ACTA ODONTOLOGICA SCANDINAVICA, 2017
VOL. 75, NO. 1, 21–29
http://dx.doi.org/10.1080/00016357.2016.1244355

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ORIGINAL ARTICLE

The effect of commercial conjugated linoleic acid products on experimental periodontitis and diabetes mellitus in Wistar rats

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ABSTRACT

Objective: The aim of present study was to determine the effects of conjugated linoleic acid enriched milk on alveolar bone loss, hyperglycaemia, oxidative stress and apoptosis in ligature-induced periodontal disease in diabetic rat model.

Methods: Wistar rats were divided into six experimental groups: 1; non-ligated (NL, $n = 6$) group, 2; ligature only (LO, $n = 6$) group, 3; streptozotocin only (STZ, $n = 8$) group, 4; STZ and ligature (STZ + L, $n = 8$) group, 5; ligature and conjugated linoleic acid (CLA) (L + CLA, $n = 8$) group, 6; STZ, ligature and CLA group (STZ + L + CLA, $n = 8$) group. Diabetes mellitus was induced by 60 mg/kg streptozotocin. Rats were fed with CLA enriched milk for four weeks. Silk ligatures were placed at the gingival margin of lower first molars of mandibular quadrant. The study duration was four weeks after diabetes induction and the animals were sacrificed at the end of this period. Changes in alveolar bone levels were clinically measured and tissues were histopathologically examined. Inducible nitric oxide synthase (iNOS) and Bax protein expressions, serum interleukin-1β (IL-1β), low-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglyceride levels and tartrate resistant acid phosphatase (TRAP)+ osteoclast numbers were also evaluated.

Results: At the end of four weeks, alveolar bone loss was significantly higher in the STZ + LO group compared to the other groups ($p < .05$). CLA decreased alveolar bone loss in L + CLA and STZ + L + CLA groups. CLA significantly decreased TRAP + osteoclast numbers and increased osteoblastic activity compared to the STZ + L group ($p < .05$). Diabetes and CLA increased Bax protein levels ($p < .05$) however CLA had no effect on iNOS expression ($p > .05$).

Conclusion: Within the limits of this study, commercial CLA product administration in addition to diet significantly reduced alveolar bone loss, increased osteoblastic activity and decreased osteoclastic activity in the diabetic Wistar rats.

ARTICLE HISTORY
Received 18 February 2016
Revised 26 September 2016
Accepted 29 September 2016

KEYWORDS
Diabetes; experimental periodontitis; conjugated linoleic acid; iNOS; TRAP

Introduction

Diabetes mellitus is a clinically and genetically heterogeneous group of metabolic disorders characterized by abnormally high levels of glucose in the blood, altered glucose tolerance, and impaired lipid and carbohydrate metabolism.[1] It is associated with a number of complications directly resulting from hyperglycaemia. These complications include retinopathy, end-stage renal disease, a variety of debilitating neuropathies, poor wound healing, enhanced risk of infection, and periodontal disease.[2–4]

Diabetes mellitus is a significant risk factor of periodontal disease and increases the severity of periodontal inflammation. A reciprocal relationship between diabetes mellitus and periodontal disease has been suggested in the literature.[5–8] In this regard, it has been shown that gingival inflammation in response to bacterial plaque was stronger in individuals with diabetes and the prevalence and severity of periodontitis increased with poor glycaemic control.[9,10] In addition to the impact of diabetes on periodontal diseases, inflammation in periodontal tissue has also a significant effect on development of diabetes and diabetic control.[11,12]

Conjugated linoleic acid (CLA) describes a group of positional and geometric isomers of polyunsaturated fatty acid (PUFA) octadecadienoic acid containing two conjugated double bonds.[13] It is also available as a dietary supplement purported to cause weight loss. The major isomers of CLA found in dietary supplements are an equal ratio of cis-9, trans-11 (c9t11)-CLA, and trans-10, cis-12 (t10c12)-CLA. CLA has some isomer specific activity such as c9t11 CLA inhibits progression [14,15] and induces regression [16] of atherosclerosis in animal models and the t10c12- CLA isomer is responsible for the adipose-lowering effects.[17,18]

In addition, it is reported that t10c12 CLA could increase bone mass. In mice fed with CLA in experimental diets, volumetric bone mineral content, bone area, and BMD of trabecular and cortical bone were increased despite decreases in body weight gain.[19] Rahman et al. [20] demonstrated

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that CLA decreased production of interleukin 6, tumour necrosis factor-α, receptor activator of NF-κβ ligand (RANKL), and bone resorption biomarker TRAP5b. It is also suggested that CLA prevents bone loss, inhibits accretion of fat mass and loss of muscle mass in aged mice.[21] The anti-osteoclastogenic effect of CLA was also shown in ovariectomized rats by lowering a marker of bone resorption, urinary pyridinium crosslinks.[22] CLA was also found to increase osteoblast differentiation in mesenchymal stem cells.[23] It is concluded that CLA protects both cancellous and cortical bone mass and has no adverse effects on bone due to loss of body weight.[24] These favourable effects of CLA might be beneficial in preventing periodontal bone loss.

