Research in Pharmaceutical Sciences, April 2018; 13(2): 142-148
Received: March 2017
Accepted: December 2017

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

*Original Article*

# The effect of betulinic acid on leptin, adiponectin, hepatic enzyme levels and lipid profiles in streptozotocin–nicotinamide-induced diabetic mice

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## *Abstract*

Diabetes mellitus is developed by lack of insulin secretion or reduction of tissues sensitivity to insulin, which lead to serious complications. The aim of this study is to evaluate antihyperlipidemic effect of betulinic acid (BA) on streptozotocin-nicotinamide (STZ-NA) induced diabetic mice. In this experimental study, seventy adult male NMRI mice (20-25 g) were divided randomly into seven groups (n = 10) of control, sham, diabetes, diabetes + BA (10, 20 and 40 mg/kg), and diabetes + metformin (200 mg/kg). Diabetes was induced by intraperitoneal (i.p.) injection of a single dose of STZ (50 mg/kg) 15 min after an i.p. administration of nicotinamide (NA) (120 mg/kg). BA and metformin were orally administered and after two weeks blood samples were taken. Blood levels of leptin, adiponectin, lipid profile and liver enzyme were then measured. One day after the last drug administration, liver was removed to evaluate the histological changes. A significant increase ($P < 0.05$) in the plasma levels of leptin, alanine-aminotransferase (ALT), aspartate-aminotransferase (AST), alkaline phosphatase (ALP), low density lipoprotein cholesterol (LDL-C), cholesterol, and a significant decrease in adiponectin and high density lipoprotein cholesterol (HDL-C) were observed in diabetic mice. The groups treated with BA indicated a significant decrease in leptin, AST, ALT, ALP, TG, cholesterol, LDL-C and an increases in adiponectin and HDL levels, while VLDL did not show significant changes. BA was found to have positive effects on liver injury. BA has an effective role on liver damage induced by diabetes through amelioration of leptin, adiponectin, liver enzyme levels and lipid profile. Since BA has a positive effect on lipid profile, adiponectin and leptin, it may improve metabolic syndrome.

***Keywords:*** Diabetes; Betulinic Acid; Streptozotocin; Nicotinamide; Liver

## INTRODUCTION

Diabetes is a common disease in the whole world, which has an increasing prevalence (1). Type 2 diabetes mellitus is a metabolic disorder that reduces the body's ability to glucose uptake. Factors causing diabetes are still unknown, although genetic factors, obesity and the absence of physical activity have an important role in diabetes (2). Several factors such as low density lipoprotein cholesterol (LDL-C) are involved in diabetes (3). One of the major risks of developing diabetes is obesity and excess adiposity (4). Some factors such as insulin resistance and possibly some adipokines (e.g. adiponectin) and hyperglycemia are involved in the pathophysiology of diabetic dyslipidemia. Adiponectin levels are decreased in type 2 diabetic patients. Low adiponectin plasma levels are correlated negatively with plasma triacylglycerols and positively with plasma high density lipoprotein cholesterol (HDL-C) and these associations are independent of insulin resistance (5,6).

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**Access this article online**

**Website:** http://rps.mui.ac.ir

**DOI:** 10.4103/1735-5362. 223796

Betulinic acid (BA) is a plant-derived Penta cyclic triterpenoids (7). BA has been extracted from various plants including *Morus Alba* root bark (8), *Bersama engleriana* (9), *Quisqualis fructus*, *Coussarea paniculata*, *Caesalpinia paraguariensis*, *Vitex negundo*, *Berlinia grandiflora*, *Ziziphus joazeiro*, *Uapaca nitida*, *Ipomea pes-caprae*, *Ancistrocladus heyneanus*, *Diospyros leucomelas*, and *Syzygium claviforum* (10). BA protects liver through antioxidant and anti-inflammatory activities (11). Interestingly BA and some of the aforementioned plants are effective in the treatment of type 2 diabetes (12,13)

There are various techniques for induction of diabetes in an animal model of type 2 diabetes. It is proven that streptozotocin-nicotinamide (STZ-NA) leads to a mild to moderate elevations in blood glucose and a reduction in insulin secretion. STZ-NA induced diabetes in mice exhibits many pathological characteristics similar to type 2 diabetes mellitus (14). In a previous study, hyperlipidemia and liver toxicity were observed in STZ-NA diabetic animals (15).

In diabetic patients, lipid profiles, leptin and adiponectin levels are altered (16) and studies on the effects of BA on these parameters are scarce. Therefore, we aimed to evaluate the effect of BA on the level of leptin, adiponectin, liver enzymes and lipid profiles in STZ-NA-induced diabetic mice.

