EFFECTS OF SUPPLEMENTATION WITH CONJUGATED DIENES OF LINOLEIC ACID ON FLUORIDE, CALCIUM, AND MAGNESIUM LEVELS IN HARD TISSUES AND SERUM OF MICE

Izabela Gutowska,[a] Irena Baranowska-Bosiacka,[b] Agnieszka Szynkowska,[a] Ewa Siwiec,[a] Małgorzata Szczuko,[a] Iwona Noceń[c] Ewa Rębacz-Maron,[d] Dariusz Chlubek,[b] Ewa Stachowska[a]

Szczecin, Poland

SUMMARY: With the recognition of their ability to promote weight loss, conjugated dienes of linoleic acid (CLA) have become the main ingredient of certain dietary supplements to counteract obesity. The results of prospective studies, however, indicate there may be long-term side effects that could be of key importance for the safety of CLA-based products. The aim of this study was to examine the effect of CLA supplementation with the effective weight-reducing *trans*-10, *cis*-12 CLA, its ineffective companion *cis*-9, *trans*-11 CLA, and 1:1 mixtures of them on the mineral composition of hard tissues and blood in mice. The study material consisted of bones, teeth, and serum of female obesity-prone C57Bl/6J mice fed MURIGRAN feed containing soy oil or soy oil plus CLA. After 4 weeks of supplementation, the animals were sacrificed and the tissues were collected for analysis. The fluoride content was determined by potentiometry, while calcium and magnesium levels were determined by atomic absorption spectroscopy. The results showed that CLA isomers significantly increased the bone fluoride content while reducing the fluoride levels in teeth and serum, which was particularly evident in case of the CLA *cis*-9, *trans*-11 isomer. With the latter, a slight reduction in the bone calcium content was also observed, accompanied by a statistically significant reduction of magnesium levels, which might have a significant impact on bone quality. It appears that calcium and magnesium are transported from bones to serum and teeth with CLA supplementation, wherein a rise in their levels was observed. In teeth, increased calcium and magnesium levels with simultaneous reduction in the fluoride levels occurred, thereby possibly reducing enamel hardness by increasing the formation of crystal defects and the presence of amorphous hydroxyapatite.

Keywords: Bone mineral changes; CLA effects on tissue fluoride; Conjugated dienes of linoleic acid (CLAs); Defective enamel apatite; Mouse model for CLAs; Serum calcium and magnesium; Tooth mineral changes.

INTRODUCTION

The need to reduce the growing amount of obesity in people, particularly abdominal obesity, has become a matter of growing importance in many countries and has led to dangerous health complications that have shown significant increases in recent years.[1,2] At the same time, the number of dietary slimming supplements has also increased to meet the demands of customers for them. Since numerous studies have shown that conjugated linoleic acids (CLAs) are effective in reducing body weight,[3,4] they have become the main ingredient in many of these supplements.

CLA-containing dietary supplements are usually mixtures of two linoleic acid (LA) isomers, *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA, in a 1:1 ratio.[5] Each

[a]Dept. of Biochemistry and Human Nutrition; [b]Dept. of Biochemistry; [c]Dept. of Medical Chemistry; Pomeranian Medical University, al. Powstańców Wlkp. 72, 70-111 Szczecin, Poland; [d]Dept. of Anthropology; University of Szczecin, ul. Wąska 13, 71-415 Szczecin, Poland.
For correspondence: D Chlubek, E-mail: dchlubek@pum.edu.pl.

of these isomers may show a wide range of mutually opposing effects,[6,7] but only the *trans*-10, *cis*-12 CLA isomer is involved in the *in vivo* delipidation processes that lead to reduction in fat tissue.[8-10] No such effect could be demonstrated for the other isomer, *cis*-9, *trans*-11 CLA, either *in vitro*, or *in vivo*.[4,11] At the same time, *in vitro* studies showed that *trans*-10, *cis* 12 CLA may favor the development of insulin resistance and lead to lipodystrophy.[12]

Since prospective studies may be of key importance for assessing the safety of CLA-based products,[12-14] C57BI/6J mice predisposed to atherosclerosis and obesity in response to high-fat diets are an obvious choice for investigating whether CLAs can affect the metabolism of hard tissues. Therefore, the aim of this study was to examine the effects of CLA supplementation on the mineral composition of bones and teeth in C57BI/6J mice.