The aim of present study is to determine the effects of conjugated linoleic acid enriched milk on alveolar bone loss, hyperglycaemia, oxidative stress and apoptosis in ligature-induced periodontal disease in diabetic Wistar rats.

Materials and methods

Animals and experimental model

Ethical approval was received from the Gaziosmanpasa University Animal Ethics Committee of School of Medicine. All experimental procedures were performed at the Gaziosmanpasa University Laboratory of Experimental Animals Research Centre. The authors declare that adequate measures were taken to minimize pain or discomfort during the study. In total, 44 Wistar male rats were used in the experiment. Rats were kept in individual cages in a room with light-dark cycles of 12 h received water and food *ad libitum*. The rat chow was consisted of at least 85% dry substance, 21% raw protein, 3.5–7% raw cellulose, 6–8% ash and provided 2600–3100 kcal/kg energy (MBD chows, MBD Ltd., Kocaeli, Turkey).

Their body weight ranged from 390 to 450g at the beginning of the experiment. The animals were randomly divided into six groups as follows:

- Non-ligated group (NL, $n = 6$)
- Ligature-only group (LO, $n = 6$)
- Ligature plus CLA group (for four weeks) (L + CLA, $n = 8$)
- Streptozotocin only group (STZ, $n = 8$)
- STZ and ligature group (STZ + L, $n = 8$)
- STZ and ligature plus CLA (for four weeks) group (STZ + L+CLA, $n = 8$)

Induction of diabetes

Diabetes was induced by a single intraperitoneal injection of 60 mg/kg body weight streptozotocin (STZ, Sigma-Aldrich, St Louis, MO) dissolved in citrate buffer (0.01 M, pH 4.5). Blood glucose levels were measured with a glucometer (IME-DC, Oberkotzau, Germany) from tail vein before the procedure and at the 3 days after diabetes induction (Table 1). The glucose level greater than 300mg/dl confirmed the presence of diabetes. Along with the blood glucose levels weights of the rats were measured before the procedure and at the 3 days after diabetes induction. Blood glucose levels and weights of the rats were measured weekly until the end of the study.

Induction of experimental periodontitis

One day after diabetes confirmation periodontal disease was induced via placing silk sutures. The rats in the STZ + LO and STZ + L + CLA groups and the rats in L + CLA and LO groups received ligature placement. The procedure was performed under general anaesthesia using ketamine (40 mg/kg, Eczacibasi Ilac Sanayi, Istanbul, Turkey). A 4–0 silk suture (Dogsan Ilac Sanayi, Istanbul, Turkey) was sub-marginally placed around the first molars of right mandibular quadrants. The sutures were checked after application, and lost or loose sutures were replaced. All ligatures placed by the same operator.

CLA product administration

CLA products were in the form of 1000 mg gel capsules (Hardline CLA). Commercial CLA products contain 1000 mg CLA in one capsule and generally recommended daily 1 to 3 capsules for an average-sized person (for three capsules the dose is approximately 40 mg/kg). CLA product was administered by drinking daily prepared CLA-enriched milk. Half capsule of CLA was dissolved in 1 litre of milk and divided into two bottles for the rats in the L + CLA and STZ + L + CLA groups. This is approximately 30 mg and rats ingested CLA-enriched milk every day for four weeks. This dosage is chosen according to the studies reported by Ortiz et al. and Giordano et al. [25,26] The CLA content of normal milk, CLA capsule and CLA-enriched milk were chemically detected.

After four weeks the animals were sacrificed and the blood samples were taken by cardiac puncture.

Table 1. Biochemical markers in Wistar rats at day 28.

Study groups	NL	LO	L + CLA	STZ	STZ + L	STZ + L+ CLA
Weight (gr) Baseline	368.00 ± 28.00	373.00 ± 35.00	409.00 ± 6.00	349.00 ± 39.00	345.00 ± 44.00	378.00 ± 34.00
Day 28	402.00 ± 40.00	413.00 ± 57.00	399.00 ± 16.00	348.00 ± 32.00	340.00 ± 56.00	349.00 ± 40.00
Glucose (pg/dL) Baseline	105.00 ± 5.00	113.00 ± 2.00	110.50 ± 9.70	397.00 ± 50.00	326.00 ± 20.00	339.00 ± 50.00
Day 28	107.00 ± 5.00	110.00 ± 3.00	110.80 ± 8.80	440.00 ± 40.00	368.00 ± 40.00	357.00 ± 79.00
IL-1β (pg/mL)	105.00 ± 37.00	77.00 ± 50.00	109.30 ± 65.10	162.00 ± 105.00	142.00 ± 59.00	97.40 ± 45.90
LDL (mg/dL)	6.00 ± 1.80	10.20 ± 1.70[a,b]	10.60 ± 2.80[b]	4.60 ± 1.20	8.60 ± 2.10[b]	8.40 ± 4.90
HDL (mg/dL)	45.10 ± 5.10	58.50 ± 7.40	56.10 ± 7.80	49.60 ± 10.10	52.30 ± 7.30	63.20 ± 9.80[a]
Triglyceride (mg/dL)	123.70 ± 21.30	92.20 ± 26.70[a]	75.20±8.50[b]	139.50 ± 55.30	74.90 ± 23.20[a]	95.70 ± 29.50

Weight, blood glucose, serum IL-1β, serum LDL cholesterol, serum HDL cholesterol and serum triglyceride levels in the study groups at the day 28 are shown.
[a] $p < .05$ vs. non-ligated group.
[b] $p < .05$ vs. STZ only group.

