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Palmitate-induced IL6 expression ameliorated by chicoric acid through AMPK and SIRT1-mediated pathway in the PBMCs of newly diagnosed type 2 diabetes patients and healthy subjects

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ABSTRACT

Inhibition of inflammation is one of the possible therapeutic approaches for Insulin resistance (IR) during type 2 diabetes mellitus (T2DM). In the current study we investigated the effects of palmitate and chicoric acid (CA) on inflammation in peripheral blood mononuclear cells (PBMCs) of newly diagnosed T2DM patients and healthy subjects and explored the mechanism by which palmitate and CA influence inflammation.

20 newly diagnosed T2DM patients and 20 healthy subjects were recruited in our study. Blood sample were collected and PBMCs were isolated. Interleukin 6 (IL6), silent information regulator type 1 (SIRT1), AMP-activated protein kinase (AMPK) and phospho-AMPK (pAMPK) were evaluated both *in vivo* and *in vitro*. PBMCs were treated with palmitate and CA to investigate their effects on inflammation. IL6 and SIRT1 genes expression were evaluated by real-time PCR. The levels of IL6 in culture medium were measured by ELISA. Proteins levels of AMPK and pAMPK in PBMCs were detected by western blotting.

IL6 expression was higher and SIRT1 expression and pAMPK levels were lower in PBMCs of diabetic patients and obese subjects compared to healthy subjects and non-obese subjects, respectively. CA significantly prevented against increased IL6 levels as well as its gene expression in PBMCs induced by palmitate. Also, CA returned reduction in SIRT1 expression and pAMPK levels mediated via palmitate to near control level.

These findings reveal that CA reduces inflammation in PBMCs probably through upregulation of SIRT1 and pAMPK. Therefore, CA would be suggested as a novel agent for the treatment of T2DM.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a common disorder around the world. The prevalence of T2DM is progressively increasing, so that it is estimated that rise to over 300 million adults by 2025 [1]. So, this is one of the serious concerns of human health worldwide. Increasing evidence reported insulin resistance (IR) as key enablers of T2DM. IR is a hallmark of T2DM. It is generally accepted that activated inflammatory signaling pathways are essential contributors to IR process in diabetic patients [2]. Various cytokines including Interleukin-1β (IL-1β), Monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor α (TNF-α) and interleukin 6 (IL6) are involved in the development of IR [3]. IL6 is an inflammatory cytokine that provoke IR by down regulation of glucose transporter-4 (GLUT-4) and insulin receptor substrate-1 (IRS-1). Several studies reported the overexpression of IL6 during IR and T2DM [4].

Infiltration of macrophages into the adipose tissue activate inflammatory pathway causes impair insulin action [5,6]. In recent years, peripheral blood mononuclear cells (PBMCs) have been identified as one of the potential sources of cytokine production in adipose tissue of diabetic patients [7,8] and serve as a good model for investigating the pathogenesis of diabetes [9]. These cells consist of lymphocytes and

Abbreviations: T2DM, Type 2 diabetes mellitus; IR, insulin resistance; PBMCs, peripheral blood mononuclear cells; IL6, interleukin 6; SIRT1, silent information regulator type 1; AMPK, AMP-activated protein kinase; pAMPK, phospho-AMPK; IL-1β, Interleukin-1β; TNF-α, tumor necrosis factor α; ANGPTL4, Angiopoietin-like protein 4; PPARγ, peroxisome proliferator-activated receptor γ; LKB1, liver kinase B1; CaMKK, calcium/calmodulin kinase kinase; NAMPT, nicotinamide phosphoribosyl transferase; FFAs, free fatty acids; CA, chicoric acid

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monocytes [10]. It has been reported that PBMCs may indicate the mechanisms of inflammation in adipose tissue [11]. So, we used PBMCs due to the non-invasive nature of procedure as compared to adipose tissue.

Various factors are involved in the insulin signaling pathway such as AMP-activated protein kinase (AMPK), silent information regulator type 1 (SIRT1), Angiopoietin-like protein 4 (ANGPTL4) and peroxisome proliferator-activated receptor γ (PPARγ) [12,13]. AMPK, metabolic master switch, acts as an energy sensor. The activity of this enzyme depends on cell's energy state so that is activated with high cellular AMP levels. AMPK is hetero trimeric Ser/Thr kinase (α catalytic subunit and β and γ regulatory subunits) which has various substrates. Phosphorylation of Thr172 within the α-subunit via liver kinase B1 (LKB1) and calcium/calmodulin kinase kinase (CaMKK) activated AMPK. Phospho-AMPK (pAMPK) affects its target by phosphorylation (In short term) and regulation of multiple transcriptional activators and coactivators (in long term) [14]. SIRT1 is an NAD^+-dependent protein deacetylase which belongs to the sirtuin family. Seven types of sirtuin (SIRT1—SIRT7) have been identified in mammals to date. SIRT1 activates AMPK with LKB1 deacetylation. Also, pAMPK increases SIRT1 activity via phosphorylation of nicotinamide phosphoribosyl transferase (NAMPT). Both molecules involve in regulation of cell metabolism, inflammation and mitochondrial function [15,16].

The serum levels of free fatty acids (FFAs) especially palmitate increase during diabetes [17]. Long term exposure to FFAs leads to IR via increasing oxidative stress, inflammation [18] and recruitment of macrophages to adipose tissue [19]. Palmitate increases macrophage inflammatory protein-1β (MIP-1β) (CCL4) production in monocytes and macrophages via activation of NF-κB/MAPK and AKT/PI3K in TLR4/MyD88 dependent pathway. Enhanced CCL4 induces production of inflammatory cytokines such as IL6. Therefore, palmitate leads to metabolic inflammation by increasing CCL4 production. Metabolic inflammation is a crucial contributor to insulin resistance [20,21]. Recently, it has been elucidated that palmitate increases inflammatory cytokines in PBMCs of T2DM patients [18].

Chicoric acid (CA) is a dicaffeyltartaric acid extracted from chicory (*Cichorium intybus L.*) [22]. CA has various biological effects such as anti-inflammatory, anti-oxidants and anti-hyperglycemic [15]. Although, the exact molecular mechanism how CA improve IR remains to be elucidated.

Decrease the inflammatory status of adipose tissue could be one of the effective strategies for improvement of T2DM. Recently it was reported that CA has anti-inflammatory effects however little is known of the effects of CA on inflammation and there was no report about the effect of CA on the regulation of SIRT1 and AMPK in PBMCs. To this end, in the present study we aimed to explore the effects of CA on inflammation and evaluate the possible mechanism by which CA influences inflammation in PBMCs of newly diagnosed diabetic patients and healthy subjects.

