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Original Research Article

Effect of L-arginine in eNOS expression in heart tissues of diabetic rats

Abstract

Aims: This study aimed to determine the role of L-arginine on some of biochemical markers: blood glucose, HDL, LDL, cholesterol, lipid peroxide (MDA) and triglyceride. Also its bioactivity on some antioxidant: reduced glutathione (GSH) and catalase.

Study design: Ninety adult male albino rats were divided into 3 groups: Group (I): control group (30 rats). Group (II*)*: Diabetic group (30 rats). Group (III): 30 rats were given L-arginine. Then the second group and the third group were injected by alloxan monohydrate in dose 80 mg/kg intraperitoneally at once. The animals were scarified and the blood was collected for determination of biochemical marker and antioxidant levels. Heart tissues was homogenized for determination of expression of eNOS gene by RT- PCR.

Results: The study showed that L arginine increases HDL, reduces glutathione, catalase and decrease LDL, TAG, Cholesterol, MDA, also increase expression of eNOS in heart tissues of diabetic rat.

Key words: Antioxidant, Diabetes mellitus, e NOS gene, L-arginine.

Introduction

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Diabetes mellitus is a serious metabolic disorder with micro and macrovascular complications that results in significant morbidity and mortality. The increasing size of the ageing population, consumption of a caloric rich diet, increasing rates of obesity and sedentary life style have led to a an increase in the number of diabetic's world wide [1].

Oxidative stress is a constant feature of uncontrolled diabetes in humans and animals. Antioxidants such as vitamin C and E are decreased in diabetes, suggesting that oxidative stress in diabetes is at least in part, due to impaired antioxidant system, additionally reports from various areas show decrease activities of catalase and reduced glutathione [2].

L. Arginine is an α-amino acid. It was first isolated in 1886. In mammals, arginine is classified as a semiessential or conditionally essential amino acid, depending on the developmental stage and health status of the individual [3].

Nitric oxide (NO) is an important cellular signaling molecule involved in many physiological and pathological processes. It is a powerful vasodilator with a short half-life of a few seconds in the blood [4].

Nitric oxide is synthesized from L-arginine in the presence of oxygen and NADPH in a reaction catalyzed by NO synthase (NOS). Three NOS isoforms are expressed in mammalian cells: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). The eNOS is constitutively expressed in endocardial cells, endothelial cells, ventricular myocytes, and other myocardial cells [5].

In diabetes, endothelial injury may be a consequence of glucometabolic and peroxidative stress. Experimental evidence suggests that the endothelium dependent relaxation is greatly reduced in the aorta of diabetic rats [6].

Materials and Methods

Experimental animals:

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Ninety adult male healthy albino rats (obtained from the national research center, Giza, Egypt) weights (200 – 220 gm).

Animals were given standard rat diet from (El- Nasr, cairo, Egypt) (protein 18 %, lipid 2.7 %, fibers 10.06 % and energy 2600 K cal) and tap water.

Animals were divided into three groups:

1. Group I: (N = 30) animals served as control group.
2. Group II (Diabetic group): (N = 30). Animals served as positive control that lift without diet over night before alloxan injection.
3. Group III (Treated group): (N = 30). Animals were given L-arginine orally for one week in dose 100 mg/kg in sterile (0.9%) Nacl before alloxan injection then L-arginine was given for another four weeks.

After that, the second group and the third group were injected by alloxan monohydrate in dose 80 mg/kg in sterile (0.9%) Nacl intraperitoneally (I.P.) at once. After 72 hour of alloxan injection, blood samples were collected from the tail vein of rats and blood glucose was determined by one touch method.

At the end of the experiment, animals were scarified and the blood was collected and centrifuged to obtain serum which collected for determination of the following parameters (glucose, serum triglycerides, cholesterol, HDL-cholesterol, LDL-cholesterol, plasma catalase, reduced glutathione and lipid peroxide by colorimetric method according manufacture instruction. Total RNA was extracted by Ribozol reagent from heart tissues for determination of expression of eNOS gene by RT- PCR.

