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Biochanin-A attenuates high-fat diet and streptozotocin-induced hyperlipidemia and oxidative stress in rats by improving antioxidant status and lipid metabolic markers

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ABSTRACT

Objective: To determine how biochanin-A (BCA) affects high-fat diet and streptozotocin-induced pathological changes in lipid metabolism and antioxidant status in diabetic rats.

Methods: Diabetic rats were orally administered BCA (10 mg/kg body weight) for 30 days to investigate its effects on lipid profiles and oxidative stress markers in the liver and kidney. In addition, the mRNA expression of antioxidant and lipid metabolism enzymes in the liver was examined.

Results: BCA attenuated hyperlipidemia by regulating mRNA expressions of HMG-CoA reductase, fatty acid synthase, carnitine palmitoyl transferase, and acetyl-CoA carboxylase. Additionally, BCA reduced high-fat diet and streptozotocin-induced oxidative stress by suppressing lipid peroxidation, improving superoxide dismutase, catalase, and glutathione peroxidase levels, and upregulating mRNA expressions of these enzymes.

Conclusions: BCA may be a promising nutraceutical for the treatment of dyslipidemia and oxidative stress associated with diabetes.

KEYWORDS: Antioxidant; Biochanin-A; Nutraceutical; Obesity; Hyperlipidemia; Type 2 diabetes mellitus; Oxidative stress

1. Introduction

The prevalence of type 2 diabetes mellitus (T2DM) is rapidly increasing worldwide. It is defined by elevated blood glucose levels

Significance

Biochanin-A is an *O*-methylated isoflavonoid found in various plants and has biological and pharmacological effects. However, there has been no solid scientific evidence on the effect of biochanin-A against high-fat diet and streptozotocin-induced pathological changes in lipid metabolism and antioxidant status. The results of the current study show that biochanin-A alleviated type 2 diabetes mellitus by attenuating hyperlipidemia and oxidative stress *via* regulating lipid metabolism and antioxidant genes. Therefore, this compound may be further investigated for use as a promising nutraceutical for the treatment of dyslipidemia and oxidative stress associated with diabetes.

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caused by a gradual loss of insulin action [insulin resistance (IR)] and failure of pancreatic beta cells[1]. Obesity, usually caused by a high-fat diet (HFD) and physical inactivity, is a significant risk factor for IR[2,3]. HFD is associated with an increase in body weight and visceral fat depots, alterations in carbohydrate and lipid metabolism, IR, and changes in adipokines[4–6]. Hyperglycemia and dyslipidemia are two of the many signs of metabolic syndrome DM. One risk factor for DM is hyperlipidemia, which is often caused by overeating and physical inactivity[7]. Therefore, optimal drugs for the treatment of DM should not only have blood glucose-lowering properties but also have a positive effect on hyperlipidemia. However, most diabetes medications have negative effects on lipid profile[8]. Therefore, it is important to search for antidiabetic drugs or foods with functional properties that can regulate both hyperlipidemia and hyperglycemia.

On the other hand, long-term consumption of HFD may induce oxidative stress by greatly reducing the activity of the liver antioxidant enzyme system and increasing the levels of lipid peroxidation products (LPO) in the liver and plasma[9]. According to some studies, oxidative stress may play a role in the etiology of metabolic disorders leading to IR, obesity[10] and DM[11]. One theory suggests that an increase in oxidative stress occurs before the onset of obesity and the metabolic disorders caused by an HFD[12]. It follows that a reduction in increased oxidative stress at this stage could prevent or limit the severity of subsequent metabolic disorders and their consequences. Moreover, oxidative stress has been shown to play an important role in organ pathophysiology and is associated with organ dysfunction[13].

Phytomedicines have long been known for their significant anti-inflammatory, antihyperlipidemic, and antioxidant effects, with few side effects[14]. Biochanin-A (BCA), an *O*-methylated isoflavonoid found in a variety of plants such as soybean, peanut, chickpea, alfalfa sprouts, *etc.*, has been associated with a number of biological and pharmacological effects[15,16]. Moreover, our previous work has shown that BCA can ameliorate the abnormalities in glucose metabolism of various vital organs (muscle, liver, and kidney) induced by HFD- and streptozotocin (STZ)-induced hyperglycemia[4]. However, there was no solid scientific evidence that BCA can protect against the pathological changes in lipid metabolism and antioxidant status induced by HFD-STZ. Therefore, in this work, we examined the associated biomarkers in rats to evaluate the therapeutic benefits of BCA against HFD-STZ-induced oxidative stress and hyperlipidemia.

2. Materials and methods

2.1. Chemicals

All of the reagents were of analytical quality, and BCA was purchased from Sigma Chemical Company (Lot # S39234V).