2. Methods

2.1. Subjects and study design

Forty subjects, aged 40–60 years, were recruited in this study. They divided into two groups: Healthy subjects (n = 20, 10 female, 10 male) and age-and gender-matched newly diagnosed T2DM subjects (n = 20, 10 female, 10 male). Diagnosis of diabetes was performed according to American Diabetes Association criteria [23]. The protocol was approved by Ethics Committee of Hamadan University of Medical Sciences (code: IR.UMSHA.REC.1396.291). All participants were enrolled from Hamadan Health Center. The written informed consent was received from all subjects prior to the study. Healthy volunteer had no history of diabetes and their fasting blood sugar (FBS) is less than 100 mg/dl. Diabetic patient refers to subjects with FBS ≥ 126 mg/dl. It is worth noting that T2DM subjects do not took any anti-diabetic drugs.

Table 1
Anthropometric indices of the study population.

Variable	Healthy subjects (n = 20)	T2DM patients (n = 20)	*p* value
Female/male	10/10	10/10	n.s.
Age (year)	46.65 ± 5.97	48.25 ± 6.69	n.s.
Height (cm)	170.45 ± 8.53	170.6 ± 10.9	n.s.
Weight (kg)	72.66 ± 6.39	76.86 ± 7.53	n.s.
BMI (kg/m2)	25.02 ± 1.63	26.48 ± 2.23	**0.024**
SBP (mmHg)	118.6 ± 5.23	121.45 ± 3.78	n.s.
DBP (mmHg)	77.4 ± 2.99	78.85 ± 3.47	n.s.

Data are expressed as Means ± SD. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure.

Subjects with other types of diabetes, inflammatory diseases, endocrine disorders, cardiovascular disease, cancer, thyroid diseases and hypertension were excluded from this study. None of the participants have the history of smoking and alcohol consumption. Also, we grouped participants of the study into obese (BMI > 25) and non-obese (BMI < 25) subjects based on BMI.

2.2. Sample collection and biochemical analysis

After an overnight fast (~12 h), anthropometric parameters of all volunteers including age, height, weight, BMI (weight/height^2), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded. The anthropometric indices are summarized in Table 1. There were no significant differences in age, height, weight, SBP and DBP between the two studied groups. However BMI were significantly higher ($p = 0.024$) in T2DM patients compared to healthy subjects. Gender distribution in each group was equal.

Blood samples of all participants (20 ml) were obtained and collected in Vacutainer EDTA-containing tubes (16 ml) and Serum-separating tubes (4 ml) for PBMC isolation and serum separation, respectively. Biochemical indices such as FBS, triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), Urea and Creatinine were determined by colorimetric methods (Pars Azmoon, Tehran, Iran) on a BIOLIS24i Premium autoanalyzer (Tokyo Boeki Machinery Ltd., Japan). Insulin was measured by ELISA kit (Monobind Inc., CA, USA). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by the formula: fasting insulin (μIU/ml) × FBS (mg/dl)/405. Hemoglobin A1C (HbA1C) was assessed using HPLC method by Tosoh G8 instrument (South San Francisco, CA).

2.3. PBMC isolation and cell culture

PBMCs were isolated immediately using Ficoll-Hypaque density-gradient centrifugation (Lympholyte-H; Cedarlane Laboratories, Ontario, Canada) as previously described [24]. After washing cells with sterile phosphate-buffered saline (PBS), PBMCs were preserved at −80 °C for *in vivo* investigation and suspended in RPMI 1640 medium (GIBCO; Invitrogen Laboratories, UK) containing 10% fetal bovine serum (FBS) (GIBCO; Invitrogen Laboratories, UK) and 1% penicillin-streptomycin (GIBCO; Invitrogen Laboratories, UK) for *in vitro* evaluation. Cell viability of purified PBMCs was evaluated by trypan blue exclusion test. Then, 2×10^6 cells/well and 5×10^6 cells/well plated in 12-well plate and 6-well plate for genes expression and western blotting analysis, respectively. Prior to treatment, cells incubated for 2 h under 5% CO2 at 37 °C. PBMCs of healthy subjects and T2DM patients were treated as follows: control groups (untreated, treated with bovine serum albumin (BSA) 1% for 12 h), CA groups (treated with 50 μM CA for 6 h), palmitate groups (treated with 500 μM palmitate for 12 h), palmitate + CA groups (pretreated with 500 μM palmitate for 12 h and then treated with 50 μM CA for 6 h). The cells and supernatant were finally

harvested by centrifugation (10 min, 2400 rpm, 4 °C) and preserved at −80 °C until later experiments.

2.4. Preparation of treatments

Sodium palmitate (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 50% (v/v) ethanol, then heated at 55 °C and diluted 1:100 in pre-warmed RPMI 1640 medium containing 1% (w/v) fatty acid free-BSA. palmitate-BSA complexes were prepared by shaking in incubator at 37 °C for 2 h. Finally, solution was sterilized by passing through 0.2 filters. CA (Sigma Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO). It is noteworthy that stock solution of 1 mM and 5 mM prepared for palmitate and CA, respectively.

2.5. MTT colorimetric assay

The cell viability was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma Aldrich, St. Louis, MO, USA) colorimetric assays to find optimum concentration and exposure time for CA and palmitate treatment. 2.5×10^5 cells/well seeded in 96-well plate and then incubated with different concentration of palmitate (100 μM, 200 μM, 300 μM, 400 μM, 500 μM, 600 μM, 700 μM, 800 μM,) and CA (10 μM, 20 μM, 30 μM, 40 μM, 50 μM, 60 μM, 70 μM, 80 μM, 90 μM, 100 μM) at 37 °C under 5% CO2 for 6, 12 and 24 h. After treatments, MTT (5 mg/ml) was added into wells followed by incubation at 37 °C for 4 h. Next, the medium was aspirated and formazan crystals were dissolved in DMSO (100 μl/sample). Finally, cell viability evaluated by measuring absorbance at 570 nm using a plate reader (BioTek Instruments Inc., Winooski, USA).

