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The Effects of Dietary Calcium and Conjugated Linoleic Acid on Bone Health

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**THE EFFECTS OF DIETARY CALCIUM AND CONJUGATED LINOLEIC
ACID ON BONE HEALTH**

A Thesis Presented

by

MICHAEL TERK

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTERS OF SCIENCE

SEPTEMBER 2007

FOOD SCIENCE

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ABSTRACT
THE EFFECTS OF DIETARY CALCIUM AND CONJUGATED LINOLEIC ACID
ON BONE HEALTH

DEGREE DATE
SEPTEMBER 2007

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Approximately 200 million people worldwide, including 25 million in the United States, suffer from osteoporosis. The pathophysiology of osteoporosis suggests that prevention through dietary intervention can be one of the most important actions. Diet can also be a successful alternative for minimizing bone loss and the need for osteoporosis related drug therapy. Conjugated linoleic acid (CLA) is the collective term used to describe positional and geometric isomers of linoleic acid with two conjugated double bonds. CLA has been shown to be biologically active in many systems, yet CLA's effect on bone mass is not clearly established. The purpose of this research is to investigate CLA's isomeric effect and synergy with dietary calcium on bone mass. CLA was observed to increase the body ash, representative of bone mass, in male ICR mice after 4-weeks of supplementation. CLA was also administered to mouse bone marrow mesenchymal stem cells during osteoblastic differentiation. CLA increased protein expression related to osteogenesis as well as increasing the calcium concentration of the osteoblastic matrix. In summary, this research provides evidence for the ability of CLA to preserve bone mass.

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CHAPTER 1

INTRODUCTION

1.1. Functional foods:

Society's belief in food having medicinal powers is not a new concept, 2,300 years ago Hippocrates is quoted, "Let food be thy medicine and medicine be thy food". The concept of food having healthful functions beyond a caloric value, has received growing interest in recent years. For example in 2005, half of the most successful U.S. consumer food and beverages listed "better-for-you" benefits (Sloan, 2006). The National Academy of Sciences has expanded the definition of functional food to include "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains" (Thomas & Earl 1994). The concept of functional foods is gaining consumer acceptance which has been shown by the increase in sales of such items (Sloan, 2006; Clydesdale, 2004). Under the focus of a dietitian the quality of a single food is strongly out weighed by the framework of a healthy diet (Herron, 1991). Technically, all food holds caloric value, but caloric value alone does not qualify for the functional food concept. Recent discoveries indicate that some foods may be beneficial by selectively altering specific pathophysiology processes that improve the quality of life or reduce the risk of disease (Milner, 2000).

Interest in the health benefits of food have been initiated by factors such as the rising costs of health care (Hanks, 1992; Lofgren *et al.* 2006), legislative changes that permit claims for foods and associated components (Clydesdale, 1997), and the emergence of new discoveries . In addition, dietary factors have been correlated to the leading causes of death among Americans; including coronary disease, and certain types

of cancers (Miniño *et al.* 2006; US Department of Health and Human Services, Public Health Service, 1998). The combination of the above factors results in consumers believing certain foods contain beneficial compounds, which is one possible explanation for the increased consumption of supplements in recent years (Radimer *et al.* 2004).

1.2. Conjugated linoleic acid

Conjugated Linoleic Acid (CLA) is a collective term used to describe positional and geometric isomers of linoleic acid (c9,c12 C_{18:2}) with two double bonds in conjugation. CLA is found naturally in foods derived from ruminant animals. Two prominent isomers of CLA are *trans*-10, *cis*-12 (t10, c12) and *cis*-9, *trans*-11 (c9, t11). The latter, is the most abundant isomer making up to 90% of the naturally consumed dietary CLA (Salas-Salvado *et al.* 2006). The biological health effects of CLA were first discovered by Dr. Pariza and his group while investigating the carcinogenic components of grilled beef. Surprisingly, in addition to carcinogens, the grilled beef extract displayed anti-carcinogenic qualities (Pariza & Hargraves 1985). Since its discovery an array of health benefits have been connected with CLA (Wahle *et al.* 2004) against diseases such as cancer, diabetes, and atherosclerosis (Table 1.1). Furthermore, CLA has shown the ability to alter body composition and increase bone mass in growing animal models (Bhattacharya *et al.* 2006; Li & Watkins 1998).

1.3. History of CLA

The discovery of CLA can be attributed to Booth *et al.* (1935), who for the first time established seasonal changes in the absorptive qualities of milk fat (Booth & Kon

1935). They reported that when cows were turned out to pasture after the winter season, the fatty acids in the milk fat showed a greatly increased absorption in the ultra-violet light region at 230nm. Four years later, Moore concluded that the absorption at 230nm was the result of two conjugated double bonds (Moore, 1939). In 1957, it was further supported that the rumen provides the environment for hydrogenation of unsaturated fatty acids (Shorland *et al.* 1957), latter termed biohydrogenation. In 1961, Bartlet and Chapman set out using the newer method of infrared spectroscopy to create efficient detection methods for the illegal addition of hydrogenated vegetable oils in butter. Inadvertently, they found a constant relationship between trans-C_{18:1} and conjugated unsaturation in a large amount of samples (Bartlet & Chapman 1961). In 1963 Reil confirmed Booth's 1935 observation by showing the seasonal changes in the fatty acid profile of Canadian milk and concluded the conjugated dienoic acid was twice as high in the summer as in the winter (Reil, 1963). In 1966, while investigating the biohydrogenation process of the rumen bacteria *Butyrivibrio fibrisolvens* with linoleic acid, Kepler *et al.* (1966) determined the reduction of linoleic acid is not a one-step process to stearic acid. In addition the group stated, a conjugated *cis-trans* octadecadienoic intermediate is formed in the rumen. A year later the same group through, partial hydrogenation and reductive ozonolysis, identified the *cis-trans* octadecadienoic intermediate to be *cis-9, trans-11* octadecadienoic acid. This was one of the first identifications of the c9, t11 intermediate in rumen fluid (Kepler & Tove 1967). In 1977, using a specific non-polar phase with liquid-gas-chromatographic analysis of milk fat, Parodi was the first to identify that the c9, t11 CLA was present in milk fat (Parodi, 1977).

1.4. Structure of CLA

CLA are a series of positional and geometric isomers of linoleic acid, where one or both of the double bonds are either in the *cis* or the *trans* configuration. The double bonds can be transposed to many positions along the hydrocarbon tail. Conjugation is a system of atoms covalently bonded with alternating single and double bonds, resulting in a general delocalization, which creates a region where the electrons do not belong to a single bond or atom, but rather a group. The electrons are shared across all the adjacent parallel-aligned *p*-orbitals of the compound. This distribution increases the stability and lowers the overall energy of the molecule. A number of different isomers of CLA have been discovered by various chemical reductive, chromatographic, and spectroscopic techniques (Yurawecz *et al.* 2006).

1.5. CLA and body composition

Park *et al.* (1997), were the first to observe that CLA has the ability to reduce lipid accumulation in weanling ICR mice supplemented with 0.5% CLA (50 % c9,t11: 50 % t10,c12) for 4 weeks. They reported a 50% decrease in fat mass while lean body mass was increased. Similar studies using enriched CLA preparations reported that the t10,c12 isomer is primarily accredited for the dramatic reduction of adipose tissue (Park *et al.* 1999). The reduction in adipose tissue generally occurs in the retroperitoneal and epididymal white adipose tissue masses in addition to other areas (DeLany *et al.* 1999). Similarly, the addition of the t10,c12 isomer in an atherogenic diet for 6 weeks reduced total fat accumulation in hamsters (Simon *et al.* 2006). Most studies that have displayed the greatest body compositional changes are conducted in young growing animals, yet

older animals also have shown to be effected (Miner *et al.* 2001). In general, mice seem to be more sensitive than other animals to the body compositional effects of dietary CLA.

1.6. Suggested mechanisms of body fat reduction

There are two suggested mechanisms of CLA's effect on fat within the animal model; increased energy expenditure through fat oxidation and uncoupling proteins, or the direct modulation of adipocyte cell size and number.

Energy expenditure, defined as basal energy requirements, can be increased due to CLA supplementation. Ohnuki, K. *et al.* (2001), studied the effect of a single oral administration of CLA on male Std ddY mice; using mixed CLA isomers and linoleic acid as control. Oxygen consumption and fat oxidation were significantly greater in the CLA-administered mice than in the control mice while there was no difference in carbohydrate oxidation. Due to the higher concentrations of noradrenalin and adrenalin hormones it was suggested that CLA enhanced sympathetic nervous activity resulting in a reduction of adipose tissue via increased energy expenditure. While investigating the effects of CLA on energy metabolism in OLETF rats Nagao *et al.* (2003) reported a decrease in hepatic triacylglycerol concentrations and an increase in oxygen consumption and energy expenditure. CLA in both low-fat (15% kcal) and high-fat (45% kcal) diets had a profound effect in male AKR/J mice, resulting in increased energy expenditure and a decrease of body fat (West *et al.* 1998). Together the above studies are examples of the many who have reported an increase in energy expenditure from CLA administration.

Uncoupling proteins (UCPs) have been of particular interest for explaining the increased energy expenditure and oxidation. Proton leak or 'uncoupling' occurs when

the proton flow is short-circuited by outward proton flow that is independent of ATP synthase, resulting in the production of heat. UCP1 is predominantly expressed in brown adipose tissue, UCP2 is ubiquitously expressed; while abundant in white adipose tissue, and UCP3 is primarily located in skeletal muscle (Adams, 2000). CLA administration has shown to increase UCP1 and UCP2 expression in mice mammary, brown adipose, white adipose, epididymal adipose, and muscle tissues (Ealey *et al.* 2002; Ryder *et al.* 2001). Individually the c9,t11 (Choi *et al.* 2004) and t10,c12 isomers have shown to elevate UCP2 and UCP3 in their respective tissues (Roche *et al.* 2002).

Table 1.1 Physiological properties of conjugated linoleic acid

Major Function	Physiological Model	Publication
Carcinogenesis	↓Mutagenic activity mediated by liver S-9 cells	(Pariza <i>et al.</i> 1979)
	↓Initiation of mouse forestomach tumorigenesis by benzo(a)pyrene	(Ha <i>et al.</i> 1990)
	↓Development of mammary tumors induced by dimethylbenz(a)anthracene in rats	(Ip <i>et al.</i> 1991)
	↑Apoptosis in cultured mammary tumor cells and premalignant lesions of the rat mammary gland	(Ip <i>et al.</i> 2000)
	↓Growth of human prostate cancer cells in SCID mice	(Cesano <i>et al.</i> 1998)
	↑Apoptosis of colonic mucosa in 1,2-dimethylhydrazine-treated rats	(Park <i>et al.</i> 2001)
	↓Colon cancer Caco-2 cell growth	(Kim <i>et al.</i> 2002)
	↓Both DMBA- and NMU-induced rat mammary carcinogenesis	(Ip <i>et al.</i> 2003; Lee <i>et al.</i> 2005)
	↓COX-2 transcription in MCF-7 breast cancer cells	(Degner <i>et al.</i> 2006)
	↓Critical G1-S cell cycle progression in DU145 human prostate carcinoma cells	(Kim <i>et al.</i> 2006)
	↓Proliferation activity against human colon SW480 and HT-29 cancer cells	(Coakley <i>et al.</i> 2006)
	↑By t10,c12 isomer of mammary tumorigenesis in transgenic mice overexpressing erbB2 in the mammary epithelium	(Ip <i>et al.</i> 2007)
	Diabetes	↓Hyperinsulinemia in the pre-diabetic ZDF rat
–Effect on fasting serum levels of lipoprotein and insulin in swine		(Stangl <i>et al.</i> 1999)
↑Insulin-stimulated glucose transport and skeletal muscle insulin action		(Ryder <i>et al.</i> 2001)
↑Hyperinsulinemia and fatty liver in the mouse		(Clement <i>et al.</i> 2002)
↑Insulin resistance in obese men with metabolic syndrome		(Riserus <i>et al.</i> 2002)
↑Fasting glucose concentrations and reduced insulin sensitivity		(Moloney <i>et al.</i> 2004)
↑Insulin resistance in normal C57BL, mildly obese/diabetic KK, and morbidly obese/diabetic KKAy mice		(Ohashi <i>et al.</i> 2004)
↓ Insulin and glucose serum levels		(Bhattacharya <i>et al.</i> 2005)
↑Expression of genes-related insulin sensitivity Zucker rats		(Inoue <i>et al.</i> 2006)
↑Oral glucose tolerance and insulinemia in fa/fa Zucker rats		(Noto <i>et al.</i> 2007)
Atherosclerosis	↓Total and LDL cholesterol and triglycerides in rabbits	(Lee <i>et al.</i> 1994)
	↓Plasma total cholesterol, LDL cholesterol and triglycerides in hamster	(Saitoh <i>et al.</i> 1995)
	↓Plasma lipoproteins and early atherosclerosis in hamsters	(Nicolosi <i>et al.</i> 1997)
	↑Fatty streak formation in the C57BL/6 mouse atherosclerosis model	(Munday <i>et al.</i> 1999)
	↓Atherogenesis and established atherosclerosis in rabbits	(Kritchevsky <i>et al.</i> 2000)
	↓Aortic lipid deposition Syrian hamsters	(Vaille <i>et al.</i> 2005)
	–Hamster plasma and liver triacylglycerol concentrations	(Porsgaard <i>et al.</i> 2006)
↑LDL receptor promoter activity and mRNA expression in HepG2 cells	(Ringseis <i>et al.</i> 2006)	
Body Composition	↓Sagittal abdominal diameter in obese men given 4.2 g/day CLA for 4 weeks	(Riserus <i>et al.</i> 2001)
	↓Fat mass in hamsters when fed 0.5 % t10,c12 for 6 weeks	(Navarro <i>et al.</i> 2003)
	↓Weight loss in healthy overweight humans supplemented for 12 months	(Gaulhier <i>et al.</i> 2004)
	– In healthy but obese humans given 6g/day CLA for 12 weeks	(Whigham <i>et al.</i> 2004)
	– In overweight humans given 1.5-3 g CLA in a dairy product for 18 weeks	(Malpuech-Brugere <i>et al.</i> 2004)
	↓ White fat pads in female genetically obese mice fed 1.5% CLA for 11 weeks	(Wargent <i>et al.</i> 2005)
	↓ Fat accumulation in hamsters from t10,c12 isomer in an atherogenic diet	(Simon <i>et al.</i> 2006)
– In Forty healthy volunteers with BMI >27 kg/m ² received CLA for 12-week	(Taylor <i>et al.</i> 2006)	

↑, increases; ↓, decreases; –, no effect

CLA treatment also influences total fat mass by reducing adipocyte cell number as well as cell size (Azain *et al.* 2000; Xu *et al.* 2003). It was shown that the t10,c12 CLA isomer can inhibit heparin-releasable lipoprotein lipase activity (Evans *et al.* 2001). CLA mixed isomer treatment has increased adipocyte apoptosis, as well as reduce proliferation and differentiation in 3T3-L1 preadipocytes (Evans *et al.* 2000; Kang *et al.* 2003; Simon *et al.* 2005). Similarly in pigs, preadipocyte proliferation rates and overall adipocyte cell sizes were lower in CLA fed groups (Corino *et al.* 2005).