Measurement of alveolar bone loss

After the decapitation of rats, the mandibles were carefully removed and the gingival tissues around the molar teeth were excised. Then mandibles were stained with aqueous methylene blue (1%, Merck & Co., Inc., Whitehouse Station, NJ) to identify the cemento-enamel junction (CEJ). The alveolar bone height was measured under a stereomicroscope by means of digital image software (16x magnification) (Stemi 2000 and Axiovison 4.8, Carl Zeiss, Jena, Germany). The measurements were performed by recording the distance from the CEJ to the alveolar bone crest at three points on both the buccal and lingual sides to quantify the alveolar bone level. A mean value for each tooth was calculated. The morphometric measurement of alveolar bone loss was performed by a single examiner who was calibrated and unaware of the identity of samples. Calibration was achieved by measuring 15 different samples three times and the results of the measurements showed *r* value of .99 meaning 99% reproducibility. After achieving 99% accuracy, the examiner measured the samples of present study.

Histopathological evaluation

Right mandibles were fixed in 10% neutral buffered formalin. The tissues were then decalcified in a fixative-added decalcification solution (GBL Co., Istanbul, Turkey) containing a fixative and ethylenediamine- tetra-acetic acid with a change twice a week for 10 weeks until decalcification was completed and then the decalcified specimens were dehydrated through an ethanol series and embedded in paraffin. The periodontal tissues in the mesial and distal part of the mandibular first molar tooth were observed. Histological analysis was performed by a single examiner who was also blinded to the identity of samples. Each sample was sliced into 5 mm continuous sections and prepared for haematoxylin and eosin (H&E) and histochemical staining for TRAP and immunohistochemistry staining for iNOS and Bax. Osteoblast cells, i.e. forming surfaces, by the visibility of active bone formation surfaces that were bordered by the osteoid and cuboidal osteoblasts in the examined area were counted.

TRAP histochemistry

Deparaffinized sections were subjected to TRAP staining, to identify osteoclasts. TRAP staining was performed according to the protocol Leong et al. demonstrated.[27] Firstly rehydrated specimens were treated with 0.2 M acetate buffer, a solution of 0.2 M sodium acetate and sodium tartrate dibasic dehydrate (Sigma-Aldrich, St Louis, Missouri, USA). After 20 min incubation at room temperature, napthol AS-MX phosphate and fast red TR salt (Sigma-Aldrich, St Louis, Missouri, USA) were added and followed by incubation at 37 °C for 1 hour. Bright red staining of the TRAP + osteoclasts was closely monitored under the microscope. Stained sections were washed in de-ionized water and sections were counterstained with Gill's hematoxylin and analyzed using light microscopy (Nikon Eclipse, E 600, Tokyo, Japan).

iNOS and Bax immunohistochemistry

iNOS immunohistochemistry was performed in order to evaluate nitric oxide activity and Bax immunohistochemistry was performed in order evaluate apoptosis. Bax is a member of bcl family and Bax/bcl ratio is considered to be an indicator of apoptosis or survival in the cell.[28,29] After deparaffinization and dehydration of the sections, antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) for 2 h at 70 °C. Then the sections were treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. After incubation with normal rabbit serum for 30 min, samples were incubated with primary antibodies overnight. The antibodies and conditions used were as follows: goat polyclonal anti-iNOS (Abcam, 1:100) and Bax (Abcam, 1:100) antibody (Abcam plc, Cambridge, UK). After washing five times with phosphate buffered saline (Abcam plc, Cambridge, UK), the sections were incubated with biotinylated immunoglobulin G for 30 min, washed several times with phosphate-buffered saline and reacted with streptavidin–horseradish peroxidase conjugated reagent (ab7403, Abcam plc, Cambridge, UK) for 30 min. Following three times 5-min washes with phosphate-buffered saline, samples were incubated with DAB chromogen to visualize the immunoreactivity. Sections were counterstained with haematoxylin and analyzed using light microscopy. Alveolar bone areas surrounding roots of the first molars were examined and iNOS evaluation was made by measuring the iNOS + areas of the bone surrounding teeth. The percentage of iNOS + area to the examined area was calculated. iNOS presence less than 25% of the areas surrounding teeth were scored as '1', %25–50 were scored as '2', %50–75 were scored as '3' and more than %75 were scored as 4. Bax evaluation was performed same as iNOS.

