Original Article

Long-term, high-dose aspirin therapy increases the specific activity of complex III of mitochondrial respiratory chain in the kidney of diabetic rats

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Abstract

Introduction: One of the main mechanisms by which diabetic complications occur is an alteration of the structure and function of proteins due to hyperglycemia. Aspirin (ASA) affects cellular pathways through different mechanisms, including glycation inhibition and antioxidant activity. The aim of the present study, as a follow up to our previous one, is to investigate the effect of long-term, high-dose ASA therapy on mitochondrial respiratory chain complexes in the kidneys and brain of streptozotocin-induced diabetic rats. Its effect on liver toxicity of the rats was also investigated.

Materials and Methods: High dose of ASA (100 mg/kg in drinking water) was administered to streptozotocin-induced diabetic rats during the twelve-week study period. After that, the rats were sacrificed under anesthesia and the tissues were retained -80 °C. Then the activity of respiratory chain complexes and the mentioned enzyme were measured in the brain, kidney, liver and serum of rats.

Results: Treatment of diabetic rats with ASA could significantly compensate for the decreased activity of complex III respiratory chain in the kidneys. In addition, the activity of the liver enzymes (ALT, AST, ALP and LDH) in the serum of diabetic rats was significantly reduced by administration of ASA. However, there were no other significant functional changes observed in the kidney and brain respiratory chains complexes and the mentioned enzymes in liver.

Conclusion: In conclusion, ASA therapy has a beneficial effect on the mitochondrial complexes and some serum enzymes in diabetic rats.

Introduction

Diabetes mellitus is a chronic metabolic disorder that is becoming a major health problem worldwide (Noguchi, 2007). The disorder is characterized by absolute or relative deficiencies in insulin secretion and/or insulin function associated with chronic hyperglycemia and disturbance/imbalance of carbohydrate, lipid, and protein metabolism (Hamden et al., 2008). Experimental and clinical evidence indicate that free radicals are produced as a result of hyperglycemia, and that this may be a common pathway in the pathogenesis of chronic diabetes, such as aging (Brownlee, 2001). Previous studies have shown that diabetes and hyperglycemia increase oxidative stress (Giugliano et al., 1996; Du et al., 2001). Mitochondria plays an important role in maintaining cell viability. Because of their continuous generation of superoxide (as a by-product of the

electron transport chain), the mitochondria are the major source of reactive oxygen species (ROS) in the cell (Rosca et al., 2005). Excess ROS production, as a consequence of diabetes, stimulates signal transduction pathways involved in diabetes pathogenesis. In addition, the resulting oxidative stress due to the imbalance in ROS production and the antioxidant defense system leads to cell damage and thus has a key role in diabetic complications (Bonnefont-Rousselot, 2002; Rabbani and Thornalley, 2008). The major mechanism underlying these events is the inactivation of the mitochondrial respiratory complexes or alteration of their critical subunits due to chronic hyperglycemia. Glycation modification of mitochondrial proteins, as with other proteins, can lead to the generation of mitochondrial oxidants, mitochondrial damage, and organ injury (Munusamy et al., 2009).

Cytochrome C reductase (complex III) is centrally located in the electron transfer process and has been implicated as one of the major sites of superoxide generation in the mitochondria during diabetes (Rosca et al., 2005; Kanwar et al., 2007; Munusamy et al., 2009). One study of diabetic rats indicated that renal complex III was an early and specific mitochondrial target after five weeks of streptozotocin administration. Interestingly, of all four respiratory complexes, only complex III activity was significantly decreased. It has also been suggested that mitochondrial dysfunction in the sensory neurons is due to abnormal mitochondrial respiratory function, resulting in diabetic neuropathy (Chowdhury et al.).

Mitochondria isolated from the brains of hyperglycemic rats contain increased ROS levels, and these are associated with reduced antioxidant barriers (Mastrocola et al., 2005). A direct relationship has also been revealed/established between the formation of intracellular advanced glycation end products (AGEs) and renal mitochondrial proteins, decline in mitochondrial function, and the formation of ROS (Rosca et al., 2005).

Aspirin (acetylsalicylic acid) is a non-steroidal, anti-inflammatory drug. It is used to cure or control many diseases. ASA is a powerful inhibitor of the post-Amadori Maillard reaction, possibly due to acetylation of the free amino groups of proteins prior to glycation.