Reduction in fat mass has recently been connected to benefits in bone health. It has been proven that the balance of the osteo-progenitor and adipocyte cellular populations within the bone marrow have shown to be an important factor associated with osteoporosis (Pei & Tontonoz 2004). Increased levels of bone marrow adipocytes and adipogenesis have been linked with a decreased osteoblastic activity (bone formation), while a decrease in adipocyte concentration can be linked to improved osteoblastic activity (Nelson-Dooley *et al.* 2005; Pei & Tontonoz 2004). Therefore it can be hypothesized that CLA can improve bone health by reducing fat mass, particularly bone marrow adipocytes resulting in a balance that favors the osteoblastic bone formation.

CHAPTER 2

CLA AND BONE HEALTH

2.1 Introduction

2.1.1. Bone – A living organ

Bone refers to a family of biological materials, all of which are composed of mineralized collagen fibril, for example dentin (the inner material of teeth) and mineralized tendons. Bone is composed of the fibrous protein collagen in a form that is also present in skin, tendons, and a variety of other soft tissues. The collagen is a main component of the three dimensional matrix into which the mineralization occurs. Functions of bone include locomotion, mechanical support, protection, support of hematopoiesis in bone marrow, reservoir for minerals; calcium in particular, and as the attachment for muscles, ligaments, and tendons.

2.1.2. Structure of bone

Structurally bone is a lightweight composite material, consisting of an organic matrix of approximately 90% type-I collagen and 10% of other organic materials. The organic matrix is combined with a mineral phase consisting of hydroxyapatite also known as carbonate apatite [$\text{Ca}_5(\text{PO}_4/\text{CO}_3)_3(\text{OH})$] (Rho *et al.* 1998/3), which are plate-shaped crystals of bone (Weiner & Price 1986). The size of these crystals is considered the smallest biologically formed crystals known (Lowenstam & Weiner 1989).

The distribution and ratio of the organic and mineral phases vary with the function of the particular tissue. For human cortical bone this ratio is roughly 1:1 by volume and 1:3 by weight (An, 2000). However, both the composition and structure is

dependant upon factors such as skeletal location, age, sex, physiological function, and mechanical loading making bone a heterogeneous material. The need for a vascular network adds to the complexity of the tissue.

In addition to minerals, bone is also composed of type-I mineralized bundled collagen. The bundled collagen is made up of fibrils that are 3 polypeptide chains each about 100 amino acids long. The chains are wound together in a cylindrically shaped triple helix (Lowenstam & Weiner 1989). These fibrils are bound and impregnated with carbonated apatite nanocrystals (Rho *et al.* 1998/3). Bone is further organized at the micro-structural scale into lamellar structures which can be described as an alternating fluid phase between repeated lamellae (Weiner & Wagner 1998). The lamellar structure not only repeats but, alternates orientation which allows bone to have great torsion strength. Cylindrical motifs are generally oriented along the long axis of bones and are termed secondary osteons, which are tunnels, created by excavation from osteoclasts. These tunnels are then almost completely filled in by osteoblastic action, leaving a narrow channel at the center which functions as a blood vessel. In fact, other even smaller capillary-like channels, termed canaliculi, are built into the structure. The canaliculi are numerous and tend to radiated out from the central blood vessel (Martin & Burr 1989).

2.1.3. Bone modeling and remodeling

Bone is a multifunctional organ which provides an environment for a heterogeneous population of cells. The heterogeneous mix of cells are controlled and organized by multiple growth factors, cytokines, hormones, and other signals (Watkins & Seifert 2000). The bone matrix is produced and mineralized through the action of

osteoblasts; while in opposition; resorption is conducted by specialized multinucleated cells called osteoclasts. Normally there is a dynamic balance between osteoblast and osteoclast activity, approximately 10% of bone is replaced each year (Cohen, 2006), yet if there is an imbalance it can result in serious consequences.

Osteoblasts develop from the mesenchymal cell lineage, initially as preosteoblasts which develop into functional osteoblasts (Minguell *et al.* 2001). Generally, osteoblasts have three functions; to produce the bone matrix proteins (Ellies & Krumlauf 2006), express necessary genes for mineralization (Franz-Odenaal *et al.* 2006), and regulation of osteoclasts (Spencer *et al.* 2006).

Osteoclasts are derived from the monocyte/macrophage lineage. The differentiation from the preosteoclast is controlled by ligand activated RANK (Receptor Activated Nuclear Factor) in addition to other ligands (Aguila & Rowe 2005). Bone resorption is also under the control of many different hormones such as: 1,25(OH)₂ vitamin D₃, parathyroid hormone (PTH), interleukin-1 (IL-1), interleukin-6 (IL-6), and estrogens (Boyle *et al.* 2003).

2.1.4. Osteoporosis

Osteoporosis results from reduced bone mass and disruption of the micro-architecture of bone. Such disruptions result in decreased bone strength and an increased risk of fracture. The risks associated with osteoporosis, include gender, heredity, nutrition, and lifestyle increase with age. Most of those affected with osteoporosis are over the age of 65 (Poole & Compston 2006). Globally osteoporotic fractures caused an estimated 5.8 million disability adjusted life years in 2000 and are associated with

increased mortality (Poole & Compston 2006). After reaching peak bone mass during the mid-thirties, both men and women start losing bone at a rate of 0.5 - 1% yearly. Simultaneously, as bone mineral density declines with age, incidence of bone fracture increases with age (McGarry & Kiel 2000). While treatment methods for osteoporosis are becoming available prevention and changes in lifestyle are considered the most important way to reduce osteoporosis (Poole & Compston 2006). Possible lifestyle changes are to increase calcium intake along with vitamin D and exercise, while simultaneously decreasing smoking and alcohol abuse (Hind & Burrows 2007). Current pharmacological intervention include bisphosphonates, hormone replacement therapy (which prevent bone resorption), strontium ranelate (uncertain mechanism of action), and parathyroid hormone peptides (anabolic action) (Poole & Compston 2006).

2.2. CLA and increased bone mass

Since the discovery of CLA to increase whole body ash (Park *et al.* 1997), which is representative of bone mass, it has been presumed CLA may enhance bone mineralization and protect against bone loss. A response from CLA administration with respect to bone mass, has been found in studies conducted with rapidly growing animals. Cook *et al.* (1997) found higher levels of bone ash in the tibia of CLA-fed chicks compared to control. Pigs supplemented with 0.5% and 1.0% CLA resulted in an increased bone weight compared to control and animals supplemented with 0.12% or 0.25% CLA (Thiel-Cooper *et al.* 2001). A positive effect was observed in growing male chicks in response to CLA fed in the form of butterfat (Watkins *et al.* 1997). It was reported that ash retention was significantly greater in mice fed 1.5% CLA compared to

restricted diet and control groups (Terpstra *et al.* 2002). Additionally, Wistar rats showed no sign of elevated bone resorption markers; while, the markers of bone formation were increased due to the CLA treatments (Kelly *et al.* 2003). Experiments have shown, BALB/C male mice fed CLA had increased cancellous and cortical bone mass in the proximal tibial metaphysis, in the lumbar, and in pure cortical bone mass in the tibia fibula junction (Banu *et al.* 2006). Another study using female C57BL/6 retired breeders treated with CLA for 10 weeks, showed increased bone mass in the fourth lumbar vertebra and the femoral diaphysis by CLA (Bhattacharya *et al.* 2006). Recently, the t10,c12 CLA isomer does dependently (0 – 0.30% - CLA) was found to increase the femur weight of male mice, but not female and control mice (Viswanadha *et al.* 2006). In summary, CLA has shown the innate ability to increase bone mass in a variety of animal models. This increase of bone mass, in general, can be explained by an increase in osteoblastic activity or a decrease in osteoclastic activity.

2.3. CLA and human skeletal health

Clinical studies that investigated CLA supplementation focusing on bone health have not been consistent. In 2005 a study conducted with 136 postmenopausal women revealed that dietary CLA intake was positively associated with forearm bone mineral density (Brownbill *et al.* 2005). Pinkoski *et al.* (2006) reported that CLA supplementation decreased cross-linked N-telopeptides of Type I collagen in urine samples compared to control, which is an indication of reduced bone resorption.

However, others reported no benefit of CLA on bone mass. CLA supplemented groups did not show statistically significant changes in bone mass (measured by dual-

energy X-ray absorptiometry) in male athletes undertaking resistance training (Kreider *et al.* 2002). Similarly, a study that investigated CLA concerning calcium and bone metabolism in humans recorded no changes with supplementation, while measuring bone resorption and formation markings in healthy male adults for 8 weeks (Doyle *et al.* 2005).

Nevertheless, it has been reported that when overweight adults were supplemented with CLA for 1 year, the mixed CLA isomers in a triacylglycerol-bound form have no effect on bone mineral mass, whereas CLA as a free fatty acid reduced bone mineral mass (Gaullier *et al.* 2004). Yet during the second year, of the same study, CLA supplementation yielded a significant improvement of bone mass (Gaullier *et al.* 2005). Thus the inconsistency of CLA on bone health may be due to the various durations and small subject number (n=11-12 per group for Kreider *et al.* 2002) for investigation on bone mass.

2.4. Increased calcium absorption

CLA has been shown to increase calcium absorption. Kelly *et al.* (2003) reported a significant increase in calcium absorption with CLA supplementation (1%) in Wistar rats for 8 weeks. Meanwhile, others reported that CLA, particularly the t10,c12 isomer, significantly increased calcium transport in human intestinal-like Caco-2 cells (Jewell & Cashman 2003). Another study conducted by the same group specifically investigated the paracellular permeability, which is the predominant route of calcium transport within the intestinal tract. The c9,t11 and t10,c12 isomers of CLA significantly increased the paracellular transport of calcium (Jewell *et al.* 2005). Recently, using micro-array techniques many molecules associated with calcium absorption were analyzed in human

intestinal-like Caco-2 cells. Although the exact mechanism is still unclear, the t10,c12 CLA isomer was found to increase calcium transport (Murphy *et al.* 2006). Others reported that the t10,c12 isomer affected the disruption of occludin which is associated with increased paracellular permeability for ions and macromolecules in intestinal Caco-2 cells (Roche *et al.* 2001).

2.5. Reduction in bone resorption

CLA supplementation reduced bone resorption rates in rats, measured by bone and urinary pyridinoline and deoxypyridinoline, compared to control (Kelly & Cashman 2004/11). Rahman *et al.* (2006a) and Banu *et al.* (2006) reported that CLA-fed mice maintained a higher bone mineral density than control-fed mice which was a result of decreased osteoclastic activity. The same group also suggested that CLA inhibited osteoclastogenesis primarily through the RANK ligand activated pathways in osteoclast-like Raw264.7 macrophage cells (Rahman *et al.* 2006b).

2.6 Possible mechanisms of CLA on bone mass

Two mechanisms were suggested for CLA's beneficial effects on bone health, alteration of the cyclooxygenase pathway and alteration of serum leptin.

2.6.1 Alteration of the Cyclooxygenase Pathway

It has been well known that prostaglandin, particularly E₂, has been involved in bone physiology. High levels of prostaglandin E₂ (PGE₂) (10⁻⁶M) inhibit bone formation, while at lower concentrations (10⁻¹⁰-10⁻⁸ M) increase bone formation. In addition PGE₂

stimulates bone resorption (Takiguchi *et al.* 1999). The enzyme cyclooxygenase (COX) catalyzes the conversion of arachidonic acid to PGE₂ among other prostanoids. There are a number of factors that induce COX (1,25(OH)₂ vitamin D₃, PTH, IL-1, IL-6, and estrogens (Boyle *et al.* 2003)), therefore such factors are suggested to indirectly increase PGE₂ and bone resorption activity (LG, 2005).

A number of reports indicated that CLA reduces prostaglandin production. In fact, CLA, mixed isomer and the t10,c12 isomer, decrease PGE₂ biosynthesis in human osteoblast-like SaOS-2 and MG-63 cells to a greater extent than the c9,t11 isomer (Cusack *et al.* 2005). It has also been demonstrated that the c9,t11 CLA isomer greatly increases the formation of mineralized bone nodules in long-term cultures of human SaOS-2 cells (Platt *et al.* 2007).

It was suggested that CLA competes with arachidonic acid in the phospholipid fraction, resulting in reduced prostaglandin production. Alternatively, CLA may directly inhibit COX (Li *et al.* 1999). Recent data shows a decrease in COX activity in cellular models due to CLA treatment (Degner *et al.* 2006; Wang *et al.* 2006).

PGE₂ is involved in the release of parathyroid hormone (PTH) (Brown & Swartz 1985). Elevated levels of PTH have been reported to reduce bone mass through osteoclastic up regulation (Boyle *et al.* 2003). Thus due to CLA's inhibitory effects on PGE₂ synthesis, CLA may decrease the osteoclastic activity through a reduction in PTH expression, which could be explained by an upstream decrease of PGE₂ levels which favors bone formation (Weiler *et al.* 2004).

