

Synergistic effect of laser irradiation and cinnamic acid as a functional food on oxidative stress in type 2 diabetes mellitus

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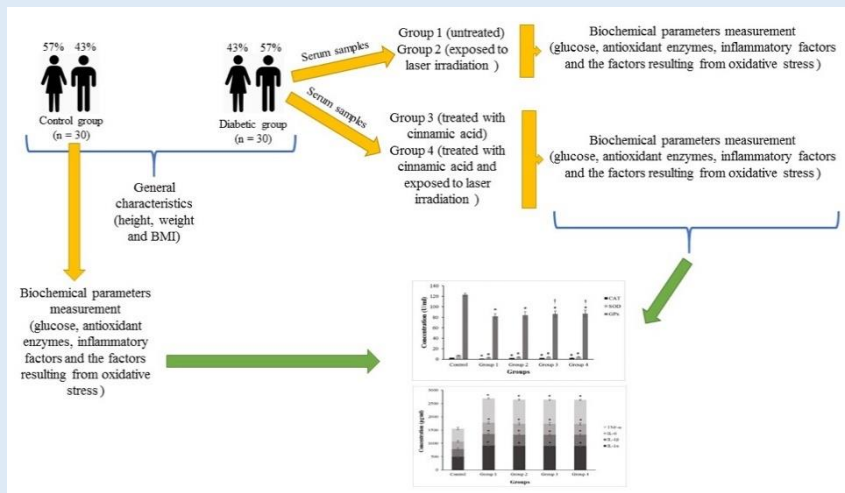
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ABSTRACT

Background: The prevalence of diabetes mellitus (DM), especially type 2, is increasing worldwide. Prevention, control and management of this chronic metabolic disease is the most important ways to avoid its consequences. The use of functional foods and bioactive compounds can be effective in preventing and controlling this

disease due to the antioxidant compounds present. Low-level laser therapy (LLLT), as an adjunct therapy, along with medication can be effective in reducing the effects of DM.

Objective: Our aim in the present study was to investigate the synergistic effects of LLLT and cinnamic acid on blood glucose, inflammatory factors, oxidative factors, and increased activity of antioxidant enzymes.



Methods: For this study, thirty healthy individuals were selected as the control group and thirty individuals with type 2 DM were selected. The levels of biochemical parameters, such as glucose, hydrogen peroxide, advanced glycation end products (AGEs), advanced oxidation protein products (AOPP), malondialdehyde (MDA) and oxidized low-density lipoprotein (Ox-LDL) were studied in the samples of different control and diabetic groups, Inflammatory factors, such as Interleukin 1 alpha (IL-1 α), Interleukin 1 beta (IL-1 β), Interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF- α) and antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were also studied.

Results: The results of all biochemical parameters showed significant differences in untreated and treated diabetic samples in compared to control group ($P < 0.001$). There was no significant difference in the reduction of inflammatory factors, glucose and hydrogen peroxide between the samples treated with both cinnamic acid and laser irradiation with the untreated diabetic sample ($P > 0.05$). A significant difference was observed in comparing the results of other biochemical factors ($P < 0.05$).

Conclusions: Concomitant use of cinnamic acid and LLLT as a complementary treatment can reduce oxidative stress and thus prevent the diabetes complications.

Keywords: Laser irradiation; Cinnamic acid; Functional foods; Oxidative stress; Diabetes mellitus.

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INTRODUCTION

DM as a non-communicable metabolic disease is one of the most important diseases affecting human societies (1). DM, with its various types, is the result of heredity, inappropriate lifestyle, inactivity, obesity, and in other cases, pregnancy and cystic fibrosis (a rare type of diabetes that known as diabetes related to cystic fibrosis) (2-4). All of these risk factors lead to an insufficient or lack of insulin production or secretion. In type 2 diabetes mellitus, insulin is produced but there is insulin resistance (5). According to the World Health Organization (WHO) in 2019, about 629 million people will be affected by DM, by 2045, if control and prevention of DM are not achieved. Each year, high blood glucose kills nearly 4 million people (6). Uncontrolled DM can lead to

