughly. Next, remove 500 µl from tube 2 and place it into tube 3, mix thoroughly. Repeat this process for tubes 4-8.

2. **Prostaglandin E_2 Acetylcholinesterase Tracer:** Reconstitute the PGE_2 tracer with 6 ml EIA Buffer. Store at 4°C until use.

3. **Prostaglandin E_2 Monoclonal Antibody:** Reconstitute the PGE_2 antibody with 6 ml EIA buffer. Store at 4°C until use.

Performing the Assay

1. **Rinse the Plates:** Immediately prior to use, open the plate packet and rinse the wells once with Wash Buffer. Then completely remove the Wash Buffer from each well.
2. **EIA Buffer:** Add 50 µl EIA Buffer plus 50 µl culture medium to Non-Specific Binding (NSB) wells. Add 50 µl culture medium to Maximum Binding (B_0) wells.

3. **Prostaglandin E_2 Standard and Samples:** Add 50 µl from each tube (1-8) into wells from bottom to top and run in duplicate. Then add 50 µl of sample per well and assayed in duplicate.
4. **Prostaglandin E_2 Acetylcholinesterase Tracer:** Add 50µl PGE_2 tracer to each well
5. **Prostaglandin E_2 Monoclonal Antibody:** Add 50 µl PGE_2 antibody to each well except the Total Activity (TA), NSB, and the blank (B) wells.
6. **Incubate the plate:** Cover the plate with plastic film and incubate for 18 hr at 4^oC.
7. **Develop the Plate:** When ready to develop the plate, reconstitute one vial of Ellman's Reagent with 20 ml of UltraWater. Empty the wells and rinse five times with Wash Buffer. Add 200 µl of Ellman's Reagent to each well and 5 µl of tracer to the TA wells. Cover the plate with plastic film., shake usinf an orbital shaker in the dark.
8. **Read the Plate:** The plate should be read at 405-420 nm. Before reading the plate, wipe the bottom of the plate with a clean tissue to remove finger prints. Check the plate periodically until the B_o wells are in the range of 0.3-0.8 A. U.

REFERENCES

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

Watkins, B. A., C-L, Shen, K. G. D. Allen, and M.F. Seifert, 1996. Dietary (n-3) and (n-6) polyunsaturates and acetylsalicylic acid after ex vivo PGE_2 biosynthesis, tissue IGF-I levels, and bone morphometry in chicks. J. bone Miner. Res. 11:1321-1332.

APPENDIX D

Extraction, Homogenization and Methylation of Bone Fatty Acids

1. Remove periosteum.

2. Use mid-shaft portion. Clean and remove marrow. Rinse bone fragments with 0.87% physiological saline and blotdry.

3. Wrap fragments in paper toweling and leave into tiny pieces. Remove any paper particles.

4. Place pieces in foil envelope and dip into liquid nitrogen for approximately 40 seconds.

5. Immediately pulverize fragments with a mortar and pestle (<1 minute grinding time).

6. Weigh samples into extraction tubes (0.3-0.5g), record weight and add 7 ml methanol and 14 ml chloroform immediately. Vortex tubes and store in refrigerator for 24 hr.

7. Rinse mortar and pestle 2 times with 2:1 chloroform:methanol after each sample.

8. After 24 hr, pour approximately 18 ml of the chloroform/methanol mixture through filter paper. Add 14 ml chloroform:mathanol into tubes and homogenize bone fragments for 45 seconds.

9. Allow bone particles to settle, then pour supernatant onto filter paper.

10. Add 12 ml chloroform:methanol (2:1) to homogenization tube and rehomogenize bone for 20 seconds. Pour homogenate onto filter paper. Rinse tube with 6 ml chloroform:methanol (2:1) and add to filter paper. Filter completely.

11. Add 10 ml 0.88% KCl to tubes, shake on low for 5 minutes, refrigerate at –20^{o}C until ready to methylate.

12. Centrifuge for 5 minutes at 1500 rpm. Remove aqueous layer, blow solvent down to approximately 1 ml, then transfer to methylation tubee and blow under nitrogen flow to dry.

13. Add 200 Dichloromethane (Methylene chloride) to each tube.

14. Add 500 µl hexane which contains 120 µg/ml 11:1 (free acid form) to each tube.

15. Add 2 ml 0.5N NaHO in Methanol to each tube. Cap tightly and vortex well.

16. Put tubes in pre-heated heating block (90-95^{o}C) for 20 minutes (Time varies with samples). During the first minute of heating, check any leaking.

17. Remove from heat and cool to room temperature,

18. Add 2 ml 14% BF_3 in Methanol to each tube. Cap tightly and vortex well.

19. Heat in heating block as previously.

20. Remove from heat block and cool to room temperature.

21. Add 1 ml deionized water FIRST and THEN 1 ml hexane to each tube. Cap tightly.

22. Shake tubes on high setting of platform shaker for 10 minutes.

23. Centrifuge for 5 minutes at 1500 rpm.

24. Add small amount of dry sodium sulfate to each crimp vial. With Pasteur pipette, transfer hexane layer (top layer) to crimp vial. Seal vials with crimp seals, label, and put into ultra low freezer (-80^{o}C) for analysis.

REFERENCES

Bligh, E. G., and W. j. Dyer, 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911-817.

Beare-Rogers, J., 1985. Methods for nutritional assessment of fats. American oil chemist' Society, Champaign, IL.

Christie, W. W., 1982. Lipids analysis: Isolation, analysis and identification of lipids. American Elsevier Publishing Co., InC., N. Y.

Nelson, G. J., 1975. Analysis of lipids and lipoproteins. (Perkins, E. G., Ed.). pp. 1-22, American oil Chemist' Society, Champaign, IL.

VITA

Dongmin Liu was born on January 21, 1968 in Jingyuan, a rural community in Gansu Province, China. In 1985, one year prior to graduation from high school, he entered the Department of Animal Sciences at Gansu Agricultural University and received a Bachelor's Degree of Science in agriculture in June 1989. At the same year, he began his graduate study and research in Poultry Science under the supervision of professor Wanping Li and earned a Master's Degree from Gansu Agricultural University.

Subsequently, he worked as an instructor and assistant professor from the year of 1992 to 1997 in the Department of Animal Science at Gansu Agricultural University. In August 1997, he came to the United States as a Ph.D candidate, studying and doing research in animal nutrition under the direction of Dr. D. Michael Denbow in the Department of Animal and Poultry Sciences at Virginia Polytechnic Institute and State University.

The candidate is a member of Gamma sigma Delta Honor Society, American Poultry Science Association, China Poultry Science Association, the recipient of a Pratt Fellowship for research in animal nutrition.

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Dongmin Liu