

Original Article

The Effect of Biochanin A as PPAR γ agonist on LDL Particles Diameter and Type 2 Diabetic Dyslipidemia

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ABSTRACT

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Key words

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Background and Aims: Small dense low-density lipoproteins (sd-LDL) particles are smaller and heavier than typical LDL ones. They can penetrate into the endothelium of coronary arteries more easily because of their small size. Diabetes mellitus is accompanied by dyslipidemia such as increasing concentration of plasma very low density lipoprotein and sd-LDL. Peroxisome proliferator activated receptor γ (PPAR γ) can decrease the level of sd-LDL in plasma. Biochanin A (BCA), a natural compound, is a PPAR γ agonist. The present study was designed to investigate the effect of BCA on sd-LDL-Colesterol level in diabetic animals.

Materials and Methods: Adult male rats (Wistar strain) were used as the animal models in this study. Animals were made diabetic by single intraperitoneal injection of Streptozotocin- Nicotinamide and then treated by 1 and 5 mg/kg of BCA for 28 days. Body weight and fasting blood glucose were also tested before and at the end of treatment. Furthermore, the size of LDL particles were measured by non-denaturing polyacrylamide gradient gel electrophoresis assay.

Results: Results of the present study indicated that BCA administration at dose of 5mg/kg decreased fasting blood glucose level and increased body weight and diameter of LDL particles in diabetic animals significantly.

Conclusions: BCA seems to be an appropriate agent in diabetes mellitus, because it improves the diabetic dyslipidemia, which is the most important complication in diabetic patients.

Introduction

Low density lipoprotein (LDL) is one of the major groups of lipoproteins in plasma. LDL particle subclasses are different in size, density, lipid composition and pathologic effects. LDL particles include light and large (LDL 1,2), intermediate (LDL 3) and small dense particles (LDL 4,5) [1]. Frequency and distribution of LDL subclasses can be influenced by multiple factors such as diet, body mass index, diabetes mellitus, metabolic syndrome and familial dyslipidemia [2, 3]. LDL particles invade the endothelium of coronary arteries which is the main cause of atherosclerosis and cardiovascular disease. Higher frequency of LDL particles with small size (sd-LDL), because of easier transport to coronary arteries endothelium, increases the risk of cardiovascular diseases [3].

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder accompanied by dyslipidemia such as increasing concentration of plasma very low density lipoprotein (VLDL) and sd-LDL [4-8]. Atherosclerosis is a common complication of T2DM; it probably arises from atherogenic effects of LDL particles. Accurate pathophysiology of diabetic dyslipidemia is still controversial. With regard to previous studies, insulin deficiency is the main cause of diabetic dyslipidemia. Insulin inhibits hormone sensitive lipase in adipocytes which will result in decreased degradation of triglycerides (TG) and migration of free fatty acids (FFA) to liver. Moreover insulin inhibits apo B100 synthesis, in this way VLDL synthesis in liver will

decrease. On the other hand, insulin stimulates lipoprotein lipase (LPL) activity, which will induce to increased intracellular oxidation of FFAs. In conclusion, in the state of insulin deficiency, VLDL synthesis increases and intracellular oxidation of FFAs decreases, which will result in diabetic dyslipidemia [9].

Peroxisome proliferator activated receptor γ (PPAR γ) transcription factor, is one of the important agents in lipid metabolism and glucose homeostasis. This is a ligand-inducible transcription factor and presents in adipocytes, colon and macrophages. Genes such as LPL, adiponectin and c-cbl associated protein are instances which are regulated by PPAR γ . These genes have important roles in lipid metabolism and glucose homeostasis. It seems that PPAR γ agonists are appropriate agents to control T2DM and the resulting dyslipidemia [10-15].

Biochanin A (BCA), a PPAR γ agonist, is an isoflavonoid found in red clover and soy beans. In several studies, the anti-diabetic effect of BCA has been approved, but its function and mechanism is still controversial. Probable anti-diabetic mechanisms that have been explained in different studies are the result of stimulation of insulin secretion from beta pancreatic cells, insulin mimetic effects, increasing of glucose uptake in peripheral tissues and regulating the key enzymes in carbohydrate lipid metabolism. So far, no study has been reported to investigate the effect of BCA on diabetic dyslipidemia. Since the BCA is a PPAR γ agonist and PPAR γ has a key role in lipid metabolism, BCA should

have a considerable effect on diabetic dyslipidemia [16-18].