2.6. Total RNA extraction and real-time PCR

Total RNA of treated PBMCs was isolated using the Hybrid-R RNA purification kit (Gene All Biotechnology, Seoul, Korea). The purity and quantity of RNA were determined by a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Also, integrity of RNA was evaluated using 1% agarose gel electrophoresis. cDNA was synthesized from 1 μg of total RNA using Revert Aid First Strand cDNA Synthesis Kit following the manufacturer's instructions (Thermo Scientific, Fermentas, USA). Real-time polymerase chain reaction (PCR) was performed using gene specific primers and SYBR Green detection kit (Ampliqon, Denmark) in the light cycler 96 (Roche Diagnostics, Germany). Primer sequences were as follows: β-actin: 5′-ACAGAGCCTCGCCTTTGC-3′ (forward) and 5′-ATCACGCCCTGGTGCCT-3′ (reverse), SIRT1: 5′-TAGGCGGCTTGAT GGTAATC-3′ (forward) and 5′-TGGCATGTCCCACTATCACT-3′ (reverse), IL6: 5′-AATCTGGATTCAATGAGGAGAC-3′ (forward) and 5′-GCATTTGTGGTTGGGTCAG-3′ (reverse). Before the assay, the standard curves were generated for all genes. β-actin was used as the housekeeping gene. The specificity of PCR products was verified by resolving in 1% agarose gels. Finally, the relative genes expression was evaluated by the $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ method.

2.7. Inflammatory cytokine determination

IL6 levels in the supernatants of treated PBMCs were evaluated using ELISA kit, with a sensitivity of 2 pg/ml and intra-assay and inter-assay CV of 3.6% and 7.7%, respectively, according to the manufacturer's instructions (Diaclone Research, Besancon, France).

2.8. Western blotting

It should be noted that cells were washed twice with PBS before freezing at −80 °C. PBMCs were lysed in RIPA buffer (Thermo Scientific, Waltham, MA, USA) supplemented with protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA) then agitated for 30 min at 4 °C. After centrifugation (10 min at 12,000*g* at 4 °C), supernatant was collected and protein concentration evaluated by bicinchoninic acid (BCA) method (BCA Protein Assay Kit, Thermo Fisher Scientific Inc., USA). Proteins were denatured with loading buffer in 95 °C for 10 min. 70μgr of total protein was loaded on 10% SDS-polyacrylamide gels. Separated proteins were transferred to 0.22 μm pore nitrocellulose membrane. The membranes were then blocked with 5% non-fat milk for 2 h at room temperature and subsequently were incubated overnight at 4 °C with diluted primary antibodies against β-actin, AMPK (Abcam, Cambridge, MA,USA) and pAMPK (Cell Signaling Technology, Beverly, MA, USA). After washing thrice with TBST (Tris-buffered saline, 0.1% Tween 20), membranes incubated with diluted goat anti-rabbit IgG HRP-conjugated secondary antibody (Abcam, Cambridge, MA, USA) for 1 h at room temperature. Then, the membranes were washed three times with TBST. Enhanced chemiluminescence (ECL) emitting kit (Bio-Rad, Germany) was used to visualize immunoreactivity of proteins. Finally, the bands intensity was quantified using ImageJ software (NIH, Bethesda, USA). β-actin was used as the internal control to normalize intensity values.

2.9. Statistical analysis

Data were analyzed using SPSS software (version 16.0, Chicago, IL, USA) and GraphPad Prism software (version 7, San Diego, CA, USA). The Two-way analysis of variance (Two-way ANOVA) was used to compare all groups. The correlation coefficients between variables were performed by Pearson's correlation tests. Results were represented as means ± standard deviation (SD). p value < 0.05 were considered as statistically significant.

3. Results

3.1. Biochemical parameters of the study subjects

As illustrated in Table 2 biochemical parameters of the study population including FBS, insulin, HOMA-IR, HbA1C, TG ($p < 0.001$) and TC ($p = 0.005$) were strongly higher in T2DM group compared with healthy control group. No significant change was observed in LDL-C, HDL-C, Urea, Creatinine, AST and ALT between the two groups.

3.2. IL6 and SIRT1 genes expression and pAMPK levels in vivo

We first compared IL6, SIRT1 and pAMPK levels between T2DM patients and healthy subjects; also we evaluated these factors in obese and non-obese subjects. There was remarkable elevation in IL6 mRNA

Table 2
Biochemical parameters of the study population.

Variable	Healthy subjects (n = 20)	T2DM patients (n = 20)	*p* value
FBS (mg/dl)	92.70 ± 6.26	178.35 ± 61.21	< **0.001**
Insulin (μIU/ml)	4.17 ± 1.49	11.68 ± 5.12	< **0.001**
HOMA-IR	0.96 ± 0.37	5.19 ± 3.31	< **0.001**
HbA1C (%)	5.67 ± 0.51	8.46 ± 2.13	< **0.001**
TG (mg/dl)	126.25 ± 49.1	244.95 ± 98.83	< **0.001**
TC (mg/dl)	163.7 ± 20.91	191.75 ± 35.99	**0.005**
LDL-C (mg/dl)	93.8 ± 19.49	107.0 ± 28.9	n.s.
HDL-C (mg/dl)	46.0 ± 10.55	42.5 ± 7.86	n.s.
Urea (mg/dl)	28.95 ± 6.43	30.8 ± 7.2	n.s.
Creatinine (mg/dl)	1.02 ± 0.17	1.05 ± 0.2	n.s.
AST (U/L)	26.4 ± 6.31	27.4 ± 6.2	n.s.
ALT (U/L)	23.20 ± 8.77	27.45 ± 9.59	n.s.

Data are expressed as Means ± SD. FBS: fasting blood glucose; HOMA-IR: Homeostasis Model Assessment-Insulin Resistance, TG: triglycerides; TC: total cholesterol; LDL-C: Low density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

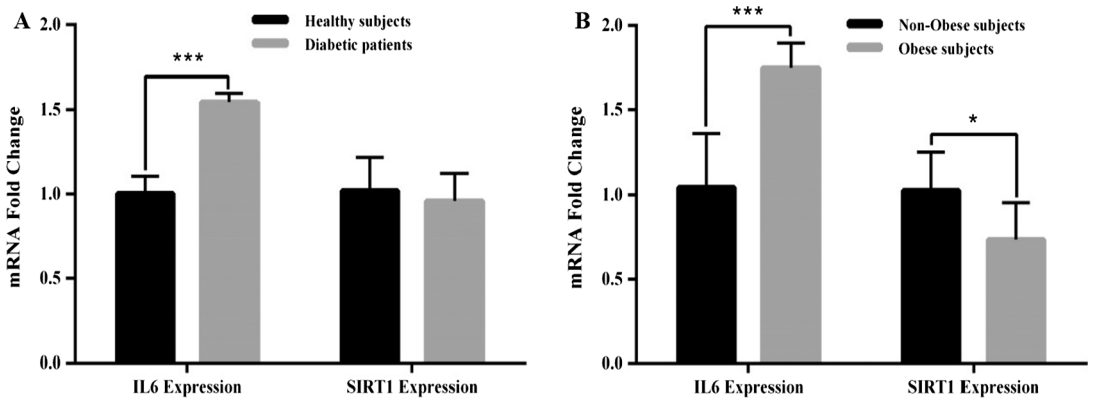

Fig. 1. mRNA Fold Change ($2^{-\Delta\Delta Ct}$) of IL6 and SIRT1 in PBMCs from (A) Diabetic patients compared to Healthy subjects (B) Obese subjects compared to Non-Obese subjects. $^{***}p < 0.001$, $^{*}p < 0.05$.