dangerous consequences such as cardiovascular disease, vision problems, kidney failure and so on. Intracellular hyperglycemia increases the production of reactive oxygen species (ROS) in mitochondria (7). ROS are involved in causing inflammatory factors and insulin resistance in people with DM (8-10). Oxidative stress plays an important role in causing the DM complications, such as atherosclerosis (11, 12). For this reason, controlling and preventing DM is one of the most important issues and challenges for researchers. There are various antioxidant enzymes in humans that protect the body against oxidative factors. SOD is the most important and first antioxidant enzyme in all aerobic organisms, which is involved in the direct reduction of active oxygen

metabolites (such as superoxide radical). CAT is a common and a heme-containing redox enzyme that is exposed to oxygen in almost all organisms. Catalase catalyzes the breakdown of hydrogen peroxide (H_2O_2) in water and oxygen. This enzyme is very important in protecting cells against oxidative damage by ROS (13, 14). GPx is another important antioxidant enzyme in humans. Its main biological role is to protect humans against oxidative damage. The biochemical function of the GPx is the reduction of lipid hydroperoxides to the relevant alcohols and the reduction of free H_2O_2 to the water. Cell lipid compounds are sensitive to free radicals and produce lipid peroxide as a result of the reaction. The GPx enzyme uses glutathione for the reduction of peroxides into alcohol and prevents the formation of free radicals. Nowadays, the use of natural antioxidants to prevent and control many diseases is recommended. Antioxidants protect the body against free radicals and oxidative stress (15). A number of functional foods that are naturally present have antioxidant properties. Medicinal herbs have a special importance among functional foods. These herbs are widely used in many countries around the world, especially in Asian countries, to relieve and treat some diseases (16, 17). Nowadays, the use of herbal medicine is spreading around the world and it has many fans. It is safe to say that herbalism is now a form of alternative medicine. A number of studies have shown that some of these herbs have anti-diabetic properties (18-20). Around the world, cinnamon is commonly considered a medicinal herb, especially in Asia (21). Cinnamic acid (Figure 1), is an unsaturated carboxylic acid and organic compound, originally isolated from cinnamon (22).

In addition to cinnamon, this compound is also extracted from other plants. Cinnamic acid is mostly found in the trans isomer form in cinnamon. A

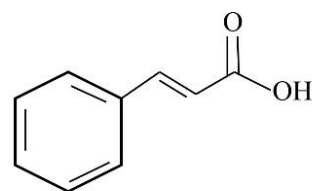


Figure 1. Trans-Cinnamic acid structure

derivative of cinnamic acid called para-hydroxycinnamic acid (p-HCA) has antioxidant, anti-cancer and anti-inflammatory properties (23). Due to the antioxidant and anti-inflammatory properties of cinnamic acid, the anti-diabetic properties of this compound have been considered in studies (24). Therefore, this derivative of cinnamon can be effective in controlling of DM as well as preventing the consequences of diabetes that are the result of oxidant factors.

In many studies, low level-laser therapy (LLLT) has been considered as a complementary treatment to improve the consequences of uncontrolled DM, such as skin ulcers (25-27). LLLT has been used for more than 50 years (28). Studies have reported that LLLT may affect oxidative stress factors. LLLT can alter the activity of antioxidant enzymes and reduce the concentration of ROS (29). Due to the increased oxidative stress and the production of ROS in people with DM, dangerous consequences of DM occur if they are not controlled. Previous studies have shown that the consumption of functional foods contain antioxidants such as cinnamic acid, as a substance to control the progression of DM can be important and helpful in preventing the consequences of DM (23). In this research, the synergistic effects of cinnamic acid, as a natural antioxidant and LLLT on reducing oxidative stress in people with type 2 diabetes mellitus were evaluated. We aimed at assessing the synergistic effect of laser therapy and cinnamic acid

administration on some inflammatory and oxidative factors in samples of patients with type 2 DM.