The present study was designed to investigate the effect of BCA on sd-LDL cholesterol (sd-LDL-c) levels and diameter of LDL particles in the serum of diabetic rats. In addition, animals' body weight and fasting blood glucose (FBG) were tested.

Materials and Methods

Animals

Initially, diabetes was induced in adult Wistar male rats with body weight of 200-230 g. Rats were purchased from central animal house of Hamadan University of Medical Sciences, Hamadan, Iran. For diabetes induction, Nicotinamide (N3376, Sigma, 120 mg/kg) and Streptozotocin (S130, Sigma, 60 mg/kg) were injected intraperitoneally in 15 minutes interval. Normal saline was used to solve the Nicotinamide; Streptozotocin was solved in 0.1 M sodium citrate buffer (pH=4.7). Nicotinamide inhibits the complete destruction of pancreatic beta cells. Seventy-two hours after injection and after 12 hours of fasting, FBG levels were tested in all rats [19-21]. The animals with FBG level of higher than 126 mg/dl were regarded as diabetic cases. This study lasted 28 days. During the study, animals were kept at temperature of 21-23°C, relative humidity of 55±10 % and were nourished by standard food and water. The study protocol was approved by the Ethics Committee of Hamadan University of Medical Sciences.

Study design

Four experimental groups were defined in this study; they were included in two treatment and

two control groups. Ten animals were assigned randomly into each group. Diabetic rats were then divided in two treatment and a control groups. Healthy rats were placed in another control group. BCA was administrated intraperitoneally in 1 and 5 mg/kg/day doses during 28 days. BCA (BCA, D2016, Sigma) was dissolved in 75% Dimethyl sulfoxide (DMSO) before administration [22, 23]. Animals' body weight, FBG, sd-LDL-c levels and diameter of LDL particles in serum were tested at the beginning and the end of the study. After 28 days, animals were anesthetized using ether. Blood sample were collected, serum was separated and maintained at -80°C. FBG levels were measured by glucometer (Glucocard 01, Arkray, Japan).

sd-LDL-C assay

For measuring the concentration of sd-LDL-c in serum, the lipoproteins which have the density lower than 1.044 g/ml should be precipitated. These lipoproteins include VLDL, IDL and large buoyant LDL (lbLDL). Precipitation reagent was composed of 15 U/ml heparin sodium salt and 90 mmol/lit magnesium chloride (MgCl₂). The serum (0.1 ml) was mixed with 0.1 ml precipitation reagent and was incubated at room temperature for 10 minutes. At the next step, the mixture was incubated on ice for 15 minutes. Finally, the mixture was centrifuged (25000×g, 15 minutes, 4°C). The supernatant included HDL and sd-LDL. As it was explained, all of the LDL particles in supernatant are sd-LDL particles. We used the direct LDL-C kit to measure the sd-LDL-c in the supernatant [24]. Next, direct LDL-c assay (LDL-c, Pars Azmun, Tehran,

Iran) was performed in the supernatant using an autoanalyzer.

Nondenaturing polyacrylamide gradient gel electrophoresis (PAGGE)

PAGGE is the most common technique to estimate the size of LDL particles. For making the gradient gel, 16% and 2% acrylamide solutions were poured in gradient maker separately and the gradient was made from 2% to 16%. For preparing acrylamide solution, Tris/Borate/EDTA (TBE), Ammonium per Sulfate 10%, NNNN-Tetramethylethylenediamin (TEMED), distilled water and acrylamide 30% (acrylamide 29.2 g- bisacrylamide 0.8 g) were used. Before loading the serum samples, electrophoresis was done at 125 volt for 15 minutes. Next, 10 mL of serum was loaded and gel was run at 70 volt for 20 minutes. Then, electrophoresis was continued at 125 volt for 24 hours at 4°C. Running buffer was composed of 0.09 mol/l Tris, 0.08 mol/l boric acid and 0.003 mol/l disodium ethylenediaminetetraacetic acid (pH=8.3). Loading buffer was made from 200 g/l Sucrose and 1 g/l Bromophenol blue. Albumin and thyroglobulin with diameter of 3.8 and 17 nm respectively were used as markers.

At the end, the gel was stained with Sudan Black for detecting the LDL bands and measuring the mobility differences [2]. The diameter of LDL particles was measured based on mobility differences of LDL particles and molecular markers on polyacrylamide gel electrophoresis.