Table 1. Composition of a diet supplemented to the animals (each ingredient in grams per kg diet).

Ingredients	Normal diet	High-fat diet
Corn starch	615	150
Sugar (Sucrose)	0	150
Cellulose	50	50
Corn oil	80	0
Lard oil	0	400
Casein	200	195
Vitamin-mineral premix	50	50
DL-methionine	3	3
Choline bitartrate	2	2
Total energy (kcal/100 g)	365.6	487

2.2. Animals

Twenty-four male Sprague-Dawley rats (body weight: 180-200 g) were obtained from Arulmigu Kalasalingam College of Pharmacy, Virudhunagar, Tamil Nadu, India, and maintained under typical laboratory conditions [temperature: (22±2)℃; humidity: 40%-60%] and a 12-hour light/dark cycle and were fed a diet and water *ad libitum*.

2.3. HFD composition

The process for making an HFD adhered to a previous study[17] basically; the ingredients of HFD are presented in Table 1[18]. The HFD was prepared in powder form and later mixed with water to form lumps. These lumps were supplemented to the rats in the space provided for the food in the cages.

2.4. Development of rat models with HFD–STZ–induced T2DM

To generate the T2DM rat model, the rats were treated with an HFD for two weeks before receiving STZ (35 mg/kg; *i.p.*) and vehicle [citrate buffer (pH 4.5); 1 mL/kg], respectively. The rats in the normal group were fed a standard diet, while the remaining rats were given an HFD for two weeks and then STZ to become diabetic. The diabetic rats were then randomly divided into three groups of six rats each and given the appropriate drugs for 30 d. Three days after receiving an injection of STZ, the rats involved in the experiment had their blood glucose levels checked. The rats were chosen for further testing because they were believed to develop diabetes if their blood glucose level was over 200 mg/dL. As soon as diabetes was identified, BCA treatment was initiated.

2.5. Experimental design

The rats were divided into the following four groups: Group 1: Normal control rats; Group 2: Diabetic control rats; Group 3: Diabetic + BCA (10 mg/kg body weight) administered in a vehicle solution (normal saline) orally for 30 d using an intragastric tube[10,15]; Group 4: Diabetic + gliclazide (5 mg/kg body weight)

administered in a vehicle solution (normal saline) orally for 30 d using an intragastric tube.

At the end of the treatment period, after rats were anaesthetized with pentobarbital sodium (40 mg/kg/body weight; *i.p.*), blood was collected using the retroorbital sinus puncture technique. The rats were then sacrificed by cervical decapitation, and the liver and kidney were immediately removed and stored at −80 ℃ for later use.

2.6. Determination of tissue lipid profile

After liver and kidney tissues were removed from the sacrificed animals, they were washed with ice-cold physiological saline, dried, and then homogenized in frozen chloroform-methanol (2∶1, *v/v*). This procedure was performed four times. The aqueous layer remained after washing the combined filtrate with 0.7% KCl. For tissue lipid analysis, the organic layer was prepared to a certain volume with chloroform. Biochemical markers, including triglycerides (TG), total cholesterol (TC), free fatty acids (FFA), phospholipids (PL), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C), were measured with kits according to the instructions for enzymatic colorimetric procedures (Nicholas Piramal India Ltd., Mumbai, India).

2.7. Measurement of lipogenesis marker enzymes in the liver

Carnitine palmitoyl transferase (CPT), total acetyl-CoA carboxylase (ACC), total fatty acid synthase (FAS), and HMG CoA reductase (HMGR) were measured using commercially available ELISA kits (R&D Systems, Inc., USA).

2.8. Determination of oxidative stress markers in the liver and kidney

The concentrations of thiobarbituric acid-reactive substances (TBARS), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were determined in liver and kidney tissues using commercially available kits from Cayman Chemical Company, USA, according to the manufacturer's instructions.

2.9. Antioxidants and lipid metabolic markers expressions by RT–PCR

Total RNA was isolated from the liver tissue of control and experimental rats using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and a DNA synthesis kit (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific™, India). Semiquantitative PCR was performed with 20 ng cDNA using specific primers (Table 2), including *CPT*, *ACC*, *FAS*, *HMGR*, *SOD*, *CAT*, and *GPx*. The corresponding primers were used in 38 cycles of PCR amplification at the following cycle temperatures: 30 s denaturation at 94 ℃, 30 s annealing at 59 ℃, and 1 min extension at 72 ℃. The housekeeping gene *β–actin* was used for standardization.

Table 2. Primer sequences for RT-PCR analysis.