Histopathology of liver

In order to evaluate the effects of CLA on diabetic and non-diabetic liver tissue, livers of the rats were removed and fixed in 10% neutral buffered formalin. After embedding in paraffin, tissues were sectioned at 5μm thickness and stained with haematoxylin and eosin (HE) for light microscopic examination. On examination, particular attention was paid to hepatocytes (vacuolation, necrosis), blood vessels (congestion, haemorrhage, fibrin aggregation, venulitis or arteritis), bile ducts (inflammation, necrosis), the presence of cellular infiltrates (neutrophils, lymphocytes, eosinophils), and interstitial tissue (fibrosis).

Serum IL-1beta levels

The effect of CLA on pro-inflammatory cytokine levels were analyzed via using rat-specific ELISA (eBioscience Inc., San Diego, CA) kit for serum IL-1beta levels, in accordance with the manufacturer's instructions. After halting colour development, the optical density was measured using a computerized micro-titre plate reader set to a wavelength of 450 nm. The cytokine levels were calculated from standard curves. The sensitivity of the IL-1beta ELISA was 4pg/mL.

Statistical analysis

Data were presented as mean ± SD or percentage as appropriate. Results and statistical analysis were elaborated with the SPSS® software (Version 20.0.0, SPSS Inc., Chicago, IL). One sample K-S test was used and the results of one sample K-S test showed that data were following normal distribution. The power of present study is 99%. Osteoclast numbers, alveolar bone loss, iNOS, Bax, osteoblast numbers, IL-1beta levels, body weight, blood glucose levels and lipid levels were evaluated with ANOVA followed by Tukey test for pair-wise comparisons. Weekly body weight and blood glucose levels were evaluated with ANOVA for repeated measures. $p < .05$ were considered statistically significant.

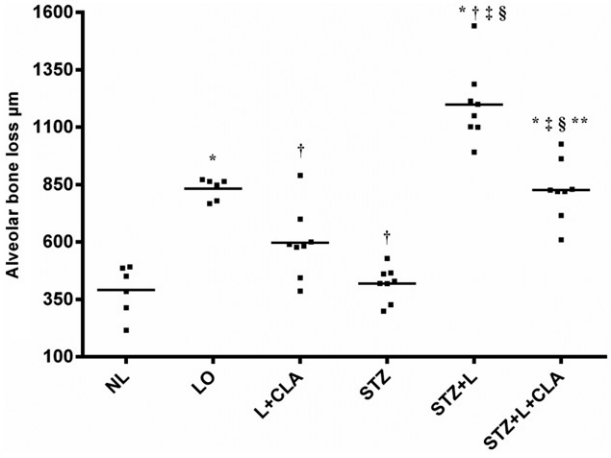

Figure 1. Alveolar bone loss in Wistar rats at day 28. Stereomicroscope measurements of the study groups at the day 28. *$p < .05$ vs. NL group, †$p < .05$ vs. LO group, ‡$p < .05$ vs. L + CLA group, §$p < .05$ vs. STZ only group, **$p < .05$ vs. STZ + L group. NL: Non-ligated group, $n = 6$; LO: ligature-only group, $n = 6$; L + CLA: ligature plus CLA group, $n = 8$; STZ: streptozotocin only group, $n = 8$; STZ + L: STZ and ligature group, $n = 8$; STZ + L+CLA: STZ and ligature plus CLA group, $n = 8$.

Results

No complications arose. Weights and blood glucose levels are shown in Table 1. CLA did not cause any positive effect on diabetic control, no significant difference was observed regarding the repeated weight and glucose level among the study groups ($p > .05$).

Morphometric measurements

The presence of the silk ligature around of the first molar induced an inflammatory reaction in the periodontal tissue. Measurement of alveolar bone loss in the mandibular molar tooth revealed significantly higher bone loss values in the STZ + L group compared to the other groups ($p < .05$) (Figure 1). Diabetes increased the severity of alveolar bone loss in the STZ + L group and CLA normalized the negative effects of diabetes on periodontal destruction. Also there was no significant difference in alveolar bone loss among the NL, STZ and L + CLA groups ($p > .05$). The difference between the LO and STZ + L + CLA groups in alveolar bone loss was not significant either ($p > .05$). CLA reduced the bone loss in L + CLA group to the control levels.

Histopathological analyses

Typical histological view for groups was shown in (Figure 2(A–C)). ICI in the NL group was significantly lower than those of the other groups ($p < .05$). The highest ICI score was observed in the STZ + L group and there were significant difference between STZ + L and LO groups and STZ + L and STZ + L + CLA groups ($p < .05$) but there was no significant difference between LO and L + CLA ($p > .05$). The ICI score of the groups are shown in the Table 2.