levels in PBMCs of T2DM patients with respect to healthy control ($p < 0.001$). No difference was revealed in SIRT1 expression between diabetic and healthy subjects (Fig. 1A). Also, we observed higher mRNA levels of SIRT1 than IL6 in both healthy and diabetic subjects (data not shown). Moreover, we found significant elevation of IL6 ($p < 0.001$) and reduction in SIRT1 ($p = 0.041$) transcription levels in PBMCs of obese subjects compared to non-obese subjects (Fig. 1B). As shown in Fig. 2 pAMPK/AMPK ratio in PBMCs from T2DM and healthy participant was not difference. However, pAMPK/AMPK ratio of obese subjects was significantly lower than non-obese subjects ($p = 0.009$).

3.3. PBMCs viability evaluation in response to CA and palmitate in vitro

Optimum concentration and exposure time for CA and palmitate treatment were obtained using MTT assay. Fig. 3A identifies PBMCs viability were significantly increased after exposure to different concentration of CA (20 μM, 30 μM, 40 μM, 50 μM, 60 μM, 70 μM, 80 μM, 90 μM, 100 μM) for 6 h compared with untreated cells. Although, no strong alteration were found in cell viability when the cells were treated with CA for 12 h (Fig. 3B). Also, we observed that treatment of cells with increasing concentration of palmitate (300 μM, 400 μM, 500 μM, 600 μM, 700 μM, 800 μM) for 12 h were significantly increased cell viability (Fig. 3C). Furthermore, palmitate increased cell viability in the concentration of 600 μM, 700 μM, 800 μM for 24 h (Fig. 3D).

Additionally, we evaluated the effects of CA (50 μM) and palmitate (500 μM) for different exposure time on target genes (IL6 and SIRT1) expression (Fig. 4). Collectively, these results revealed that optimum concentration and exposure time for next experiments are 50 μM CA for 6 h and 500 μM palmitate for 12 h (cell viability > 95%).

3.4. Effects of palmitate and CA on IL6 expression in vitro

PBMCs were treated with palmitate and CA to evaluate the effect of CA on IL6 expression in palmitate-treated cell. Compared to untreated cells, IL6 expression was strongly increased in PBMCs exposed to palmitate by 1.99 and 2.34 fold in non-diabetic volunteers and T2DM patients, respectively ($p < 0.001$). Inversely, CA dramatically leads to decrease IL6 gene expression in PBMCs of healthy volunteer and T2DM patients ($p < 0.001$). Palmitate-increased IL6 transcription levels is reversely regulated by CA in healthy subjects (from 1.99 fold to 1.62) ($p = 0.002$) and T2DM patients (from 2.34 fold to 1.8) ($p < 0.001$) (Fig. 5A). It is worth to mention that we also evaluated target genes expression in cells treated with BSA 1% and untreated cells. We observe no difference between these groups (data not shown). Furthermore, as expected, IL6 secretion into culture medium was found to be increased in response to palmitate in PBMCs of normal subjects and diabetic patients approximately 1.95 and 1.87 fold, respectively ($p < 0.001$). Along with IL6 gene expression results, secretion of IL6 into medium was decreased during CA stimulation ($p < 0.001$). Also, our study indicated that CA significantly ameliorates palmitate-induced IL6 levels in PBMCs of both healthy control and T2DM patients ($p < 0.001$). As depicted in Fig. 5B, medium IL6 levels was markedly higher in untreated PBMCs of T2DM patients than healthy volunteer (381.03 pg/ml vs 320.95 pg/ml) ($p = 0.003$).

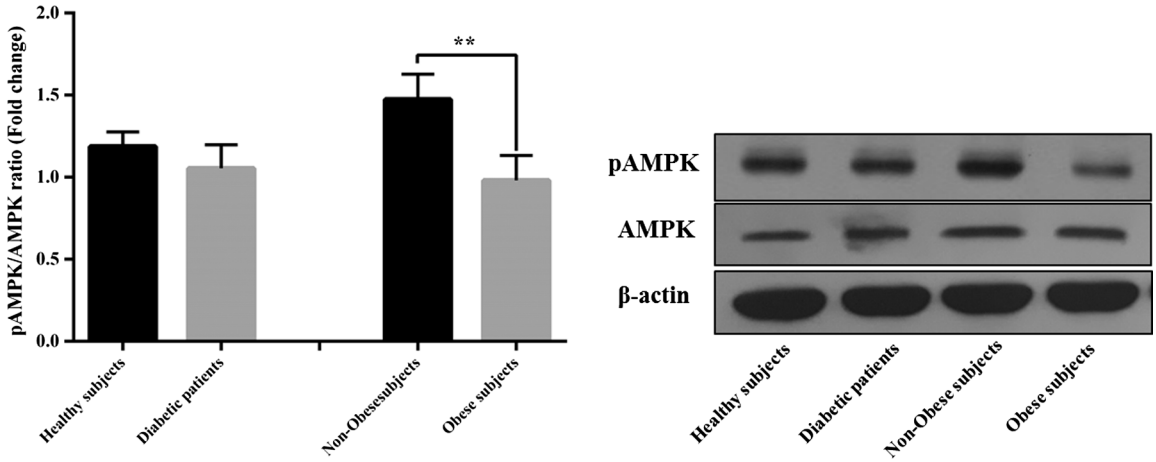

Fig. 2. pAMPK/AMPK ratio in PBMCs of Diabetic patients compared to Healthy subjects and Obese subjects compared to Non-Obese subjects. $^{**}p < 0.01$.