## MATERIAL AND METHODS

### *Experimental design*

In the experimental study 70 male NMRI mice (5 weeks old, 25-35 g) were purchased from animal center of Ahvaz Jundishapur University of Medical Sciences (AJUMS). The Ethic Committee for Animal Experiments approved the study (IR.AJUMS.REC.1394. 145-6794) and all experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. Mice were housed in cages under controlled conditions at 25 ºC and a 12 h light–dark cycle and 10% humidity. Mice were receiving food pellets and water *ad libitum*. Betulinic acid (Sigma-Aldrich, USA) and metformin (Sigma-Aldrich, USA) were orally administered 2 weeks after confirmed diabetes induction. The mice were randomly divided into seven groups of 10 animals each as follows: group 1, normal control; group 2, sham (0.1 mg/kg saline intraperitoneal, i.p.); group 3, untreated type 2 diabetic (STZ 50 mg/kg i.p., 15 min later a single dose of NA 120 mg/kg i.p.); group 4 to 6, type 2 diabetic animals received BA at 10, 20 and 40 mg/kg by gavage) (16); group 7, type 2 diabetic animals received metformin (200 mg/kg, by gavage) (13).

### *Induction of type 2 diabetes mellitus*

Streptozotocin was dissolved in citrate buffer with pH 4.5 and nicotinamide in normal saline (17). Type 2 diabetes was induced in mice through a single administration of 50 mg/kg streptozotocin followed by 120 mg/ kg nicotinamide 15 min later. Seven days after the injections, blood glucose levels were evaluated. Mice with blood glucose more than 200 mg/dL were used in the following experiments (11).

### *Biochemical assessment*

Plasma glucose levels were determined by glucometer (Elegance CT-X10, convergent technologies, Germany). Plasma leptin was determined by ELISA kit (Labor Diagnostika Nord GmbH, Germany) and adiponectin was measured by ELISA kit (Mediagnost, GmbH, Germany). Lipid profile and plasma levels of liver enzymes were measured using an Autoanalyzer device (BT3000, Italy) and biochemical assay kits (Pars Azmoon, Iran). Lipid profile values were expressed in mg/dL (8). Very low density lipoprotein cholesterol (VLDL) concentration was calculated using VLDL = TG/5 (18).

Ahmadi, *et al*. showed that when triglyceride is <100 mg/dL, Friedewald equation may overestimate LDL-C concentration and it should be calculated by a modified Friedewald equation. Therefore, LDL-C concentration was calculated using formula LDL (mg/dL) = TC/1.19 + TG/1.9 – HDL/1.1 – 38 (19). The atherogenic index (AI = Log (TG/HDL-C)) was defined as the zone of atherogenic risk (20).

### *Histology analysis*

After blood collection, the mouse liver was removed immediately and fixed in 10% formalin solution. Then excised liver was dehydrated in graded alcohol concentrations and embedded in paraffin. Sections of 4-6 μm were prepared and stained with hematoxylin and eosin (H&E). Six microscopic slides per animal were examined for assessment of histological changes such as congestion of erythrocytes and infiltration of inflammatory cells. Mean of 10 fields was considered for each slide and were read in a "blind" fashion (21).

### *Data analysis*

Data are expressed as mean ± SEM. Statistical comparison between different groups were done using one-way analysis of variance (ANOVA) followed by post-hoc LSD test; ($P < 0.05$) was regarded as significant.

## RESULTS

### *Effect of betulinic acid on plasma leptin levels*

The leptin levels in all groups of animals are shown in Table 1. Leptin was increased significantly in diabetic group ($P < 0.01$) and the mean level of plasma leptin significantly decreased in mice treated with 20 and 40 mg/kg BA ($P < 0.01$). Metformin-treated group compared to the diabetic control indicated significant decrease in plasma leptin levels ($P < 0.01$).

### *Effect of betulinic acid on plasma adiponectin levels*

The results showed that plasma adiponectin levels decreased in diabetic mice when compared to the control and sham groups ($P < 0.01$) and increased in diabetic mice treated with BA 40 mg/kg ($P < 0.01$) unlike diabetes group (Table 1).

### *The effect of betulinic acid on lipid profile and atherogenic index*

Plasma levels of cholesterol increased in the diabetic group compared to the control and sham groups. Further administration of BA at 20 and 40 mg/kg ($P < 0.01$) indicated decreased cholesterol in comparison to diabetic group (Table 1).

The concentration of LDL increased in the diabetic group compared to the control and sham groups ($P < 0.05$), but in the experimental groups receiving 20 and 40 mg/kg of BA ($P < 0.05$) and metformin ($P < 0.01$), a significant reduction was observed comparing to the diabetic group (Table 1).