METHODS

Cinnamic acid, as a trans isomer, was purchased from sigma (Sigma-Aldrich, St. Louis, USA). SOD antioxidant enzyme assay kit was purchased from Biovision (BioVision Incorporated, USA). Diagnostic kits for the other antioxidant enzymes, CAT and GPx, were purchased from Biocore (BiocoreDiagnostik Ulm GmbH, Ulm, Deutschland). Human glucose (with an intra- and inter-assay CV < 8% and < 10%, respectively) enzyme-linked immunosorbent assay (ELISA) kits were purchased from MyBioSource Inc. (San Diego, CA 92195-3308, USA). The H₂O₂ assay kit was purchased from ZellBio (ZellBio GmbH, Ulm, Germany). The concentration of H₂O₂ was investigated by colorimetric method. SOD, CAT, and GPx enzymes were also measured by colorimetric method. Moreover, measurement of AOPP and AGEs parameters were determined according to the instructions described previously by spectrophotometric method (30). MDA assay kit was purchased from Cayman chemical company (Cayman Chemical, 1180 East Ellsworth Road, Ann Arbor, Michigan 48108 USA). The evaluation of MDA was based on the colorimetric method. The concentration of Ox-LDL was determined by the kit purchased from Mercodia Company (Uppsala, Sweden). The assay was performed using the ELISA method. In this study, the concentration of inflammatory factors IL-6, IL-1 α , IL-1 β and TNF- α were investigated by ELISA method. Human ELISA assay kit for IL-6 was purchase from MyBioSource Inc. (San Diego, CA 92195-3308, USA). Assay kits for IL-1 α and IL-1 β were purchased from Diaclone (25020 Besancon cedex, France). TNF- α assay kit was purchased from R&D Systems, Inc. (614 McKinley Place NE Minneapolis, MN 55413).

Microplate reader (Mindray, model MR-96A, Germany) and microplate spectrophotometer (model Fluostar, bmglabtech, Germany) were used in ELISA and Colorimetric methods, respectively.

Participants: In this study, 30 healthy individuals, as a control group and 30 patients with type 2 DM were selected among the persons referred to Vali-Asr medical laboratory in Tehran, Iran. Samples from 30 patients were divided into four groups:

Group 1: Untreated samples with cinnamic acid and without laser irradiation (diabetic).

Group 2: Samples exposed to laser irradiation (diabetic + LLLT).

Group 3: Samples of patients treated with cinnamic acid (diabetic + Cinnamic acid).

Group 4: Treated samples with cinnamic acid and exposed to laser irradiation (diabetic + Cinnamic acid + LLLT).

In explaining the division of groups, it should be noted that blood samples were taken from patients with diabetes (before taking cinnamic acid). The biochemical factors described above were measured once before laser irradiation (as group 1) and again after laser irradiation (as group 2). Then, the same patients were treated with cinnamic acid (at a dose of 40 mg/kg per day) for 3 months. After three months, a blood sample was taken from them. Again, biochemical factors were measured once before laser irradiation (as group 3) and again after laser irradiation (as group 4). People with type 1 DM, as well as other conditions such as cardiovascular disease, were excluded from the study. According to the WHO criteria, people with fasting plasma glucose \geq 126 mg/dL or glycated hemoglobin (HbA1c) \geq 6.5% were considered with type 2 DM. Written consent

was obtained from all participants. During this time, the patients also took their main medication.

General Features and Sampling: Age, sex, weight, height and body mass index (BMI) of all participants in the study were recorded. Blood samples were obtained from all participants after 12 hours of overnight fasting. After collecting the blood sample, they were centrifuged (250 g for 10 min). Then, the serum was separated from centrifuged specimens. Isolated serums were used to evaluate the biochemical parameters and antioxidant enzymes described above. After 24 hours, samples were irradiated by low level green LASER diode and then examined for a second time.

Laser Irradiation: We used a diode laser pointer for irradiation. The type of laser was a low level green laser diode with a wavelength of 532 nm at 100 mw in a continuous wave mode with divergence < 1.5 mRad, beam mode (TEM₀₀), beam diameter at aperture ~1.5, crystal type Nd:VVO4:KTP, and power source 1 × 3V CR2 alkaline batteries. The power density of the laser was 509.55 mW/cm² at a distance of 6.5 cm from the laser device from serum inside the tube, and the diameter of the laser spot was set to 0.5 cm. Like our previous study (29), Irradiation was applied for 8 seconds. Green diode pumped solid state (DPSS) Laser Pointer (model RLP-532, 1040 Vienna, Austria) was used for LLLT.

