Original Article

Effect of Dietary Supplementation with Conjugated Linoleic Acid on Bone Mineral Density, Bone Metabolism Markers and Inflammatory Markers in Healthy Post-menopausal Women: a Randomized Double Blind Placebo Controlled Trial

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Abstract

Introduction: Conjugated linoleic acid (CLA) has been shown to positively influence calcium and bone metabolism in experimental animals and cell culture, but there are limited human data available.

Material and Methods: The study consisted of a double-blind, placebo-controlled trial in which 76 healthy post-menopausal women (aged 55.1) were randomly assigned to receive daily either four capsules CLA G80 containing 3.2 g isomer blend (50:50% cis-9, trans-11: trans-10, cis-12 isomers) or four capsules containing high oleic sunflower oil as placebo for 12 weeks. Urine and blood samples were collected at weeks 0 and 12 and were analyzed for biomarkers of calcium and bone metabolism and inflammatory markers (TNF-α and IL-6). Subjects completed 3-days dietary records during the trial, in weeks 0 (baseline), 6 and 12.

Results: Supplementation with 3.2 g CLA isomer blend (50:50% cis-9,trans-11:trans-10,cis-12 isomers) for 12 weeks had no significant effects on markers of bone formation (serum osteocalcin, bone-specific alkaline phosphatase) or bone resorption (urine C-telopeptide-related fraction of type 1 collagen degradation products), PTH, urinary calcium, urinary creatinine and CTP to creatinine ratio. And serum interleukine-6 did not change significantly over 12 weeks in postmenopausal women.

Conclusion: Under the conditions tested in this double-blind, placebo-controlled trial in postmenopausal women, 3.2 g CLA isomer blend (50:50% cis-9, trans-11: trans-10, cis-12 isomers) did not affect markers of bone metabolism and calcium.

Key Words: Conjugated linoleic acid (CLA), post-menopausal women, bone mineral density, IL-6

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### Introduction

Women normally spend one-third of their lives in postmenopausal phase\(^1\). Especially in the first decade after menopause, bone density loss is observed\(^2\). Shortage of ovarian hormones, particularly estrogen efficiency, is the most common cause in bone density loss after menopause. This factor exposes postmenopausal women to higher risk of osteoporosis\(^3\). In the elders, especially in women, it has been seen that osteoporosis due to bone density loss and high bone fragility lead to osteoporotic fractures\(^4\).

Today, hormone therapy and inhibitory medicines of bone re-absorption are used in order to prevent and treat osteoporosis\(^5\). But, due to possible consequences of estrogen therapy such as breast cancer and endometrial adenocarcinoma, the adoption of this kind of treatment in postmenopausal is very low and only 3% to 8% of the women accept this kind of treatment\(^6\). Prevention of osteoporosis is easier than its treatment. Nutrition and lifestyle management are used in order to minimize bone density loss, decrease the need for medication and prevent osteoporosis. The effect of nutritional interventions on bone density regulation is well known\(^7\).

Over the past few years, there has been considerably much attention to biological benefits of conjugated linoleic acid (CLA) on health care\(^8\), for example anti-obesity function in animals\(^9\)\-\(^11\) and humans\(^12\)\-\(^17\), anti-cancer effects, anti-tumor effects\(^18\)\-\(^21\), the risk reduction of atherosclerosis\(^22\)\-\(^25\), reduction of the risk factors of diabetes and high blood pressure\(^26\)\-\(^27\), energy metabolism, positive effects on immune performance\(^28\) and anti-inflammatory properties\(^29\)\-\(^32\). New properties of conjugated linoleic acid (CLA) are the increase of immune functions and positive properties in formation of bone in animal models.