There was no significant difference in osteoblast numbers between the NL and STZ groups ($p > .05$). Also, the

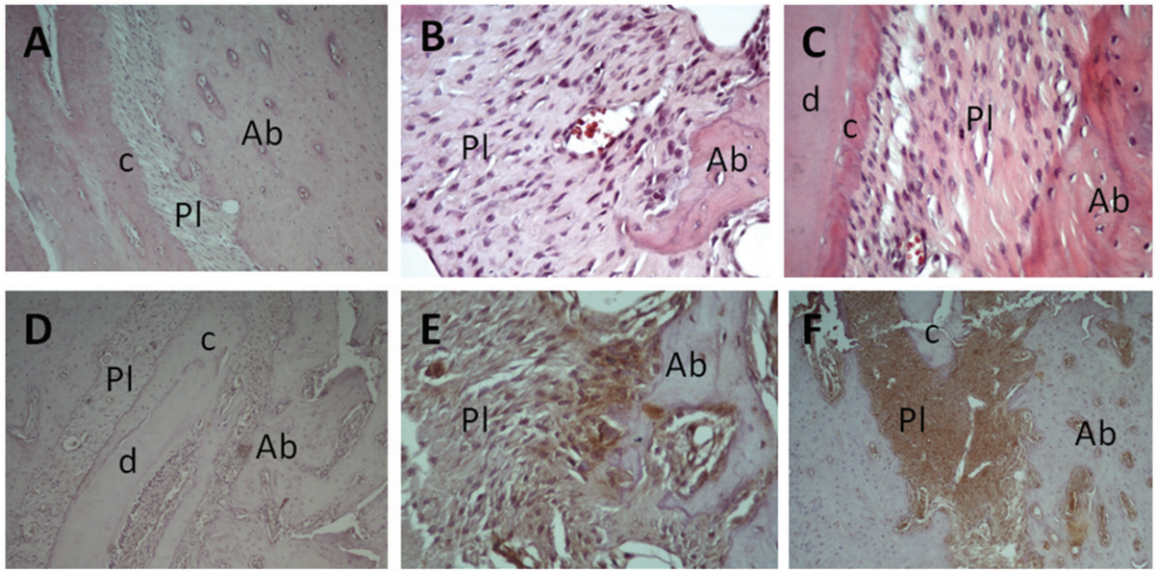

Figure 2. Representative samples of Bax-stained periodontium from Wistar rats. Histological images at the day 28. (A–C) Haematoxylin-eosin staining of the study groups. (D–F) Bax immunohistochemistry staining. (A) NL group (HE, ×100), (B) STZ + L group (HE, ×400), (C) STZ + L+CLA group (HE, ×400), (D) NL group (×100), (E) STZ + L group (×400), (F) STZ + L+CLA group (×100). Ab: alveolar bone; c: cementum; d: dentin; Pl: periodontal ligament.

osteoblast numbers in the STZ + L group was significantly lower than those of the NL and L + CLA groups ($p < .05$) (Table 1). There was no significant difference in osteoblast cell counts among the other groups ($p > .05$).

TRAP histochemistry

View of the representative slides presents TRAP + osteoclasts (Figure 3(A–F)). The osteoclast numbers of the study groups were shown in the Figure 4. The osteoclast numbers were highest in the STZ + L group and lowest in the L + CLA and STZ + L + CLA groups. The differences were statistically significant ($p < .05$). CLA-enriched milk significantly decreased TRAP + osteoclast numbers in L + CLA and STZ + L + CLA groups compared to the other groups ($p < .05$). There were no significant difference between L + CLA and STZ + L + CLA groups regarding to osteoclast numbers ($p > .05$).

iNOS and Bax immunohistochemistry

Histological view of the representative slides were shown in the Figures 2 and 3 (Figure 2 (D–F), Figure 3(G–I)).There were no significant difference in iNOS scoring among the groups ($p > .05$). Bax protein expressions were significantly higher in the STZ + L + CLA group than NL and LO groups ($p < .05$). Periodontitis had no effect on apoptosis however diabetes increased Bax protein levels in STZ, SZ + L and STZ + L + CLA groups. Diabetes and CLA both increased Bax expressions. iNOS and Bax expression levels are shown in the Table 2.

Histopathology of liver

The liver of the NL group had normal structure with well-organized hepatocytes around central veins. In group LO, liver morphology showed small aggregates of polymorphonuclear

Table 2. Quantitative immunohistological and morphometric measures in Wistar rats at day 28.

Groups	NL	LO	L + CLA	STZ	STZ + L	STZ + L+CLA
iNOS	1.10 ± 1.40	1.00 ± 0.90	1.30 ± 1.20	2.00 ± 0.90	2.00 ± 0.40	1.30 ± 0.50
Bax	0.33 ± 0.51	0.50 ± 0.54	2.00 ± 0.89[a,b]	1.66 ± 0.51[a]	1.66 ± 0.51[a]	2.16 ± 0.75[a,b]
Osteoblast Numbers	25.50 ± 2.90	21.50 ± 2.90	37.60 ± 8.70[b]	24.20 ± 13.30	18.70 ± 4.50[a]	34.60 ± 2.80
Inflammatory cell infiltration	0 ± 0[b]	1.30 ± 0.50[b]	2.30 ± 0.50[a]	2.00 ± 0.60[b]	3.30 ± 0.50	1.30 ± 0.50[a,b]

iNOS expressions, osteoblast numbers and inflammatory cell infiltrate scores of the study groups at the day 28 were shown.
[a] $p < .05$ vs. non-ligated group.
[b] $p < .05$ vs. STZ + L group.