Statistical Analysis: Statistical analysis was performed utilizing SPSS (version 23, IBM, USA) software for Windows. All results were expressed as mean ± standard deviation. Independent-sample T-test was used to compare the mean of general characteristics of the participants. Statistical significance was analyzed by one-way ANOVA to compare the mean of the obtained data. After one-way ANOVA test, Tukey post hoc was used. The Kolmogorov-Smirnov test was used to analyze the normal distribution of data. P-values < 0.05 were considered significant.

RESULTS

General Features: The age of participating volunteers was between 65 and 80 years old. In control group, 43% were male (n = 13) and 57% were female (n = 17). In the group of people with type 2 DM (diabetic group), 43% were female (n = 13) and 57% were male (n = 17). The results for the general features of control and diabetic groups are given in Table 1. As shown in Table 1, the height, weight, and BMI of control individuals were compared with those of diabetics. Statistically, there was no significant difference in comparison of general features between control and diabetic groups.

Table 1. Participant's general features

Feature	Control group n = 30	Diabetic group n = 30	P value
Height (Cm)	166.7±6.6	167.6±6.2	0.59
Weight (Kg)	82.4±6.9	84.7±6.1	0.18
BMI (Kg/m ²)	29.7±3.6	30.2±3.3	0.57

Data are given as mean ± SD. P value < 0.05 is significant. BMI, body mass index

Biochemical Parameters: In the serum of healthy individuals (as the control group) as well as patients with diabetes (as the diabetic group), some important factors were measured. These biochemical parameters included blood glucose, antioxidant enzymes such as CAT, SOD and GPx, oxidizing agent (H_2O_2), inflammatory factors such as IL-1 α , IL-1 β , IL-6 and TNF- α , and factors created by oxidative stress and high blood glucose, such as Ox-LDL, MDA, AOPP and

AGEs, the latter is caused by an uncontrolled increase in blood glucose. The levels of expressed biochemical parameters were assessed in the control group and the samples of people with type 2 DM (groups 1 to 4). A comparison of the results between the control group and four diabetic groups is shown in Table 2. As shown in Table 2, there was a statistically significant difference in the results of groups 1 to 4 compared to the control group.

Table 2: Comparison between the levels of biochemical parameters of the control group with other groups

Biochemical Parameters	Groups					P value
	Control	Group 1	Group 2	Group 3	Group 4	
Glc ($\mu\text{g/ml}$)	311.3 \pm 27.2	430.9 \pm 22.4	427.9 \pm 20.8	426.9 \pm 14.5	426.1 \pm 14	< 0.001
H_2O_2 ($\mu\text{M/ml}$)	228.6 \pm 18.6	338.5 \pm 27.5	328.7 \pm 14.7	327.7 \pm 22.6	326.7 \pm 15.1	< 0.001
CAT (U/ml)	2.8 \pm 0.07	1.1 \pm 0.05	2.2 \pm 0.1	2.4 \pm 0.1	2.5 \pm 0.09	< 0.001
SOD (U/ml)	7.4 \pm 0.3	3.3 \pm 0.3	4.1 \pm 0.4	4.3 \pm 0.3	4.4 \pm 0.3	< 0.001
GPx (U/ml)	122.9 \pm 2.4	82 \pm 5.2	84.2 \pm 6.7	86 \pm 6.6	87.2 \pm 6.3	< 0.001
AGEs (AU)	36.1 \pm 5.1	85.1 \pm 5.9	76.8 \pm 6	74.6 \pm 5.9	73.6 \pm 5.9	< 0.001
AOPP ($\mu\text{mol/l}$)	113.1 \pm 8.7	197.4 \pm 8.8	186.3 \pm 16	184.9 \pm 15.6	183.7 \pm 15.7	< 0.001
MDA ($\mu\text{M/ml}$)	2.0 \pm 0.09	3.3 \pm 0.2	3.0 \pm 0.2	2.9 \pm 0.2	2.8 \pm 0.2	< 0.001
Ox-LDL (mU/l)	10.1 \pm 0.8	18.8 \pm 1.2	15.2 \pm 0.9	14.6 \pm 0.8	13.6 \pm 0.9	< 0.001
IL-1 α (pg/ml)	492.5 \pm 32.5	918.8 \pm 27.9	905.5 \pm 27.4	904.3 \pm 27.6	903.1 \pm 26.6	< 0.001
IL-1 β (pg/ml)	294.4 \pm 41.3	428.9 \pm 36.1	417.5 \pm 41.4	416.7 \pm 41.6	415.7 \pm 41.6	< 0.001
IL-6 (pg/ml)	284 \pm 36.9	427.3 \pm 41.9	415.3 \pm 45.5	414.5 \pm 45.7	413.3 \pm 41	< 0.001
TNF- α (pg/ml)	482.3 \pm 50.5	918 \pm 27.5	908.8 \pm 25	907.3 \pm 23.2	906.2 \pm 23.1	< 0.001