2.6.2 Serum leptin and CLA

CLA's positive effects on bone mass may also be explained by a down regulation of leptin (Corino *et al.* 2002; Pruzanski *et al.* 1998). CLA has shown to reduce leptin in humans by a reduction in fat mass (Medina *et al.* 2000/7). Leptin is linked to the production of the RANK ligand (Receptor Activated Nuclear Factor) (Elefteriou *et al.* 2005; Elmquist & Strewler 2005), which regulates bone resorption through osteoclast differentiation and activation (Elefteriou *et al.* 2005; Rahman *et al.* 2006b; Rahman *et al.* 2001). Thus reduction of leptin by CLA will decrease RANKL, which subsequently reduces bone resorption. In fact, Rahman *et al.* (2006) showed that CLA fed mice displayed an increased bone mineral density and reduced osteoclast activity, explained by reduced leptin and IL-6 (interleukin-6) in the serum. In conclusion as CLA reduces leptin, osteoclastic activity is reduced, thereby preserving bone mass.

Similarly, leptin has been shown to play a role in the regulation of both the pro-inflammatory cytokines, such as IL-6 and IL-1, these pro-inflammatory cytokines stimulate osteoclast activity, thereby inducing resorption (Kang & Pariza 2001). Since CLA has proven to reduce both IL-6 (Burguera *et al.* 2001) and IL-1 (Thomas & Burguera 2002) secretion *in vivo*, it is possible that the reduced leptin results in decreased IL-1 and IL-6, which in turn limit osteoclasts' action indirectly.

CHAPTER 3

THE ACTIVE ISOMER OF CLA ON IMPROVING BODY ASH

3.1. Introduction

Approximately 200 million people worldwide, including 25 million in the United States, suffer from osteoporosis. Osteoporosis is a condition of bone in which the bone mineral density is reduced, thus bones are more susceptible to fracture. Osteoporosis is largely a problem of the elderly and post-menopausal women (May *et al.* 1994). After reaching peak bone mass during the mid thirties, both men and women start irreversible bone loss at a rate of 0.5% - 1% yearly resulting in an increased risk of bone fracture with age (McGarry & Kiel 2000). Such declines in bone mineral density are an inevitable part of the aging process and require new strategies for its prevention. Since pharmacological treatment of osteoporosis has had either limited success or adverse effects, it is recommended that prevention may be the best choice of action. Changes in diet and lifestyle are some successful alternatives to minimize bone loss and decrease the requirement of osteoporosis related drug therapy (Poole & Compston 2006).

Conjugated linoleic acid (CLA) is a collective term used to describe positional and geometric isomers of linoleic acid (c-9, c-12 C_{18:2}) with two double bonds in conjugation. CLA is formed during the biohydrogenation process of linoleic acid to stearic acid in the rumen by *Butyrivibrio fibrisolvens* (Kepler *et al.* 1966). Two prominent isomers of CLA – *trans*-10, *cis*-12 (t10,c12) and *cis*-9, *trans*-11 (c9,t11) – are naturally found in the dairy and meat products of ruminant animals. The latter, c9,t11 CLA is the most abundant isomer making up to 90% of the naturally consumed CLA

(Salas-Salvado *et al.* 2006). Over the last ten years, considerable attention has been focused on CLA's beneficial effects including anticarcinogenic, reduction in the severity of atherosclerosis, hypertension, and diabetes (Belury, 2002). CLA has also shown to alter body composition; specifically, CLA reduces body fat and increases lean mass along with bone mass in growing animal models (Bhattacharya *et al.* 2006; Li & Watkins 1998).

CLA's ability to enhance body ash, which is representative of improvements to bone mass, have recently received attention. Brownbill *et al.* (2005) reported that dietary CLA may positively benefit bone mineral density in post-menopausal women. Additionally, CLA has shown to increase the osteoblastogenic markers (osteocalcin and alkaline phosphatase) in osteoblasts-like cells (Watkins *et al.* 2001). However, the effects of CLA on bone health are not consistent. As mentioned in Chapter 2, clinical studies have shown positive effects of CLA consumption on bone health, while others have shown no such effects (Banu *et al.* 2006; Bhattacharya *et al.* 2006; Kelly *et al.* 2003; Li *et al.* 1999; Terpstra *et al.* 2002; Thiel-Cooper *et al.* 2001; Thiel-Cooper *et al.* 2001; Viswanadha *et al.* 2006; Watkins *et al.* 1997).

Thus, inconsistent effects of CLA, particularly human studies, led us to hypothesize that there are conditions or dietary interactions under which CLA may improve bone mass efficiently. Finding these conditions will allow us to use CLA efficiently for improving bone mass. Based on the observation that CLA positively benefits bone mineral density in postmenopausal women supplemented with calcium (Brownbill *et al.* 2005). Along with, CLA's ability to improved calcium absorption *in vivo* and in the human colon adenocarcinoma CaCo2 cell line, we hypothesized that CLA

may enhance calcium's effect on bone mass (Jewell & Cashman 2003; Jewell *et al.* 2005; Kelly *et al.* 2003; Murphy *et al.* 2006; Roche *et al.* 2001). In fact, a preliminary study showed the relationship between CLA and increased dietary calcium (Park *et al.* 2006). As an extension, this study was designed to examine the specific isomeric effect of the c9,t11 and c10,t12 CLA isomers with dietary calcium focusing on total body ash which is representative of bone mineral density.

3.2. Materials and Methods

3.2.1. Materials

Mice were acquired from Charles River Laboratories (Wilmington, MA). The custom formulated semi-purified diet, TD04460, was prepared by Harland Teklad (Madison, WI). All CLA preparations were obtained from Natural Lipid (Hovdebygda, Norway). The mixed isomer consisted of 38% c9, t11 and 38% t10, c12, with 14% oleic acid (c9, C_{18:1}) and 3% steric acid (C_{18:0}). The t10,c12 isomer was 94 % pure, with 2% c9,t11 isomer and 3% other conjugated isomers (C_{18:2}). The c9,t11 isomer was 90% pure, with 4% t10,c12 isomer, 2% other conjugated isomers (C_{18:2}), and 3% oleic acid (c9, C_{18:1}). Kjeldahl catalyst tablets consisting of 3.5 g K₂SO₄ and 0.4 g CuSO₄ x 5H₂O were purchased from FOSS analytical (Eden Prairie, MN). Hydrochloric Acid, sulfuric acid, nitric acid, anhydrous ethyl ether, Kjel-Sorb Solution (saturated boric acid solution with indicator) were purchased from Fisher Scientific (Atlanta, GA). All reagents were of ACS or reagent grade.

3.2.2. Methods

3.2.2.1. Animals and Diets

Three-week-old male ICR mice were fed a semi-purified adaptation diet (Table 3.1) for one week. At the conclusions of the adaptation period the mice were, grouped into 8 groups of 9 animals based on body weight. The mice were then housed individually in wire bottom cages and fed a semi-purified diet containing a normal (0.5 %) or a high (1.0%) calcium level. Each calcium level consisted of four groups; 0.5 % CLA mixed isomer, 0.2 % c9, t11 isomer (c9,t11), 0.2% t10, c12 isomer (t10,c12), and no CLA as control. Diets were stored at -20 °C and provided freshly three times a week. Mice were provided with water and food *ad libitum* and maintained on a 12-h light/dark cycle in ambient temperatures of 22-25 ° C. University of Massachusetts Amherst's Institutional Animal Care and Use Committee procedures and guidelines were strictly followed and all studies were approved by the University's Animal Care Office. Body weight and feed intake were measured in all mice, one and three times a week, respectively.

3.2.2.2. Organ and serum analysis

At the end of the 4-week feeding period the animals were sacrificed through CO₂ asphyxiation. Immediately following death the blood was collected through a lower sternum cardiac puncture. A ventral incision down the midsagittal plane was made, allowing access to the abdominal cavity. From which the spleen, liver, kidneys, and adipose tissue (epididymal, retroperitoneal, and mesenteric) were isolated and individually weighed. The empty carcass weight, which included all the tissue and

organs except for the gastrointestinal contents and blood, was recorded. Each mouse was individually wrapped and stored at -80°C. Blood samples were centrifuged at 5,000 rpm for 10 minutes. The separated blood plasma from each sample was removed and stored at -80°C.

Table 3.1 Composition of experimental diets.

Ingredient	0.5% Ca diet (%)	1.0% Ca diet (%)
Casein	19.4	19.4
L-Cystine	0.30	0.30
Corn Starch	38.1	37.6
Maltodextrin	13.2	13.2
Sucrose	8.75	8.75
Soybean Oil+ CLA ^A	10.0	10.0
Cellulose	5.00	5.00
Mineral Mix, w/o Ca (TD 04374)	3.50	3.50
Vitamin Mix (TD 94047)	1.00	1.00
Choline Bitartrate	0.25	0.25
TBHQ	0.002	0.002
CaCO ₃	0.5	1.0

^A 0.5% soybean oil substituted with CLA mixed isomers or 0.2% substituted with either the t10,c12 isomer or the c9,t11 isomer

Composition of experimental diets. Diets were composed by Harland Teklad and contained 10% fat, with two levels of calcium (0.5% and 1.0%). Within each level of calcium there were 4 CLA treatments 0.5 % CLA mixed isomer, 0.2 % c9, t11 isomer, 0.2% t10, c12 isomer, and no CLA as control, which was substituted with soybean oil. TBHQ; tertiary butylhydroquinone.

3.2.2.3. Body composition analysis

Water content was determined through a repeated freeze dry process (Virtis, Gardiner, NY) until no further water was lost from the sample. The samples were homogenized into particles using a high velocity grinder (Jarden Corporation, Rye, NY). Ash was determined from dried samples by gravimetric methods after a 12-hour ashing in

a muffle furnace (Fisher Scientific, Atlanta, GA) at 550°C. Fat was extracted from dried samples with anhydrous ethyl ether in a semi-automated Soxhlet system (Foss, Hillerod, Denmark) for a minimum time of 8 hours. Nitrogen content was determined by the Kjeldahl Method which was converted into the total protein content. Samples were briefly digested with catalyst and concentrated sulfuric acid in a heated digestion system, then the distillation process was completed on a semi-automated Kjeltec system (Foss, Hillerod, Denmark) with a saturated boric acid solution. The resulting ammonium-borate complexes were titrated with 0.2N hydrochloric acid. The percent nitrogen $[14.01 \times (\text{ml titrant} - \text{ml blank}) - (\text{N of titrant}) \times 10] / \text{sample wt.}]$ was multiplied by 6.25.

3.2.2.4. Bone analysis

The left tibia was isolated from each animal. The ash content of each tibia was determined after a 12- hour ashing in a muffle furnace (Fisher Scientific, Atlanta, GA) at 550°C. The ash was then dissolved in 5% trace metal free nitric acid. Calcium was analyzed using an atomic absorption spectrophotometer (Perkin Elmer model 2380, Waltham, Massachusetts). The calcium concentration was determined from a standard curve generated from known concentrations of CaCO₃ in 5% nitric acid. The phosphate content of the bone was determined according to the method of Anderson and Davis (1982) with minor modifications. The samples were diluted into water 500 times. To this 0.5 mL of ammonium molybdate (10.1 mmol/l) and 0.5 ml of ascorbic acid (0.28 mol/l) was added and vortexed. Following mixing, the samples were heated in a boiling water bath for 10 minutes, cooled and then the absorbance was read at 822nm. The

phosphate concentration was determined from a standard curve generated from known concentrations of H_3PO_4 (Anderson & Davis 1982).

3.2.2.5. Statistical analysis

All data were analyzed by two-way analysis of variance (calcium and diet) by SAS statistical software (Version 9.0; SAS institute Inc., Cary, NC, USA). Mean separations were conducted with Duncan's New Multiple Range Test ($p < 0.05$).

3.3. Results

3.3.1. Body weight and food intake

There were no differences of food consumption in all treatment groups compared to control (Figure 3.1). There was no effect of calcium on feed intake. The mixed isomer group resulted in a decreased food consumption compared to the c9,t11 isomer but was not different from the t10,c12 isomer and control groups. Consumption of the t10,c12 isomer resulted in a decreased food consumption compared to the c9,t11 isomer but was similar to the mixed isomer and control groups.

The calcium level had no effect on body weight (Figure 3.2). The CLA-mixed isomer and the t10,c12 isomer groups had similar body weights and were less than the c9,t11 isomer and control groups. The c9,t11 isomer group exhibited a similar body weight to the control group.

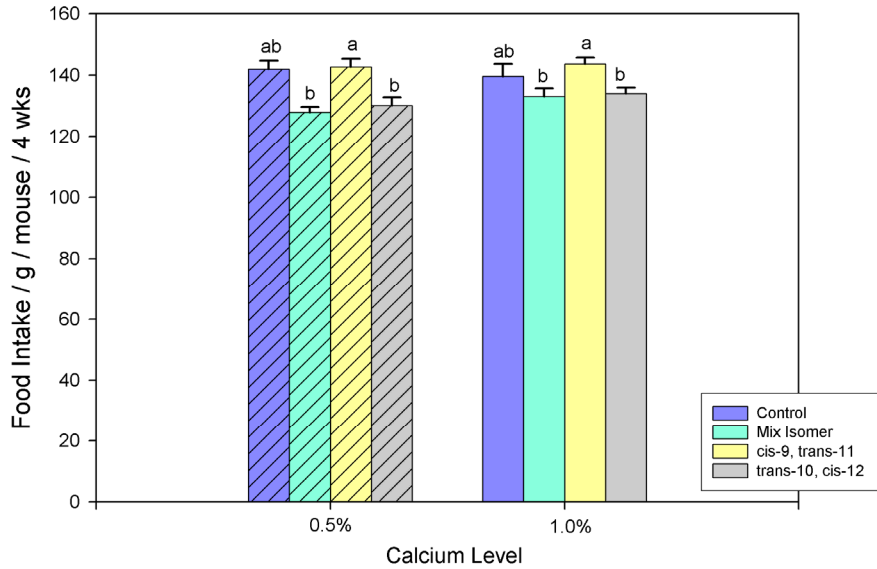


Figure 3.1. Total Feed Intake. 4-week-old male ICR mice were fed treatment diets for 4 weeks. Treatments included two levels of calcium that consisted of 4 CLA preparations (0.5% CLA-mixed isomer, 0.2% c9,t11 and t10,c12 CLA isomers, and control). Each bar represents the mean \pm SE of 9 animals. Means with the same letter are not significantly different ($P < 0.05$).