Useful properties of conjugated linoleic acid (CLA) are not provided by diet lonely and there is need for food supplements. There are 28 different isomers in conjugated linoleic acid (CLA) which main form of this acid is found in food, cis-9 and trans-11 as it is called Rummenic Acid. This isomer is 90% of conjugated linoleic acid in food\(^33\). Health benefits of conjugated linoleic acid isomers are mainly related to two kinds of isomers cis-9, trans-11 and cis-12.

According to previous studies, there is an incoherence which isomers of conjugated linoleic acid (CLA) is more effective on bone metabolic bone\(^34\). Recent surveys on animals show that conjugated linoleic acid (CLA) can minimize bone density loss by decreasing the level of prostaglandin E2 in the bone issue\(^35\)\-\(^36\). Prostaglandin E2 is synthesized from arachidonic acid by cyclophosphamid oxygenase enzyme and effects on bone metabolism. The restrain or stimulation in bone formation and re-absorption is dependent on the prostaglandin E2 concentration. It leads to increased insulin- like growth factor (IGF) at low levels and regulates protein expression to insulin- like growth factor (IGFBP) and thus stimulates bond formations\(^37\)\(^,38\). In high doses, it causes restraining the synthesis of collagen, decreasing mRNA in the bone, restraining osteoblast function and finally decreases the formation and density of bones\(^37\).

On one hand, increasing the age and on the other hand, decreasing estrogen in postmenopausal women along with increase in producing prostaglandin E2 and inflammatory cytokines such as Tumor necrosis factor alpha, interleukin-6 and beta 1 by immune cells\(^39\)\-\(^42\) results to the increase the activity of osteoclast and bone re-absorption in postmenopausal\(^43\). These cytokines lead to increase cyclo-oxygenase expression 2 in osteoblastic and the production of prostaglandin E2\(^44\)\(^,45\). On the other hand, conjugated linoleic acid (CLA) causes to increase the absorption of the calcium\(^46\)\(^,47\). In previous studies, there are shown the decreasing effects of inflammatory cytokines activities by conjugated linoleic acid (CLA) in animal models\(^48\). Therefore, due to mentioned subject, the evidences about the effect of linoleic acid (CLA) on bone density and its metabolism in animal and human model are mysterious. On the other hand, there is not performed any survey based on this research in order to evaluate those effects on postmenopausal women. This survey is conducted to determine the supplementary effect of conjugated linoleic acid (CLA) with similar mixture from known isomers trans- 10, cis-12, cis- 9, cis-11 on the bone mineral density, its metabolism and inflammatory indexes in healthy postmenopausal women.
Materials and Methods

In this clinical trial 76 women in the age of 45-65 with at least one and maximum 10 years spent their menopause period and their body mass index were 20-32 kg/m², were studied. Exclusion criteria included sever osteoporosis (t score below -2.5 for hip and spine), bone disease, smoking, using alcohol and drugs, risk of fracture in recent three months, heart disease/cardiovascular, hepatic disease, gastrointestinal, diabetes, hypothyroidism or hyperthyroidism, hormone therapy, dietary supplements for weight loss, omega 3 supplements, medicines increasing bone, bone re-absorption inhibitors, corticosteroids, anticonvulsant medicines and lipid and blood sugar lowering drugs.

The written informed consents were obtained from all patients. Patients were randomly divided into two groups. First group received CLA supplement, daily 4 capsules (each capsule 1 gram, totally 3.2 gram from the mixture 2 isomers cis- 9, trans- 11and trans- 9 and cis- 12 conjugated linoleic acid) and second group received some placebo, daily 4 capsules (each capsule was one gram containing sunflower oil with oleic acid). In this survey, the patients received the supplements and placebos for twelve weeks. CLA supplements used in this study were as follows: Clarinol G80 coated with a transparent gel coat containing 80% CLA (Dutch firm Lipid Nutrition Loders Croklaan B. V.). The patients were trained to take 3 capsules with each meal and 1 capsule before going to bed.