Statistical analysis

Data are presented as Mean±SD and one-way ANOVA analysis, followed by post hoc Tukey's test. P value <0.05 was accepted as significant.

Results

The effect of BCA administration on body weight of the diabetic rats is shown in Table 1. According to this table, body weight significantly increased in 5mg/kg BCA treated rats compared with that of the rats treated with 1 mg/kg BCA and the diabetic control group ($p<0.001$), but it could not reach the level of the healthy control group ($p<0.01$). There was no significant difference in body weight between diabetic control group and the rats treated with 1mg/kg BCA ($p>0.05$).

Table 1. Effect of BCA administration on animal's body weight

| | Healthy Control | Diabetic Control | Diabetic + 1mg/kg BCA | Diabetic + 5mg/kg BCA |
|--|------------------------|---|---|--|
| Pre treatment day 0 | 220.43±12.52 | 224.97±11.8 ($p_a=0.32$) | 221.63±13.37 ($p_a=0.34$, $p_b=0.23$) | 229.48±12.24 ($p_a=0.27$, $p_b=0.41$, $p_c=0.35$) |
| 7 days after diabetes induction | 225.93±12.6 | 183.17±14.34 ($p_a=0.18$) | 178.5±15.42 ($p_a=0.15$, $p_b=0.19$) | 186.31±14.76 ($p_a=0.33$, $p_b=0.16$, $p_c=0.29$) |
| 28 days after treatment | 257.3±14.54 | 131.74±12.5 ^{a*} ($p_a=0.0005$) | 129.62±17.83 ^{a*} ($p_a=0.0002$, $p_b=0.12$) | 220.51±13.44 ^{a#,b*,c*} ($p_a=0.0005$, $p_b=0.0004$, $p_c=0.0005$) |

Mean±SD body weight (gram) in healthy control (n=10), diabetic control (n=10) and BCA-treated animals (1 and 5mg/kg; n=10). a: compared with healthy control; b: compared with diabetic control; c: compared with diabetic rats treated by 1mg/kg BCA. #: $p<0.01$, *: $p<0.001$.

BCA= Biochanin A

Table 2 indicates the effect of BCA injection on FBG level of diabetic rats. FBG level decreased significantly in diabetic animals treated with 5 mg/kg compared to the diabetic animals treated with 1 mg/kg and diabetic control group ($p < 0.001$). FBG level was still remarkably lower in healthy control group compared with the diabetic animals ($p < 0.01$). Considerable difference was not observed in FBG level between diabetic control group and treated group with 1 mg/kg BCA.

The influence of BCA consumption on sd-LDL-c level and diameter of LDL particles in diabetic rats are demonstrated in Table 3. There was significant difference in sd-LDL-c level ($p < 0.001$) between treated group by 5 mg/kg BCA and the group that was treated with 1 mg/kg BCA and diabetic control group

in sd-LDL-c level ($p < 0.001$). It should be indicated that sd-LDL-c in the healthy control group was considerably lower than that of diabetic groups ($p < 0.01$). Obviously, the use of 1 mg/kg BCA could not be effective in lowering the sd-LDL-c level compared with diabetic control group ($p > 0.05$). Use of 5 mg/kg BCA in diabetic rats, increased significantly the diameter of LDL particles compared with the group treated by 1mg/kg BCA and control diabetic group ($p < 0.01$). As is shown in Table 3, diameter of LDL particles in healthy control was remarkably larger than that of diabetic groups ($p < 0.01$). No significant difference was observed between the control diabetic group and animals treated with 1 mg/kg BCA ($p > 0.05$).

Table 2. Effect of BCA administration on animals' fasting blood glucose

| | Healthy Control | Diabetic Control | Diabetic + 1mg/kg BCA | Diabetic + 5mg/kg BCA |
|--|-----------------|--|---|---|
| Pre treatment day 0 | 81.64±9.87 | 83.24±10.23 ($p_a=0.14$) | 84.18±10.46 ($p_a=0.17, p_b=0.11$) | 82.76±11 ($p_a=0.15, p_b=0.09, p_c=0.22$) |
| 7 days after diabetes induction | 82.73±11.33 | 195.57±14.61 ($p_a=0.41$) | 190.38±11.42 ($p_a=0.25, p_b=0.38$) | 197.53±13.67 ($p_a=0.14, p_b=0.27, p_c=0.23$) |
| 28 days after treatment | 81.9±10.78 | 200.78±15.82 ^{a*} ($p_a=0.28$) | 197.48±12.3 ^{a*} ($p_a=0.0003, p_b=0.19$) | 108.9±14.1 ^{a#,b*,c*} ($p_a=0.007, p_b=0.0001, p_c=0.0005$) |

