Original Article

Attenuation of Hyperinsulinemia-induced DNA Damage of Peripheral Lymphocytes by Carvedilol

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Abstract

Context: Most people with diabetes suffer from cardiovascular problems; however, increased oxidative stress caused by diabetes can increase the risk of DNA damage and cancer. Carvedilol is a third-generation beta-blocker that can both improve heart function and prevent oxidative stress. **Aims:** The present study aimed to assess carvedilol's genoprotective effects against hyperinsulinemia-induced DNA strand break in rats. **Materials and Methods:** To evaluate the extent of DNA damage caused by high insulin concentrations and the effect of carvedilol on these lesions, isolated lymphocytes of high-fat type 2 diabetic rats were evaluated using the comet method. **Results:** Our results in this study using the comet method showed that hyperinsulinemia and hyperglycemia of high-fat diet have significant genotoxic parameters in rats (tail length 84.35 ± 0.23 vs. 0.90 ± 0.02, % DNA in tail 16.09 ± 0.09 vs. 7.63 ± 0.04, and tail moment 13.58 ± 0.09 vs. 0.07 ± 0.01) compared with the control group ($P < 0.001$). In rats receiving carvedilol, we observed the genoprotective effect in a dose-dependent manner, which is predicted due to the antioxidant activity of carvedilol and its metabolites. **Conclusion:** It does not have an adverse effect on the blood sugar profile of diabetics and reduction of cardiovascular complications of the disease; carvedilol can prevent genetic damage and cancer risk in hyperinsulinemia induced by the high-fat diet.

Keywords: *Carvedilol, comet assay, diabetes, genotoxicity, hyperinsulinemia*

Introduction

In diabetic patients, it has been shown that in addition to vascular dysfunction, increased oxidative stress and oxidant levels can damage the genome and increase the risk of cancer.[1,2] Hyperinsulinemia caused by type 2 diabetes mellitus increases IGF-1, which, due to its induction of cell growth and inhibition of apoptosis, can cause or progress to cancer or even increase cancer mortality.[3,4]

In addition, hyperinsulinemia can lead to an irregular cycle of oxidative stress and impaired insulin signaling.[5] Imbalances in the levels of oxidants and antioxidants are called "oxidative stress."[6] Insulin can increase the production of ROS and peroxynitrite by increasing the activity of the enzyme NADPH oxidase (Nox2). It has been shown that initially with increasing insulin, superoxide dismutase enzymes increase in response to superoxide increases, but with continued ROS, due to irreversible inactivation of this enzyme with hydrogen peroxide, they decrease and then proteolyze. In short, hyperinsulinemia via Nox2 can cause oxidative stress.[5] Hyperglycemia can also cause oxidative stress and damage to DNA and other peptides and lipids by upsetting the balance between oxidants and antioxidants.[6]

Carvedilol is a non-selective beta-blocker and is metabolized to 5-hydroxyphenylcarvedilol (5OHC), 4-hydroxyphenylcarvedilol (4OHC), and O-desmethylcarvedilol (DMC).[7] Unlike other beta-blockers, carvedilol has not been shown to induce insulin resistance but can reduce insulin resistance by sensitizing insulin tyrosine kinase receptors and inhibiting the sympathetic nervous system.[7,8] Additional data showed that carvedilol is a potent antioxidant. Studies have shown that carvedilol can inhibit mitochondrial dysfunction and histopathological lesions in cardiac tissue due to stress caused by doxorubicin.[9] It is also suggested that carvedilol can inhibit lipid peroxidation through its antioxidant properties and ROS removal.[10] Carvedilol and its metabolites have a carbazole arm that can transport a hydrogen atom to the ROS production chain and inhibit this cycle. Hydroxy metabolites can have 50–80 times more antioxidant activity; also, in comparison with carvedilol and its metabolites with tocopherol, it has been shown that carvedilol metabolites can be 1000 times more potent in

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terms of antioxidant activity.[11,12] In summary, carvedilol and metabolites can reduce lipid peroxidation, inhibit free radicals, prevent the depletion of endogenous antioxidants, release iron in ferric ion oxidation reactions, and increase levels of antioxidant enzymes such as catalase, GSH-Px, and SOD.[11,13]