Data are given as mean \pm SD. P value < 0.05 is significant. Group1: Diabetic. Group 2: Diabetic + LLLT. Group 3: Diabetic + cinnamic acid and group 4: Diabetic + cinnamic acid + LLLT.

Glc, glucose; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; AGEs, advanced glycation end products; AOPP, advanced oxidation protein products; MDA, malondialdehyde; Ox-LDL, oxidized Low-Density Lipoprotein; IL-1 α , Interleukin 1 alpha; IL-1 β , Interleukin 1 beta; IL-6, Interleukin 6 and TNF- α , tumor necrosis factor alpha.

Comparisons between biochemical parameter levels were studied among samples of patients with type 2 DM under untreated conditions, treatment with cinnamic acid, laser irradiation, as well as treatment with cinnamic acid and laser irradiation. To evaluate the effect of LLLT and the effect of cinnamic acid, as well as the effect of both LLLT and cinnamic acid on the biochemical parameters, a comparison of the results was done between the groups. Multiples comparisons were made by Tukey HSD Post Hoc test (Table 3). Increasing the concentration of CAT, SOD

and GPx in treated groups with both cinnamic acid and exposed to laser irradiation with untreated diabetes group was significant (Figure 2). Statistically, there was no significant difference in reduction of inflammatory factors in comparison between the groups (Figure 3).

A significant difference was observed in the comparison of AGEs, AOPP, MDA and Ox-LDL in the group treated with both cinnamic acid and exposed to laser irradiation with the untreated diabetic group.

Table 3: Multiple comparisons between the levels of biochemical parameters between the groups

Groups	Biochemical parameters						
	Glc (µg/ml)	H ₂ O ₂ (µM/ml)	AGEs (AU)	AOPP (µmol/l)	MDA (µM/ml)	Ox-LDL (mU/l)	
1	2	0.92	0.26	< 0.001	0.01	< 0.001	< 0.001
	3	0.83	0.18	< 0.001	0.005	< 0.001	< 0.001
	4	0.74	0.12	< 0.001	0.002	< 0.001	< 0.001
2	1	0.92	0.26	< 0.001	0.01	< 0.001	< 0.001
	3	0.99	0.99	0.48	0.98	0.25	0.09
	4	0.98	0.98	0.15	0.9	0.002	< 0.001
3	1	0.83	0.18	< 0.001	0.005	< 0.001	< 0.001
	2	0.99	0.99	0.15	0.98	0.25	0.09
	4	0.98	0.99	0.9	0.99	0.29	0.001
4	1	0.74	0.12	< 0.001	0.002	< 0.001	< 0.001
	2	0.98	0.98	0.15	0.9	0.002	< 0.001
	3	0.99	0.99	0.9	0.99	0.29	0.001

P value < 0.05 is significant. Group1: Diabetic. Group 2: Diabetic + LLLT. Group 3: Diabetic + cinnamic acid and group 4: Diabetic + cinnamic acid + LLLT.

Glc, glucose; AGEs, advanced glycation end products; AOPP, advanced oxidation protein products; MDA, malondialdehyde; Ox-LDL, Oxidized Low-Density Lipoprotein.