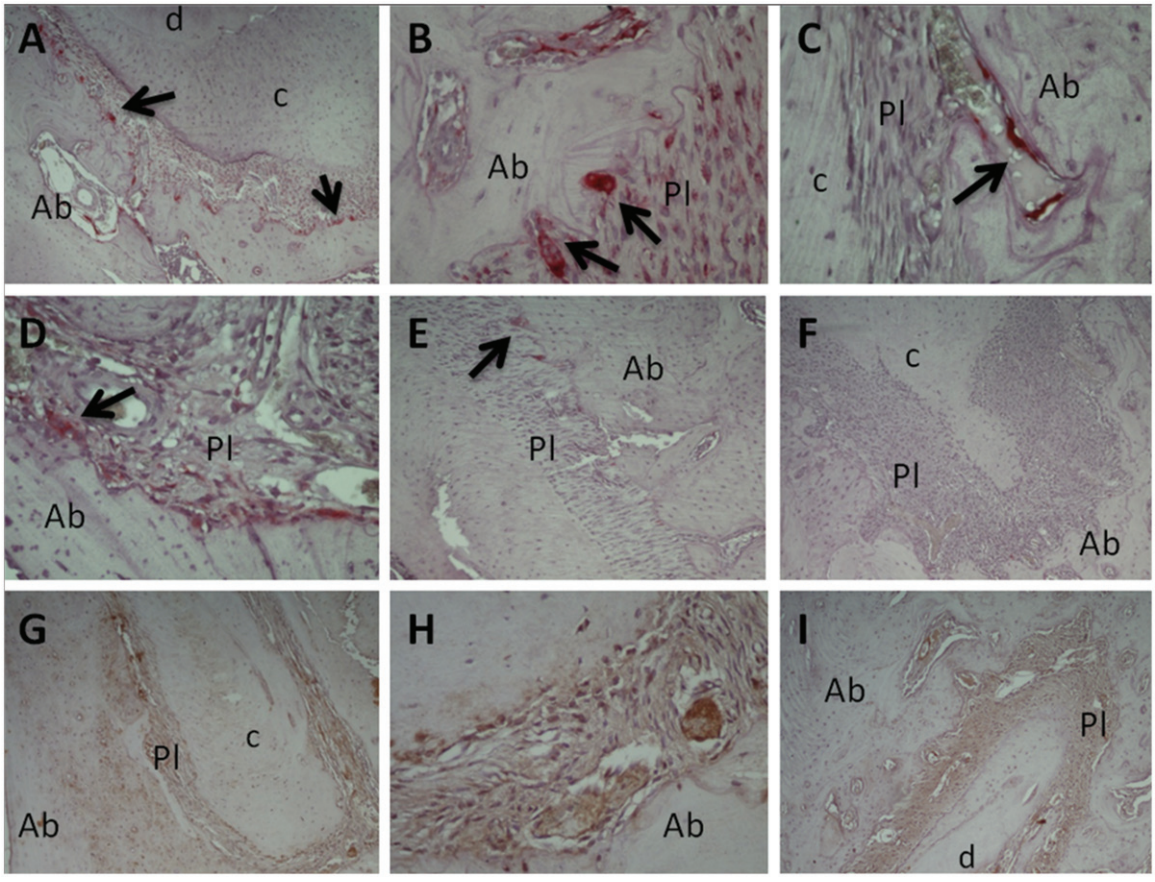

Figure 3. Representative samples of TRAP- and iNOS-stained periodontium from Wistar rats. Histological images at the day 28. (A–F) TRAP enzymohistochemistry staining of the study groups, (G–I) iNOS immunohistochemistry staining. (A) Control group (×100), (B) LO group (×400), (C) STZ group (HE, ×400), (D) STZ + L group (×400), (E) L + CLA group (×100), (F) STZ + L+CLA group (×100), (G) STZ group (×100), (H) STZ + L group (×400), (I) STZ + L+CLA group (×100). Ab: alveolar bone; c: cementum; d: dentin; Pl: periodontal ligament. Black arrow points at osteoclast cell.

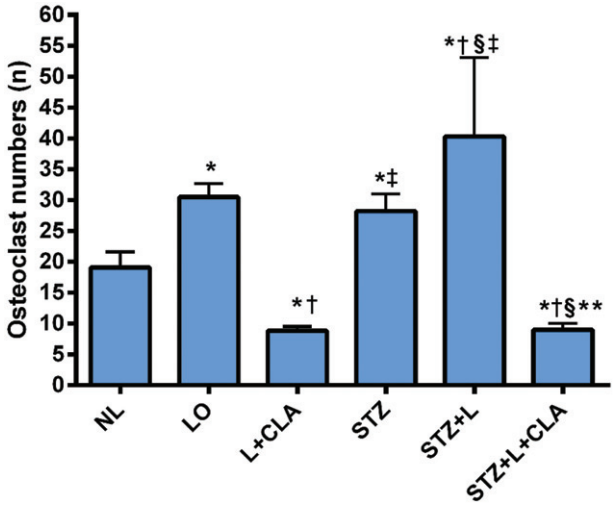

Figure 4. Osteoclast numbers in Wistar rats at day 28. Histomorphometric osteoclast counts at day 28. *$p < .05$ vs. NL group, †$p < .05$ vs. LO group, ‡$p < .05$ vs. L + CLA group, §$p < .05$ vs. STZ only group, **$p < .05$ vs. STZ + L group. NL: Non-ligated group, $n = 6$: LO: ligature-only group, $n = 6$; L + CLA: ligature plus CLA group, $n = 8$; STZ: streptozotocin only group, $n = 8$; STZ + L: STZ and ligature group, $n = 8$; STZ + L+CLA: STZ and ligature plus CLA group, $n = 8$.