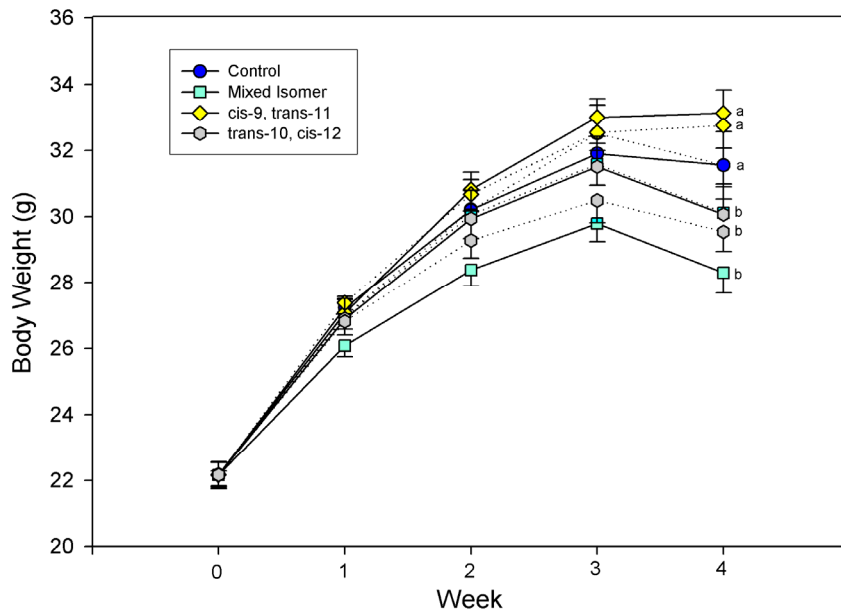


Figure 3.2. Body Weight. 4-week-old male ICR mice were fed treatment diets for 4 weeks. Treatments included two levels of calcium (solid line represents 1.0% Ca, broken line represent 0.5% Ca) that consisted of 4 CLA preparations (0.5% CLA-mixed isomer, 0.2% c9,t11 and t10,c12 CLA isomers, and control). Each data point represents the mean \pm SE of 9 animals. Means with the same letter are not significantly different at each time point ($P < 0.05$).

3.3.2. Effects on total body ash

The 1.0% level of calcium generally increased the total amount of body ash in all groups, compared to the 0.5% level (Figure 3.3). CLA-mixed isomer improved total body ash in both 0.5% and 1% calcium. Supplementation of the t10,c12 CLA isomer yielded a higher amount of total body ash, which was further elevated by an increased amount of calcium in the diet. The t10,c12 isomer displayed similar amounts of ash to the mixed isomer group and an increased amount of ash compared to the c9,t11 isomer and control groups among both calcium levels.

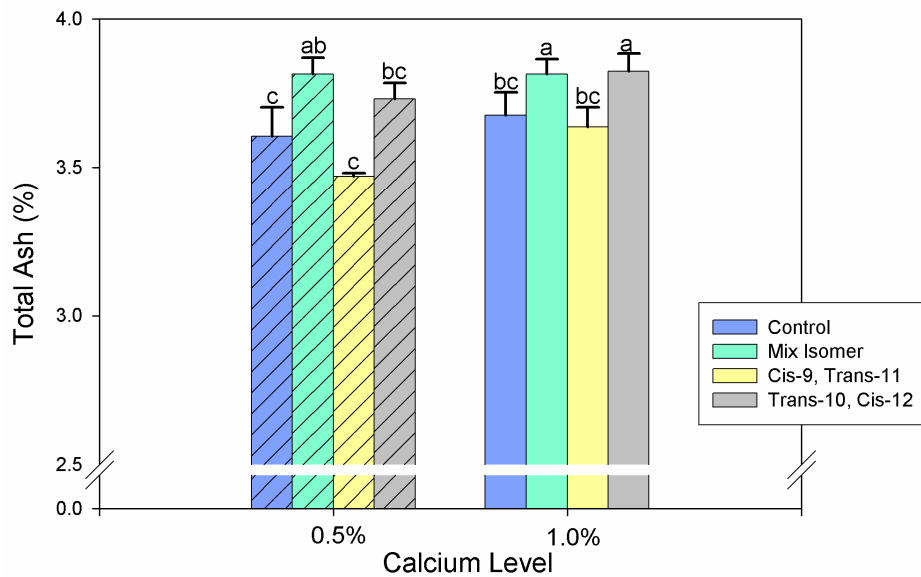


Figure 3.3. Total Body Ash. 4-week-old male ICR mice were fed treatment diets for 4 weeks. Treatments included two levels of calcium that consisted of 4 CLA preparations (0.5% CLA-mixed isomer, 0.2% c9,t11 and t10,c12 CLA isomers, and control). Ash was determined by gravimetric methods after a 12-hour ashing in a muffle furnace at 550°C. Each bar represents the mean \pm SE of 9 animals. Means with the same letter are not significantly different ($P < 0.05$).

3.3.3. Effects on bone composition

The amount of calcium in the tibia showed more variation at the 1% calcium level, specifically the CLA mixed isomer treatment increased the calcium compared to the t10,c12 isomer and control groups (figure 3.4: A). This result is constant to a result in chapter 4 (figure 4.1), in which the calcium content of osteoblastic cells treated with CLA show an increase of calcium with the mixed isomer treatment over the t10,c12 isomer and control treatments. The percent of calcium in the tibia (A) and the percent of calcium in the tibia ash (B) followed similar trends. The treatment groups at the 0.5 % calcium level were of similar value, except the c9,t11 groups were comparatively less. At the 1.0% calcium level the CLA mixed isomer group showed a greater calcium percent followed by the t10,c12 group (P-value of 0.460, determined by least square of means), while the c9,t11 group was similar to control. This data shows that the mixed isomer is needed to increase the calcium content of the mouse tibia.

Contradictory to the total body ash data (figure 3.3), the overall percent of ash recovered from the bones (C) was decreased in the mixed isomer and the t10,c12 groups at the 1% calcium level. The control and the c9,t11 groups showed increased levels of ash in the tibia at the 1% calcium level.

The phosphate content of the bones followed similar trends to the calcium content (figure 3.5). The percent phosphate in the tibia at the 0.5 % calcium level was similar among all the treatments. At the 1.0 % calcium level the phosphate in the tibia ash was significantly elevated by the CLA mixed isomer and the t10,c12 isomer compared to the c9,t11 group which was similar to control. The percent phosphate in the tibia ash at the 0.5 % calcium level was similar among all treatments with the c9,t11 group displaying

the lowest percent. At the 1.0% calcium level the CLA mixed isomer had a significantly greater phosphate percent, followed by the t10,c12 group, while the c9,t11 group was similar to control.

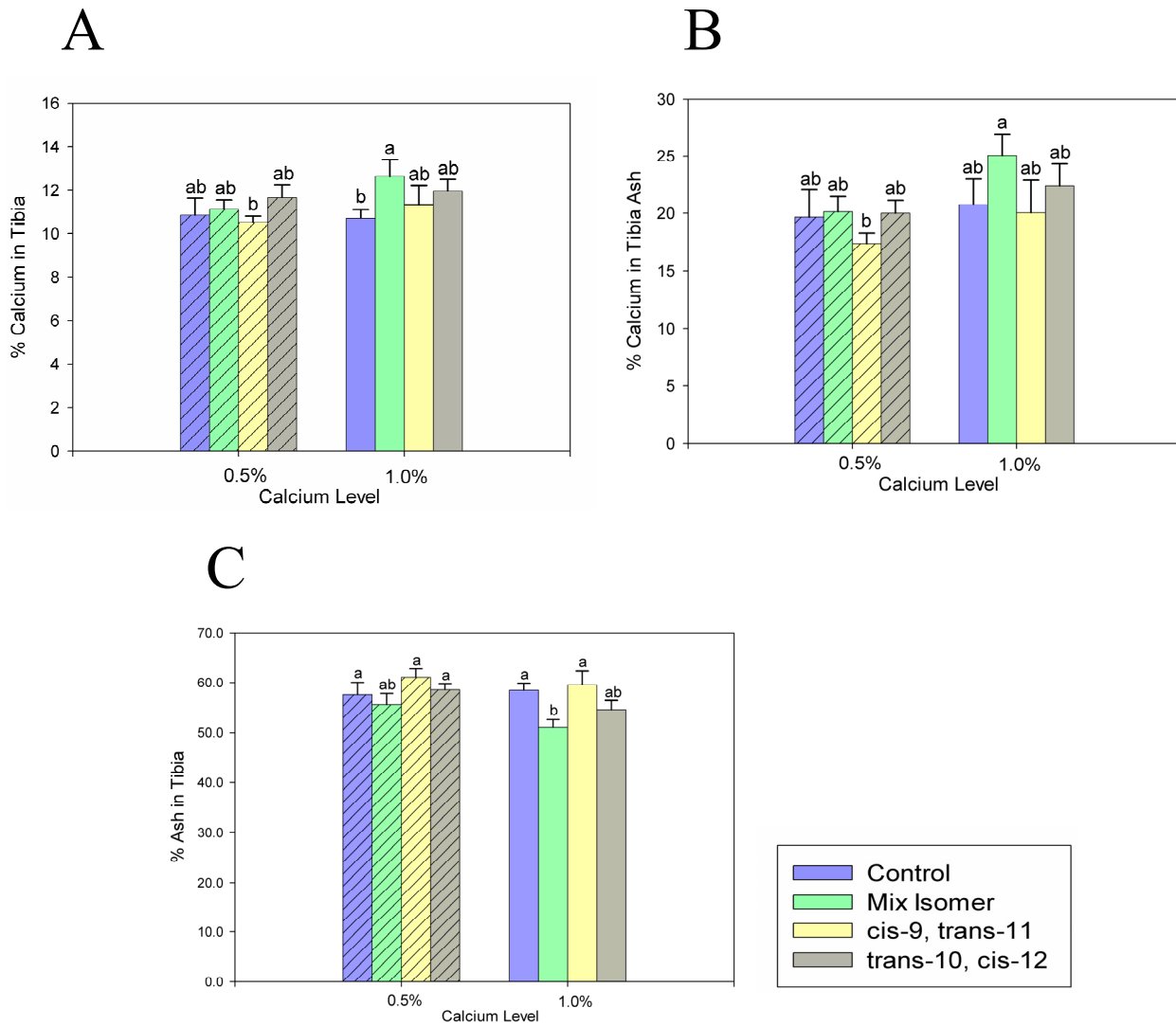


Figure 3.4. Bone Calcium Content. 4-week-old male ICR mice were fed treatment diets for 4 weeks. Treatments included two levels of calcium that consisted of 4 CLA preparations (0.5% CLA-mixed isomer, 0.2% c9,t11 and t10,c12 CLA isomers, and control). The ash of the left tibia was determined by incineration and calcium concentration was determined by atomic absorption spectrometry. (A) Percent calcium in the tibia, (B) percent calcium in the ashed tibia, (C) percent ash in the tibia. Each bar represents the mean \pm SE of 9 animals. Means with the same letter are not significantly different ($P < 0.05$).

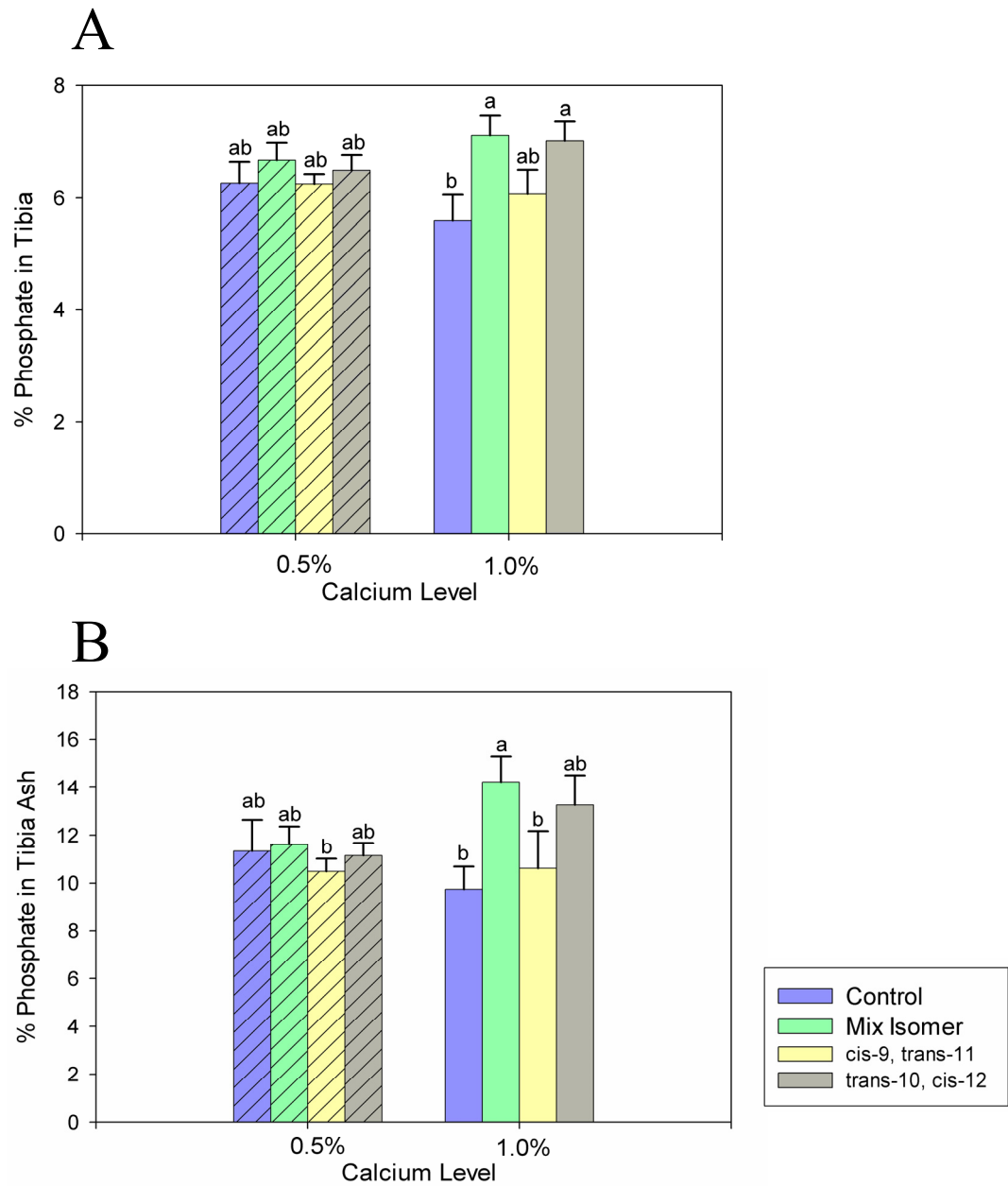


Figure 3.5. Bone Phosphate Content. 4-week-old male ICR mice were fed treatment diets for 4 weeks. Treatments included two levels of calcium that consisted of 4 CLA preparations (0.5% CLA-mixed isomer, 0.2% c9,t11 and t10,c12 CLA isomers, and control). The ash of the left tibia was determined by incineration and phosphate concentration was determined by spectrophotometric analysis of reduced molybdate-phosphate complexes. (A) Percent phosphate in the tibia, (B) percent phosphate in the ashed tibia. Each bar represents the mean \pm SE of 9 animals. Means with the same letter are not significantly different ($P < 0.05$).