The amounts of supplement acceptance by the patients were followed up by weekly calls and capsules remaining in packages were investigated at each visit, also patients were questioned about their problems. In these contacts, some issues and probable problems were addressed such as intolerance to supplements and other problems, probable change in use of foods, new disease and changes in physical activity. If any condition would happen, the patient would be omitted from the survey or his results would not be used for final analysis. At the end of the sixth week and twelfth week, the capsules were evaluated in terms of patients’ compliance, by counting remaining capsules.

Blood Biochemical Assessment

Serum concentrations of inflammatory markers, tumor necrosis factor alpha, interleukin 6, metabolic markers of the bone including bone alkaline phosphate, osteocalcin, C- urinary telopeptide (CTP) and parathyroid hormone (PHT), urinary calcium and urinary creatinine were measured.

The levels of the serum IL-6 and TNF-α were investigated by ELISA which is the research kit (ELISA kit for human IL-6 and ELISA kit for human TNF-α, French Diaclon Firm). The measurement method sensitivity was lower than 2 pg.ml and 8 pg.ml, respectively. The accuracy of in-test in mentioned measurement was 5.3 and 6.1 respectively based on the percentage of in-test changes. C- telopeptide level in urine samples was determined by EIA and the bone-specific alkaline phosphate levels in serum were determined by using of immune enzymatic by research kits of Inc. IDS Bolton, UK. The sensitivity of measured method was 50 and 0.7 micrograms per liter. In-test accuracy in above measurement was 4.1 and 4.9, respectively based on the variation coefficient. Serum osteocalcin levels were determined by using ELISA by research kits of IDS Inc. Bolton, England. The sensitivity of mention method was 0.5 ng.ml. The accuracy of in-test in mentioned measurement was 1.7 based on the variation coefficient.

The levels of serum parathyroid hormone (PTH) were measured by research kits of IDS Inc, Bolton, England by using of ELISA method. The sensitivity of mention method was 0.5 pm.l. The accuracy of in-test in mentioned measurement was 5.5 based on the variation coefficient.

Urinary creatinine level was measured by chemical colorimetric method (Pars Test Inc., Tehran, Iran). The sensitivity of mention method was 6 mg.dl. The accuracy of in-test in measured measurement was 1.2 based on the variation coefficient. Urinary calcium was measured by chemical colorimetric method (Pars Test Inc., Tehran, Iran). The sensitivity of mention method was 0.2 mg.dl. The accuracy of in-test in mentioned measurement was 2.3 based on the variation coefficient.

Statistical Methods

To compare results, there were used t-test, Wilcoxon, Mann-Whitney U, Wilcoxon and ANOVA. The
meaningful level was considered as 0.05. There was used statistical software SPSS, version 16 to analysis data.

Results
First of all, 76 healthy postmenopausal women participated in this survey. There were 38 women in the placebo group and 38 women in the group of supplement receivers. The number of 4 women from the first group (2 persons because of headache, 2 persons because of digestive problems or bellyache) and 5 women from second group (4 persons because of digestive problems and 1 person for traveling) were excluded from the survey. Therefore, total women participated in this survey were 67 persons. The amounts of capsules based on their counting were determined for first group (receiving CLA supplements) 85% and for second group (placebo group) 75%.

Patients in two groups were similar in terms of age, time duration of menopause, height, body mass index, waist circumference, physical activity, systolic and diastolic blood pressure. In this survey, the changes related to bone mineral density and bone mineral content were not significant in two groups (Table 1). Tumor necrosis factor- alpha levels decreased after 12 week consuming CLA supplements (1.97 pg.ml). But the rate of changes of IL-6 was not statistically significant (Table 2, Figure 1).

| Table 1: Bone mineral density in post-menopausal women initially and after 12 weeks. |
|---------------------------------|-----------------|-----------------|
| Body Composition                | Placebo (n=33)  | CLA supplement (n=34) |
|                                 | Baseline        | After 12 weeks   | Baseline         | After 12 weeks |
| Bone mineral content (gr)       | 1997 ± 31264    | 1967 ± 2151      | 1942 ± 851294    | 1936 ± 278     |
| Bone mineral density (gr/cm²)  | 1.1 ± 0.09      | 1.1 ± 0.09       | 1.08 ± 0.09      | 1.09 ± 0.09    |