ASA is also known as a powerful antioxidant that can act directly and indirectly (Oberle; et al., 1998; Shi et al., 1999). We previously showed the beneficial effects of long-term, high-dose ASA therapy on lipid profile and HDL functionality, in terms of LCAT and PON1 activities, in diabetic rats (Jafarnejad et al., 2008b). Recently, the beneficial effect of current dose of ASA has been shown on glycogen metabolism in rat. ASA inhibited glycogen synthesis and glucose depression, decrease in diabetic rat heart (Dervisevik et al., 2014). Continuing on, we here present other results relating to the effect of ASA therapy on mitochondrial complexes and liver function. This study aimed to investigate the effect of long-term, high-dose ASA therapy on the specific activity of mitochondrial chain complexes I, III, and IV in the kidneys and brains of diabetic and normal rats. In addition, we investigated the possible liver toxicity caused by ASA through determination of the activity of important liver function enzymes (ALT, AST, ALP, and LDH) in both liver and serum of rats.

Materials and methods

Chemicals
ASA, STZ, and all other materials were purchased from Sigma-Aldrich Co. Assay kits for liver enzymes, and glucose were purchased from Pars Azmoon Co., Tehran, Iran.

Experimental

Animal models
The investigations were performed with 36 male Wistar albino rats (Pasteur Institute, Tehran) aged 8 weeks and weighing 240 ± 20 g; all of the rats were housed under controlled temperature conditions with a 12-h light/dark cycle. After 3 weeks, the rats were divided into four groups. Two groups (the Diabetic and Diabetic + ASA) were administered STZ 50 mg/kg body weight in Na^+ citrate buffer (pH 4.5) by intraperitoneal (i.p.) injection (Jafarnejad et al., 2008b). If the blood glucose level was < 270 mg/dl after 4 days, the injection was repeated once; thereafter, only rats with blood glucose levels ≥ 270 mg/dl were included in our experiments.

Two other groups (Control or normal and Control+ ASA) were injected with vehicle alone.

The experimental protocol was approved by the Animal Ethical Committee in accordance with the guidelines for the care and use of laboratory animals prepared by Tarbiat Modares University.

Treatment of the diabetic and control groups (Diabetic + ASA and Control + ASA) with 100 mg/kg of ASA in the drinking water was initiated after 1 week of STZ administration. This dose of ASA was selected for its ability to induce Hsp70 in rats (Jafarnejad et al., 2008b); the drinking water was replaced twice a day. The study continued up to the end of week 12. Thereafter, all animals were anesthetized and sacrificed; their organs were snap-frozen in liquid nitrogen and stored at -80°C for further study.

Blood samples were taken from each rat by puncturing the retro-orbital plexus every 4 weeks; samples were allowed to clot for 60 min at room temperature. Serum was separated by centrifugation at 3000 rpm and 25°C for 15 min, and was used to determine biochemical parameters (Saeed et al., 2008). Mitochondria were extracted from the brains and kidneys of both normal and STZ-treated rats as explained previously (Rosca et al., 2005).

Biochemical measurements

Liver homogenates were prepared by suspending tissues in ice-cold 0.25 M sucrose solution to obtain a 5-fold dilution and homogenizing the resulting mixture with a Homogenizer. Serum and liver ALP, ALT, AST, and LDH activity and blood glucose were measured using a spectrophotometric diagnostic kit obtained from the Pars Azmoon Company (Tehran, Iran). The protein concentration in the liver homogenates and mitochondria was determined by the Bradford method (Bradford, 1976).

Mitochondrial respiratory chain complex assays

Mitochondria were extracted from the brains and kidneys of normal and STZ-treated rats, according to the method previously explained (Rosca et al., 2005; Benard et al., 2006). Mitochondrial complex I catalyzes the oxidation of $NADH+H^+$ to NAD^+, ultimately reducing ubiquinone to Ubiquinol. Complex I activity was measured as the rate of NADH oxidation at 340 nm and 37°C. The reaction mixture contained: 10 μl dimethylformamide or ASA at a given concentration; 1 mM of KCN; 0.2 mM of NADH; and 100 μg of mitochondria (45 μl) in a final volume of 1 ml respiratory buffer. After 1 min, the reaction was initiated by the addition of decylubiquinone (150 μM final concentration) and measured at 340 nm against a blank containing all the components except decylubiquinone.