3.3.4. Effects on body composition

There was no effect of the calcium level on total water content (Figure 3.6). The mixed isomer group resulted in greater water content compared to the control group in both calcium levels. The trend of the mixed isomer to increased water content was also observed in the t10,c12 isomer group. There was no effect observed by the c9,t11 isomer on water content in both calcium levels, which was similar to control.

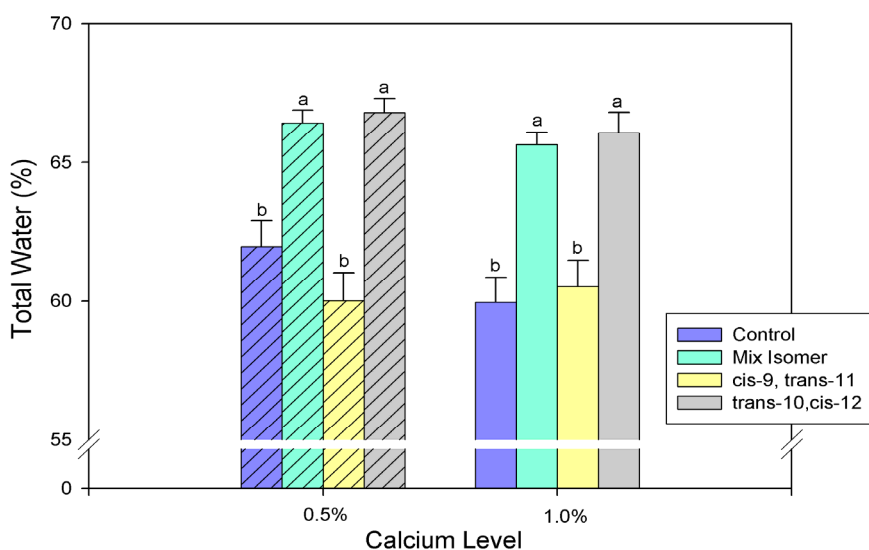


Figure 3.6. Total Water Content. 4-week-old male ICR mice were fed treatment diets for 4 weeks. Treatments included two levels of calcium that consisted of 4 CLA preparations (0.5% CLA-mixed isomer, 0.2% c9,t11 and t10,c12 CLA isomers, and control). Water content was determined through a repeated freeze dry process until no further water was lost from the sample. Each bar represents the mean \pm SE of 9 animals. Means with the same letter are not significantly different ($P < 0.05$).

Total protein content in these animals was not affected by dietary calcium levels (Figure 3.7). However, the CLA mixed isomer significantly improved total protein content compared to control regardless of calcium level. This was also observed with the

t10,c12 isomer groups but not the c9,t11 isomer group. Again, the c9,t11 isomer group did not have any effect on total protein content in both calcium levels.

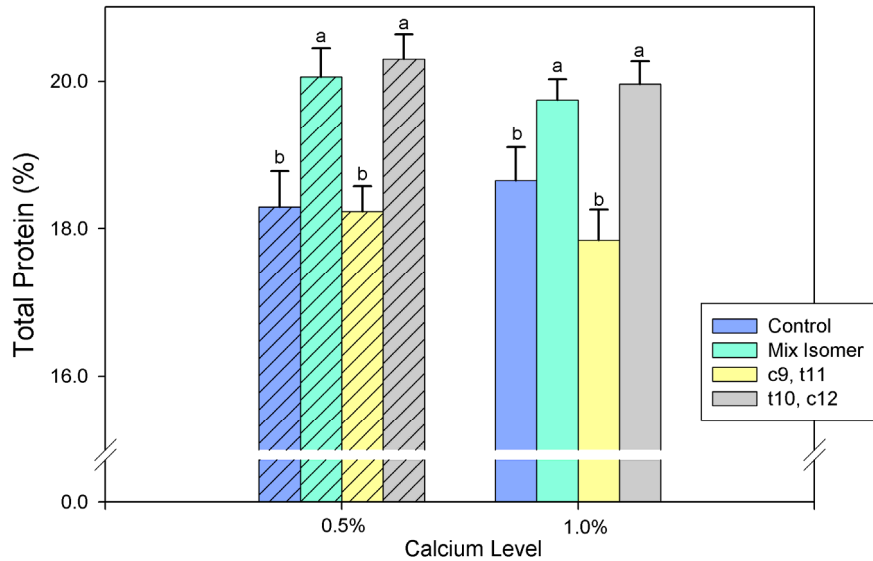


Figure 3.7. Total Protein Content. 4-week-old male ICR mice were fed treatment diets for 4 weeks. Treatments included two levels of calcium that consisted of 4 CLA preparations (0.5% CLA-mixed isomer, 0.2% c9,t11 and t10,c12 CLA isomers, and control). Nitrogen content was determined by the Kjeldahl Method which was converted to the total protein content. Each bar represents the mean \pm SE of 9 animals. Means with the same letter are not significantly different ($P < 0.05$).

No differences in total fat were observed in the two levels of dietary calcium (Figure 3.8). As previously reported, CLA mixed isomers significantly decreased total fat compared to control, 49% reduction at 0.5% calcium and 47% reduction at 1% calcium. The reduction of total body fat was observed by the t10,c12 isomer group, but not the c9,t11 isomer group. The c9,t11 isomer group at 0.5% calcium level, actually had higher fat than control.

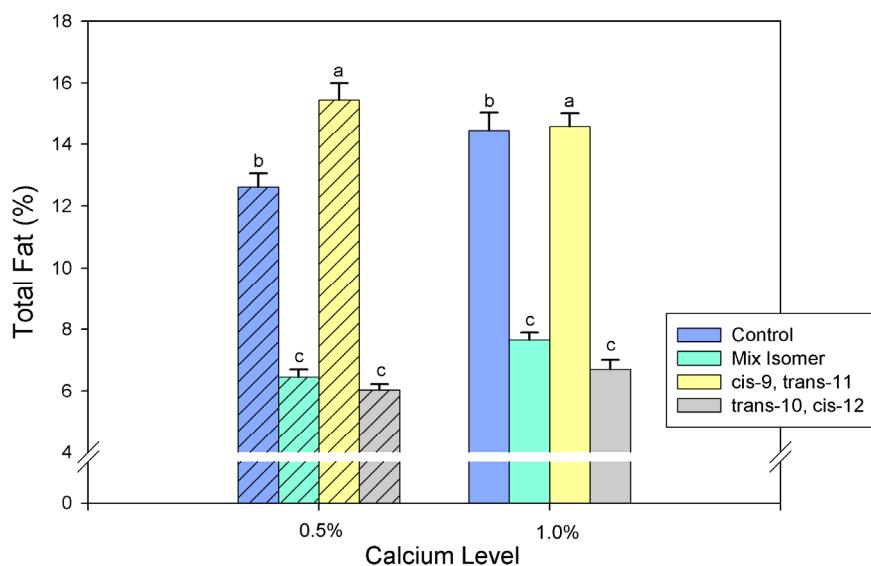


Figure 3.8. Total Fat Content. 4-week-old male ICR mice were fed treatment diets for 4 weeks. Treatments included two levels of calcium that consisted of 4 CLA preparations (0.5% CLA-mixed isomer, 0.2% c9,t11 and t10,c12 CLA isomers, and control). Fat was extracted with anhydrous ethyl ether in a semi-automated Soxhlet system for 8 hours. Each bar represents the mean \pm SE of 9 animals. Means with the same letter are not significantly different ($P < 0.05$).

3.3.5. Effect on organs and adipose tissues weights

Organ weights are shown in figure 3.9. There were no significant differences in any of the organs (liver, kidney, and spleen) among all treatments. Conversely, significant differences were found in adipose tissue weights (Figure 3.10). There was no effect of calcium on the adipose tissue weights. The mixed isomer group significantly reduced all adipose tissue weights, epididymal, mesenteric, retroperitoneal, and total, compared to control regardless of the dietary calcium level. Similar trends were observed with the t10,c12 isomer group, but not with the c9,t11 isomer groups.

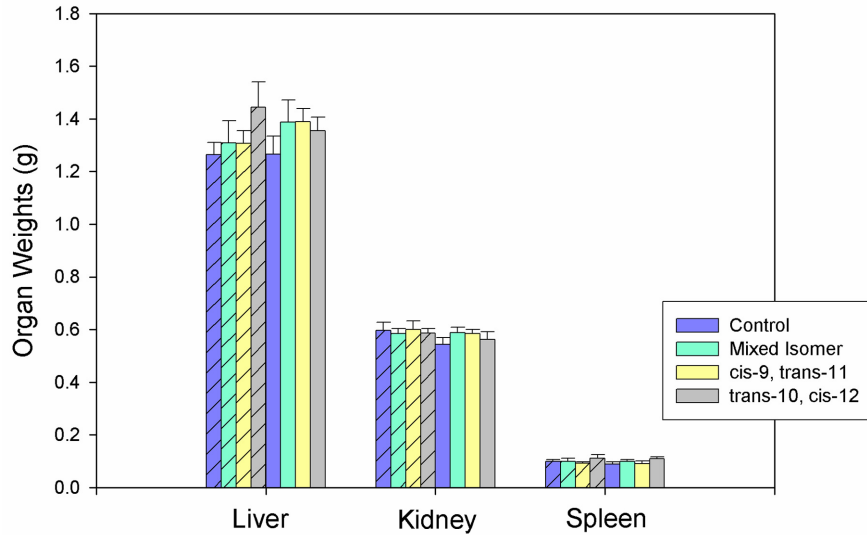


Figure 3.9. Organ Weights. 4-week-old male ICR mice were fed treatment diets for 4 weeks. Treatments included two levels of calcium (0.5% Ca; striped bars, 1.0% Ca; full bars) that consisted of 4 CLA preparations (0.5% CLA-mixed isomer, 0.2% c9,t11 and t10,c12 CLA isomers, and control). Each bar represents the mean \pm SE of 9 animals.

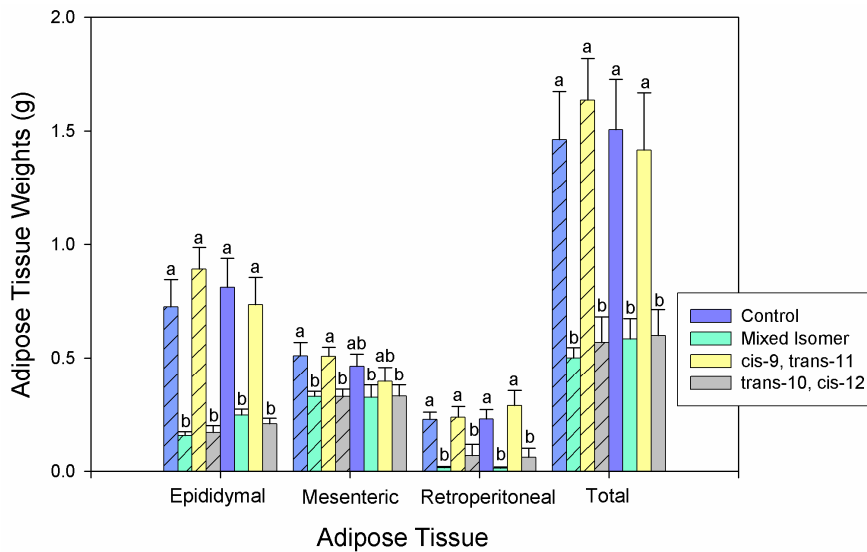


Figure 3.10. Adipose Tissue Weights. 4-week-old male ICR mice were fed treatment diets for 4 weeks. Treatments included two levels of calcium (0.5% Ca; striped bars, 1.0% Ca; full bars) that consisted of 4 CLA preparations (0.5% CLA-mixed isomer, 0.2% c9,t11 and t10,c12 CLA isomers, and control). Weights of 3 adipose tissues (epididymal, mesenteric, and retroperitoneal) and total adipose tissue weight. Each bar represents the mean \pm SE of 9 animals. Means with the same letter are not significantly different ($P < 0.05$).

3.4. Discussion

The ash, created by incineration, is mainly composed of inorganic minerals salts and trace metals. Since bone is the largest reservoir of calcium and phosphorus, a connection between bone mineral density and body ash can be drawn. Increased body ash by CLA supplementation can be associated with an increase in bone mineral density. Furthermore, the CLA mixed isomer treatment was shown to increase the calcium and phosphate content in the tibia of mice compared to control. Along with the changes in body ash, the present investigation confirms that CLA alters body composition in male ICR mice. The present study also shows a similar interaction to the previous observation in which CLA supplementation along with increased calcium intake significantly improved body ash (Park *et al.* 2006). In addition, it was determined that the t10,c12 isomer of CLA is responsible for increasing the amount of total body ash in the mouse model which is in agreement with previous findings (Belury, 2002; Bhattacharya *et al.* 2006; Mensink, 2005).