Gene	Sequence	Product size (bp)
HMGR	F 5′-GGGACCAACCTTCTACCTCAG-3′ R 5′-GACAACTCACCAGCCATCAC-3′	411
FAS	F 5′-GAGGACTTGGGTGCCGATTAC-3′ R 5′-GCTGTGGATGATGTTGATGATAGAC-3′	111
ACC	F 5'TCTATTCGGGGTGACTTTC3' R 5'CTATCAGTCTGTCCAGCCC3'	109
CPT	F 5'AACCTTGGCTGCGGTAAGACTA3' R 5'AGTGGGACATTCCTCTCTCAGG3'	184
SOD	F 5′-CATTCCATCATTGGCCGTACT-3′ R 5′-CCACCTTTGCCCAAGTCATC-3′	62
CAT	F 5′-GTACAGGCCGGCTCTCACA-3′ R 5′-ACCCGTGCTTTACAGGTTAGCT-3′	57
GPx	F 5′-GCGCTGGTCTCGTCCATT-3′ R 5′-TGGTGAAACCGCCTTTCTTT-3′	56
β–actin	F 5′-GGCACCACACTTTCTACAAT-3′ R 5′-AGGTCTCAAACATGATCTGG-3′	259

F: forward; R: reverse.

2.10. Statistical analysis

SPSS software (version 20.0) was used for statistical analysis. Data are expressed as mean±SD. One-way analysis of variance (ANOVA) and least significant difference (LSD) test were applied to determine the significance of differences between groups. The *P* value less than 0.05 was considered statistically significant.

2.11. Ethical statement

All studies were conducted in accordance with the guidelines of the Institutional Animal Ethical Committee of Arulmigu Kalasalingam College of Pharmacy (approval number: AKCP/IAEC/83/20-21).

3. Results

3.1. BCA attenuates hyperlipidemia in the liver and kidney

The lipid profiles of the liver and kidney of control and diabetic rats are shown in Figure 1. HFD-STZ developed significant hyperlipidemia, as evidenced by the increased levels of TC (Figure 1A), TG (Figure 1B), FFA (Figure 1C), LDL-C (Figure 1D), and PL (Figure 1E) and the concomitant decrease in HDL-C (Figure 1F) in renal and hepatic tissues. By returning the lipid profiles to the normal range similar to the normal control group, diabetic rats treated with BCA markedly ($P<0.05$) reduced the hyperlipidemia in renal and hepatic tissues.

3.2. BCA improves HFD– and STZ–induced abnormal lipid metabolism of the liver

Figure 2 shows the activity of lipogenesis marker enzymes in the liver of normal and diabetic rats. After HFD and STZ treatment, the HMGR (Figure 2A), FAS (Figure 2B), and ACC (Figure 2C) activities of rats were significantly increased, whereas their CPT

activity (Figure 2D) in the liver was simultaneously decreased. Interestingly, a significant (P<0.05) improvement in the altered activity of these enzymes was observed in diabetic animals treated with BCA for 30 d compared with untreated diabetic rats.

3.3. Effect of BCA on oxidative stress status in the liver and kidney

The oxidative stress status in normal and diabetic rats is shown in Figure 3. According to the results, diabetic rats had significantly (P<0.05) increased TBARS levels (Figure 3A) and concomitantly decreased GSH levels (Figure 3B), and CAT (Figure 3C), SOD (Figure 3D), and GPx activities (Figure 3E). Animals treated with BCA successfully reduced oxidative stress by increasing GSH levels and the activity of SOD, CAT, and GPx in diabetic rats.

3.4. Effect of BCA on mRNA expressions of markers of oxidative stress and lipid metabolism

Figure 4 shows the mRNA expressions of biomarkers of lipid metabolism in the livers of diabetic and control rats. The HFD- and STZ-induced diabetic rats showed a remarkable upregulation of *HMGR* (Figure 4A), *FAS* (Figure 4B), and *ACC* (Figure 4C) expression and a downregulation of CPT (Figure 4D). In contrast, considerable (P<0.05) recovery of these abnormal mRNA expressions in the liver was shown after BCA administration.

The effects of BCA on the mRNA expressions of the innate enzymatic antioxidants *SOD*, *CAT*, and *GPx* in control and diabetic rats are shown in Figure 5. The expressions of *SOD* (Figure 5A), *CAT* (Figure 5B), and *GPx* (Figure 5C) in the liver of diabetic control rats were significantly downregulated after the administration of