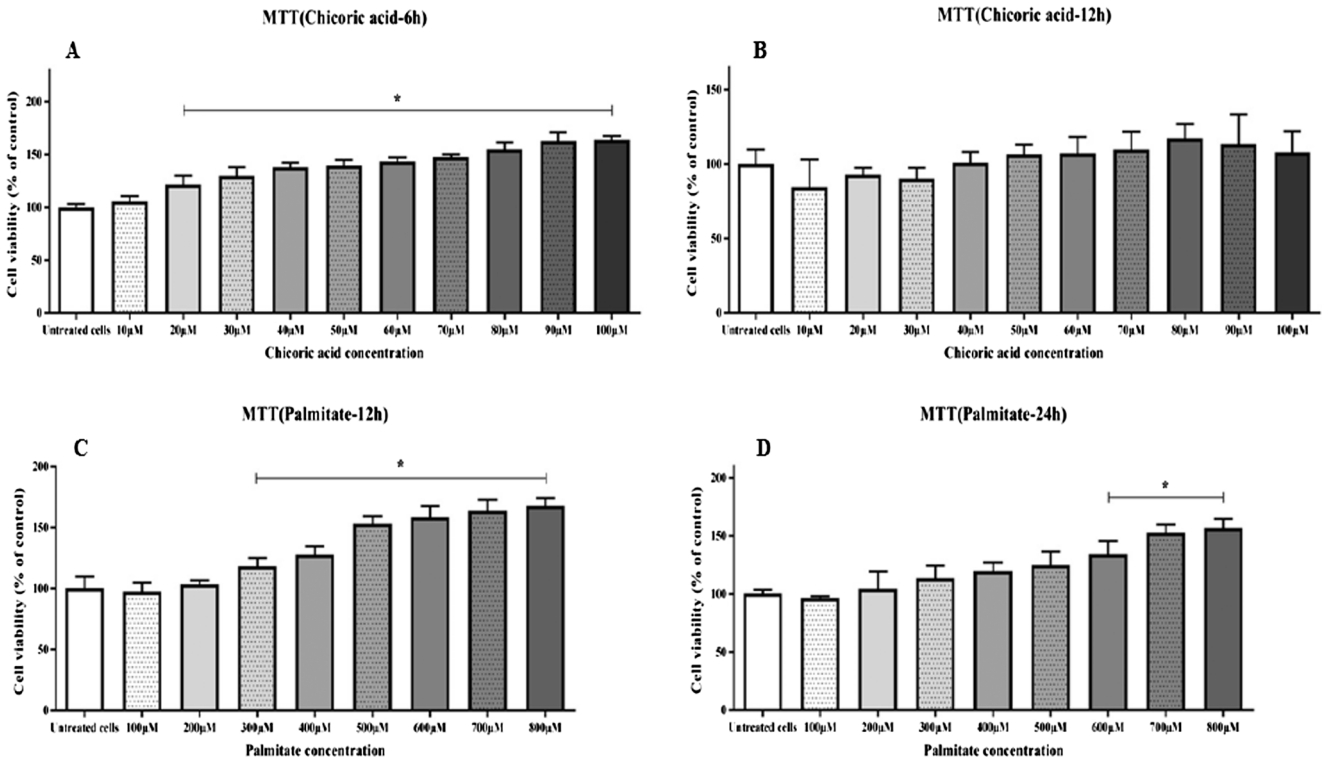

Fig. 3. Cell viability evaluation by MTT assay. Cells were treated with various concentrations of CA and palmitate for different exposure time. Data are expressed as mean ± SD of four replicates in three independent experiments. $^{*}p < 0.05$.

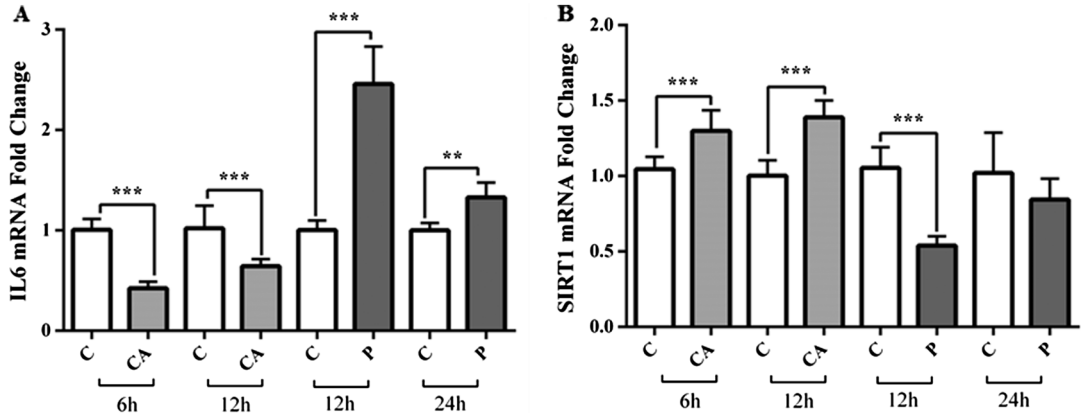

Fig. 4. Effects of CA (50 μM) and palmitate (500 μM) for different exposure time on target genes expression ($2^{-\Delta\Delta Ct}$). C: Control, CA: Chicoric acid, P: Palmitate. Data are shown as mean ± SD. The experiment was performed in triplicate. $^{***}p < 0.001$, $^{**}p < 0.01$.

3.5. Effects of palmitate and CA on SIRT1 expression in vitro

SIRT1 expression in PBMCs was evaluated in response to palmitate and CA. Treatment of PBMCs with palmitate caused a decrease in SIRT1 gene expression in healthy subjects and T2DM patients ($p < 0.001$). Unlike palmitate, CA treatment of PBMCs from healthy subjects and T2DM patients remarkably increased SIRT1 genes expression as compare to untreated cells approximately 2.02 and 1.52 fold, respectively ($p < 0.001$). In healthy control, decreased SIRT1 gene expression induced by palmitate significantly has been restored via CA (from 0.35 fold to 0.77) ($p < 0.001$). Moreover, SIRT1 mRNA levels in untreated cells were not difference in PBMCs taken from T2DM patients with respect to normal subjects ($p = 0.96$) (Fig. 6).

3.6. Effects of palmitate and CA on AMPK and pAMPK levels in vitro

To elucidate whether the anti-inflammatory effects of CA is mediated through AMPK pathway we evaluated phosphorylation of AMPK in PBMCs of all groups using western blotting. Our findings revealed that palmitate treatment resulted in a significant reduction in pAMPK/AMPK ratio of PBMCs in diabetic group (0.28 fold, $p < 0.001$). However, pAMPK/AMPK ratio was increased in the CA treated cells, when compared with untreated cells in healthy subjects and T2DM patients with almost 1.88 and 1.68 fold, respectively ($p < 0.001$). Moreover, we found that CA significantly returned reduction in pAMPK/AMPK ratio in PBMCs were pretreated with palmitate to control level (from 0.28 fold to 0.87) among diabetic patients ($p < 0.001$).