As it can be observed in Table 1, in diabetic group the plasma HDL concentration was decreased, but in the experimental group receiving 10, 20 and 40 mg/kg of BA, HDL concentrations showed a significant increase ($P < 0.01$). Also, metformin-treated group showed a significant increase compared to the diabetic group ($P < 0.001$).

**Table 1.** The effect of different doses of betulinic acid on leptin, adiponectin levels, and lipid profiles in mice with diabetes induced with streptozotocin-nicotinamide.

| Groups | Leptin (ng/mL) | Adiponectin (ng/mL) | Triglyceride (mg/dL) | LDL (mg/dL) | HDL (mg/dL) | Cholesterol (mg/dL) | AI |
|---|---|---|---|---|---|---|---|
| Control | 0.134 ± 0.01 | 221.29 ± 12.88 | 102.40 ± 5.60 | 39.07 ± 3.27 | 68.20 ± 1.28 | 96.60 ± 2.76 | 0.174 ± 0.02 |
| Sham | 0.166 ± 0.02 | 200.68 ± 17.05 | 103.10 ± 4.40 | 38.77 ± 5.77 | 66.28 ± 2.50 | 100.4 ± 5.31 | 0.191 ± 0.01 |
| Diabetes | 0.238 ± 0.01$^{\#\#}$ | 151.32 ± 11.27$^{\#\#}$ | 115.66 ± 3.36 | 60.55 ± 4.76$^{\#}$ | 60.00 ± 1.04 | 113.0 ± 4.89 | 0.284 ± 0.01$^{\#}$ |
| Diabetes + 10 mg BA | 0.190 ± 0.02 | 161.23 ± 12.16 | 113.62 ± 3.62 | 47.97 ± 5.67 | 75.00 ± 1.15$^{**}$ | 108.0 ± 2.51 | 0.179 ± 0.01$^{**}$ |
| Diabetes + 20 mg BA | 0.187 ± 0.01$^{*}$ | 162.13 ± 4.830 | 101.50 ± 3.24$^{*}$ | 38.94 ± 2.36$^{*}$ | 72.00 ± 5.04$^{**}$ | 95.28 ± 3.16$^{**}$ | 0.151 ± 0.03$^{**}$ |
| Diabetes + 40 mg BA | 0.171 ± 0.01$^{**}$ | 191.01 ± 2.730$^{**}$ | 105.60 ± 3.76 | 38.52 ± 9.20$^{*}$ | 72.00 ± 3.64$^{**}$ | 90.28 ± 6.31$^{**}$ | 0.164 ± 0.02$^{**}$ |
| Metformin | 0.151 ± 0.02$^{**}$ | 161.32 ± 6.220 | 112.57 ± 7.42 | 37.01 ± 8.58$^{**}$ | 77.00 ± 4.90$^{***}$ | 96.14 ± 10.71 | 0.161 ± 0.04$^{**}$ |

LDL, low-density lipoprotein; HDL, high-density lipoprotein; AI, atherogenic index; BA, betulinic acid. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs* diabetic group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs* control. n = 10, mean ± SD, one-way ANOVA and Post Hoc LSD tests.

As seen in Table 1, in diabetic group the atherogenic index increased compared to the control and sham groups ($P < 0.05$), but in the groups receiving 10, 20 and 40 mg/kg of the BA, atherogenic index exhibited a significant decrease ($P < 0.01$). Also, metformin-treated group showed a significant decrease in atherogenic index compared to the diabetic group ($P < 0.01$).

The concentration of triglyceride was increased ($P < 0.05$) in the diabetic group compared with the experimental group receiving 20 mg/kg of BA (Table 1).

### *Effect of betulinic acid on the concentration of AST, ALT and ALP*

The results indicated that injection of STZ-NA exerted a significant increase in plasma levels of aspartate-aminotransferase (AST) ($P < 0.05$) in diabetic mice; administration of 20 ($P < 0.05$), 40 mg/kg ($P < 0.01$) BA and metformin decreased significantly the AST plasma levels compared with diabetic group (Table 2).

In the diabetic group the plasma alanine-aminotransferase (ALT) concentrations significantly increased ($P < 0.05$), but in the experimental groups, ALT concentrations decreased after receiving the 10, 20 and 40 mg/kg BA ($P < 0.01$). Metformin-treated group also indicated significant ($P < 0.05$) decrease in ALT compared to the diabetic group (Table 2). Injection of STZ-NA showed significantly increased plasma levels of alkaline phosphatase (ALP) ($P < 0.01$) in diabetic mice and administration of 20 ($P < 0.05$) and 40 mg/kg ($P < 0.01$) BA decreased the ALP plasma levels compared to the diabetic group. Metformin-treated group indicated significant ($P < 0.01$) decrease in ALP compared to the diabetic group (Table 2).