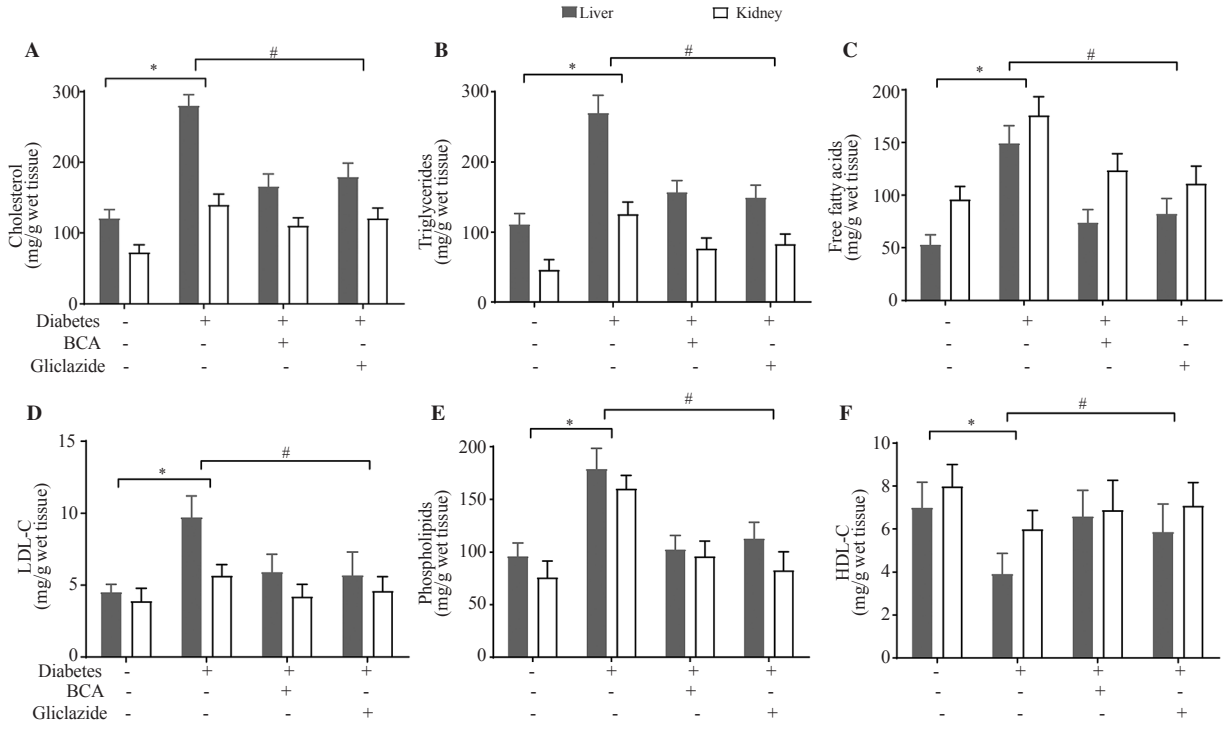

Figure 1. Effect of biochanin-A (BCA) on (A) total cholesterol, (B) triglycerides, (C) free fatty acids, (D) LDL-C, (E) phospholipids, and (F) HDL-C in hepatic and renal tissues of diabetic rats. The values are expressed as mean±SD, n=6, $^{*}P$<0.05 *vs.* the normal control, $^{\#}P$<0.05 *vs.* the diabetic control. LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol.

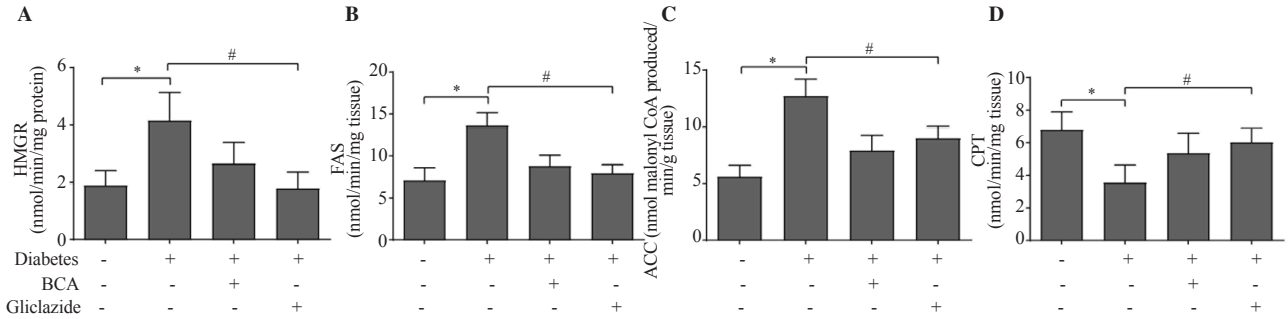

Figure 2. BCA treatment improves the activities of lipid metabolic enzymes (A) HMGR, (B) FAS, (C) ACC and (D) CPT in high-fat diet (HFD) and streptozotocin (STZ)-induced diabetic rats. The values are expressed as mean±SD, n=6, $^{*}P$<0.05 *vs.* the normal control, $^{\#}P$<0.05 *vs.* the diabetic control. HMGR: HMG CoA reductase; FAS: fatty acid synthase; ACC: acetyl-CoA carboxylase; CPT: carnitine palmitoyl transferase.