MATERIALS AND METHODS

Animals: The study material consisted of bones, teeth, and serum from female mice, type C57BL/6J that can develop obesity and atherosclerosis from their diet. After one week of adaptation, 46 of these mice, 6 weeks old, were divided into five groups: control (n = 6) and four experimental equal groups of 10 each (total = 40).The animals were fed MURIGRAN rodent chow containing 6.5% of soy oil (control group) or 5.5% of soy oil with the addition of 1% CLA mix composed of the *cis*-9, *trans*-11 isomer (CLA9,11) and the *trans*-10, *cis*-12 isomer (CLA10,12) (experimental groups). After 4 weeks of supplementation, the animals were sacrificed by administration of ketamine and the tissues were removed and collected for analysis. The study was approved by the Local Ethics Committee for Scientific Experiments on Animals in Szczecin (Poland).

Sample preparation and analysis: Blood samples were obtained from the deceased mice by collection directly from the heart. They were centrifuged 30 min after collection, and the serum was separated. Femurs and teeth were separated from soft tissues and boiled three times in distilled water. Next, the samples were dried for 24 hr at 60ºC and pulverized in an agate mortar.

Fluoride (F) concentrations were determined by the potentiometric method using an Orion F ion-selective electrode. Calcium (Ca) and magnesium (Mg) concentrations were determined using an atomic absorption spectrophotometer 9100X (PHILIPS).

Statistical analysis: The results were statistically analysed using the software package Statistica 6.1. The arithmetical mean and the standard deviation (SD) were calculated for each of the studied parameters. Because most of the distributions differed from the normal distribution (Shapiro-Wilk test), further analysis involved non-parametric tests. To assess the differences between the studied groups, the non-parametric Mann-Whitney test was used. The level of significance was taken as $p \leq 0.05$.

RESULTS

CLA supplementation of the mice led to an increased accumulation of F in femoral bones in all study groups (Figure 1a). In addition, statistically significant differences were observed between the CLA mix (as used in dietary supplements) and the two CLA9,11 and CLA10,12 isomers used separately.

Opposite results were observed in the teeth (Figure 1b) and in the serum (Figure 1c), wherein supplementation with the CLA isomers led to a reduction in F levels.