Changes in serum osteocalcin, serum bone alkaline phosphatase, PTH, C-telopeptide, urinary calcium, urinary calcium to creatinine ration were not statistically significant after 12 week consuming CLA supplements (Table 3).

| Table 2: Level of inflammatory markers in post-menopausal women initially and after 12 weeks. |
|---------------------------------|-----------------|-----------------|
| Inflammatory markers            | Placebo (n=33)  | CLA supplement (n=34) |
|                                 | Baseline        | After 12 weeks   | Baseline         | After 12 weeks |
| TNF-a (pg/ml)                   | 7.8 ± 3.9       | 8.7 ± 3.9/4      | 6.8 ± 3.7       | 4.7 ± 2.2/2     |
| IL-6 (pg/ml)                    | 0.95 ± 0.8      | 1.12 ± 0.7       | 1.11 ± 0.7      | 1.03 ± 0.6/6    |

| Table 3: Serum and urinary bone metabolism marker levels in post-menopausal women initially and after 12 weeks. |
|---------------------------------|-----------------|-----------------|
| Bone metabolism markers         | Placebo (n=33)  | CLA supplement (n=34) |
|                                 | Baseline        | After 12 weeks   | Baseline         | After 12 weeks |
| Osteocalcin (ng/ml)             | 15±7/9          | 15 ± 7±8/3       | 15±7/2          | 15/7±6/5       |
| Bone alkaline phosphatase (pg/l)| 19/5±8          | 18/6±6/1         | 18/7±6          | 18/2±7/5       |
| PTH (pg/ml)                     | 2.3±1±9         | 2.9±1±2/2        | 2.7±1±6/1       | 3.2±1±8        |
| Urinary creatinine (mmol/l)     | 12/3±5/1        | 12/9±5/1         | 12/2±6          | 14/4±3/3       |
| Urinary calcium (mmol/l)        | 4/2±1/2         | 3/7±0/9          | 3/8±1/0/7       | 3/4±0/8        |
| Urinary calcium to creatinine ratio | 0/35±0/0/3      | 0/35±0/0/2       | 0/25±0/0       |
| Urinary creatinine (pg/ml)      | 2903/5±1819     | 3402/4±2041      | 2702±1353       | 3339±1639      |
| Urinary CTP/Cr (pg/mmol)        | 236/1±106       | 266±208/9        | 250/5±128/9     | 234/1±116/7    |

Discussion
In this survey, CLA supplement did not have any effect on urinary calcium in postmenopausal women participated in the survey. The only survey on the effect of CLA supplement on serum calcium and
urinary calcium is related to Doyle et al in 2005 and its results are similar to ours. In Kelly et al study, the calcium intestinal absorption in young mice were significantly increased by using of CAL supplements and food diet rich in fatty acid PUFA, n-3. But, this effect was not observed in food diet rich in fatty acid PUFA, n-6. CLA effects on bones are dependent on the amount of PUFA fatty acids in food diets. Both fatty acids n-3 and n-6 affect on cytokines, prostaglandins and, as a result, on the metabolism of bones. CLA is a competitive inhibitor with fatty acid PUFA, n-6. It prevents elongation and reduces the availability of substrate for the enzyme cyclo-oxygenase and causes decreasing prostaglandin E2 production. In the present study, the amount of intake the fatty acid PUFA n-6 was higher than fatty acid PUFA n-3. Lack of effect of CLA on bone metabolism markers and urinary calcium, despite of the decline in serum tumor necrosis factor-alpha, may be due to the inhibitory effect of high levels of fatty acid PUFA, N-6.