HFD and STZ, but BCA significantly ameliorated these changes (P<0.05), which was supported by an increase in the mRNA levels of these enzymes in the liver.

4. Discussion

The current study compared the effects of BCA with those of the second-generation sulfonylurea gliclazide, which is safe and

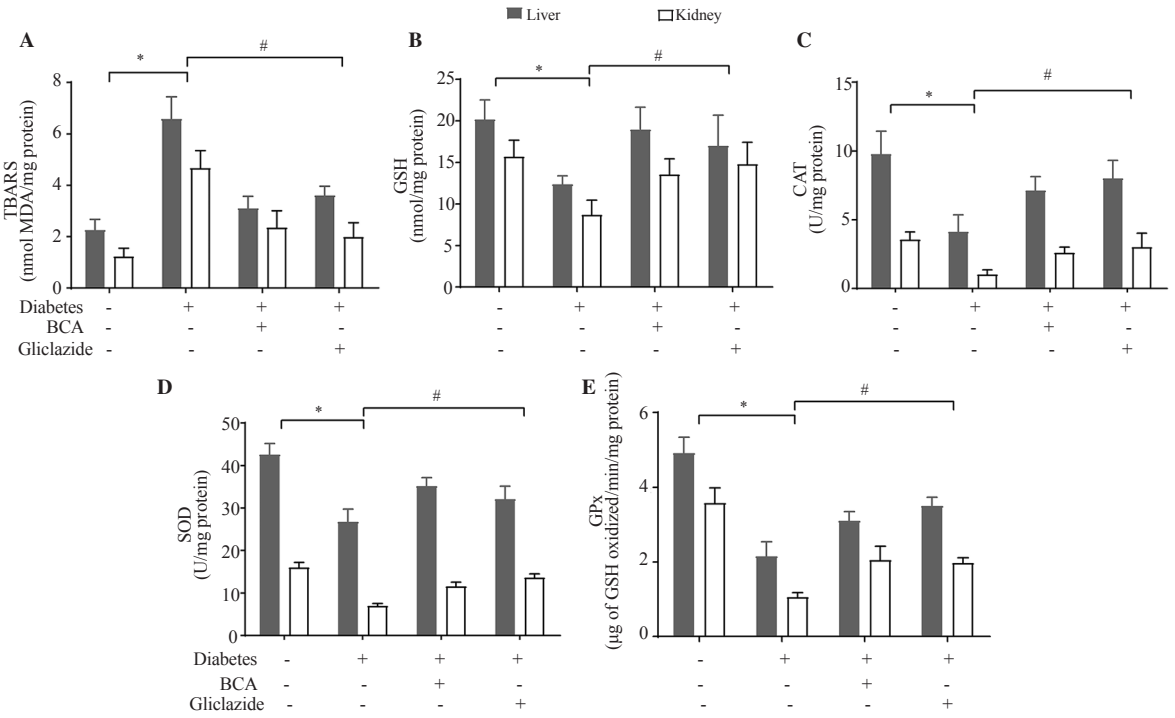

Figure 3. BCA supplementation reverses the altered oxidative stress indicators including (A) TBARS, (B) GSH, (C) CAT, (D) SOD, and (E) GPx in the liver and kidney of HFD-STZ-induced diabetic rats. The values are expressed as mean±SD, n=6, $^{*}P$<0.05 *vs.* the normal control, $^{\#}P$<0.05 *vs.* the diabetic control. TBARS: thiobarbituric acid reactive substances; GSH: glutathione; CAT: catalase; SOD: superoxide dismutase; GPx: glutathione peroxidase.