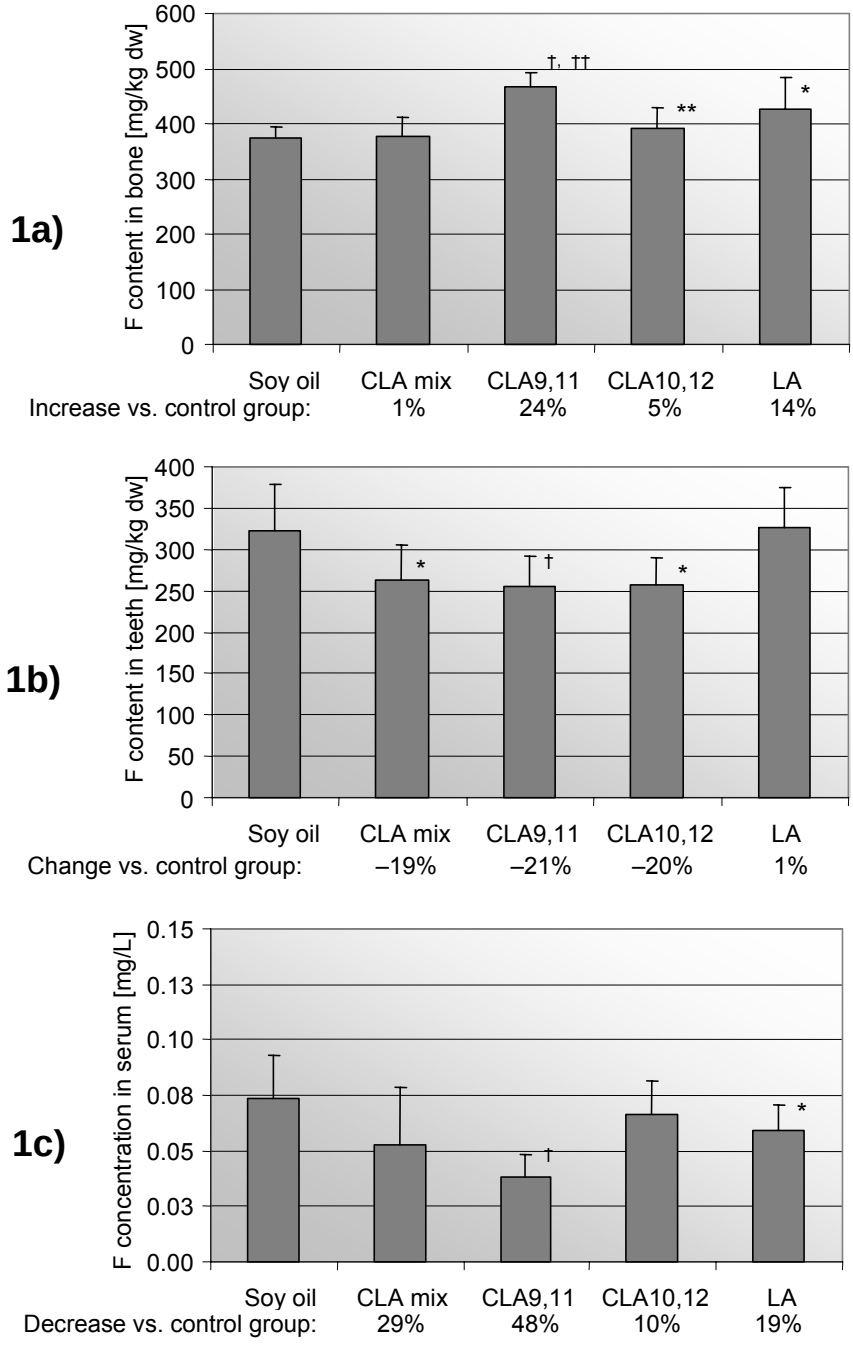


Figure 1. Content of fluoride in the bone (a), teeth (b), and serum (c) of control and experimental mice. *p<0.03; †p<0.01 - significant difference vs. control group **p<0.05; ††p<0.002 - significant difference vs. CLA mix group.

Bone Ca content increased slightly in the group supplemented with the CLA mix, and while the isomers led to reductions in bone Ca, statistical significance was observed only for LA (Figure 2a). In the teeth, the CLAs caused a significant

increase in Ca content, although statistically significant differences were observed only for the CLA mix and CLA9,11. However, no statistically significant differences were observed between the groups supplemented with CLA and its individual component isomers (Figure 2b). Serum Ca levels also increased but only after supplementation with the CLA mix, CLA9,11, and CLA10,12 (Figure 2c).