For assay of complex III, the reaction mixture (1 ml) contained: 10 mM Tris-HCl, pH 7.2; 0.2 mM ADP; 3 μM rotenone; 0.3 mM KCN; 10 mM succinate; and 40 μg of mitochondria. The reaction was initiated by the addition of 40 μM cytochrome c in the assay cuvette. After 1 min, 10 mM of malonate was added and the inhibition rate was measured for a further 1 min; 50 μM decylubiquinol was then added and the rate measured for a further 1 min (Morin et al., 2002). For assay of complex IV, oxidation of cytochrome c was monitored at 550 nm and 30°C (Benard et al., 2006).

Statistics

All results are presented as mean ± S.D. Differences between, values were analyzed for significance by one-way ANOVA and non-parametric analyses (Mann-Whitney test) using SPSS 16.0. Significance was set at $p < 0.05$.

Results

Our data (Fig.1A) indicate that serum glucose levels in the diabetic groups were significantly increased by STZ administration. ASA administration caused a non-significant decrease in serum glucose level. The body weight of the diabetic groups was also significantly lower than that of the control groups. ASA administration had no effect on this parameter in both diabetic and normal rats (Fig.1B and Table 1). The weights of some of the rat organs were also measured at the end of the experiment, and the data are shown in Fig.3 and Table 1. There were no significant changes. The specific activity of complexes I, III, and IV of the respiratory chain in the kidneys and brains of all groups of rats is shown in Figs.2A and 2B, respectively. After diabetes induction, the specific activity of all of the

Table 1: Weight of the body, kidney and brain of all rats in different experimental groups.

Groups	Weight (g)	Body	Kidney	Brain
Control (Mean±S.D.)		273.2±14	2.63±0.4	1.6±0.1
Control+ASA (Mean±S.D.)		272.3±24	2.62±0.5	1.7±0.1
Diabetic (Mean±S.D.)		232±25.8*	3.05±0.4	1.5±0.3
Diabetic+ASA (Mean±S.D.)		240±36*	3.00±0.1	1.6±0.1

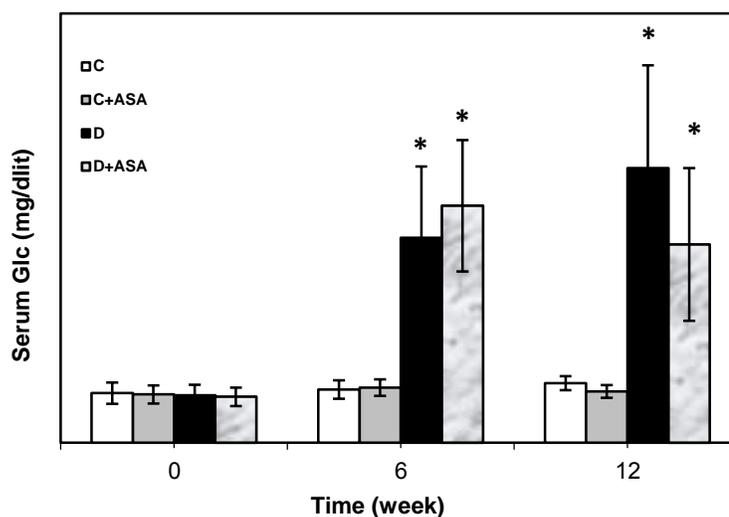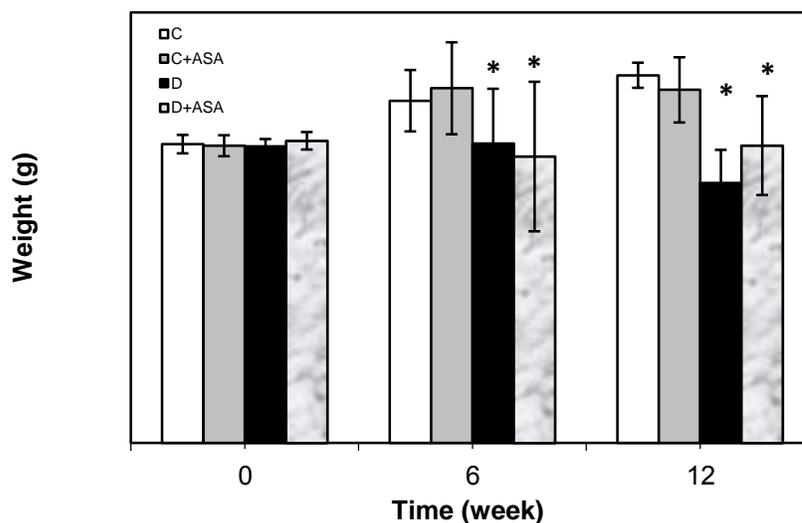∗ Significance (p≤ 0.05) of data comparing diabetic or Diabetic+ASA groups with other groups.