### *Histological analysis*

Normal liver tissue was observed in the control and sham groups. Lobular structures of the liver were damaged in diabetic mice. Accumulation of erythrocytes in central vein and sinusoids and infiltration of inflammatory cells were observed in diabetic group. Congestion of erythrocytes and infiltration of inflammatory cells were observed in 10 and 20 mg/kg BA-treated mice while these histological changes were effectively reversed by the dose of 40 mg/kg of BA. Metformin-treated mice showed normal histology similar to that of the control animals (Fig. 1).

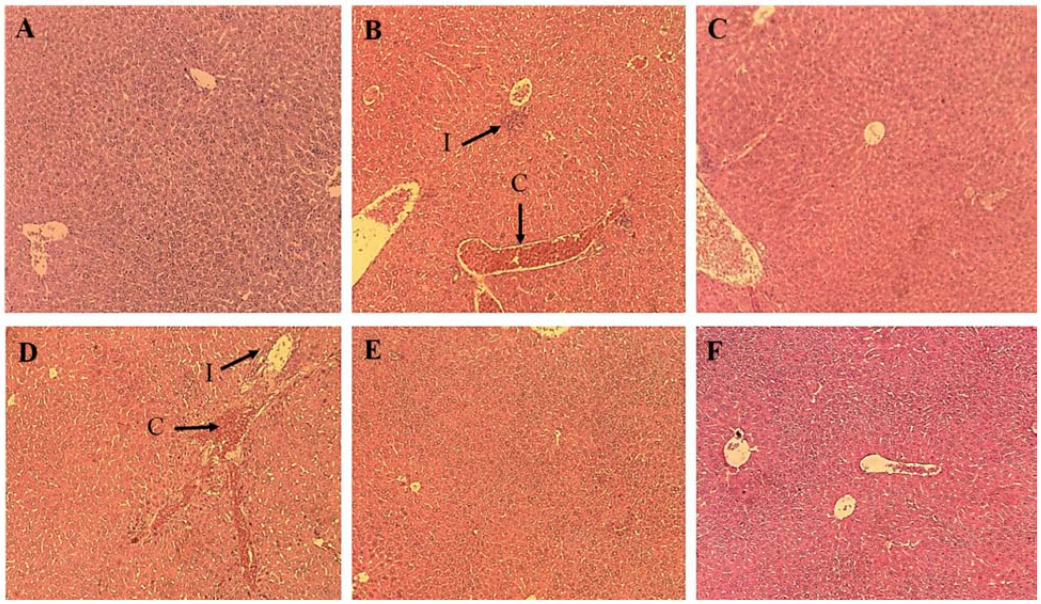


**Fig. 1.** Effect of the different doses of betulinic acid on liver histological analysis in diabetic mice induced by streptozotocin-nicotinamide. A, control; B, diabetes; C, D, and E, diabetes + betulinic acid (10, 20 and 40 mg/kg); F, diabetes + metformin, (hematoxilyn and eosine stain, × 400). Arrows indicate, I: infiltration of inflammatory cells; C: congestion of erythrocytes.

**Table 2.** The effect of the different doses of betulinic acid on liver enzymes in diabetic mice induced by streptozotocin-nicotinamide.

| Groups | AST | ALT | ALP |
|---|---|---|---|
| Control | 359.6 ± 12.96 | 78.25 ± 8.69 | 115.14 ± 6.50 |
| Sham | 382.16 ± 14.98 | 78.40 ± 7.03 | 119.23 ± 6.41 |
| Diabetes | 444.28 ± 18.58$^{\#}$ | 95.28 ± 4.24$^{\#}$ | 145.67 ± 6.84$^{\#\#}$ |
| Diabetes+10 mg BA | 409.00 ± 23.43 | 74.00 ± 3.22$^{**}$ | 135.34 ± 5.48 |
| Diabetes+20 mg BA | 387.00 ± 26.32$^{*}$ | 72.50 ± 4.05$^{**}$ | 128.11 ± 4.98$^{*}$ |
| Diabetes+40 mg BA | 335.71 ± 28.84$^{**}$ | 76.83 ± 3.68$^{**}$ | 115.42 ± 4.57$^{**}$ |
| Metformin | 300.16 ± 16.47$^{***}$ | 81.5 0± 4.48$^{*}$ | 113.76 ± 3.87$^{**}$ |

AST, aspartate-aminotransferase; ALT, alanine-aminotransferase; ALP, alkaline phosphatase; BA. betulinic acid. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, *vs* diabetic. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs* control. n = 10, mean ± SD, one-way ANOVA and Post Hoc LSD tests.