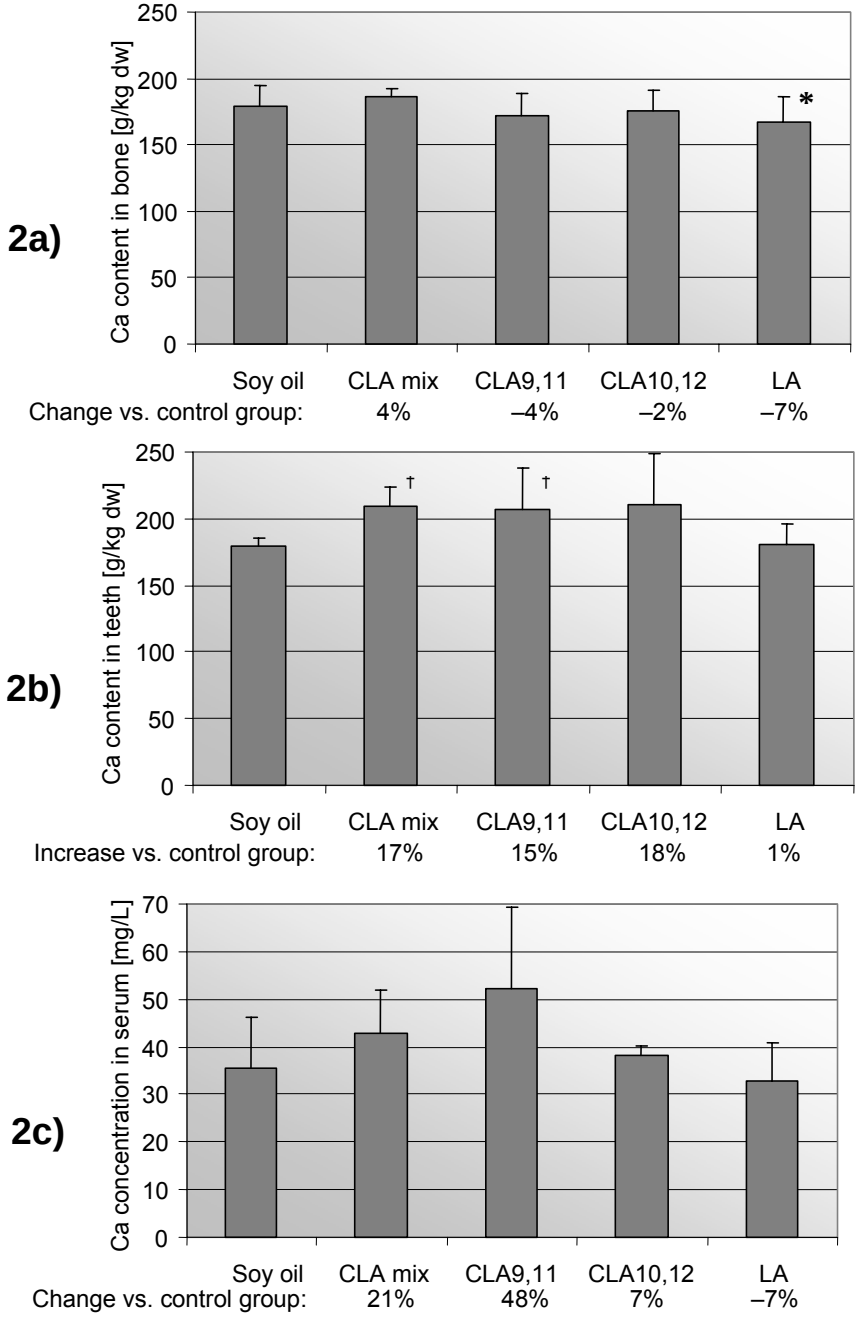
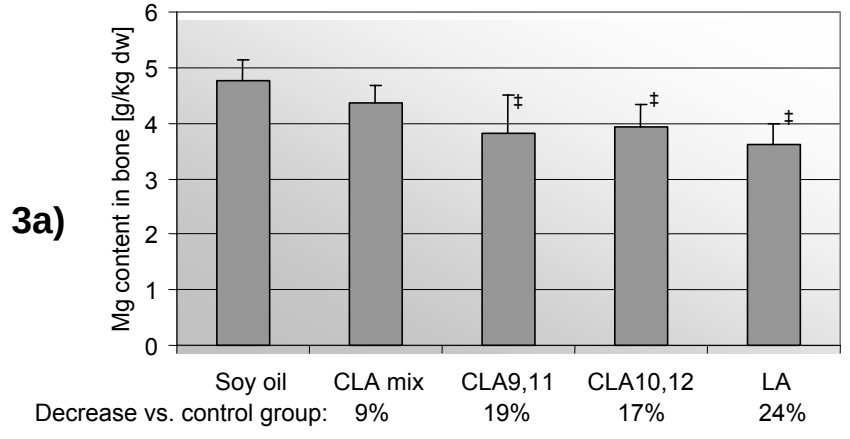
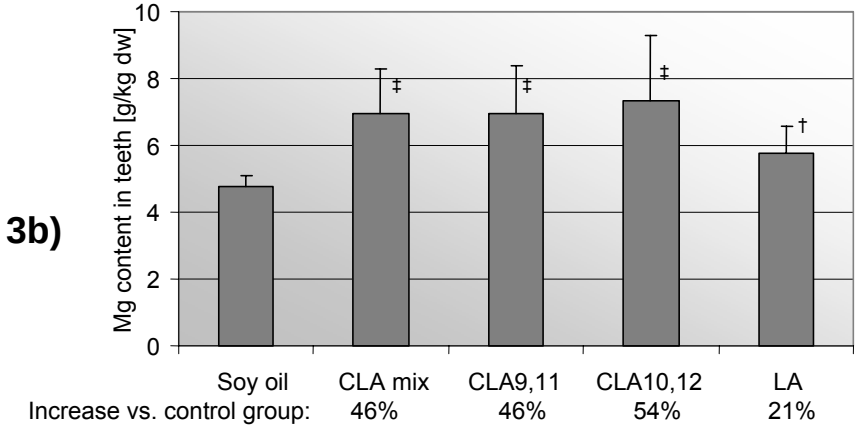