scattered throughout the sinusoidal spaces. The histopathological sections of the liver of the diabetic rats in both STZ and STZ + L groups showed degenerative changes in the hepatocytes represented by disorganization of the hepatic cords, cytoplasmic vacuolization, congestion of the central veins with mild hepatocellular necrosis and the sinusoids were infiltrated by mild nonspecific inflammatory cells. Livers of the rats in the L + CLA group showed normal liver morphology. In STZ + L + CLA group, the hepatocytes showed morphological change such as pyknosis and cytoplasmic vacuolization. In addition, there was no evidence of inflammatory cells infiltration in the livers of the CLA-treated diabetic rats.

Biochemical analyses

Serum IL-1beta levels

There were no significant differences in serum IL-1beta levels among the study groups ($p > .05$).

Serum LDL, HDL, triglyceride levels

LDL, HDL cholesterol and triglyceride levels in serum are shown in Table 1. LDL cholesterol levels were lowest in the STZ group and highest in the L + CLA group. The differences between NL and LO, NL and L + CLA groups were statistically significant ($p < .05$). Besides, LDL cholesterol levels were statistically lower in STZ group than LO and L + CLA groups ($p < .05$).

Serum HDL cholesterol levels were significantly lower in NL group than LO and STZ + L + CLA groups ($p < .05$).

Serum triglyceride levels in NL group was significantly higher than LO and STZ + L groups ($p < .05$). There were no significant differences among the other groups ($p > .05$).

Discussion

The present study evaluated the effects of CLA supplementation on experimental periodontitis and found that CLA supplementation significantly reduced alveolar bone loss in experimental periodontitis in both diabetic and non-diabetic rats. This study is the first study to report that CLA, as a dietary supplement agent, reduced periodontal destruction. Bone destruction is the result of disrupted bone remodelling caused by a relative increase in osteoclastic activity and/or decrease in osteoblastic activity. In present study, CLA reversed disrupted bone remodelling in diabetic rats with periodontitis by decreasing osteoclastic activity along with the inflammatory cell infiltration and increasing osteoblast counts. These results support the morphometric results showing that CLA prevented alveolar bone loss.

This preventive effect of CLA on bone resorption observed in our study is supported by the literature reporting that CLA supplementation reduced the rate of bone resorption in osteoporotic rats and prevented bone loss by inhibiting excessive bone resorption and stimulating new bone formation in osteoporotic mice.[22,30] The mechanism of inhibiting bone resorption might be due to modulating RANKL induced osteoclastic activity which was revealed by Rahman et al. demonstrating the inhibition of RANKL signalling in monocytic cells.[31] Banu et al. also demonstrated that CLA caused a significant decrease in fat mass and an increase in muscle mass in addition to decrease in osteoclastic activity, RANKL, IL-6 and TNF-α levels of muscle mass in aged mice.[21] CLA also increased bone mineral content and BMD along with a decrease in body weight gain.[19] In our study, diabetes increased osteoclastic activity in all diabetic groups and CLA caused a dramatic decrease in TRAP + osteoclast numbers along with the increase in osteoblast cells.

Diabetes is a significant risk factor of periodontal disease and aggravates the severity of the periodontal destruction. Persistent oxidant-antioxidant imbalance results in oxidative stress and contributes to pathogenesis of periodontitis and diabetes through the formation of advanced glycation products (AGE) and increased production of ROS.[32,33] In order to understand the association of diabetes and chronic periodontitis, there are some important points to consider. The effect of diabetes on periodontal tissues are caused mostly by hyperglycaemia and it has some adverse effects on functions of neutrophil and periodontal tissue cells.[34,35] Another cell group affected by diabetes is monocytic cells. Phagocytic functions of these cells are altered by AGE and the receptors of AGE.[36,37] Therefore, host response against bacterial attack is impaired in diabetes and pro-inflammatory cytokines also participate in this interplay by over expression of pro-inflammatory cytokines and mediators as a result of deterioration in innate immunity.[38–40] Several studies supported the role of diabetes and hyperglycaemia in increased production of cytokines and elevated cytokine levels in gingival crevicular fluid.[40–42] Studies also showed that periodontal inflammation was higher and more persistent in diabetes.[42–44] Moreover, inhibition of AGE and TNF provided significant improvement in diabetic complications.[42,43] In order to evaluate any possible beneficial effect of CLA on diabetes and periodontal disease we evaluated morphometric bone loss, Bax and iNOS expressions in bone, serum IL-1β levels, blood glucose measurements,

serum lipid profile and histopathological changes in liver and periodontal tissues.