Drugs, chemicals, and kits; Alloxan (Biotechnology Co., Egypt).

- Catalase, reduced glutathione, cholesterol, triglycerides, HDL, LDL and malondialdehyde kit (Biodiagnostic Co., Egypt).
- Glucose kit (BioMed Diagnostic Co., Egypt).

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- L-arginine (Sigma-Aldrich Co., Egypt).

Detection of the level of NO

Nitric oxide colorimetric assay kit (cat No K262-200). Bio Vision (USA) according to manufacture instruction

mRNA analysis (rt-PCR).

Total RNA was extracted from homogenates of heart tissue using RiboZol solution (Amresco, Solon, USA) according to the manufacturer's instructions. RNA samples were stored at -80°C until analysis.

RT-PCR was performed according to manufacture instructions (Qiagen one step). Reaction tube of RT-PCR was containing 5 µl of RT-PCR buffer 5× 10 pM of specific primers for eNOS or GADPH, 5 µg of total RNA template, 1 µl dNTPs mix (100 mM), 1 µl enzyme mix (Hot Star Taq DNA polymerase, Omniscipt and Sensiscript reverse transcriptases).

Polymerase chain reaction (PCR) amplification was performed as at the following temperature steps: 5 min, 95 ^{o}C; 94 ^{o}C for 20 sec; 55 ^{o}C for 30 sec; 72 ^{o}C for 40 sec (30 cycles); and 72 ^{o}C for 5 min. The $MgCl_2$ concentration used for rat eNOS cDNA amplification was 2.0 mM. The following primer pairs were used: 5'-CGA GAT ATC TTC AGT CCC AAG C-3' (sense) and 5'-GTG GAT TTG CTG CTC TCT AGG-3' (antisense) for rat eNOS. PCR was performed with rat GADPH as an internal standard (Thermo scientific) USA .Sense5'-CAGTGCCAGCCTCGTCTCAT-3', antisense 5'- GGGGCCATCCACA GTCTTC-3', sample for rat GADPH has same condition for rat eNOS except the annealing temperature was 54 ^{o}C, in a thermal cycler (Progene, Techne (Cambridge) LTD., UK).

Detection of PCR product.

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Polymerase chain reaction products (5 µl) were detected by electrophoresis on a 1.5 % agarose gel containing ethidium bromide. Location of a predicted product was confirmed by using 100 bp ladder (Gene Ruler, Thermo scientific, USA)) as a standard size marker. The negative control tubes containing all component as in RT-PCR reaction tubes except it was not containing total RNA template.

Statistical analysis

The data were expressed with SPSS version 13 soft ware as the mean ± standard error (SE), analysis of variance (ANOVA) indicated a significant at ($P < 0.05$). Independent t-test for parametric quantitative data.

The density of PCR product was measured using Scion Image J Soft Ware (Scio Cooperation, Fredrick, Maryland) and expressed as mean ± SE

Results

The result shows that L-arginine reduces the blood glucose level by 49% from 266.7 mg/dl in alloxan group to 135.1 mg/dl in treated group (Table 1).

Also the result shows that L-arginine reduces the triglyceride level by 25.5% from 200.8 mg/dl in group I to 149.6 mg/dl in treated group. And also reduces the LDL level from 210.2 mg/dl in group I to 164.4 mg/dl in treated group which means L-arginine reduced LDL level by 22%.

Finally the result shows that L-arginine improves HDL level by 97% from 17.5 mg/dl in group I to 34.5 mg/dl in treated group (Table 1).

L-arginine decrease cholesterol level from 291.2 mg/dl in group I to 241.7 mg/dl in treated group which means L-arginine reduced triglyceride level by 17%.

The result shows that L-arginine increase the reduced glutathione level by 80% from 0.5 mg/dl in alloxan group to 0.9 mg/dl in treated group (Table 1).