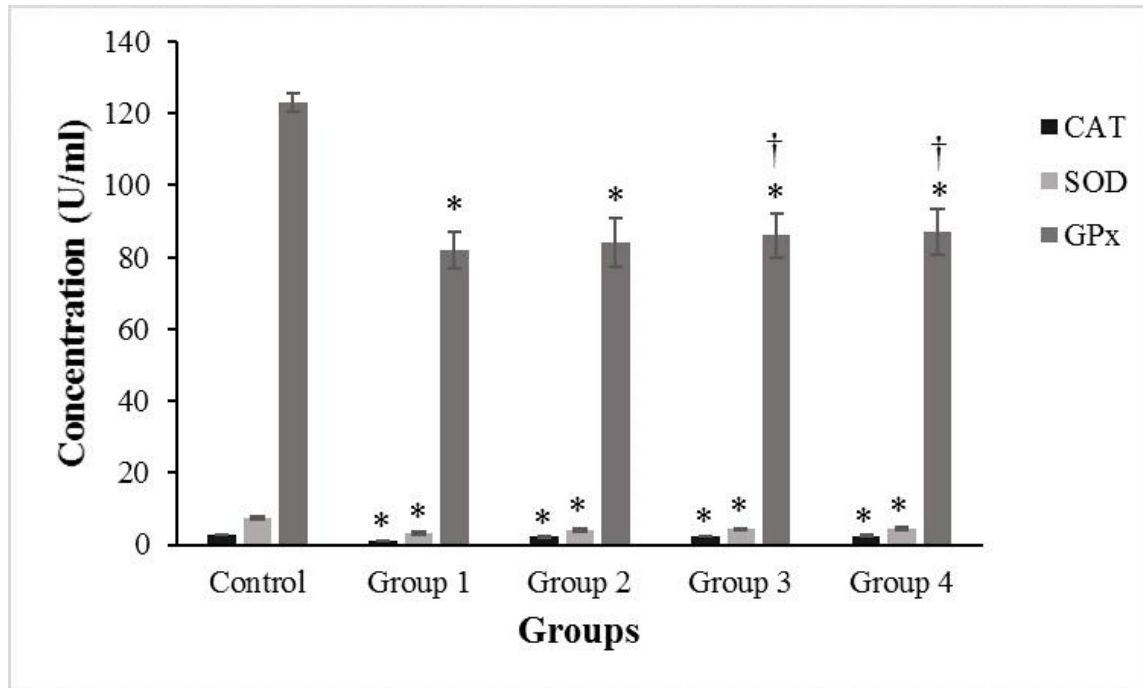


Figure 2. Changes in the serum antioxidant enzymes levels in the control group and diabetic samples in different conditions. * Significances of data comparing diabetic samples vs. the control group ($p < 0.05$). † Significances of data comparing treated samples with cinnamic acid and both cinnamic acid and laser irradiation vs. the other of groups.