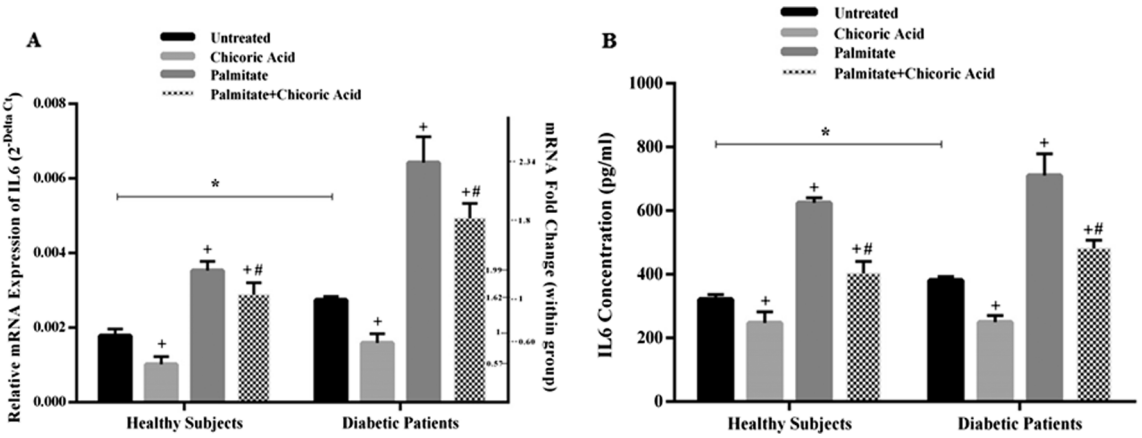

Fig. 5. (A) Effects of palmitate and CA on IL6 genes expression in PBMCs of healthy subjects and diabetic patients. Left side of the graph shows mRNA expression ($2^{-\Delta Ct}$) and right side of the graph shows mRNA fold change ($2^{-\Delta\Delta Ct}$). The non-dotted and dotted lines show fold change for healthy subjects and diabetic patients, respectively. (B) Effects of palmitate and CA on IL6 concentration in PBMCs of healthy subjects and diabetic patients. Data are shown as mean ± SD. $^{*}p < 0.05$ (between group), $^{+}p < 0.05$ compare to untreated cells (within group), $^{\#}p < 0.05$ compare to palmitate treated cells (within group).

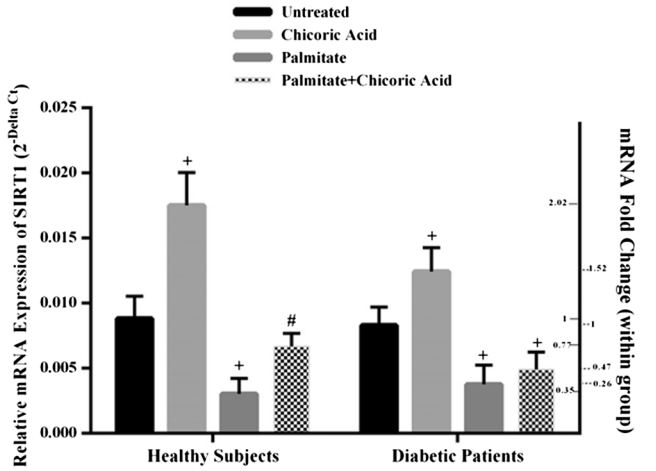

Fig. 6. Effects of palmitate and CA on SIRT1 genes expression in PBMCs of healthy subjects and diabetic patients. Left side of the graph shows mRNA expression ($2^{-\Delta Ct}$) and right side of the graph shows mRNA fold change ($2^{-\Delta\Delta Ct}$). The non-dotted and dotted lines show fold change for healthy subjects and diabetic patients, respectively. Data are shown as mean ± SD. $^{*}p < 0.001$ (between group), $^{+}p < 0.001$ compare to untreated cells (within group), $^{\#}p < 0.001$ compare to palmitate treated cells (within group).

Therefore, according to the results mentioned above, SIRT1 and pAMPK may involve in the anti-inflammatory effects of CA. The between group investigation showed that pAMPK/AMPK ratio in untreated cells was higher in healthy control with respect to the diabetic patients although it was insignificant ($p = 0.947$) (Fig. 7).

3.7. Association of IL6 with SIRT1 expression and pAMPK/AMPK ratio in vitro

Correlation of parameters among total study population showed that IL6 concentration positively correlated with IL6 expression ($r = 0.839$, $p < 0.001$) and negatively correlated with SIRT1 expression ($r = -0.841$, $p < 0.001$) and pAMPK/AMPK ratio ($r = -0.836$, $p < 0.001$) (Fig. 8A and B). Also, there was a significant positive and inverse correlation of pAMPK/AMPK ratio with SIRT1 expression ($r = 0.902$, $p < 0.001$) and IL6 expression ($r = -0.812$, $p < 0.001$), respectively (Fig. 8C). Furthermore, we correlated the medium IL6 levels with IL6 and SIRT1 genes expression and pAMPK/AMPK ratio in PBMCs of each group, separately. As represented in Table 3, strong inverse correlation was observed between IL6 concentration and pAMPK/AMPK ratio in CA-treated PBMCs ($r = -0.717$, $p = 0.020$) in healthy control participants. Additionally, we observed a positive association between IL6 concentration and IL6 expression in untreated PBMCs of healthy subjects ($r = 0.603$, $p = 0.045$). No significant correlation was noted between IL6 concentration and SIRT1 expression in

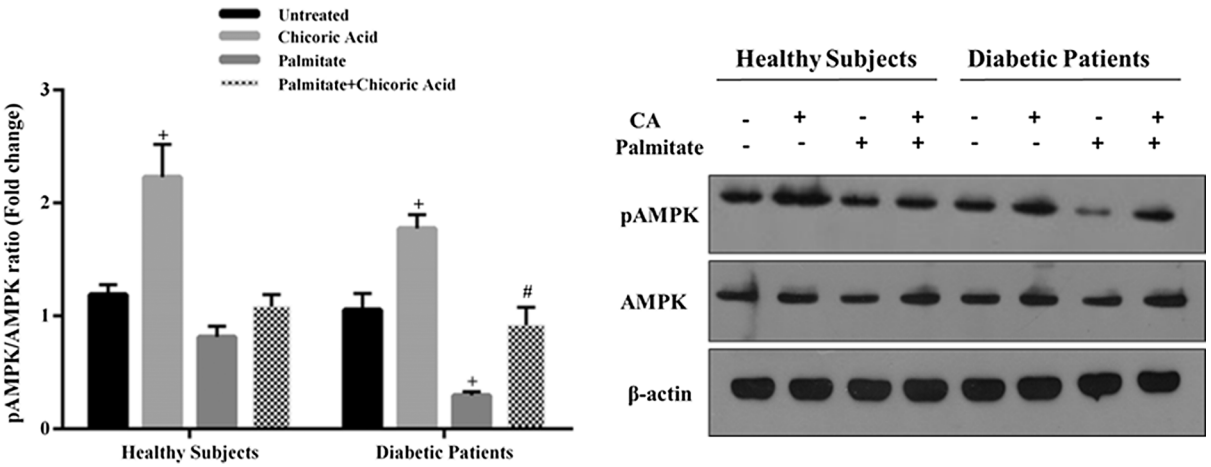

Fig. 7. Effects of palmitate and CA on pAMPK/AMPK ratio in PBMCs of healthy subjects and diabetic patients. Data are shown as mean ± SD. $^{*}p < 0.001$ (between group), $^{+}p < 0.001$ compare to untreated cells (within group), $^{\#}p < 0.001$ compare to palmitate treated cells (within group).