In addition to the relationship between total body ash and bone mineral density, respective to bone strength, the anisotropic properties of bone should also be considered. The contribution of collagen and its underlying architecture have shown to impact the mechanical strength of the bone (Boskey *et al.* 1999). In a study of the equine radius, collagen fiber orientation was highly correlated with mechanical strength properties (Riggs *et al.* 1993). Additionally, it has been reported that in the equine radius, longitudinally oriented collagen was correlated with greater modulus and monotonic strength, and remodeling was associated with more transverse collagen (Martin & Burr 1989). In this study, the total ash levels of the tibia in the mixed isomer and the t10,c12

groups were lower than control. As the level of ash being only one marker of bone biology, the administration of CLA could possibly alter the architecture (Puustjarvi *et al.* 1999), amount of collagen-crosslinks (Oxlund *et al.* 1996), and/or protein expression levels in the bone environment (Uitterlinden *et al.* 1998) which all have previously shown to be correlated with increased bone strength.

The exact mechanism in which CLA, particularly the t10,c12 isomer, increases the total body ash is unclear, yet there are several hypotheses. CLA has been shown to enhance the absorption of calcium in the intestines of Wistar rats during an 8-week experimental phase (Kelly *et al.* 2003). Similarly, others reported that CLA, significantly increased calcium transport and paracellular permeability in human intestinal-like Caco-2 cells (Jewell & Cashman 2003; Jewell *et al.* 2005). The t10,c12 isomer disrupted occludin, a protein responsible for membrane integrity and the permeability of ions, which was also associated with an increased paracellular permeability of calcium (Roche *et al.* 2001).

Alternatively, it is purported, that CLA improves bone mass by inhibiting the cyclooxygenase (COX) enzymatic pathways. COX plays an important role in bone formation as well as bone resorption through interaction with 1,25(OH)₂ vitamin D₃, PTH, IL-1, IL-6, and estrogens (Boyle *et al.* 2003; Cusack *et al.* 2005; Li *et al.* 1999).

The reduction of the hormone leptin has also been associated with CLA supplementation (Medina *et al.* 2000/7). A decrease in leptin is responsible for a decreased production of RANK (Receptor Activated Nuclear Factor) ligand which regulates bone resorption through osteoclast differentiation and activation. This can result in improved bone mass accumulation (Corino *et al.* 2002; Elefteriou *et al.* 2005;

Pruzanski *et al.* 1998; Rahman *et al.* 2001). CLA has also shown to modulate RANK in osteoclast differentiation of RAW264.7 cells (Rahman *et al.* 2006b). In addition, elevated levels of parathyroid hormone (PTH) have been reported to reduce bone mass through increased osteoclastic activity (Boyle *et al.* 2003). Weiler *et al.* (2004), reported a 60% reduction of PTH in male rats that were fed CLA with in their diet. The reduction of PTH could be explained by an upstream decrease of enzymatic COX activity by CLA (Degner *et al.* 2006; Wang *et al.* 2006). These findings imply that CLA may decrease the osteoclastic activity through a reduction in PTH expression.

In this study, CLA is shown to increase total body ash, which is representative of bone mineral density, in male ICR mice during a 4-week feeding period. This increase was linked with the t10,c12 isomer, but not the c9,t11 isomer. In addition the calcium and phosphate content in the tibia was higher in the mixed isomer group compared to control. This suggests the possible beneficial effects of CLA as a dietary supplement to help those susceptible to osteoporosis.

CHAPTER 4

THE EFFECT OF CLA ON OSTEOBLASTOGENESIS OF MOUSE BONE MESENCHYMAL STEM CELLS

4.1. Introduction

Osteoporosis results from reduced bone mass and disruption of the micro-architecture of bone. Such disruptions result in decreased bone strength and an increased risk of fracture (Poole & Compston 2006). After reaching peak bone mass during the mid-thirties, both men and women start losing bone at a rate of 0.5 - 1% yearly. Simultaneously, as bone mineral density declines with age, incidence of bone fracture increases with age (McGarry & Kiel 2000). While treatment methods for osteoporosis are becoming available prevention and changes in lifestyle are considered the most important way to reduce osteoporosis (Poole & Compston 2006). Possible lifestyle changes are to increase calcium intake along with vitamin D and exercise, while simultaneously decreasing smoking and alcohol abuse (Hind & Burrows 2007).

Bone is a tissue that goes through remodeling throughout life. Remodeling is the balance between bone formation and bone resorption. Bone formation can be described as osteoblastic activity (Minguell *et al.* 2001), and conversely, bone resorption can be described as osteoclastic activity (Aguila & Rowe 2005). Osteoblasts originate from bone marrow mesenchymal stem cells which have multipotent capabilities of differentiating into a variety of different cell types including osteoblasts, adipocytes, and chondrocytes (Yin & Li 2006). In opposition, osteoclasts originate from monocyte/macrophage precursor cells from a hematopoietic lineage (Boyle *et al.* 2003).

Conjugated linoleic acid (CLA) is a collective term used to describe positional and geometric isomers of linoleic acid (c9,c12 C_{18:2}) with two double bonds in conjugation. CLA is found naturally in foods derived from ruminant animals. Two prominent isomers of CLA are *trans*-10, *cis*-12 (t10, c12) and *cis*-9, *trans*-11 (c9, t11). The latter, is the most abundant isomer making up to 90% of the naturally consumed dietary CLA (Salas-Salvado *et al.* 2006). The biological health effects of CLA were first discovered by Dr. Pariza and his group while investigating the carcinogenic components of grilled beef. Surprisingly, in addition to carcinogens, the grilled beef extract displayed anti-carcinogenic qualities (Pariza & Hargraves 1985). Since its discovery an array of health benefits have been connected with CLA such as fat mass reduction and against diseases such as diabetes and atherosclerosis(Wahle *et al.* 2004).

Since the discovery of CLA to increase whole body ash, while reducing body fat (Park *et al.* 1997), it has been confirmed that CLA can enhance bone mineralization and protect against bone loss in different animal models (Banu *et al.* 2006; Bhattacharya *et al.* 2006; Cook *et al.* 1997; Kelly *et al.* 2003; Terpstra *et al.* 2002; Thiel-Cooper *et al.* 2001; Watkins *et al.* 1997). However, other studies have reported no effect of CLA on bone mineral composition (Ostrowska *et al.* 2003; Weiler *et al.* 2004) .

Epidemiological studies have shown that CLA supplementation focusing on bone health have not been consistent. A study conducted with postmenopausal women reported CLA was positively associated with forearm bone mineral density (Brownbill *et al.* 2005). In addition, Pinkoski *et al.* (2006) reported that CLA supplementation decreased markers of bone resorption in subjects during resistance training. CLA supplementation also yielded a significant improvement of bone mass, during the second

year of supplementation in overweight adults (Gauillier *et al.* 2005). However, others reported no benefit of CLA on bone mass (Doyle *et al.* 2005; Kreider *et al.* 2002).

It has been reported that the balance of osteoblasts and adipocytes within the bone marrow are an important factor associated with osteoporosis (Pei & Tontonoz 2004). Increased levels of bone marrow adipocytes and adipogenesis have shown to decrease the amount of osteoblastic activity. While a decrease in bone marrow adipocytes concentration can increase osteoblastic activity (Nelson-Dooley *et al.* 2005; Pei & Tontonoz 2004). Since there are a wealth of studies, indicating that CLA reduces adipogenesis *in vivo* and *in vitro* (Bhattacharya *et al.* 2006; Brownbill *et al.* 2005; DeLany *et al.* 1999; Evans *et al.* 2001; House *et al.* 2005; Park *et al.* 1997; Simon *et al.* 2006), it is possible that CLA could reduce the adipocyte population within the marrow environment, resulting in a balance that favors osteoblastic bone formation. Thus, through a direct and/or indirect action CLA could possibly decrease adipogenesis in bone, while simultaneously increasing the amount of cellular bone production.

Based on this theory, the objective of this study is to investigate the molecular action of CLA on bone formation by using mouse bone marrow mesenchymal stem cells. Others have reported CLA can indeed have an impact on the proliferation and differentiation of bone cells (Cusack *et al.* 2005; Platt *et al.* 2007; Watkins *et al.* 2003). CLA treatments increased alkaline phosphatase activity, a signal of osteoblast activity, in human osteoblast-like cell lines (Cusack *et al.* 2005). In rodent calvarial cells transcription factor core binding factor alpha 1 (Cbfa1) also known as runx-2 was increased with administration of CLA (Watkins *et al.* 2003). More recently, the c9,t11 isomer of CLA increased in mineralized bone nodule formation which was accompanied

by a variable increase in alkaline phosphatase activity in bone cells of human origin (Platt *et al.* 2007). The purpose of this study is to investigate the effect of individual isomers of CLA on the osteoblastogenesis differentiation of rodent bone marrow mesenchymal stem cells.

4.2. Material and methods

4.2.1. Materials

Mouse bone marrow mesenchymal stem cells were obtained from ATCC (American Type Cell Culture, Manassas, Virginia). TRIzol reagent, SYBR-greenER super-mix, dNTP mix, and Oligo(dT)12-18 were attained from Invitrogen (Carlsbad, California). Arsenazo III liquid stable reagent was purchased from Thermo Electron (Louisville, CO). Bio-Rad D/C protein assay kit was purchased from Fisher Scientific (Atlanta, GA). All CLA preparations were obtained from Natural Lipid (Hovdebygda, Norway). The mixed isomer consisted of 38% c9, t11 and 38% t10, c12, with 14% oleic acid (c9, C_{18:1}) and 3% steric acid (C_{18:0}). The t10,c12 isomer was 94 % pure, with 2% c9,t11 isomer and 3% other conjugated isomers (C_{18:2}). The c9,t11 isomer was 90% pure, with 4% t10,c12 isomer, 2% other conjugated isomers (C_{18:2}), and 3% oleic acid (c9, C_{18:1}). The linoleic free fatty acid was purchased from Sigma Aldrich (St. Louis, MO) and was greater than 99% pure. All Chemicals and serum, unless otherwise noted were acquired from Sigma Aldrich (St. Louis, MO) and were of cell culture grade.

4.2.2. Methods

4.2.2.1 Proliferation and osteoblastogenic media

The cells were cultured in Dulbecco's Modified Eagle's Medium with high glucose, 3.7 g/L sodium bicarbonate, 10% heat inactivated fetal bovine serum, 100 units/ml penicillin, and 100 ng/ml streptomycin. Upon confluence, the cells were differentiated by osteoblastogenic differentiation media (ODM). ODM includes proliferation media supplemented with 0.1 μ M dexamethasone, 10mM β -glycerol phosphate, and 50 μ M L-ascorbic 2-phosphate. Osteoblastogenesis was conducted for a minimum of 21 days, in order to allow maturation of the osteoblastic cells.

4.2.2.2. Preparation of treatment

Fatty acids were complexed with albumin at a final concentration of 50 μ M. In experiment one there were 8 treatment groups; undifferentiated, two concentrations of CLA mixed isomer treatment (100 μ M and 50 μ M), two concentrations of linoleic acid (100 μ M and 50 μ M), one concentration of each c9,t11 and t10,c12 CLA isomer (50 μ M), and albumin only as control. The second experiment was a dose response study which only investigated the effects of the t10,c12 CLA isomer treatment. There were 6 concentrations; 0, 5, 10, 20, 50, or 100 μ M.

Fatty acid-albumin complexes were prepared as previously described (Calder *et al.* 1990). An exact amount of free fatty acid was dissolved in methanol and the required amount of fatty acid/methanol solution was transferred into a clean vial. The methanol was then evaporated under nitrogen. The fatty acid residue was dissolved with 0.1 M potassium hydroxide. Albumin in 0.1M PBS, was added to the solublized fatty acid

solution. The albumin-fatty acid solution was then incubated at 4°C overnight. After incubation the pH of the solution was adjusted to 7.2 with 0.05N hydrochloric acid. The volume was adjusted and then sterilized, using a syringe driven 0.22µm filter in a sterile environment. This fatty acid-albumin stock solution was diluted 20x with culture media before use. Final concentrations of fatty acids were either 50 or 100µM while albumin was 50µM. All samples were cultured in triplicate.

4.2.2.3. mRNA isolation

Total mRNA was isolated from osteoblasts, with phenol based TRIzol reagent using approximately 1mL/100mg to lyse the cells. In a fresh microcentrifuge tube 0.5 mL chloroform was added per mL of homogenous lysate. The lysate solution was kept at room temperature for 10 minutes, before centrifugation at 12,000 g for 10 minutes at 4°C. After centrifugation the supernatant was removed while not disturbing the lower phenol layer. Isopropanol was added to the supernatant, in a 1:1 ratio with the initial amount of TRIzol reagent and centrifuged again. The isopropanol was removed and the mRNA pellet was washed by adding 75% ethanol in the same volume as isopropanol. The pellet was allowed to air dry for 5 minutes. The isolated mRNA was then resolubilized in water. The quantification of the isolated mRNA relative to the amount of protein was determined through spectrophotometric analysis at 260 nm and 280 nm, which represent nucleic acid and protein respectively. If the ratio of absorbance of 260nm/280nm was greater than 1.5, the sample was assumed to have enough pure mRNA for the next steps.

4.2.2.4. Creation of cDNA

In a clean RNase free micro-centrifuge tube, the following was added to each; 1µl 10mM dNTP mix, 1µl oligo(dt) 12-8, 10µl RNase free water, and 1µl mRNA with approximately 10pg – 500ng of mRNA. The tubes were gently mixed and briefly centrifuged to combine all contents at the bottom of the tubes. Then the tubes were incubated at 65°C for 5 minutes and then briefly placed on ice. The following was added to each tube: 400µl 5x first-strand buffer, 100µl 0.1M DTT, 100µl RNase-out (40U/µL), and 100µl superscript III RT (200U/µL). Next the samples were incubated at 25°C for 5 minutes, directly followed by incubation at 50°C for 60 minutes. Finally, the transcriptase was denatured by heating at 70 °C for 15 minutes.