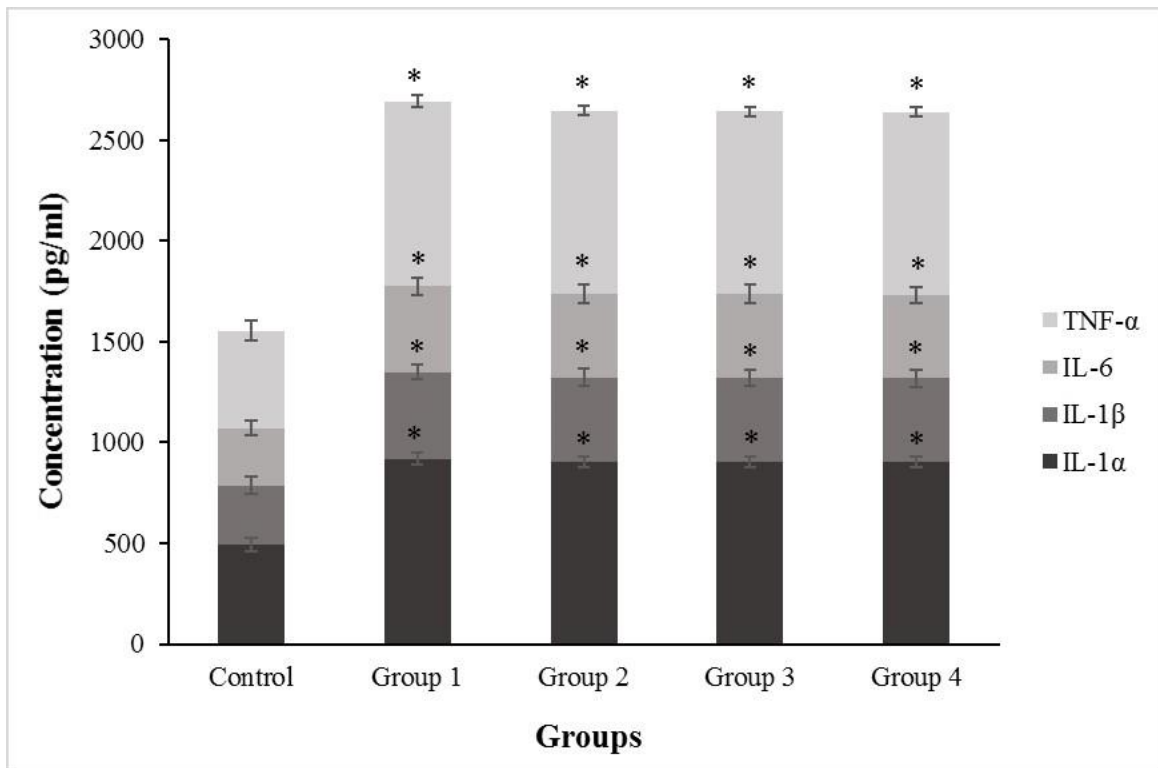


Figure 3. Changes in the serum inflammatory agents' levels in the control group and diabetic samples in different conditions. * Significances of data comparing diabetic samples vs. the control group ($p < 0.05$).

DISCUSSION

Our aim in this study was to evaluate some biochemical parameters in samples of patients with type 2 DM. In the sample of these patients, the effects of both LLLT and cinnamic acid (as a bioactive compound) was studied with the biochemical parameters mentioned above. In our previous study, we reported the effect of LLLT on antioxidant enzymes and minerals (29). Functional foods-based diet as a novel dietary approach has been studied in type 2 DM management (31). The anti-diabetic effects of cinnamon are due to its derivatives, which can also have antioxidant properties. The antioxidant effects of ferulic acid (a derivative of cinnamic acid) have previously been reported in diabetic mice (32). The effect of p-methoxycinnamic acid (another derivative of cinnamic acid) on plasma glucose and insulin concentration in diabetic rats was studied by Yibchok-anun et al. They reported that p-methoxycinnamic acid reduces plasma glucose by stimulating insulin secretion from the pancreas and can have hypoglycemic effects (33). The role of GLUT4, an insulin-regulated glucose transporter, is considered in type 2 DM (34). Based on the results of a study by Lakshmi et al., cinnamic acid plays a role in the activity of GLUT4 protein and so it regulates glucose transport (35).

The anti-diabetic and anti-hyperlipidemic effects of p-hydroxycinnamic acid on diabetic rats were studied and evaluated by Ambika et al (36). Based on the results, they reported that treatment of diabetic rats with p-hydroxycinnamic acid increased the concentration of antioxidant enzymes such as CAT and SOD in liver and kidney. Decreases in lipid hydroperoxides were also observed in treated rats in compared to untreated rats. In terms of increasing the concentration of SOD and CAT, our study agreed