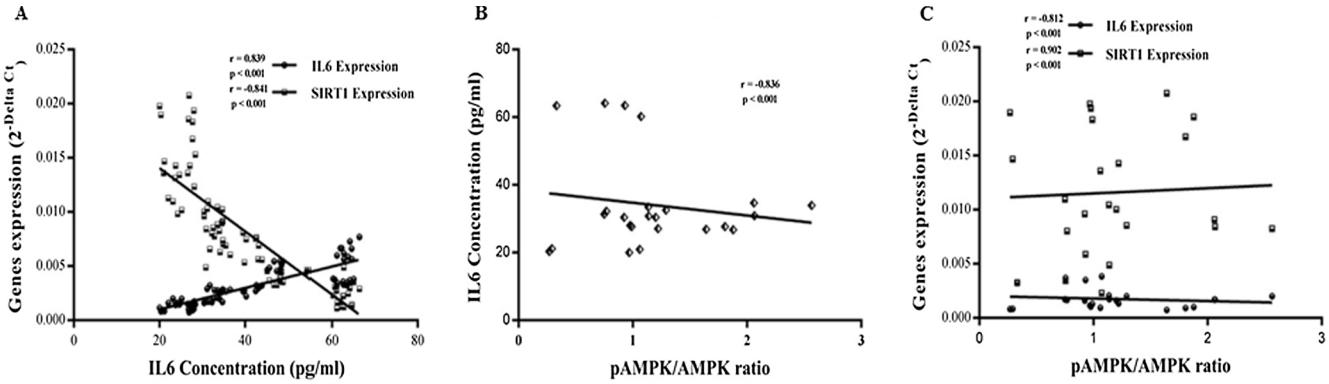

Fig. 8. Pearson correlation analysis between (A) IL6 concentration with IL6 and SIRT1 genes expression (B) pAMPK/AMPK ratio with IL6 concentration (C) pAMPK/AMPK ratio with IL6 and SIRT1 genes expression in total study subjects.

Table 3
Correlation coefficients of IL6 concentration with IL6 and SIRT1 genes expression and pAMPK/AMPK ratio in each group, separately.

		IL6 concentration			
		Untreated PBMCs	Chicoric acid treated PBMCs	Palmitate treated PBMCs	Palmitate + Chicoric acid treated PBMCs
IL6 expression	Healthy subjects	**0.603***	0.306	0.065	−0.006
	Diabetic patients	−0.589	−0.124	0.465	−0.169
SIRT1 expression	Healthy subjects	0.789	0.197	0.509	0.026
	Diabetic patients	−0.438	0.445	0.235	0.399
pAMPK/AMPK ratio	Healthy subjects	0.028	**−0.717***	−0.539	−0.322
	Diabetic patients	0.310	−0.217	0.365	0.181

Bold texts are statistically significant. *$p < 0.05$.

both untreated and treated groups ($p > 0.05$).

4. Discussion

The role of inflammation in IR and diabetes has been well documented. Moreover, inflammation mediates various complications of diabetes. Therefore, the attenuation of inflammation is one of the best strategies for treating diabetes. Targeting IL6 as a well-known inflammatory cytokine is of considerable interest for T2DM treatment. Recent studies have indicated CA as anti-inflammatory agent; however there is great need to clarify the mechanism of this effect. So, in this study we evaluated the possible mechanism by which CA affects IL6 as a valuable molecular biomarker of inflammation in PBMCs of newly diagnosed T2DM.

Firstly, we revealed higher IL6 both in mRNA and protein levels in PBMCs from newly diagnosed diabetic patients compared to healthy volunteer. A similar result was observed by Volpe et al. (2014) that represented higher levels of IL6 in plasma of T2DM compered to healthy control [18]. Also Gupta et al. (2017) reported no difference in serum pro-inflammatory cytokine levels but elevated inflammatory cytokine expression in monocytes of T2DM patients [25]. Elevated FFAs in plasma during diabetes might be a key factor for increasing inflammatory cytokine such as IL6 in PBMCs [2]. However, a recent study investigated that IL6 expression in PBMCs had no difference between T2DM and healthy subjects [7]. Such discrepancies could be attributed to duration of the disease and anti-diabetic drugs usage while T2DM patients in our study are newly diagnosed and they have elevated TG levels in serum.

SIRT1 expression was comparable in two studied groups however it remained not significant. Previous studies revealed that SIRT1 expression decreased during T2DM [26,27]; also in another study we reported lower SIRT1 expression in children with IR [12]. These results corroborate the role of SIRT1 in insulin signaling pathway and diabetes. Also, we observed higher mRNA levels of SIRT1 than IL6 in both healthy and diabetic subjects; some possibilities can be present. Participants in the study were fast and this is reported that SIRT1 up regulated in response to the fasting and calorie restriction [28].

Our research failed to show difference in pAMPK and AMPK levels between PBMCs of newly diagnosed diabetic patients and healthy control participants. In accordance with our present observation generated from healthy and T2DM subjects, in the similar population Alizadeh et al. (2018) have showed that pAMPK in PBMCs had no difference between T2DM and healthy group [7]. Although Zhu et al. (2017) revealed lower levels of pAMPK in liver tissue of STZ-induced diabetic mice [15]. To support this notion, it has been reported that insulin decrease phosphorylation of AMPK in L6 myotubes, conditions that seen in diabetics [29]. This difference in results may have occurred due to small sample size of our study.

Concordant with recent reports, we found higher IL6 and lower SIRT1 genes expression and pAMPK levels in obese subjects compared to non-obese subjects. These differences may be due to high calorie intake that is present in obesity. The low levels of AMP/ADP during obesity reduces the activity of AMPK and then down regulate SIRT1 [12,30].