4.2.2.5. qRT - PCR analysis

For analysis of the cDNA the following was added to each micro-tube; 1µl of template cDNA, 12.5µl SYBR-greenER super-mix, 0.5µl ROX reference dye, 10.5 RNase-free water, and 0.5µl of each the left and right primer of the gene of interest (Table 4.1), totaling a 25µl reaction volume. The reaction wells were then moved into a real-time PCR machine (Stratagene, La Jolla, CA) for the following cycles: 50° C for 2 minutes, 95° C for 10 minutes, 40 cycles of 95° C for 15 seconds - 60° C for 60 seconds, followed by an optional melting curve analysis.

Table 4.1. Primer sequences used for quantitative real-time PCR

Target Gene		Primer Sequence
Runx-2	sense	5'-AAACCGTCA AAGGTGTTGTC-3'
	antisense	5'-GGTTTCAAAGCACAGAGAGG-3'
Osteopontin	sense	5'-CAGA ATGCTGTGTCCTCTGA-3'
	antisense	5'-CTCATCCGAATGGTGAGATT-3'
Osteocalcin	sense	5'-CTTGAAGACCGCCTACAAAC-3'
	antisense	5'-ATACTTGCAGGGCAGAGAGA-3'
GAPDH	sense	5'-CAATGAATACGGCT ACAGCA-3'
	antisense	5'-GATGGAAATTGTGAGGGAG A-3'

Runx-2, runt-related gene 2, also known as Cbfa1, core binding factor alpha 1; GAPDH, glyceraldehydes 3-phosphate dehydrogenase

4.2.2.6. Quantification of calcium in osteoblast matrix

After 21 days of differentiation with CLA treatments the cultures were washed with PBS then incubated at 37° C with 2mL tris-buffer (25mM tris base and 0.5 % triton-X 100) for 30 minutes. The cell monolayer was scraped into a clean centrifuge tube. Upon collection of all cellular matter, each tube was sonicated (Misonix incorporated, Farmingdale, NY) in an ice bath for 30 seconds. Then, 0.5ml of the cell lysate was then transferred to an acid washed crucible and placed in a muffle furnace (Fisher Scientific, Atlanta, GA) at 550 °C for a minimum time of 4 hours. Following the ashing procedure, 2mL of 1.0N acetic acid was added to the crucible to solublize the remaining ash matter. The acetic acid mixture was then transferred to a clean centrifuge tube and normalized with concentrated sodium hydroxide. The calcium concentration was quantified in a 1:50 - sample: reagent - ratio, using the metallochromogen Arsenazo III reagent which combines with calcium ions ($K^{\circ} = 1 \times 10^{-7}$) (Bauer, 1981) to form a highly colored chromophore, which was measured at 650nm on an automated spectrophotometric plate

reader (Bio-Tek instruments, Winooski, VT). Finally the determined calcium concentration was normalized by the total protein concentration from each sample. Protein concentration was determined using the Bio-Rad DC Protein Assay kit.

4.2.2.7. Histochemical analysis of osteoblasts

After 28 days of differentiation with CLA treatments the cultures were washed with PBS and fixed with 10% formaldehyde. The samples were then washed with 95% alcohol and allowed to dry. The Alizarin red S stain (1 % alizarin red in deionized water and the pH adjusted to 7.0 with 1% ammonium hydroxide) was used to visualize the calcium deposits (DAHL, 1952). The stain was applied for 5 minutes then rinsed with water and optionally counter stained with fast green FCF solution (0.2 % fast green FCF and acetic acid in deionized water is a 4.5x concentrated solution) (Penney *et al.* 2002). The samples were then rinsed and dehydrated with alcohol.

4.2.2.8. Statistical analysis

Statistical analysis was computed with SAS statistical software (Version 9.0; SAS institute Inc., Cary, NC, USA). Data was analyzed by two-way analysis of variance (treatments and experiments). Mean separations were conducted with Duncan's New Multiple Range Test ($p < 0.05$). The calcium concentration in osteoblasts (figure 4.1) was analyzed with the log value.

4.3 Results

4.3.1 Experiment 1: Effects of CLA and linoleic acid on osteoblasts

4.3.1.1 Quantification of calcium in osteoblast matrix

The CLA mixed isomer increased the calcium content compared to the other groups. Followed by the linoleic acid treatment groups and then the individual isomer and control groups. Increasing the concentration of the CLA mixed isomer treatment resulted in an increased amount of calcium, which was not observed with the linoleic acid treatment. The t10,c12 isomer showed an increased calcium accumulation compared to the c9,t11 isomer.

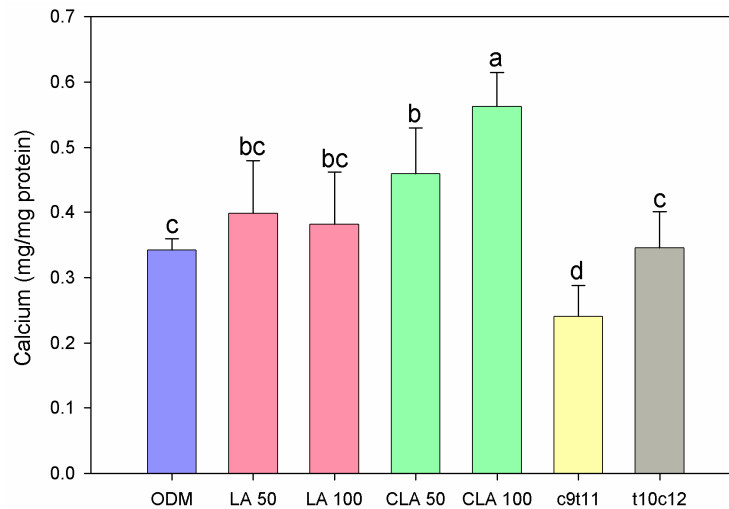


Figure 4.1. Quantification of Calcium in Osteoblast Matrix; Experiment 1. Effects of CLA and its isomers on calcium concentration from murine bone marrow stromal stem cells. Cells were differentiated with osteogenic differentiation media (Dulbecco's Modified Eagle's Medium, 10 % fetal bovine serum, 0.1 μ M dexamethasone, 10mM β -glycerol phosphate, 50 μ M L-ascorbic acid 2-phosphate) for 4 weeks. Fatty acids were treated throughout differentiation period as albumin complexes (50 - 100 μ M linoleic acid (LA), 50 - 100 μ M CLA-mixed isomer (CLA), 50 μ M cis-9,trans-11, or trans-10,cis-12 isomer with 50 μ M albumin for all treatments). Calcium was determined by the colorimetric Arsenazo III method. Each bar represents the mean \pm SE N=9, collected from 3 independent experiments. Means with the same letter are not significantly different ($P < 0.05$).

4.3.1.2. qPT-PCR analysis

The t10,c12 isomer of CLA significantly increased the expression level of osteopontin, osteocalcin, and runx-2 compared to other treatments (figure 4.2).

Osteopontin expression was significantly increased due to the t10,c12 treatment, the CLA mixed isomer treatment at 100 μ M induced the second highest level of expression. All other treatments were similar to control. Osteocalcin expression was also significantly increase by the t10,c12 and the 100 μ M CLA mixed isomer treatments. All other treatments were similar to control. A similar trend was shown in expression of Runx-2, the t10,c12 and 100 μ M of CLA mixed isomer treatments increased expression. The other treatments were either similar or reduced when compared to the control group. Overall the t10,c12 and the 100 μ M concentration of CLA mixed isomers are responsible for the increase of relative quantity of mRNA expression in osteoblastic cells.

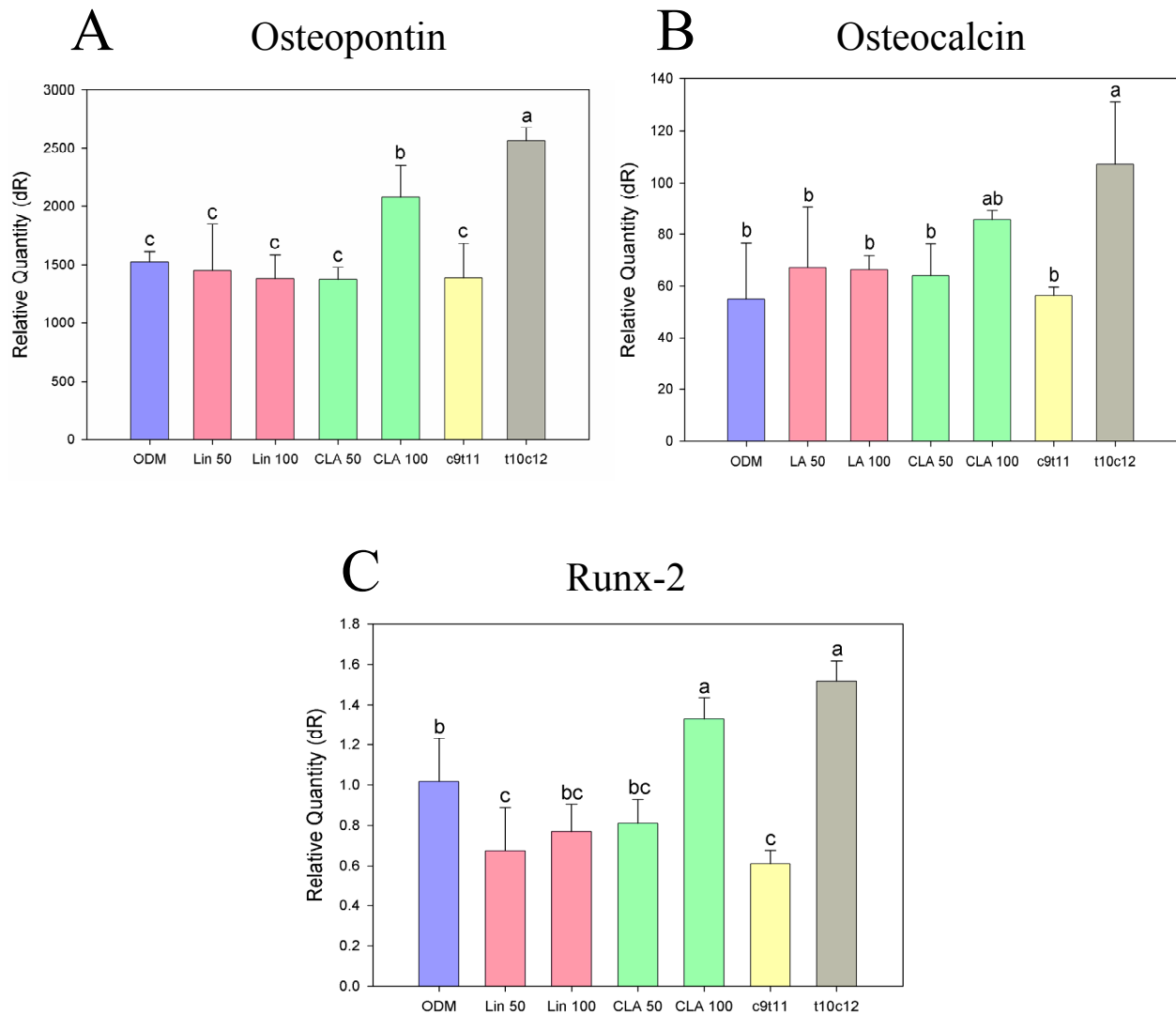


Figure 4.2. Relative RNA Expression; Experiment 1. (A) Osteopontin (B) Osteocalcin (C) Runx-2. Effects of CLA and its isomers on RNA expression from murine bone marrow stromal stem cells. Cells were differentiated with osteogenic differentiation media (Dulbecco's Modified Eagle's Medium, 10 % fetal bovine serum, 0.1 μ M dexamethasone, 10mM β - glycerol phosphate, 50 μ M L-ascorbic acid 2-phosphate) for 4 weeks. Fatty acids were treated throughout the differentiation period as albumin complexes (50 - 100 μ M linoleic acid (LA), 50 - 100 μ M CLA-mixed isomer (CLA), 50 μ M cis-9,trans-11, or trans-10,cis-12 isomer with 50 μ M albumin for all treatments). RNA expression determined through qRT-PCR methods and normalized by GAPDH expression. Each bar represents the mean \pm SE of 7 samples. Means with the same letter are not significantly different ($P < 0.05$).

4.3.1.3. Calcium deposit visualization

The t10,c12 isomer of CLA visually increased the mineralization of the cell cultures. The CLA-mix treatment also increased cellular mineralization, compared to control and the c9,t11 treatment. After the four-week differentiation period all treatment groups showed mineralization compared to the undifferentiated cultures.