Figure 2. Content of calcium in the bone (a), teeth (b) and serum (c) of control and experimental mice. *$p<0.05$; †$p<0.008$ - significant difference vs. control group.

Bone Mg levels decreased with all the CLA isomers (Figure 3a). Opposite results from CLA supplementation occurred in the teeth, in which a marked, but not a statistically significant increase in Mg levels was observed in all study groups (Figure 3b). In all study groups, serum Mg levels increased, with statistical significance being observed for the CLA mix, CLA9,11, and CLA10,12 (Figure 3c).





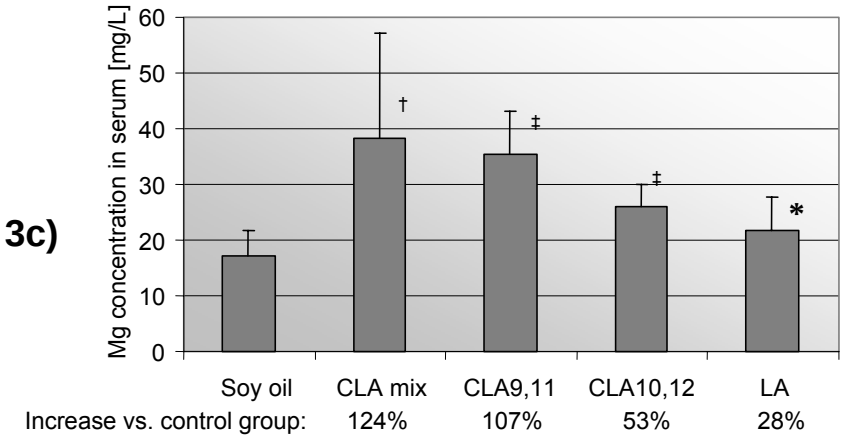


Figure 3. Content of magnesium in the bones (a), teeth (b), and serum (c) of control and experimental mice. *p<0.05; †p<0.03; p<0.005 - significant difference vs. control group.