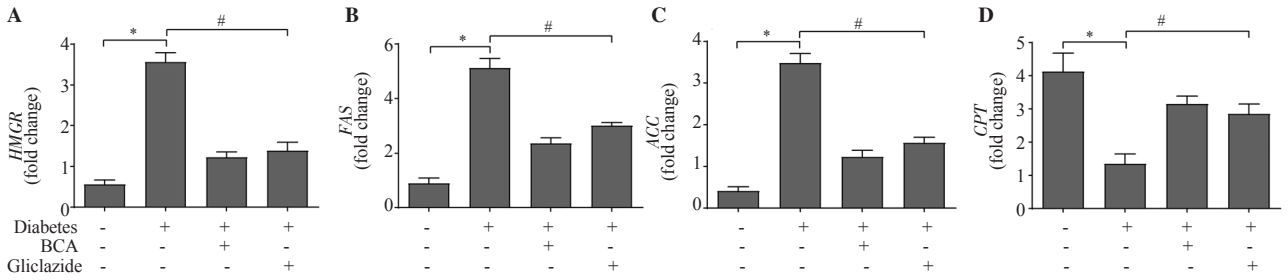

Figure 4. BCA treatment downregulates (A) *HMGR*, (B) *FAS*, and (C) *ACC*, and upregulates (D) *CPT* mRNA expressions in HFD-STZ-induced diabetic rats. The values are expressed as mean±SD, n=6, $^{*}P$<0.05 *vs.* the normal control, $^{\#}P$<0.05 *vs.* the diabetic control.

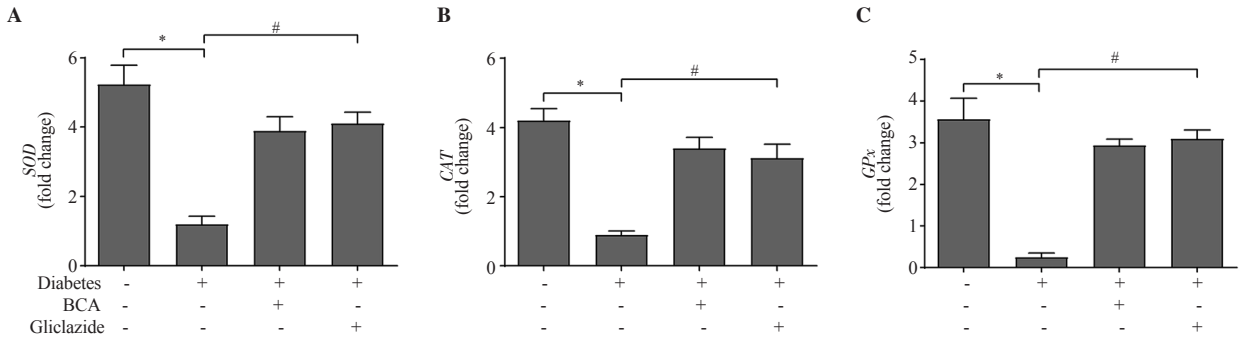

Figure 5. BCA supplementation enhances the antioxidant status by upregulating the mRNA expressions of (A) *SOD*, (B) *CAT*, and (C) *GPx* in HFD-STZ-induced diabetic rats. The values are expressed as mean±SD, n=6, $^{*}P$<0.05 *vs.* the normal control, $^{\#}P$<0.05 *vs.* the diabetic control.