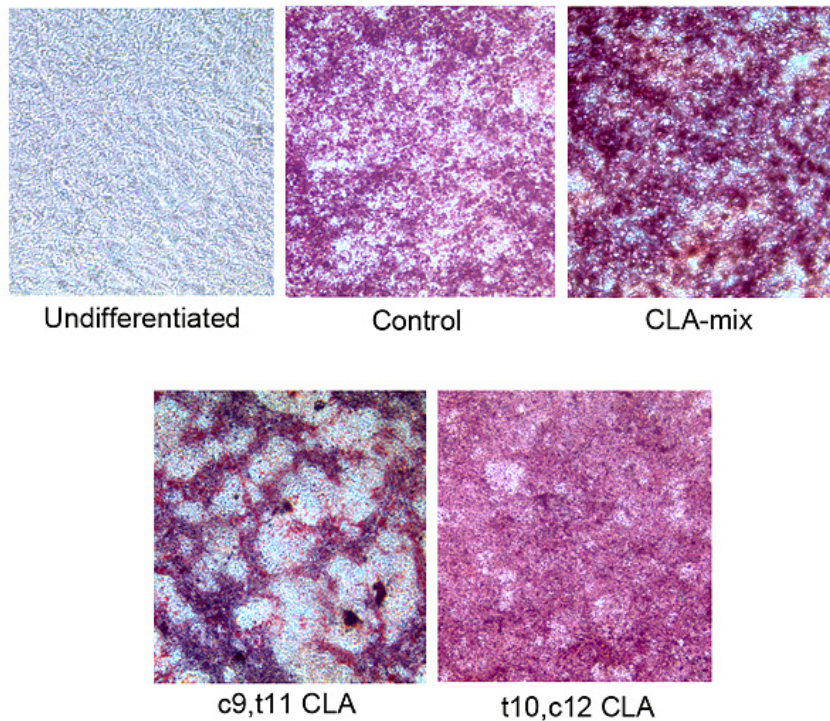


Figure 4.3. Calcium Deposit Visualization; Experiment 1. Effects of CLA and its isomers on bone formation from murine bone marrow stromal stem cells. Cells were differentiated with osteogenic differentiation media (Dulbecco's Modified Eagle's Medium, 10 % Fetal Bovine Serum, 0.1 μ M Dexamethasone, 10mM β - Glycerol Phosphate, 50 μ M L-Ascorbic acid 2-phosphate) for 4 weeks. Undifferentiated cells were not treated with osteogenic differentiation media. Fatty acids were treated throughout the differentiation period as albumin complexes (100 μ M CLA-mixed isomer, 50 μ M cis-9,trans-11 or trans-10,cis-12 isomer with 50 μ M albumin for all treatment). Cells were stained with Alizarin Red S stain, a method for the visualization of the nodular pattern and calcium deposition of osteoblastic cell culture which selectively binds to calcium and is red in color.

4.3.2 Experiment 2: t10,c12 CLA dose response

4.3.2.1 Quantification of calcium in osteoblast matrix

The t10,c12 isomer increased the calcium content compared to the other treatment concentrations (figure 4.4). The 25 μ M treatment was similar to control. While, the 5 μ M, 10 μ M, and 100 μ M treatments slightly decreased the calcium content. The maximum beneficial concentration of the t10,c12 isomer is shown to be 50 μ M.

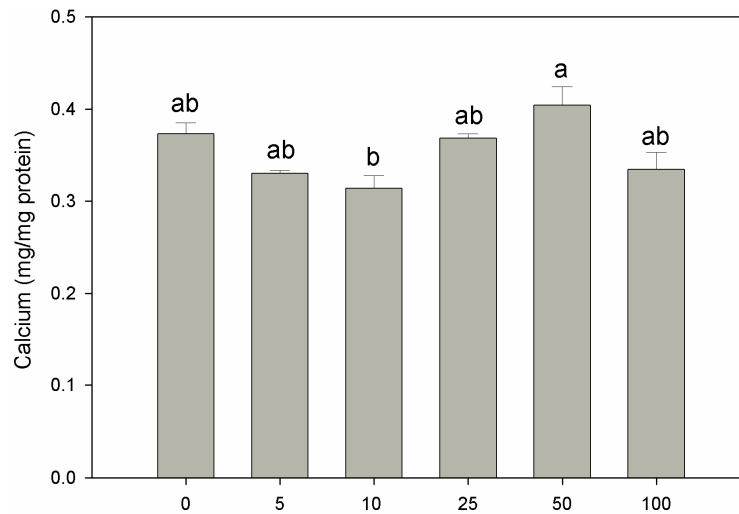


Figure 4.4. Quantification of Calcium in Osteoblast Matrix: Experiment 2. Effects of increasing concentration of the t10,c12 isomer on calcium concentration from murine bone marrow stromal stem cells. Cells were differentiated with osteogenic differentiation media (Dulbecco's Modified Eagle's Medium, 10 % fetal bovine serum, 0.1 μ M dexamethasone, 10mM β - glycerol phosphate, 50 μ M L-ascorbic acid 2-phosphate) for 4 weeks. Fatty acids were treated throughout the differentiation period as albumin complexes; 5 μ M, 10 μ M, 20 μ M, 50 μ M, 100 μ M of trans-10, cis-12 isomer with 50 μ M albumin for all treatments). Calcium was determined by the colorimetric Arsenazo III method. Each bar represents the mean \pm SE N=3, collected from one experiment. Means with the same letter are not significantly different (P< 0.05).

4.3.2.2. Calcium deposit visualization

The different concentrations of the t10,c12 isomer had little effect on the visualization of the calcium within the cultures. The density of the color in the 50 μ M treatment group was slightly stronger compared to the other groups; which is supported by the calcium analysis (figure 4.4).

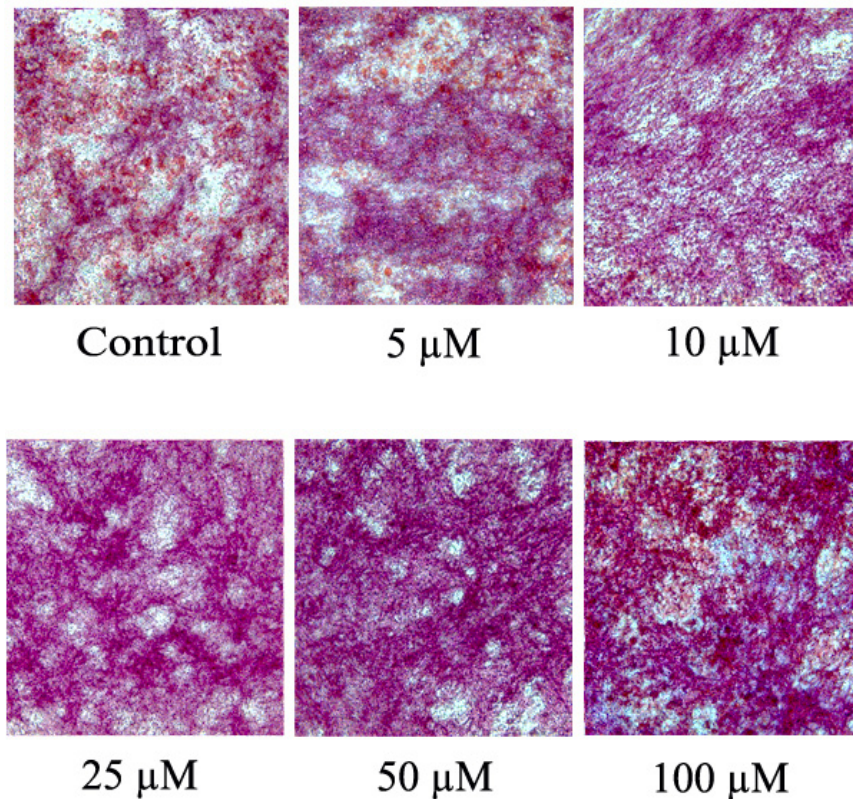


Figure 4.5. Calcium Deposit Visualization; Experiment 2. Effects of CLA and its isomers on bone formation from murine bone marrow stromal stem cells. Cells were differentiated with osteogenic differentiation media (Dulbecco's Modified Eagle's Medium, 10% Fetal Bovine Serum, 0.1 μ M Dexamethasone, 10mM β - Glycerol Phosphate, 50 μ M L-Ascorbic acid 2-phosphate) for 4 weeks. Fatty acids were treated throughout the differentiation period as albumin complexes; 5 μ M, 10 μ M, 20 μ M, 50 μ M, 100 μ M of trans-10, cis-12 isomer with 50 μ M albumin for all treatments). Cells were stained with Alizarin Red S stain, a method for the visualization of the nodular pattern and calcium deposition of osteoblastic cell culture which selectively binds to calcium and is red in color.

4.4. Discussion

This present study has shown that CLA has the ability *in vitro* to modulate the osteoblastogenesis of murine mesenchymal stem cells. The t10,c12 isomer of CLA was shown to increase the osteogenic gene expression of osteocalcin, osteopontin, and runx-2 compared to other treatments. This effect was accompanied by the CLA mixed isomer treatment increasing the calcium concentration of the cellular mineralization compared to other treatments. Furthermore the dose response of the t10,c12 isomer show that an optimal concentration of the isomer is 50µM to enhance the calcium in the osteoblast matrix. These results suggest the beneficial role CLA may have on bone health.

Osteoblastic production of Runx-2 (also known as Cbfa-1), an essential protagonist for osteogenesis (Komori *et al.* 1997), has also been reported to increase as a result of CLA supplementation (Watkins *et al.* 2003). Osteocalcin is expressed throughout the matrix maturation and osteopontin characterizes the post differentiation phase (Aubin, 2001; Liu *et al.* 2003)

It has been well known that prostaglandin, particularly E₂, has been involved in bone physiology. High levels of prostaglandin E₂ (PGE₂) (10⁻⁶M) inhibit bone formation, while at lower concentrations (10⁻¹⁰-10⁻⁸ M) increase bone formation. In addition PGE₂ stimulates bone resorption (Takiguchi *et al.* 1999). A number of reports have indicated that CLA reduces prostaglandin production. In fact, the CLA mixed isomer and the t10,c12 isomer, decrease PGE₂ biosynthesis in human osteoblast-like SaOS-2 and MG-63 cells to a greater extent than the c9,t11 isomer (Cusack *et al.* 2005). This PGE₂ reduction by the mixed isomer and t10, c12 isomer treatments, resulting in increased bone formation is consistent with our results.

CLA has shown the ability to increase whole body ash (Park *et al.* 1997), which is representative of bone mass. Cook *et al.* (1997) found higher levels of bone ash in the tibia of CLA-fed chicks compared to control. Pigs supplemented with 0.5% and 1.0% CLA resulted in an increased bone weight compared to control and animals supplemented with 0.12% or 0.25% CLA (Thiel-Cooper *et al.* 2001). A positive effect was observed in growing male chicks in response to CLA fed in the form of butterfat (Watkins *et al.* 1997). It was reported ash retention was significantly greater in mice fed 1.5% CLA compared to restricted diet and control groups (Terpstra *et al.* 2002). Additionally, Wistar rats showed no sign of elevated bone resorption markers; while, the markers of bone formation were increased due to the CLA treatments (Kelly *et al.* 2003). Experiments have shown, BALB/C male mice fed CLA had increased bone mass (Banu *et al.* 2006). A study using female C57BL/6 retired breeders treated with CLA for 10 weeks, showed increased bone mass by CLA (Bhattacharya *et al.* 2006). Recently, the t10,c12 CLA isomer dose dependently (0 – 0.30% - CLA) was found to increase the femur weight of male mice (Viswanadha *et al.* 2006). In summary, CLA has shown the innate ability to increase bone mass in a variety of animal models. This increase of bone mass, in general, can be explained by an increase in osteoblastic activity or a decrease in osteoclastic activity.

CLA supplementation reduced bone resorption rates in rats, compared to control (Kelly & Cashman 2004/11). Rahman *et al.* (2006a) and Banu *et al.* (2006) reported that CLA-fed mice maintained a higher bone mineral density than control-fed mice which was a result of decreased osteoclastic activity. The same group also suggested that CLA

inhibited osteoclastogenesis primarily through the RANK ligand activated pathways in osteoclast-like Raw264.7 macrophage cells (Rahman *et al.* 2006b).

In this study, CLA is shown to increase the expression of osteoblastogenic markers, which are representative of osteoblastic activity, in murine mesenchymal stem cells. CLA treatments also increased total calcium concentration, determined visually and chemically, within the cellular cultures. This increase was linked with the mixed CLA isomers and the t10,c12 isomer, but not the c9,t11 isomer. Also the t10,c12 isomer was shown at a 50 μ M concentration to increase the calcium accumulation in culture. This suggests the cellular action of CLA to increase osteoblastic differentiation suggesting the possible beneficial effects of CLA as a dietary supplement for the prevention of osteoporosis.

CHAPTER 5

FUTURE REASEARCH

In order to compliment this current research future work should continue to focus on the *in vivo* and *in vitro* research models. Furthermore, future research should record effects that may indirectly influence the formation or more importantly the resorption of bone.

In this current research the bone ash of the tibia was decreased in treatment groups compared to control. Other research has shown that after intense long term-exercise bone density was significantly reduced without changing the bone's mechanical properties. This suggests that changes in the structural orientation of the collagen network contribute to the retention of strength even though a reduction in the mineral density occurs (Puustjarvi *et al.* 1999). The possibility of CLA to structurally reorganize bone tissue is worth exploration. Analysis could include physical strength testing and visualization of the bone tissue's structure when supplemented with CLA versus control. In addition, the ability of CLA to increase bone density could be evaluated throughout the experiment. Specifically, through the use of dual energy X-ray absorptiometry (DEXA), which is a nondestructive analytical method to evaluated bone density (Rahman *et al.* 2006a). DEXA could provide data that would identify the period at which CLA significantly increases the bone density. This type of 'time-course' experiment could also be carried out in animals of different age or animals which have undergone an ovariectomy to simulate the post-menopausal period. These experiments would determine if the effects of CLA on bone mass are dependent on the age of the animal or simply the amount of time and level at which CLA is consumed.

Future research investigating the effects of CLA on bone resorption, would give further insight to how consumption of CLA results in a higher bone density. By establishing if an increase in bone formation or a decrease in bone resorption occurs. One possible assessment could be to quantify the activity or inhibition of osteoclastic cells, measured by urinary pyridinoline and deoxypyridinoline (Kelly & Cashman 2004/11; Zhan *et al.* 1999), as a result of CLA consumption with increased calcium.

The next phase of the cell culture research is to conduct time dependent studies with CLA on differentiating osteoblasts. Following the time dependent studies, other similar cell lines from different species could be used to determine the specie specific effects of CLA. This current research focused on the ability of CLA to enhance osteoblastogenesis, yet it would also be beneficial to simultaneously monitor other gene expression. Specifically, genes related to adipocyte tissue and collagen proteins.

Research could also be conducted to determine the inhibition or up-regulation of other cellular processes by CLA administration. The mesenchymal stem cell has the ability to differentiate into adipocytes or chondrocytes, therefore studies should be conducted to determine the effects of CLA on the different differentiation possibilities of these pluripotent stem cells. The studies would be similar to the present study although different cofactors would be used in the media to induce alternate differentiation. The culmination of CLA's effects on the different pathways of the mesenchymal stem cell would allow for a better understanding of the increased bone mass in the biological environment.

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