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The result shows that L-arginine reduces the MDA level by 47% from 56.9 nmol/ml in alloxan group to 30.3 nmol/ml in treated group. (Table 1).

Also there was negative correlation between catalase, reduced glutathione and blood glucose (Fig. 2, 3)

Fig. (4) Shows mRNA eNOS RT-PCR analysis. The band of mRNA for eNOS in the diabetic heart (Group II) appears significantly decreased in comparison to that in the control lane (with GADPH as internal standard). Also there was significant increase in expression in treated group (III) as compared with group I.

Table (1): Show the average values of each group separately.

	Group I (control) Mean ± SE	Group II (Diabetic) Mean ± SE	Group III (Treated) Mean ± SE	P value
Glucose (mg/dl)	94.2 ± 2.6	266.7± 7.6	135.1 ± 1.4	<0.001
TG (mg/dl)	3.4 ± 5.6	200.8± 3.9	49.6 ± 1.8	<0.001
LDL (mg/dl)	81.9 ± 4.3	210.2± 3.4	120.4 ± 2.03	<0.001
HDL (mg/dl)	53.9 ± 2.8	17.5 ± 4.9	34.5 ± 1.2	<0.001
CHL (mg/dl)	177.8 ± 3.7	291.2± 2.4	241.7 ± 1.4	<0.001
Glutatione (mg/dl)	1.2 ± 0.01	0.5 ± 0.01	0.9 ± 0.01	<0.001
Catalase (U/l)	28.4± 0.5	11.9 ± 0.3	18.5 ± 0.3	<0.001
MDA (nmol/ml)	56.2± 0.2	56.9± 0.4	30.3± 0.3	<0.001
Cardiac NO (nmol/mg protein)	43 ± 18	21 ± 9	25 ± 6	<0.001

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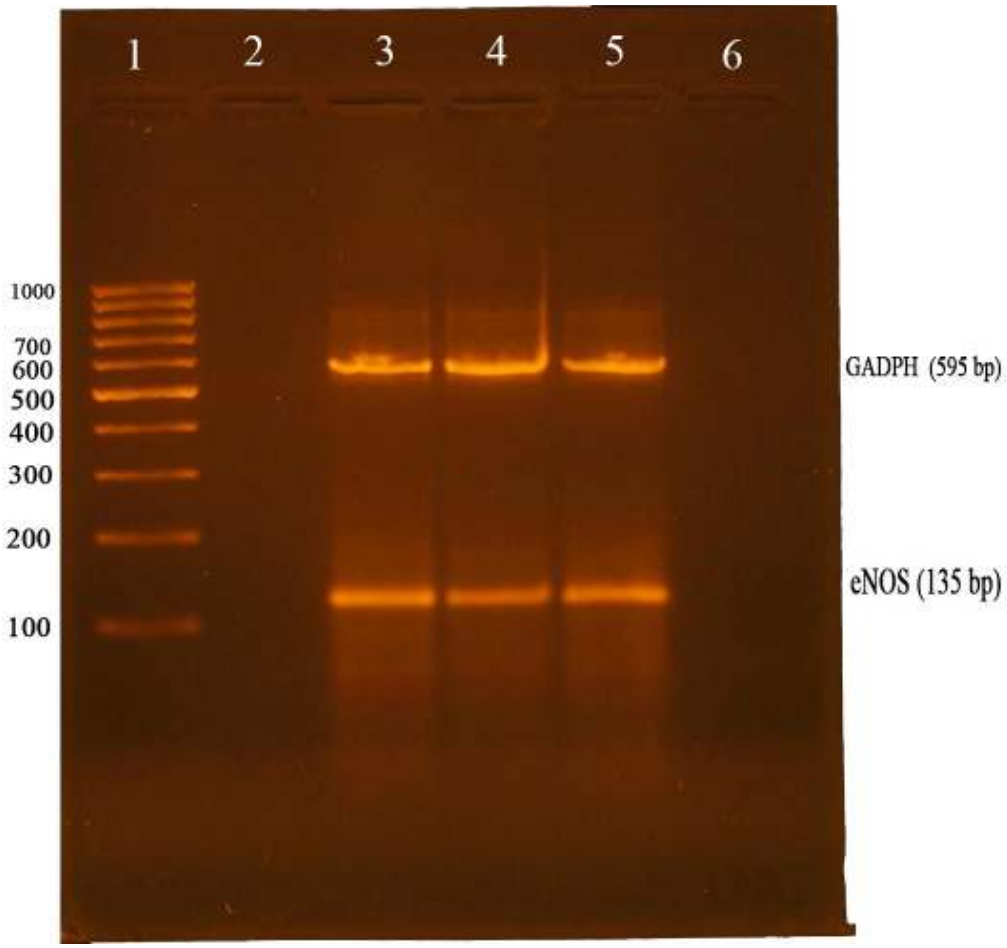