The comet method evaluates the extent of damage and breakage in DNA.[14,15] The measurement mechanism is such that after isolating the DNA, it places it in a magnetic field and forces the pieces to migrate; the parts move and settle according to their weight; the comet tail size indicates the amount of fracture in DNA. After Ostling and Johansson, Singh *et al*. showed that alkalizing the test conditions instead of the neutral conditions could increase this method's capability so that the neutral conditions could only show double-stranded DNA fractures. However, in the alkaline conditions, damage with less severe breaks can be investigated, such as failure in single strands or alkali-labile sites and DNA–DNA or DNA–protein cross-linking. The comet method is beneficial for toxicological studies and can be considered a worthy technique in identifying diagnostic, prognostic, and predictive biomarkers in oncology.[16-18] According to the studies, we intend to investigate carvedilol's effect on the damage caused in DNA due to high concentrations of insulin in diabetic rats.

Materials and Methods

Chemicals

Carvedilol was purchased from Farabi Pharmaceutical Co. NaCl, NaOH, EDTA, H_2O_2, Na_2CO_3, NaH_2PO_4, Tris, and Triton X-100 were obtained from Merck Co. (Germany). Low melting point agarose (LMA) and normal melting point agarose (NMA) were provided by Cinnagen Co. (Iran). Na_2HPO_4, KCl, and ethidium bromide were from Sigma Co. (USA). The food of the mice came from Isfahan Royan Institute. Ficoll was provided by Inno-Train Diagnostik GmbH (Germany).

Experimental design

Rats of Wistar breed, both male and female, weighing between 217 and 277 g, were purchased from the center nest of the animal laboratory of the Faculty of Pharmacy, Isfahan University of Medical Sciences. All animals were kept in a typical health center, with a standard temperature (22–25°C) and relative humidity of 55% with a regular diet. All animal testing protocols were reviewed and approved by the Ethics Committee of Isfahan University of Medical Sciences.

A total of 30 rats were randomly divided into five groups of 6 animals: (1) control group (NC), (2) type 2 diabetic model group (T2DM), (3) type 2 diabetic group treated with 5 mg/kg of carvedilol for 3 days, (4) type 2 diabetic rats treated with 10 mg/kg of carvedilol for 3 days, and (5) normal rats treated with 10 mg/kg of carvedilol for 3 days.

Induction of type 2 diabetic mellitus in rats

In three groups, a high-fat diet (with 60% kcal of fat) was induced by feeding for at least 5 months. Then, after anesthesia with ether, blood samples were taken from the corner of the rat's eye using a heparinized capillary tube and retro-orbital puncture method. Their diabetes was confirmed by measuring blood sugar and insulin. We confirmed the induction of diabetes by measuring glucose and blood insulin levels. A glucometer device measured blood glucose levels (EasyGluco) and rats with a glucose level greater than 227 mg/dL were considered diabetic. The electrochemiluminescent immunoassay technology measured insulin concentration (Cobas e 411 analyzer). The other two groups received a normal diet during the experiment.[19]

Sample preparation

Fresh blood was used to isolate the lymphocytes. To separate the lymphocytes, the Ficoll and phosphate-buffered saline (PBS) have to put it at room temperature beforehand to be at the same temperature as the environment. Then the PBS was gently added to 2 mL of the Ficoll inside the Falcon and covered with foil to prevent any damage caused by light. The samples were centrifuged at 2800 rpm for 30 min. The second layer (cloudy and white) containing lymphocytes was carefully separated with a sampler and diluted with PBS with a ratio of 1:3 and centrifuged for the second time at 1500 rpm for 15 min. After centrifugation, the cells adhered to the Falcon floor, the supernatant was drained, and the previous step was repeated. The comet method with a cell concentration of 10^6 per mL is required. At high cell concentrations, the sample was diluted with PBS.

For animal sampling, food was unavailable for 12 h before the blood glucose concentration was better displayed.