DISCUSSION

CLA-containing products are widely available commercially, and interest in them has grown rapidly since the discovery of the role of CLAs for weight reduction or slimming in various research models.[8-10] However, as more is learned about CLAs, it is becoming evident that the safety of CLAs for weight reduction is largely dependent on concomitant pathologies.[15] To the best of our knowledge, the present study is the first to examine the effects of CLA isomers on the accumulation of F, Ca, and Mg in hard tissues, where they play a key role as building blocks in those tissues.

Our results show that CLA isomers may increase, in a statistically significant manner, the F content in murine bones with simultaneous reduction of F levels in the teeth and serum. This effect is most pronounced for *cis*-9, *trans*-11 CLA, which has no delipidation properties.[4,11] The excessive accumulation of F in bones caused by this isomer may interrupt the apatite crystal nucleation process. Crystallization would occur continuously toward the C-axis at peripheral areas, whereas the central area would remain amorphous, resulting in crystal defects.[16] In addition, excessive accumulation of F in trabecular bone may increase the bone volume and thickness without a concomitant increase in trabecular connectivity, which may reduce bone quality despite the increase in bone mass.[17] On the other hand, the mix of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA isomers at ratios used in slimming supplements caused no significant changes in the bone F content, probably due to the presence of the *trans*-10, *cis*-12 CLA which masked the negative effect of *cis*-9, *trans*-11 CLA.[6,7] However, despite the lack of change in the F content of bones in mice supplemented with a mix of CLA isomers, we observed a significant reduction in F levels in teeth and serum caused by both the CLE mix and its two component isomers used separately.

Excessive leaching of F from teeth may weaken the tooth structure, thus leading to faster initiation and development of caries.[18] Probably, the reduction in F levels in the teeth and serum of mice in all study groups was due to F being transported to bones and inhibition of osteoclastogenesis by modulated RANKL signaling.[19] In addition, the process may be augmented by CLAs increasing the levels of the bone formation markers osteocalcin and alkaline phosphatase, as demonstrated by Watkins et al. in a murine osteoblastic cell line study.[20]

The available literature has only limited information on the effect of CLAs on Ca metabolism in hard tissues.[19,21] In one of these studies, supplementation of mice with a 1:1 mix of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA caused no significant changes in the Ca accumulation and intensification of osteogenesis in the bone tissue.[21] Those results appear to agree with our own findings of the lack of significant change in the bone Ca levels in mice supplemented with the CLA mix. However, besides a slight reduction in the amount of Ca in the bones of our mice, we found a statistically significant reduction of Mg levels, which might have a significant impact on bone quality. Probably, Ca and Mg are transported from bone to blood (plasma) and teeth, where an increase in their levels was observed. In teeth, increased Ca and Mg levels with simultaneous reduction in F may reduce

enamel hardness by formation of crystal defects and increasing the amount of amorphous (immature) hydroxyapatites.[16,17]

As is well known, the problem of obesity is particularly important in the perimenopausal period, since at this stage of life, obesity is often accompanied by disorders related to excessive body weight, such as arterial hypertension, hyperglycemia, hyperinsulinism, and atherosclerosis. In the available literature, long-term use of CLA as a supplements to reduce body weight not only helps to maintain weight loss and to affect the bone mineral density,[22,23] but it also may intensify the above-mentioned disorders.[24,25] Owing to such undesirable side effects, researchers are examining other natural substances that reduce the body weight that will not result in adverse effects. Two of these substances are raspberry ketones[26-28] and astaxanthin found in red salmon.[29] In addition, possible connections between the growing prevalence of obesity generally, particularly of the abdominal type, and changes in the nutritional quality of today's food are in urgent need of greater investigation.

In conclusion, this work has shown that dietary supplementation with the various isomers of linoleic acid caused changes in bone and teeth mineral composition in the mice. However, the greatest changes were observed with *cis*-9, *trans*-11 CLA, which does not exert a delipidation effect either *in vitro* or *in vivo*, but is added to slimming diet supplements in 1:1 proportion.[30]

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