Figure 4: Agarose gel electrophoresis of RT-PCR product showing rat eNOS expression (135 bp) in heart tissue of different groups.

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Lane 1: DNA ladder (100 bp), lane 2: negative control, lane 3: control group, Lane 4: group II (diabetic group). Lane 5: group III (L.arginine treated group), with GADPH as internal standard at 595 bp

Discussion

This study reveals a number of important findings on the relationship between diabetes and the consumption of L-arginine. Poorly controlled long standing metabolic disorders diabetes mellitus is frequently results in cardiovascular complications [9].

L-arginine administration reduces lipid peroxide levels in diabetic patients, due to direct scavenger of superoxide anions. L-arginine might enhance relaxation by increasing NO release, which in turn scavenges enhanced rates of superoxide anion production that are elevated in diabetic arteries [10].

The results which were obtained showed that treatment of diabetic rats with the L-arginine leads to significant reduction in blood glucose level and this could be attributed to its effect on the enzymes that digest carbohydrates and hence decrease glucose absorbance from intestine and these data were in line agreement with Broca et al 2004 [11].

The level of MDA was significantly reduced which indicate that the reduction in the lipid preoxidation as a result of the treatment. Also the antioxidant enzymes, catalase and reduced glutathione were significantly increased as result of the treatment indicating the reduction of oxidative stress and ROS in the treated groups which may be attributed an antioxidation effect of the L-arginine [12].

Lipid profile results show high efficacy of L-arginine in reducing triglyceride and LDL levels and improving HDL level and these data were in line with Fu et al 2005 [13].

Antioxidant enzymes as reduced glutathione and catalase were improved by L-arginine according to result. L-arginine increase reduced glutathione. from 0.5 mg/dl in diabetic group to 0.9 mg/dl in

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treated group, which means that after treatment by L-arginine reduced glutathione improved by 80% and these data were in line with Li et al 1994 [14].

L-arginine increase catalase by 55.5% from 11.9 U/L in diabetic group to 18.5 U/L in treated group and these data were in line with Li et al 1994 [14].

A defect in nitric oxide function develops very early in diabetes mellitus and leads to altered vascular reactivity [15]. The association of diabetes mellitus with atherosclerotic cardiovascular disease may be a consequence, in part of the decrease of eNOS protein in endothelial cells.

NO has important cardiovascular effects and modulates cardiac function. The contraction of cardiac muscle responds to chemical transmitters, oxygen tension and physical factors by modifying transmembrane calcium currents, intra-cellular calcium storage and the contractile proteins [16].

Reports indicate that diabetes induces alterations of calcium handling and impairs nitric oxide production in coronary endothelial cells, due to tetrahydrobiopterin deficiency and in the contractile proteins that limit myocardial function independent of altered metabolism [17].