Fig. 1A

Fig. 1B

Fig.1. Changes in the serum glucose levels (A) and weight (B) of all animals at the first week, fourth week and twelfth week of the experiment. * Significances of data comparing D (diabetic) and D+ASA (diabetic rats treated with ASA) vs. the C (control group) and C+ASA (control group treated with ASA) (p < 0.05).

complexes was decreased, but only the changes in the kidney were significant. ASA showed relative compensation/ exerted a compensatory impact against the harmful effects of diabetes on all complexes in these tissues. Except for complex III in the kidneys, none of the changes was significant.
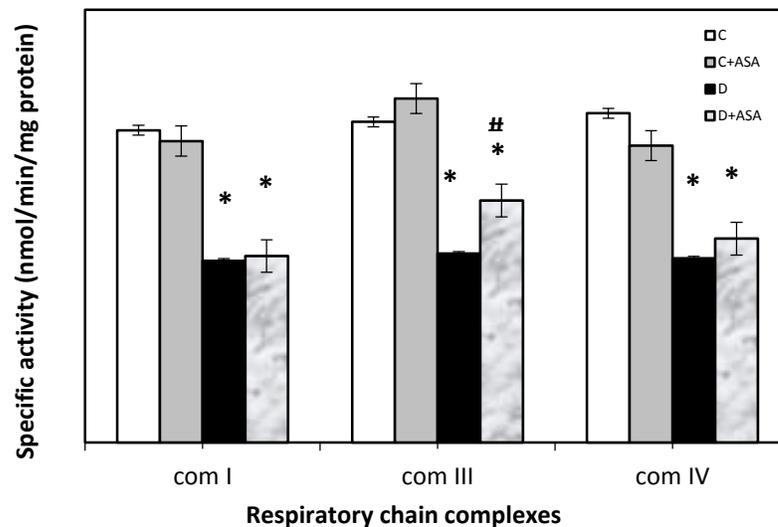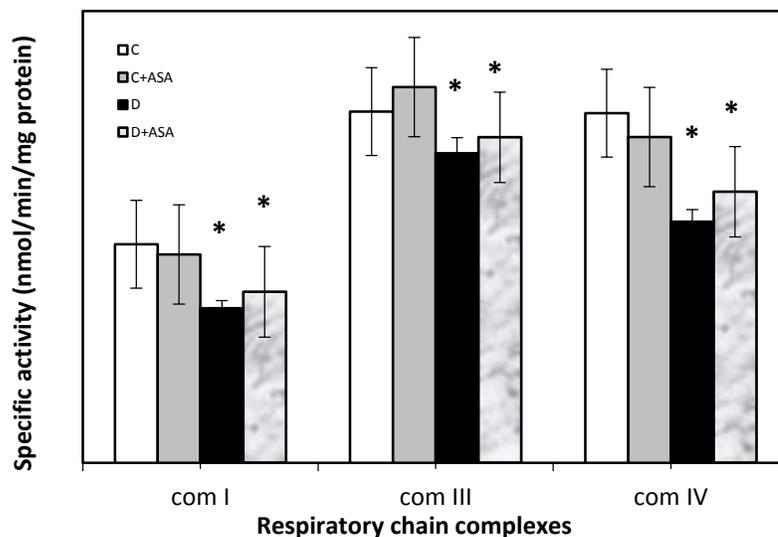
Fig. 2A

Fig. 2B

Fig.2. Changes in the specific activity of mitochondrial respiratory chain complexes in the (A) kidney and (B) brain of all animals in the study. * Significances of data comparing D (diabetic) and D+ASA (diabetic rats treated with ASA) vs. the C (control group) and C+ASA (control group treated with ASA) (p < 0.05). # Significances of data comparing diabetic rats treated with ASA vs. diabetic groups (p < 0.05).