Due to available direct and indirect evidences about probable effects of CLA on bone metabolism, we can conclude as follows:

1- Decreasing preinflammatory cytokines such as TNF-a, IL-1 and IL-6 and increasing prostaglandin E2 and osteoclastogenesis
2- Regulating leptin and decreasing re-absorption and increasing bone formation
3- Regulating serum insulin level
4- Decreasing serum parathyroid hormone level and reducing the osteoclast activity

Osteoporosis or progressive loss of bone mass is happened due to the increase of bone osteoclastic activities comparing to its osteoplastic activities. However, the aging process is associated with increased pre-inflammatory cytokines such as TNF-a, IL-1, IL-6 and IL- Beta 1. These cytokines are known as main regulators of osteoclastic activities and bone re-absorption increase. These cytokines lead to increase the expression of cyclo-oxygenase 2 in stroma and osteoplastic cells and produce prostaglandin E2 as an essential factor in osteoclastogenesis. In recent study, after 12 weeks using of supplementation with CLA, the level of TNF-alpha increased significantly but the changes of IL-6 was not statistically significant. In some surveys, CLA leads to decrease TNF-a levels. On the other hand, some reports showed some increase or any change in TNF-a after using CLA. Such a disharmony in reported results is related to the differences between isomers kinds, their purity, using different ratios of two isomers, duration of study and empirical models.

Another hypothesis about CLA is that prostaglandin E2 has involved in secretion of PTH and bone metabolism. One of the undesirable effects of estrogen deficiency, after menopause, is the negative balance of calcium which leads to form secondary hyper-parathyroidism. This condition causes to increase bone re-absorption. In conducted study on mice with polycystic kidney, there was observed the feeding with CLA for 8 weeks, compared to control group, in all mice induced the reduction of 60% in PTH and the secretion reduction of prostaglandin E2. If CLA leads to decrease the biosynthesis of prostaglandin E2 in parathyroid tissue, there will be a risk to reduce the secretion of PTH. However, to prove this hypothesis, there is need to
perform some survey in animals and humans. In recent study, CLA supplement, compared to the placebo, has no effect on the secretion of PTH in postmenopausal women.

One of the limitations in present study is the measurement of conjugated linoleic acid levels in participants’ serums showing the acceptance rates of patients correctly. The numbers of capsules used in this study were high because of low purity of CLA (about 80%). It was suggested, by using of supplements with higher purity, the used capsules are reduced in order to increase acceptance rates of persons and reduce their digestive problems. Due to the counting of capsules, the acceptance rates of capsules was 75% in placebo group and 85% in the group received supplement.

One of the main reasons in the various obtained results from CLA effects on bone and body composition in human and animal studies is using of CLA supplement dose. In human studies, used dose is daily between 3-6 g; while in animal studies, used dose is 30 times higher than human studies51. If we are used the supplements with high purity, we can use upper dose from this supplement. Another reason for the lack of CLA effects on bone formation markers (such as osteocalcin, bone- specific alkaline phosphate) is short duration of this research (12 weeks). The time of re-monitoring of bone formation markers is 6 months after food and medicine interventions and it is suggested to consider bone re-absorption markers about 3-6 months after food and medicine intervention.

One of the advantages of this study is to investigate bone turnover markers in postmenopausal women as an aim group because there are little studies about this group. It is suggested to investigate bone turnover markers in postmenopausal women in the first few years after menopause, when bone turnover rate is high. As a result, this study suggests that consuming 3.2 g CLA supplement containing equivalent isomers such as cis-9, trans-10, trans-11 and cis-12 with ClarinolTM is safe in this particular group for 3 months.

**Conclusion**

Under the conditions tested in this double-blind, placebo-controlled trial in postmenopausal women, 3.2 g CLA isomer blend (50:50% cis-9, trans-11: trans-10, cis-12 isomers) did not affect markers of bone metabolism and calcium.

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