Although dysfunction of the NO pathway may promote atherogenesis, NO supplied exogenously to activated endothelium down regulates pro-inflammatory activity such as chemokine secretion and functional activity such as transendothelial migration of neutrophils [18]

Conclusion

The study show that L arginine increase HDL, reduced glutathione, catalase and decrease LDL, TAG, Cholesterol, MDA, also increase expression of eNOS in heart tissues of diabetic rat.

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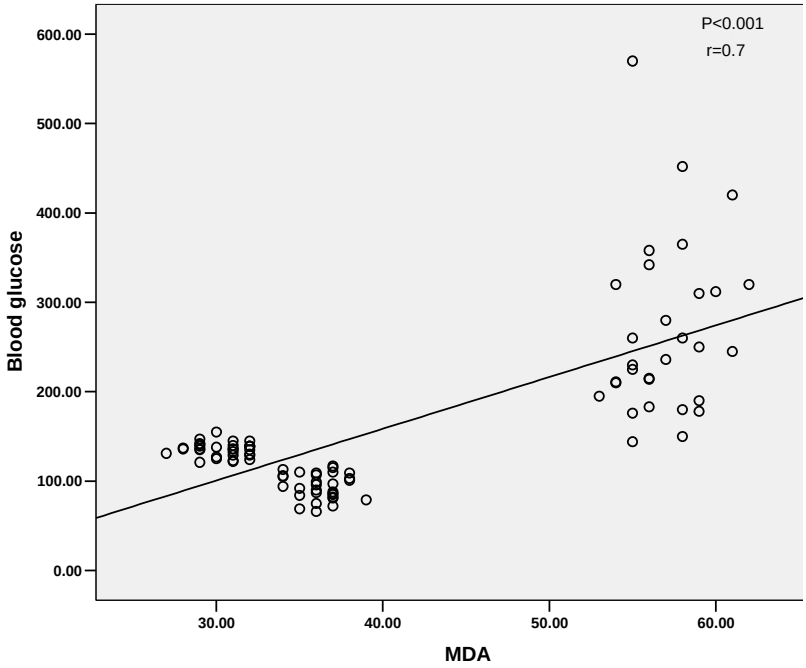

Figure (1): Show positive correlation between MDA and blood glucose.

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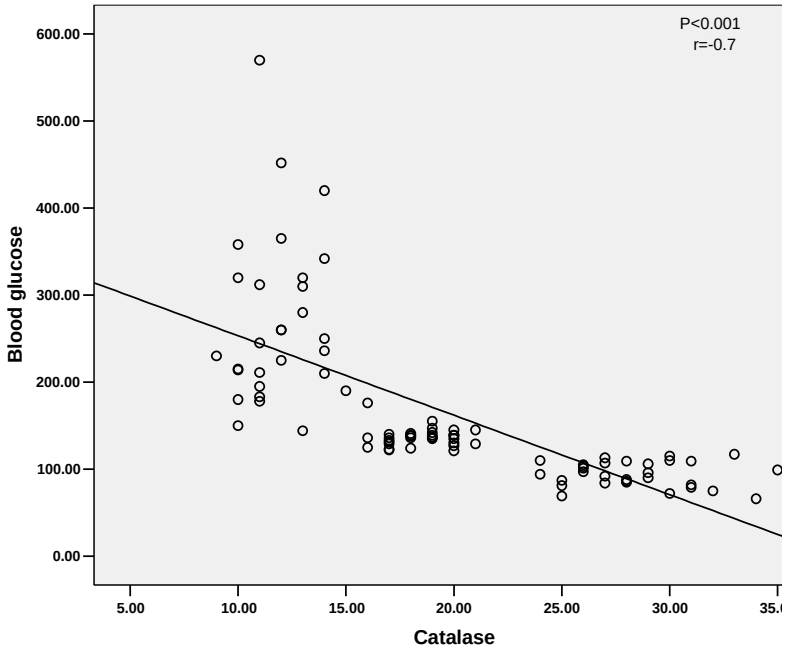