Single-cell gel electrophoresis (the comet assay)

It has been proven that various parameters such as the composition of solutions, buffers, and the duration of different steps are effective in the comet. We first coated the glass slides with NMA layer for better cell adhesion. After lymphocyte separation, the cell suspension with 1% LMA diluted with PBS in a 1:1 ratio was mixed and implanted on the agar layer. The slides were then put in a lysis buffer (pH = 10.0) for 40 min to release cell DNA. After 40 min, the slides were slowly washed with deionized water to remove membrane cellular protein. After DNA release, the slides were placed in the electrophoresis buffer (pH> 13) for 40 min, after which the slides were transferred to the electrophoresis tank and electrophoresed in the same buffer for 40 min (at 25 V and 300 mA). Finally, the slides were removed from the tank and neutralized with Tris solution. Ethidium bromide solution (20 g/mL) was used to stain the slides for 5 min after drying, and then the slides were washed once with PBS and twice with deionized water for 10 min. For each group, three slides and a total of 100 comets were randomly selected, and the % DNA in the tail, length tail, and tail moment were measured.[20]

Statistical analysis

Each experiment was carried out at least three times separately. Data are presented as means ± SEM. Statistical comparison between different groups was done using one-way analysis of

variance (ANOVA) followed by Tukey's *post hoc* test, using GraphPad Prism 8 (Graph Pad Software, Inc., CA, USA). Statistically significance was set at $P < 0.05$.

Results

Investigation of physiological characteristics of the studied animals

By measuring blood glucose, insulin, and body weight in rats, we examined changes in physiological characteristics over 5 months due to the consumption of normal or high-fat diets. As the results shown in Table 1, after 5 months of high-fat diet, insulin (0.8 against 0.08) and blood glucose (156.66 against 117.58) levels increased significantly.

Comet assay results

The comet assay method was used to find the safe or non-genotoxic concentration of carvedilol and to evaluate DNA damage due to insulin and hyperglycemia and the genoprotective potential of different carvedilol concentrations in lymphocyte cells of rats. We evaluated three genotoxic parameters, including tail length, percent of DNA in the tail, and tile moment. The analysis was based on one-way ANOVA, followed by Tukey's *post hoc* test. The control group included rats that received a regular diet until the end of the study and did not receive any carvedilol treatment.

Results of diabetic group

It included comparing the control group with rats that received a high-fat diet for at least 5 months. This group's amount of DNA damage is indicated by tail length (84.35 ± 0.23 against 0.90 ± 0.02), % DNA in tail (16.09 ± 0.09 against 7.36 ± 0.04), and tail moment (13.58 ± 0.09 against 0.07 ± 0.01). The results are mean ± SEM of three replications for six rats in this group and shown in Table 2.

Confirmation of non-genotoxic concentration of carvedilol

To evaluate carvedilol's safety or non-genotoxic concentration, a group of rats that received a regular diet was treated with carvedilol at 10 mg/kg for 3 days and after the comet test, three parameters tail length, % DNA in tail, and tile moment were measured. The results showed that carvedilol with maximum doses did not cause harm to genome and improved the parameters of genetic toxicity evaluation (data not provided).

Results of the diabetic group that received carvedilol

To assess carvedilol's protective potential against insulin-induced DNA damage, two groups of rats on a high-fat diet, whose diabetes was confirmed, were evaluated. Rats were treated with carvedilol 5 and 10 mg/kg by gavage for 3 days. Blood sampling was performed on the 3rd and 6th days (to investigate probable carvedilol metabolites' effect) after carvedilol administration. Three studied parameters including tail length, % DNA in tail, and tail moment showed significant protective effects. The results are mean ± SEM of three replications for six rats in this group. The results are shown in Figure 1.

Discussion

People with diabetes often suffer from cardiovascular problems, including high blood pressure, which has been shown to progress with hyperglycemia, insulin resistance, and hyperinsulinemia, which have a stimulating effect on the renin–angiotensin–aldosterone system. Antihypertensive drugs can be beneficial, but due to beta-blockers' adverse effect on blood sugar control, doctors refuse to prescribe them. However, carvedilol has been shown not to affect hemoglobin A1c (HbA1c) levels and, in addition to controlling cardiovascular risk factors, can control blood sugar in people with diabetes and reduce insulin resistance.[21-25] Hyperinsulinemia caused by insulin resistance in diabetic patients can increase oxidative stress by acting on antioxidant enzymes and ROS producers, reducing nitric oxide bioavailability and impairing endothelial function and hypertension.[23,26]

Studies have shown that malondialdehyde (MDA) level increases in diabetes, which indicates an increase in lipid peroxidation. Furthermore, increased exposure to hydrogen

Table 1: A high-fat diet is compared with the control group with a regular diet on blood glucose, body weight, and blood insulin levels in rats after the 5th month