In the present study, periodontal disease and related alveolar bone loss was induced by ligature method which is considered to be a useful experimental model.[4,34,45] Furthermore, experimental diabetes was induced by injection of a single dose of streptozotocin (2-deoxy-2-(3-methyl-3-nitrosoureido)-d-glucopyranose, STZ) [46] which is also a widely used experimental model for diabetes.[34,45,47,48] Ligature model caused formation of bacterial plaque and induced an inflammatory response, reproducing human periodontal disease in this study and diabetes was successfully achieved and plasma glucose levels increased in STZ-treated rats. As a result, we found that alveolar bone loss significantly increased in ligature groups and we also demonstrated that diabetes increased bone loss significantly as the highest bone loss was observed in the STZ + L + CLA group but diabetes did not cause periodontal disease alone. In addition, CLA decreased periodontal tissue destruction in STZ + L + CLA and L + CLA groups significantly. These results might have clinical significance in terms of preventing periodontal bone destruction along with decreasing inflammation and risk of diabetes. Commercial CLA capsules (3–6g daily, approximately 32–45 mg/kg) [49–53] are used to decrease body weight, increase muscle strength and mass. Therefore, CLA could be beneficial in overweight and obese people or patients with type 2 diabetes by decreasing adipose tissue, improving weight control and decreasing the risk of periodontal bone loss.

CLA is shown to have beneficial effects on hyperglycaemia and diabetes in animal studies [54,55] and to decrease insulin sensitivity.[29] But the results are contradictory. Another study reported that c9t11 CLA was inversely correlated to the prevalence of diabetes, fasting plasma triglycerides, and fasting blood glucose concentrations.[56] In contrast CLA was shown to increase blood glucose levels and HDL cholesterol while decreasing LDL cholesterol and triglyceride levels in normoglycemic rats. Latter reported an increase in insulin resistance suggesting that CLA did not have any effect on hyperglycaemia.[57] In sedentary women with metabolic syndrome, supplementation with CLA improved glycaemic control and decreased body fat but, there were no change in serum lipids or blood pressure.[58] Noone et al. [59] observed that the daily intake of 3 g CLA supplement decreased LDL cholesterol levels non-significantly. On the other hand, von Loeffelholz [60] suggested that CLA supplementation increases LDL cholesterol. However, our results are supporting the literature not reporting any positive effect of CLA on diabetes. We did not find any beneficial effect of CLA on hyperglycaemia, weight of the rats or diabetic control. Furthermore, regarding the serum lipid profile, CLA caused no specific changes. There was only a significant increase in HDL cholesterol levels in STZ + L + CLA. In addition, we observed that diabetes caused pathological changes in rat liver such as disorganization of the hepatic cords, cytoplasmic vacuolization and inflammation and CLA reversed pathological changes induced by diabetes.

Nitric oxide (NO) is a reactive radical and high levels of NO are mostly generated by inducible NO synthetase (iNOS, NOS2). iNOS suppresses osteoblast proliferation and differentiation and also contributes to certain physiological processes.[61,62] It is found that CLA inhibited iNOS expression and NO synthesis *in vitro*.[63] In contrast, studies reported contrasting results such as that CLA had no effect on NOS activity [64] or increased NOS expression.[65] Regarding iNOS expressions and serum IL-1 β levels, we observed slight changes but the differences were not statistically significant.

In terms of apoptosis, it is suggested that effect of CLA on lowering adipose tissue is due to apoptosis of adipocyte cells.[66] It is also reported that CLA increased the p53 protein and Bax protein levels but suppressed the expression of Bcl-2 protein levels *in vivo*.[28,67] There is no study reporting any apoptotic effect of CLA on periodontal tissues. We found that both diabetes and CLA increased apoptosis in periodontal ligament and alveolar bone while periodontitis alone did not cause any apoptotic reaction.[68,69] However, Gamonal et al. demonstrated that apoptotic cells were higher in chronic periodontitis than healthy controls [70] however, periodontitis did not increase pro-apoptotic protein expressions in our study.

Within the limits of this study, commercial CLA product administration significantly reduced alveolar bone loss, increased osteoblastic activity and decreased osteoclastic activity in the diabetic Wistar rats. CLA describes a group of positional and geometric isomers of polyunsaturated fatty acid PUFA and the favourable effects of CLA might result from proresolving mediators. Future studies in this aspect are necessary to reveal mechanisms of CLA activity in human body.

Acknowledgements

This study was supported by the Scientific Research Project Fund of Gaziosmanpaşa University. The authors also thank Prof. Dr. Ramazan ERENLER (Gaziosmanpaşa University, Faculty of Arts and Science, Department of Chemistry, Tokat, Turkey) and Dr. Osman DEMIR (Gaziosmanpaşa University, Faculty of Medicine, Department of Biostatistics) for their assistance in implementation of this study.

Disclosure statement

The authors declare that there are no conflicts of interest in this study.

Funding

No external funding apart from the support of the Gaziosmanpasa University Unit of Scientific Research Projects was available for this study.

This study was supported by the Scientific Research Project Fund of Gaziosmanpaşa University.

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