Figure (2): Show negative correlation between catalase and blood glucose

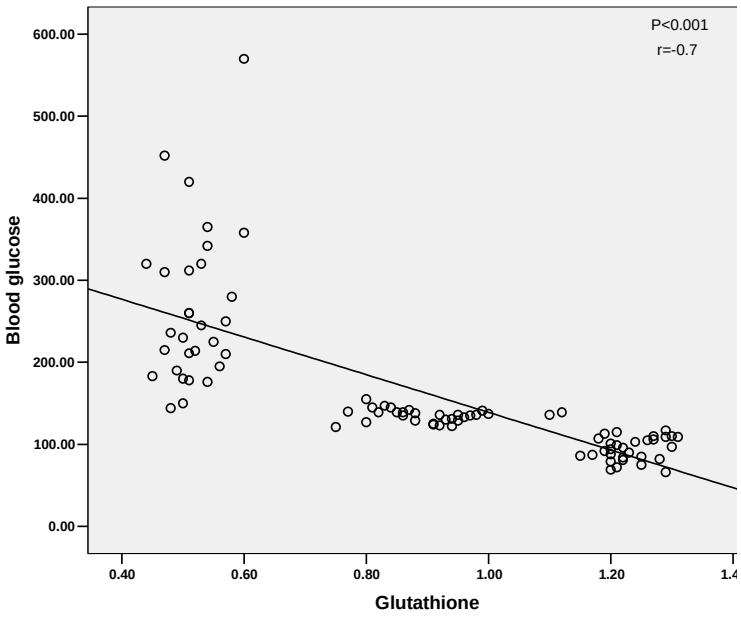

Figure (3): Show negative correlation between reduced glutathione and blood glucose.

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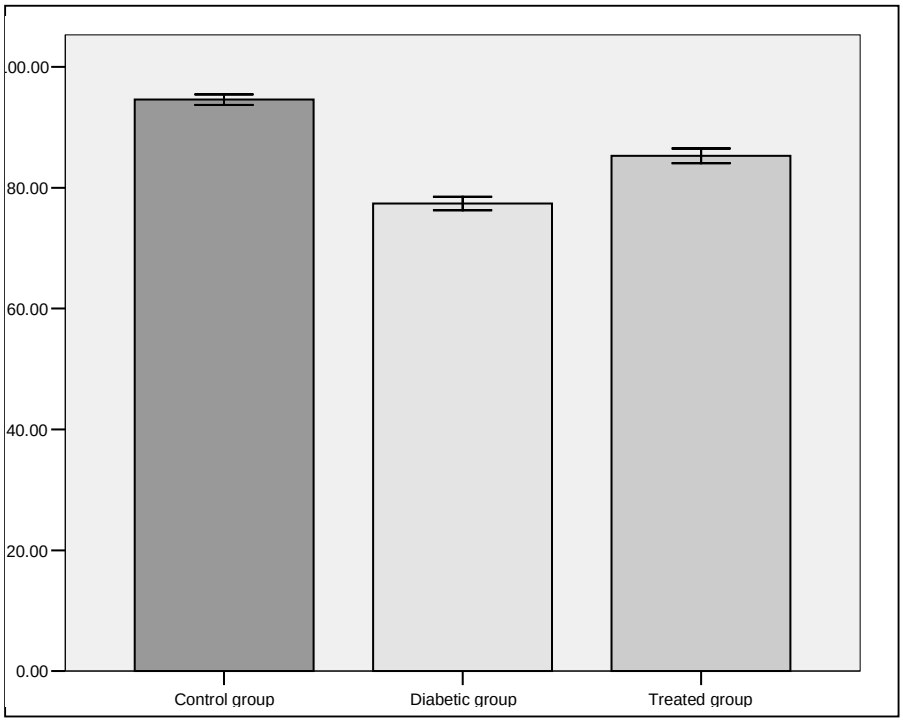

Figure 5: Show eNOS expression in heart tissues in different group.

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