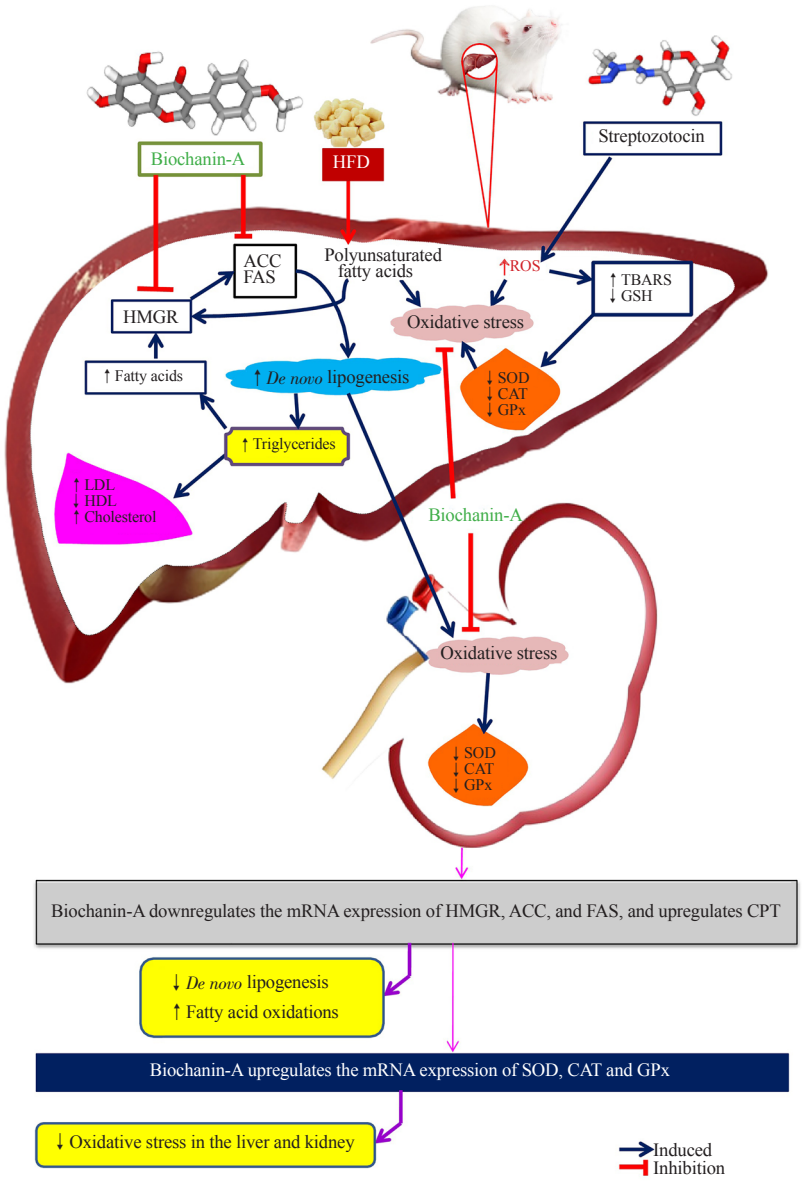

Figure 6. The mechanisms of action of BCA against HFD and STZ-induced diabetes.

effective in clinical trials, on hyperlipidemia and oxidative stress in HFD-STD-induced diabetic rat models[19]. The results showed that BCA improved the antioxidant status and lipid profile in diabetic rats that was equivalent to that of gliclazide. In a previous study, we discovered that BCA may be able to treat abnormalities in carbohydrate metabolism by reversing hyperglycemia and IR[4]. Moreover, this study demonstrates the therapeutic efficacy of BCA against pathological changes associated with diabetes, such as hyperlipidemia and oxidative stress in the liver and kidney.

The association between hyperlipidemia and DM and its numerous consequences has been confirmed according to recent studies[20–22], in addition to the well-known association with cardiovascular disease. In particular, hyperlipidemia is considered one of the major risk factors for the development of diabetes complications[22,17]. The main factor causing abnormal lipid levels in diabetic patients is IR[23]. Peripheral IR promotes the release of FFA from adipose tissue, which is then absorbed by the liver, increasing the production of TG. The formation of TG-rich VLDL-C is then stimulated by TG synthesis in the liver[24]. Through the action of cholesterol ester transfer protein, TG-rich VLDL enriches LDL and HDL, making them richer in cholesterol. After digestion by hepatic or lipoprotein lipase, these TG-rich LDL molecules are converted into small dense LDL. Therefore, it is difficult to rely on conventional methods to reduce pathologic risk because the lipid abnormalities associated with diabetes are broad and go beyond a mere increase in LDL[25].

The results of the current study support the hypothesis that dyslipidemia can be successfully developed by HFD and STZ in rats, as evidenced by the dramatic change in biochemical indices of tissues (liver and kidney). In particular, rats in the diabetic control group showed increased lipid levels in both organs, including TG, PL, FFA, and LDL, and a parallel decrease in HDL levels. Inconsistencies in lipid metabolism were found to be a

pathogenesis-inducing factor for IR in this study, confirming the findings of our previous studies that IR was present in HFD-STZ-induced diabetic rats. Consequently, our results are consistent with these findings[23,26]. In contrast to untreated diabetic rats, our study showed that treatment with BCA in diabetic rats reduced abnormal lipid levels in the liver and kidney to normal levels. This underscores the ability of BCA to combat hyperlipidemia.

In addition, we investigated how BCA affects the enzyme markers of lipid metabolism HMGR, FAS, ACC, and CPT. Adipocytes maintain lipid homeostasis through lipolysis and lipogenesis, and significant lipogenesis is strongly associated with obesity, IR, and T2DM[5,21]. It is generally accepted that the liver plays an important role in increasing cholesterol production in both T2DM and obesity. The rate-limiting step in the *de novo* production of cholesterol is catalyzed by HMGR, a peroxisomal enzyme bound to the endoplasmic reticulum. The amount of cell-associated cholesterol decreases as HMGR activity decreases, activating signaling pathways controlled by sterol regulatory element-binding protein 2[27]. The most important enzyme involved in *de novo* lipogenesis and a physiological stopper of CPT, producing malonyl CoA in the liver, is ACC[28]. Consequently, these enzymes are crucial for hepatic lipid metabolism, and alterations in them could cause dyslipidemia[29]. In the present study, we discovered increased HMGR, FAS, ACC, and concomitantly decreased CPT activity in the liver of obese rats, suggesting increased lipogenesis and it could be the cause of abnormal lipid profiles in both blood and tissues. Interestingly, BCA supplementation restored these abnormal enzyme activities to the normal range in diabetic rats, suggesting that BCA may limit lipogenesis and thus protect the liver. The therapeutic efficacy of BCA against hyperlipidemia was also evidenced by significantly decreased expressions of HMGR, FAS, and ACC, as well as increased CPT in the liver of diabetic rats. These results support the hypothesis that BCA regulates lipid metabolic enzymes at the mRNA level and protects the liver from lipotoxicity.