LP, AST, ALT, and LDH activities were also measured in the liver homogenates of all of the rats. The specific activity of each enzyme was calculated after determination of total protein in the tissue homogenates. The results of these assays are shown in Fig.4. In addition, the activity of these enzymes was measured in the sera of all of the rats, and the results are shown in Fig.5. Relative compensation of serum enzyme activity altered by diabetes induction was observed following ASA administration.

Discussion

In the present study, i.p. injection of STZ into adult rats induced diabetes type I, as in previous studies (Jafarnejad et al., 2008b; Dervisevik et al., 2014). The results indicated a significant decrease in the specific activity of complexes I, III, and IV of the respiratory chain in the kidneys and brains of diabetic rats. Treatment of the diabetic groups with ASA increased the specific activity of the complexes, with a remarkably significant increase in the complex III of rat kidneys. The administered dose of ASA did cause any toxicity on the function of liver enzymes, but did decrease the serum level of them, in diabetic rats.

The central role of mitochondria in diabetes, from acute hyperglycemia to a chronic disease has been
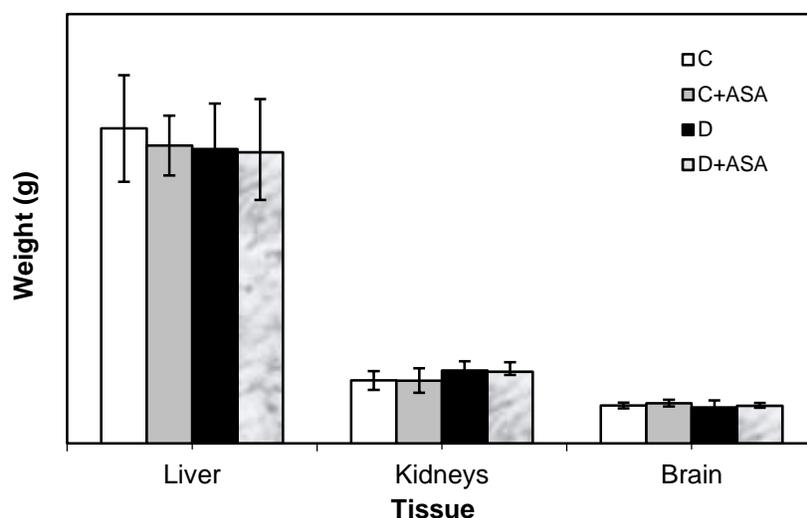
Fig.3. Kidneys, liver and brain weight of the all animals; D (diabetic) and D+ASA (diabetic rats treated with ASA) vs. the C (control group) and C+ASA (control group treated with ASA).
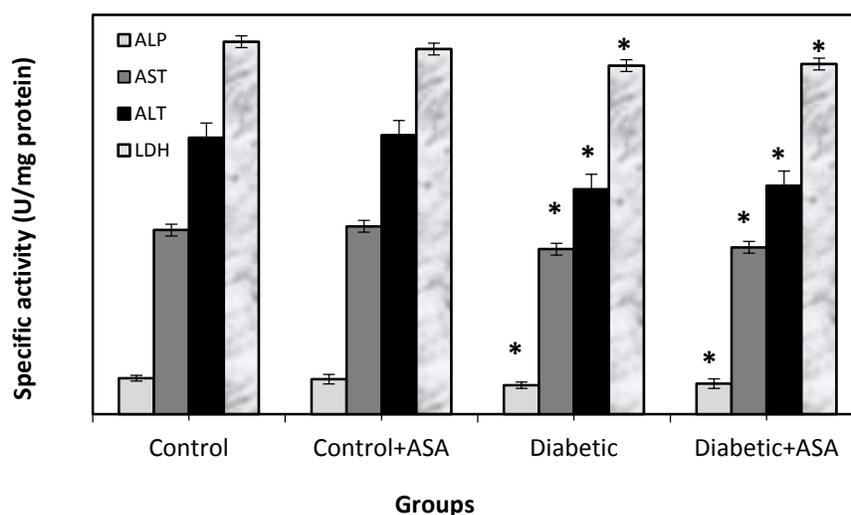