	Blood insulin (ng/mL)		Blood glucose (mg/dL)		Bodyweight (g)	
	The first day	Fifth month	The first day	Fifth month	The first day	Fifth month
Regular diet group	0.08	0.08	111.58	117.58	196.5	274.58
High-fat diet group	0.08	0.8***	112.16	156.66***	196.33	230.91***

Data are shown as mean ± SEM. ***Mean value was significantly different from the regular diet group ($P < 0.001$)

Table 2: The effect of hyperinsulinemia and hyperglycemia in the diabetic group compared with control groups on tail length (pixels), % DNA in the tail, and tail moment

Treatment	Tail length (pixel) Mean ± SEM	% DNA in tail Mean ± SEM	Tail moment Mean ± SEM
Control group	0.90 ± 0.02	7.63 ± 0.04	0.07 ± 0.01
Diabetic groups	84.35 ± 0.23***	16.09 ± 0.09***	13.58 ± 0.09***

Data are shown as mean± SEM. The sign *** shows significantly different results from the control group ($P < 0.001$)

peroxide in the hearts of diabetic rats leads to an increase in catalase levels and a decrease in GSH-Px and SOD, resulting in oxidative stress and enhancement of MDA levels in diabetic patients.[27-29] Another study on the liver, heart, and kidneys of diabetic rats showed that diabetes-induced hyperglycemia increased the plasma oxidative stress biomarker such as 8-OHdG and that oxidative stress caused DNA damage and genome instability.[28] In addition to hyperglycemia, diabetes can also cause oxidative stress through hyperinsulinemia, shown in studies of human colon cells and lymphocytes to increase free radical production and oxidative stress as insulin concentrations increase, which causes damage to DNA. In this study, in addition to reducing the level of internal antioxidants and increasing free radicals, it is suggested that oxidative DNA damage can be through the PI3 kinase-Akt-tuberin pathway.[30]

Carvedilol, a third-generation non-selective beta-blocker, and its metabolites have been reported to be up to 10 times more effective than vitamin E as an antioxidant; so they can reduce free radicals, prevent the peroxidation of lipids and low-density lipoproteins, increase GSH, and increase the activity of antioxidant enzymes.[21,31] Among other beta-blockers, carvedilol is better tolerated in diabetic patients in weight and blood sugar control, which suggests that carvedilol may be a good option for controlling cardiovascular problems in people with diabetes.[6] A study on the effectiveness of carvedilol on the oxidative stress caused by diabetes in the hearts of diabetic rats has shown that carvedilol increases the activity of CAT, SOD, and GSH-Px enzymes and also improves heart function.[27,32] Ramzy *et al*.[33] also found that carvedilol could increase GSH level and decrease MDA by its antioxidant activity and decrease caspase-3 and reduce lipid peroxidation in the testis preventing apoptosis which will help prevent damage to the testicles caused by diabetes.

This study examined the protective effect of carvedilol on DNA damage caused by elevated insulin and hyperglycemia of diabetes rats. We first showed that the increase in insulin from high-fat diets in rats could cause DNA damage in lymphocyte cells, significantly different from the control group that received a normal diet during this research. Also, receiving a high-fat diet for 5 months has increased blood sugar and weight changes in rats when compared with the regular diet group.

Based on the previous findings, it can be said that carvedilol and its metabolites can prevent DNA damage or reduce the