Moreover, an increase in oxidative stress in the liver and kidney of HFD- and STZ-induced diabetic rats was observed. Many studies showed an increase in oxidative stress biomarkers in patients with diabetes[1,11]. Diabetes is a chronic metabolic disorder in which mitochondria play a key role as the most common source of ROS production. There is an important connection between high blood sugar levels and the development of oxidative stress. Therefore, one strategy for T2DM therapy is to control the production of ROS. TBARS is a well-known biomarker of oxidative stress resulting from the LPO of polyunsaturated fatty acids by ROS[1]. In the current study, HFD supplementation and STZ injection significantly increased TBARS levels, while GSH levels decreased, indicating oxidative stress in diabetic rats. In addition, the diabetic state leads to oxidative stress due to free radical generation through protein glycosylation, glucose autooxidation, and polyol pathways[30]. Persistent cellular damage is caused by a decrease in antioxidant levels and/or an irregular increase in ROS levels. Reportedly, the onset and development of the consequences of diabetes are highly influenced by oxidative stress[11,31,32].

The deleterious consequences of oxidative stress can be reduced by enzymatic antioxidant systems that are known to scavenge ROS. Antioxidant enzymes such as SOD, CAT, and GPx can inhibit ROS-mediated damage. SOD is the key antioxidant enzyme in cells and instigates the neutralization of ROS-induced toxicity and steps up the neutralization and dismutation of the harmful superoxide anion (O_2) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2)[11]. To reduce the toxic effects of H_2O_2 on tissues, CAT can increase the degradation of H_2O_2 into water and molecular oxygen, completing the detoxification process similar to SOD[33]. However, in mammals, CAT is restricted to peroxisomes, so interaction with SOD-produced H_2O_2 is unlikely[34]. CAT could degrade peroxynitrite and oxidize nitric oxide to nitrite to balance the oxidation of NO[35,36]. In addition, GPx is an important intracellular antioxidant enzyme that can also promote the degradation of H_2O_2 to H_2O and lipid peroxides to their corresponding alcohols, mainly in mitochondria[11]. The present experiment showed that the antioxidant defense systems were altered by HFD and STZ. The change in the potential of antioxidant enzymes suggests the role of ROS in the pathogenesis of diabetes-related oxidative stress. Our study showed that administration of BCA to diabetic rats significantly increased the activities of these antioxidant enzymes. On the other hand, we analyzed the mRNA expression of these enzymes in the liver using RT-PCR to confirm these results. This study confirmed that the downregulation of *SOD*, *CAT*, and *GPx* genes in the liver contributes to the development of oxidative stress in the liver. The current study also found that BCA may be able to reduce the effects of oxidative stress caused by diabetes. This was evidenced by the fact that BCA reduced LPO in the liver and kidney, increased GSH levels, and upregulated mRNA expressions of antioxidant enzymes in the liver, thereby maintaining homeostasis and preventing oxidative stress.

Overall, the results of this study suggest that BCA could reduce dyslipidemia by regulating the activity of lipid metabolism enzymes such as HMGR, FAS, and ACC and increasing the activity of CPT, which plays an important role in *de novo* lipogenesis and fatty acid oxidation, respectively. In addition, BCA reduced oxidative stress by increasing the activities of antioxidant enzymes (SOD, CAT, and GPx) *via* upregulation of their corresponding mRNA expressions in both liver and kidney. Our study suggests that BCA may attenuate diabetes-induced hyperlipidemia by reducing *de novo* lipogenesis and promoting fatty acid oxidation, and promote the native antioxidant status by attenuating oxidative stress. The mechanisms of actions of BCA against HFD-STZ-induced dyslipidemia and oxidative stress are shown in Figure 6. However, BCA may not be suitable as an alternative medicine for the treatment of T2DM until further preclinical studies are conducted to investigate a wide range of mechanisms of action. Furthermore, we only examined specific

genes responsible for *de novo* lipogenesis and fatty acid oxidation. In addition, the effect of BCA on the expression of genes responsible for fatty acid catabolism, fatty acid synthesis and absorption needs to be investigated.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

PPS and VVSU wrote the manuscript; PPS, VVSU, PC, SS and PT performed experiments and collected the data; PP, SV, MS and MSB analyzed the data; PPS, VVSU and GS designed the study; GS reviewed the manuscript. All authors read and approved the final manuscript.

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