Mean±SD fasting blood glucose (mg/dl) in healthy control (n=10), diabetic control (n=10) and BCA-treated animals (1 and 5 mg/kg; n=10). a: compared with healthy control; b: compared with diabetic control; c: compared with diabetic rats treated with BCA. #: $p < 0.01$, *: $p < 0.001$. BCA= Biochanin A

Table3. Effect of BCA administration on animals' sd-LDL-c level and LDL particle diameter in serum

| | Healthy Control | Diabetic Control | Diabetic + 1mg/kg BCA | Diabetic + 5mg/kg BCA |
|------------------------------------|-----------------|--|---|--|
| sd-LDL-c (mg/dl) | 35.75±3.68 | 68.41±6.9 ^{a*} ($p_a=0.22$) | 70.22±7.53 ^{a*} ($p_a=0.31, p_b=0.27$) | 49.39±4.82 ^{a#,b*,c*} ($p_a=0.32, p_b=0.15, p_c=0.42$) |
| LDL particles diameter (nm) | 28.73±1.9 | 18.94±3.48 ^{a#} ($p_a=0.33$) | 18.64±4.38 ^{a#} ($p_a=0.004, p_b=0.13$) | 23.6±3.91 ^{a#,b#,c#} ($p_a=0.007, p_b=0.001, p_c=0.005$) |

Mean±SD of sd-LDL-C and LDL particles diameter in healthy control (n=10), diabetic control (n=10) and BCA-treated animals (1 and 5mg/kg; n=10). a: compared with healthy control; b: compared with diabetic control; c: compared with diabetic rats treated with 1 mg/kg BCA. #: $p < 0.01$, *: $p < 0.001$. BCA= Biochanin A

Discussion

Recently, sd-LDL is considered as an important risk factor for cardiovascular diseases. For their small size, sd-LDL particles stay longer in blood stream, penetrate the arteries and are more subject to oxidation. Insulin deficiency and diabetes mellitus are important agents which accelerate the conversion of VLDL to sd-LDL. Based on the previous studies, in the case of insulin deficiency, VLDL synthesis increases and LPL activity decreases, which will result in formation of TG rich VLDL particles. Hepatic lipase converts the TG rich VLDL particles to sd-LDL particles [9, 25-27]. It seems that LPL plays an important role in pathophysiology of diabetic dyslipidemia. Several studies have reported that there is a PPAR γ - responsive element (PPRE) in promoter region of LPL gene. Therefore, PPAR γ agonists like BCA will be useful agents in controlling diabetic dyslipidemia [12, 28].

Wang et al. reported that PPAR γ transcription factor decreases the blood level of total cholesterol and triglyceride and has an important role in lipid metabolism [11]. Ashok Kumar et al. indicated that there is a PPRE in promoter region of LPL gene [28]. Ahmadian et al. stated that PPAR γ agonists are useful agents in preventing congestive heart failure in type 2 diabetic patients by decreasing the blood level of LDL-c. [29]. Kerstan et al. demonstrated that PPAR γ transcription factor increases the intracellular oxidation of fatty acids via increasing the LPL activity thus decreasing the blood level of LDL-c was

decreases [12]. Azizi et al. showed that administration of BCA decreased the FBG and LDL-c levels in diabetic rats [18].

The results of the present study, consistent with previous studies, revealed that intraperitoneally injection of BCA in diabetic animals is effective in decreasing the sd-LDL-C level in diabetic rats and increasing the diameter of LDL particles. Furthermore, FBG decreased and body weight increased. It means that the administration of BCA can be useful in preventing cardiovascular diseases in diabetic patients. It should be added that to make BCA an effective drug in dealing with diabetic dyslipidemia, there are still several untouched problems such as administration form, effective dose and its side effects. This study was the first one that was designed to investigate the effect of BCA on diabetic dyslipidemia.

Conclusion

According to the results of the present study, it seems that BCA has a dose dependent effect on improving the diabetic dyslipidemia. Decreased level of sd-LDL-C was accompanied by decreased level of FBG and heightened weight loss in diabetic rats. In conclusion, BCA seems to be an appropriate agent in diabetes mellitus, because it improves the diabetic dyslipidemia that is the most important complication in diabetic patients.

Conflict of Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work to influence its outcome.

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