with the study of Ambika et al. The reducing effect of blood glucose by cinnamic acid in diabetic rats was studied by Kasetti et al (37). In their study, diabetic rats were treated with cinnamic acid (50 mg/kg b.w). After 4 hours, the reduction in blood glucose in the treated rats was significantly higher than in the untreated diabetic rats (185 mg/dl vs. 266 mg/dl). Cinnamic acid also plays an important role in altering the enzymes involved in carbohydrate metabolism. In our study, a decrease in blood glucose was observed in samples of treated patients with cinnamic acid and samples exposed to laser irradiation (LLLT). It has been reported that ferulic acid modulates IL-1 β expression. Ferulic acid is effective in reduction of cholesterol, triglycerides, creatinine and urea, and increasing plasma insulin in diabetic rats (38). Cinnamomum cassia derivatives (for example cinnamic acid) are effective in reducing anti-inflammatory activity in mice in which inflammation was induced. Cinnamic aldehyde increases the activity of SOD, CAT and GPx in inflammatory mice and reduces IL-6 and TNF- α . It also lowers MDA levels (39). In vitro studies show that cinnamic acid and its derivatives suppress fructose-mediated protein glycation (40). Therefore, the use of cinnamic acid and its derivatives can be helpful in preventing of AGEs-mediated DM consequences. The effects of low-level laser irradiation on the control and treatment of diabetes damages have been discussed (41-43). In the present study, concomitant use of cinnamic acid and LLLT was not effective in reducing the levels of inflammatory factors (such as IL-1 α , IL-1 β , IL-6 and TNF- α) in diabetic patients' samples.

The effects of cinnamic acid on the consequences of diabetes were studied by Anlar et al (44). In their study, diabetic rats were treated at a dose of 50 mg/kg b.w of cinnamic acid. Antioxidant

enzymes such as CAT, SOD, and GPx and as well as MDA levels were measured in the plasma, liver, and kidney tissues of treated with cinnamic acid diabetic rats. As a result, cinnamic acid plays an important role in modifying the activity of antioxidant enzymes and decreased MDA levels. These effects of cinnamic acid were also observed in our study. It has been shown that cinnamic acid, not cinnamic aldehyde, lowers blood glucose and improves glucose tolerance in diabetic rats. Cinnamic acid can stimulate glucose-induced insulin secretion in type 2 diabetic rats (45). In the present study, as in our previous study, there was no significant reduction in glucose and H₂O₂ concentrations in the treated diabetic samples by laser irradiation. In this study, the results of treated samples with cinnamic acid and treated samples with cinnamic acid under LLLT were not significant. It can be said that LLLT probably has no effect on reducing glucose and H₂O₂ levels in the short term. In uncontrolled diabetes, the production of ROS can have an effect on the brain. One of the consequences of diabetes on the brain is cognitive impairment. It has been shown that the use of cinnamic acid is effective in lowering the levels of MDA and ROS, as well as increasing the levels of CAT and SOD. The effect of cinnamic acid on reducing blood glucose and increasing blood insulin concentrations is significant. Regarding the effect of cinnamic acid on lowering blood glucose, our study was inconsistent with the study of Hemmati et al. (46).

CONCLUSION

Based on the obtained results, it can be concluded that cinnamic acid as an important bioactive and antioxidant compound can play an important role in reducing the factors caused by oxidative stress. This

natural compound is also involved in increasing the activity of antioxidant enzymes. The synergistic effects of using cinnamic acid and LLLT can also be effective in reducing the oxidative effects of DM and thus preventing the consequences of DM. Of course, more studies need to be done.

LIST OF ABBREVIATIONS: LLLT, low level-laser therapy; DM, diabetes mellitus; ROS, reactive oxygen species; Glc, glucose; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; AGEs, advanced glycation end products; AOPP, advanced oxidation protein products; MDA, malondialdehyde; Ox-LDL, oxidized Low-Density Lipoprotein; IL-1 α , Interleukin 1 alpha; IL-1 β , Interleukin 1 beta; IL-6, Interleukin 6 and TNF- α , tumor necrosis factor alpha

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COMPETING INTERESTS: The authors declare that there are no conflicts of interest.

AUTHOR'S CONTRIBUTIONS: Danik Martirosyan participated in the study design. He also edited the article. Mohammad Reza Ashoori participated in the writing and analysis of the results. Hossein Mirmiranpour contributed to the original idea of the paper, doing the experimental work and, data collection. The authors read and approved the final version before its submission.

HUMAN AND ANIMAL STUDIES: This article contains human studies. All of which were consented and humane. No animal studies were conducted.

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