## DISCUSSION

In the present study, type 2 diabetes was established by injecting STZ-NA in mice. Various animal models of type 2 diabetes have so far been reported of which STZ-NA induced diabetes are more common. In this model of diabetes islet β-cells are partially destroyed. This is due to the protective effect of nicotinamide on islet β-cells (22). STZ increases blood glucose through the pancreatic β-cells oxidative stress (23). STZ destructs the β-cells rapidly and subsequently reduces insulin secretion. NA acts as an antioxidant and inhibits the nitric oxide secretion, thus protects β-cells and prevents the destructive effects of STZ (7). Protective effects of pretreated nicotinamide on islets have already been reported (24). This combination by reducing caspase-3 delays the destruction of the islets. The present study showed that induction of diabetes led to an increase in fasting blood glucose levels in diabetic mice compared to the control group. The effects of nicotinamide in diabetic rats showed that treatment with nicotinamide through inhibition of apoptosis reduces and prevents the destructive effects of streptozotocin (25). BA treatment showed improved levels of leptin, adiponectin, lipid profile and liver enzymes. In agreement with our results, *Morus alba* root bark extract, which is rich in BA, was effective in reducing blood glucose and lipid peroxidation (8). In another study, BA increased insulin secretion and muscle glycogen (26). In one study, BA caused antidiabetic activity by reducing insulin resistance and potentiating β-cell mass and function. Previous studies indicated that BA improves glucose metabolism and enhances insulin-stimulated glucose uptake in the adipose tissues and liver of diabetic rats (27). In the present study, we observed an increase in the liver enzyme levels in untreated diabetic mice, which were decreased in mice treated with BA indicating the hepato-protective effect of BA by reducing elevated serum levels of ALT, AST and cholesterol. Yi, *et al*. showed that treatment with BA reduces the levels of ALT and AST in a dose-dependent manner in mice that were used to alcohol. They determined that BA pretreatment conferred protection against alcohol-induced fatty liver (28).

In the present study, the diabetic mice treated with BA, showed a reduction in plasma cholesterol, TG, LDL, VLDL and atherogenic index. Also during 2 weeks of treatment at 10, 20 and 40 mg/kg BA, an increase in adiponectin and HDL were found but leptin levels and atherogenic index were decreased. Therefore, there is a negative relationship between adiponectin and lipid profile, atherogenic index and leptin. Consistent with current study, de Mello, *et al*. examined high fat diet (HFD) adult mice during the 15 weeks. They indicated that the mice treated with BA reduced body weight, abdominal fat accumulation, plasma glucose, TG and total cholesterol. In addition, treatment with BA increased leptin and insulin plasma levels (29). It seems that BA, through a decrease in plasma leptin of NA-STZ model, prevents insulin resistance, whereas De Mello, *et al*. showed that BA increases the leptin levels in diabetic HFD mice. Enhances in leptin levels in HFD mice probably can prevent increasing adipose tissue and obesity. As a result, BA may

improve leptin levels. Metformin is one of the appropriate drugs for diabetes that lowered blood glucose through inhibiting of gluconeogenesis and increased utilization of glucose in the liver (30). In the present study, metformin treatment reduced lipid profile, atherogenic index, leptin and liver enzymes, also showed an increase in the adiponectin levels. ALP and ALT exist in large quantities in the hepatocyte. Therefore, metformin is suitable for the treatment of type 2 diabetes (30). Liver parenchymal damage increased the levels of ALP and ALT enzymes in the blood stream. Excessive weight loss is associated with decreased plasma adiponectin (31). In histological assessments we found that diabetes led to inflammation in the liver. BA at the dose of 40 mg/kg had positive effects on liver histology. Probably BA administration led to appropriate changes in the plasma lipid profiles due to BA antioxidant properties. Therefore, it could be useful in the hyperlipidemia treatment.

## CONCLUSION

Present data indicated that BA improves lipid profiles and also hepatic enzymes activity of diabetic mice. It could be useful in control of type 2 diabetes in traditional medicine, even though, further studies are required to fully understand the exact mechanism of BA on diabetic mice.

## ACKNOWLEDGMENTS

The content of this paper is extracted from the M.Sc. thesis (No. D-94-11) submitted by Razieh Shabani which was financially supported by Health Research Institute, Diabetes Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, I.R. Iran.

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