Fig.4. Changes in the specific activity of liver enzymes of all animals in the study. * Significances of data comparing diabetic and diabetic rats treated with ASA vs. the other of groups (p < 0.05).

emphasized in this study. Acute hyperglycemia increased mitochondrial superoxide formation as a result of increased substrate, increased reducing equivalents, and mitochondrial membrane hyperpolarization (Rosca et al., 2005). Complexes I and III are the main sites of reactive oxygen species (ROS) production by intact renal mitochondria (Gredilla et al., 2004). The direct correlation between the increased concentration of glycated proteins (methylglyoxal-induced modifications) and the inhibition of complex III in the kidney in chronic diabetes is consistent with prior work linking ROS production to complex III in intact mitochondria (Chen et al., 2003; Rosca et al., 2005). Renal mitochondrial respiration is

decreased in diabetes. The magnitude of the decrease is related to the inhibition of complex III (Rosca et al., 2005). However, normally I kidney mitochondria have an excess amount complex III and demonstrate a high biochemical threshold after experimental inhibition (Rossignol et al., 1999). In chronic diabetes, the diminution of complex III activity, which is due to both post-translational modifications and the quantity of complex III components, cause a decline in oxidative phosphorylation (Turko and Murad, 2003). It has been also shown that the significant decrease of complex III (cytochrome c reductase) activity was due to incorrect assembling of its subunits (Munusamy et al., 2009). All of the mentioned reports indicated the importance of
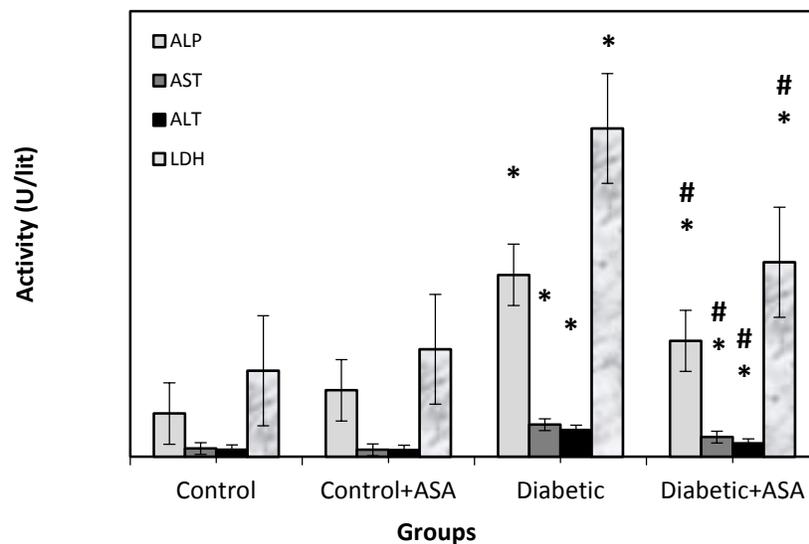
Fig.5. Changes in the activity of the mentioned enzymes in the serum of all animals in the first week, fourth week and twelfth week of the experiment.

* Significances of data comparing diabetic and diabetic rats treated with ASA vs. the other of groups (p < 0.05).

Significances of data comparing diabetic rats treated with ASA vs. diabetic groups (p < 0.05).

glycation and misfolding of the complex III components, which result in its inactivation.

Clinical evidences indicated inhibition of protein glycation and/or an increase in protein stability can significantly reduce the risk of diabetic complications (Harding and Ganea, 2006; Urios et al., 2007a). Thus, there have been numerous attempts to pharmacologically prevent or slow down the glycation of proteins and control the pathogenesis of this harmful disease. ASA as well as some other anti-glycating agents have been used to inhibit or delay protein glycation (Nathan, 1995; Hadley et al., 2001; Urios et al., 2007a; Urios et al., 2007b; Jafarnejad et al., 2008a; Jafarnejad et al., 2008b; Jafarnejad et al., 2008c; Angiolillo and Suryadevara, 2009). ASA can acetylate the ε-NH_2 groups of lysine residues in proteins (Shojaeian and Bathaie, 2004); hence, it blocks the potential sites for glycation and inhibits this process (Caballero et al., 2000; Hundal et al., 2002; Urios et al., 2007a; Jafarnejad et al., 2008b). ASA also induces Hsp70; therefore, it can indirectly protect against and control protein folding under stressful conditions like diabetes (Jafarnejad et al., 2008b). In addition, ASA, as an antioxidant protect biomacromolecules against oxidative stress (Caballero et al., 2000; Lapshina et al., 2006; Jafarnejad et al., 2008b; Bathaie et al., 2010). The data obtained here also indicated that ASA significantly altered the activity of complex III in the

kidney of diabetic rats. A similar result was obtained in kidneys using amino guanidine (Rosca et al., 2005).