Further, we revealed that treated cells with palmitate result in elevation of IL6 expression as well as its secretion levels. In line with this result several literatures confirm our findings. IL6 expression was reported to be increase in palmitate-induced cells [18,31,32]. Palmitate induces inflammation through NF-κB pathway [3]. So, induced inflammation occurs as a result of palmitate enhancement, conditions that seen in T2DM. The results also demonstrated that CA decrease inflammatory cytokine including IL6 both in mRNA and protein levels in PBMCs of newly diagnosed T2DM and non-diabetic volunteers. Also, CA reduced overexpression of IL6 induced by palmitate in healthy and T2DM groups. This is the first study examined the effect of CA on inflammation in PBMCs of newly diagnosed diabetic patients. Recently anti-inflammatory effect of CA was identified both *in vivo* and *in vitro* studies. Our data is in harmony with Zhu et al. (2017) results who

reported that CA decreases liver inflammatory cytokines such as IL6 in diabetic mice [15]. In another study by them, CA alleviated IL6 expression in glucosamine induced HepG2 cells [33]. CA has the anti-inflammatory effect by reducing ROS and then decreasing NF-κB activity. As already reported, it is plausible to speculate that CA reduces IL6 expression in PBMCs through inhibition of NF-κB pathway [34,35]. Accumulating evidence support the mediatory role of NF-κB as a key regulator of inflammation [36]. Anti-inflammatory effects of CA indicating a protective role for insulin resistance. It was revealed that CA has improved insulin resistance [33], however the molecular mechanism is still unknown. Investigation the localization of GLUT4 could clarify beneficial effects of CA on IR.

Also we evaluated SIRT1 expression and pAMPK levels which are molecules may involve in the inflammation pathway. Our results showed that SIRT1 expression was decreased following PBMCs stimulated with palmitate. Studies on other cells type have also showed that the expression and activity of SIRT1 down regulated with palmitate [37,38]. Down regulation of SIRT1 via palmitate are probably mediated by increasing oxidative stress and decreasing NAD^+ levels [38]. SIRT1 knockdown in macrophage leads to recruitment of these cells into the adipose tissue and subsequent increase multiple inflammatory pathways, concomitant with the enhancement of insulin resistance [27,39], whereas overexpression of SIRT1 opposite these effects [40]. Although, one study demonstrated that SIRT1 inhibition do not effect on inflammation in skeletal muscle cells [3].This is probably due to different cell type. CA treatment up regulated gene expression of SIRT1 in palmitate-induced cells in healthy individuals. Effects of CA on SIRT1 expression has not been previously evaluated in T2DM patients. However, an overexpression of SIRT1 in response to CA in HepG2 cells treated with glucosamine has been earlier reported [15]. It appears that increased SIRT1 expression induced by CA is account for the alleviation of inflammatory cytokine levels which then improve insulin sensitivity.

We also observed that palmitate reduce phosphorylated form of AMPK. This data are consistence with Sun et al. (2008) study in which palmitate decrease both total AMPK and pAMPK in β-cells of rats, also they revealed that fenofibrate increase AMPK and pAMPK that down regulated by palmitate [41]. Results of one study is contradict with our finding which reported increased pAMPK levels in palmitate-induced MIN6 cells, they suggested that phosphorylation of AMPK protect β cell from lipotoxicity [42]. This contradiction in result may derive from culture medium, types of cells and exposure duration. Present study revealed for the first time that CA returned pAMPK levels to near control in palmitate treated cells of diabetic patients. These findings are in well supportive with recent studies showing that CA induces phosphorylation of AMPK and promotes its activation [14,15]. Studying the liver tissue of STZ-induced diabetic mice showed that CA compensate the reduction of pAMPK levels [15]. It was also reported that oleate attenuate inflammation induced by palmitate through AMPK activation [43]. These findings raise the intriguing possibility that AMPK is a key mediator of CA function in the reduction of inflammation. Therefore, CA can be introduced as a new AMPK activator along with metformin and resveratrol that used for T2DM therapy [44].

Based on Kochumon et al. (2018) results, palmitate increases inflammation through NF-κB activation. So that, binding of palmitate to TLR4 engage MyD88. This interaction activates the NF-κB/MAPK that induces the production of inflammatory cytokines such as IL6 [20]. Although, in this study palmitate increases IL6 production through down regulation of AMPK and SIRT1. Palmitate inhibits AMPK by Protein Phosphatase 2A (PP2A) activation [45]. Inhibited AMPK leads to inactivation of SIRT1 and then acetylation of NF-κB. Acetylated NF-κB induces IL6 expression [36]. Therefore, inhibition of AMPK and SIRT1 via palmitate activates NF-κB probably in a MyD88 independent pathway. Also, we hypothesized that AMPK and SIRT1 attenuated inflammation in CA-exposed PBMCs probably through decreasing ROS, p53, AKT and NF-κB [36,38].

Another interesting finding in present study is that CA prevented against down regulation of SIRT1 expression and pAMPK levels induced by palmitate in healthy subjects and diabetic patients, respectively. We can conclude that mechanism of CA effects on inflammation is a little difference in healthy subjects and diabetic patients. In non-diabetic volunteers CA relieved inflammation caused by palmitate through increasing SIRT1 expression while in diabetic patients the effects of palmitate were suppressed by CA through activation of AMPK. Further investigations could explain this discrepancy in more detail.

Taken together, the results revealed that down regulation of SIRT1 and AMPK may involve in activation of inflammation pathway. We presented evidence that CA ameliorates inflammation probably through activation of AMPK and overexpression of SIRT1. So, SIRT1 and AMPK may serve as a key mediator in anti-inflammatory activity of CA in healthy subjects and diabetic patients, respectively. Overall, considering the anti-inflammatory effects of CA on PBMCs, CA as a natural agent maybe improve inflammation related conditions such as IR and T2DM. However, the precise molecular mechanisms underlying how CA improves IR and T2DM deserve further study.

Importantly, we observed that decreased IL6 level was accompanied by increased SIRT1 expression and pAMPK/AMPK ratio. These results emphasize that AMPK and SIRT1 have a strong relationship with each other and involved in reducing inflammation. AMPK activates SIRT1 by increasing NAD^+ level and SIRT1 increases AMPK activity by deacetylation of LKB1 [16].

5. Conclusion

Findings from our study suggest that CA could be a potential candidate for the treatment of T2DM. CA can confer protection against inflammation through SIRT1 and AMPK activation. However, other factors involved in this pathway should also be investigated to further support this notion. Also, other potential mechanisms of regulation could be evaluated.

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Conflicts of interest

The authors declare no conflict of interest.

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