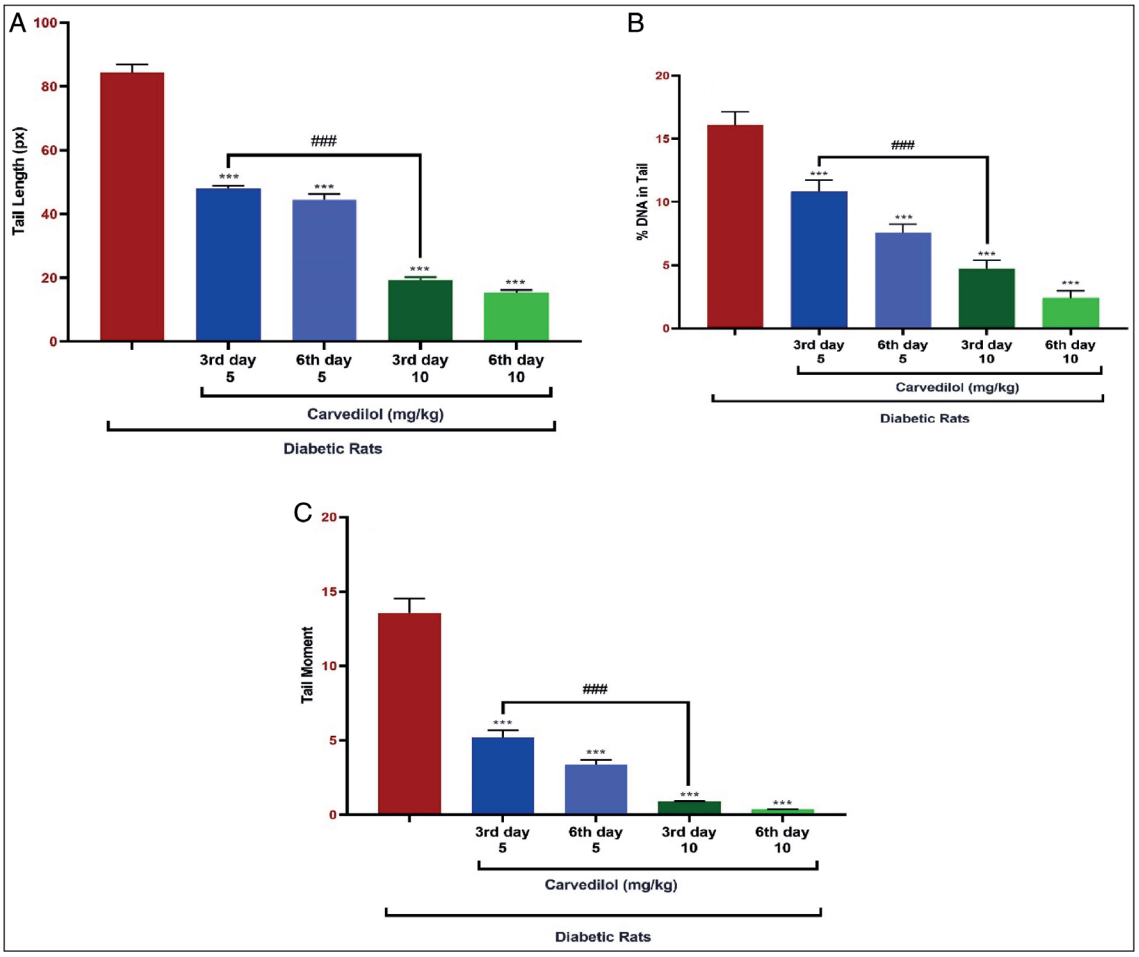

Figure 1: The genoprotective effect of carvedilol and its metabolites in diabetic rats. A. Tail length. B. % DNA in tail. C. Tail moment. Each graph has been represented as mean ±SEM. The sign (***) indicates a significant difference with diabetic rats, and the sign (###) indicates a significant difference between the two groups receiving different carvedilol doses (*P* <0.001)

injury caused by its antioxidant activity.[27] We showed that carvedilol could reduce insulin-induced genotoxicity in rats on a high-fat diabetic diet with the comet assay method. We suggest that carvedilol can reduce the genomic adverse effects of insulin resistance and, on the other hand, with its antioxidant properties, inhibit ROS production and detoxify the produced ROS. Also, in this work, we confirmed antigenotoxic effects of carvedilol and its metabolites according to DNA damage parameters of tail length, percent of DNA in the tail, and tail moment related to increasing the level of antioxidant enzymes. We also showed that the drug's presence in the body for a more extended time increases the possibility of producing metabolites that can significantly reduce the damage compared with carvedilol, indicating that the carvedilol metabolites have better antioxidant properties than the parent compound.

Given that diabetic patients often have cardiovascular problems such as hypertension and that the prevalence of various cancers in these patients is increasing, it can be suggested that these patients can improve these complications by taking carvedilol and preventing damage to the genome and probable tumors and cancers.

Conclusion

We showed that diabetes can cause DNA damage through hyperinsulinemia and hyperglycemia by increasing oxidative stress with the comet assay. In groups treated with carvedilol in a concentration-dependent manner, this damage was reduced, which is predicted due to carvedilol's antioxidant activity and its metabolites.

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Conflicts of interests

There are no conflicts of interest.

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