Our data also indicate no toxic effect of ASA on the mitochondrial complexes of normal rat kidneys. As well as no toxic or beneficial effects of ASA on these complexes in the brains of normal and diabetic rats.

To investigate the possible/potential toxicity of ASA in rat liver, the activity of AST, ALT, ALP, and LDH in the serum and liver tissue of all of rat groups was determined before and after treatment with ASA. The results indicate that the activity of these enzymes was significantly higher in the serum of diabetic rats in comparison with the control group. Similar results have been reported previously in diabetic rats (Hanna et al., 1997; Hamden et al., 2008; Moharib and El-Batran, 2008; Saeed et al., 2008). A significant increase in serum ALP, AST and LDH activity has also been observed in patients with diabetes mellitus (Goldberg et al., 1977).

Oral administration of ASA significantly decreased the activity of the serum enzymes in the diabetic rats in this study. Similar reduction was reported following oral administration of 17β-estradiol, and insulin to diabetic rats (Hamden et al., 2008). Dietary fiber has also been shown to reduce serum ALP, ALT, and AST activity in STZ-induced diabetic rats (Moharib and El-Batran, 2008).

We also measured the activity of the above- mentioned

enzymes (ALP, ALT, AST, and LDH) in the liver tissue of all rat groups. Our data revealed a significant decrease in ALP, ALT, AST, and LDH specific activity in the livers of all diabetic animals after 12 weeks. Decreases in ALP, AST, and ALP activity have also been observed in the livers of STZ-induced diabetic rats by other researchers (Saeed et al., 2008). These results are compatible with the observed increase in the serum activity of these enzymes in diabetic rats. If the decreased liver enzyme activity was due to liver toxicity resulting from a COX-2-dependent pathway, upon ASA administration it would be treated (Chavez et al., 2012) and the activity of the liver enzymes would return to normal/base values. However, ASA therapy had no significant effect on the level of liver enzymes of both control and diabetic rats in this study. These results indicate that long-term, high-dose ASA therapy not only has no toxic effect on normal rat liver, but also preserves liver function, possibly through its antioxidant activity. The protective effect of ASA in STZ-induced diabetic rats has also been suggested previously (Caballero et al., 2000).

We showed that long-term, high-dose ASA therapy slightly decreased serum glucose levels after twelve weeks of the experiment. These changes were not statistically significant. However, in our previous study a significant decrease in blood glucose after 5 months of ASA therapy was observed in diabetic rats (Jafarnejad et al., 2008b). As reported previously, it is possible that ASA affects intestinal absorption of glucose (Arvanitakis et al., 1977) or reduces hepatic gluconeogenesis (Hundal et al., 2002).

The weight of diabetic animals was also decreased due to diabetes induction. The effect of ASA administration in weight decrease was not significant. A similar result was also observed in our previous study (Jafarnejad et al., 2008b). Furthermore, no changes in the weight of the kidneys, brain and liver of rats were observed following diabetes induction, regardless of whether ASA treatment was initiated or not. However, Lapshina *et al.* showed that the weight of the livers and hearts of diabetic rats was considerably higher in comparison with control rats. Their studies used various doses of ASA (5–50 mg/kg daily injection for up to 9 weeks), and showed that only the lower doses of ASA increased liver weight, and the other doses had no effect

(Lapshina et al., 2006).

It can be concluded from the present study that long-term, high-dose ASA therapy increases complex III levels in the kidneys of rats. It also reduces the level of AST, ALT, ALP, and LDH activity in the serum of diabetic rats, with no significant effect on the function of liver enzymes; that may happen as a result of to its direct interaction with and inactivation of serum enzymes. Thus, ASA, as an antioxidant and anti-glycating agent with chemical chaperone-like activity could be a useful therapy for reducing the toxicity induced by diabetes mellitus.

Acknowledgments

Conflict of interest

All authors declared that there is no conflict of interest.

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