

Effect of L-arginine on the expression of endothelial

2 ***nitric oxide synthase (eNOS) in heart tissues of***

3 ***diabetic rats***

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Abstract

14 **Aims:** This study aimed to determine the role of L-arginine on eNOS expression and
15 its role on the level of some antioxidants as reduced glutathione and catalase in
16 **alloxan-induced diabetic rats.**

17 **Study design:** Ninety adult male albino rats were divided into three (3) groups of
18 thirty rats (30) each: Group (I): control group, Group (II): Diabetic control group,
19 Group (III): L-arginine treatment group. The second and the third groups were made
20 diabetic by intraperitoneal administration of 80 mg/kg alloxan monohydrate, while L-
21 arginine (100 mg/kg in sterile 0.9% NaCl) was given orally to rats in group III for one
22 week before alloxan injection, and a further four weeks after induction of diabetes.
23 The animals were sacrificed and blood collected for the determination of biochemical

antioxidant markers. Heart tissues were homogenized for determination of expression of eNOS gene by RT-PCR.

Results: The study showed that L-arginine increases HDL, reduces glutathione and catalase and decreases LDL, TAG, Cholesterol and MDA. It also increased the expression of eNOS in heart tissues of diabetic rats.

Keywords: Antioxidant, Diabetes mellitus, eNOS gene, L-arginine.

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Introduction

Diabetes mellitus (DM) is an important metabolic disorder that associated with micro- and macrovascular problems that affect the morbidity and mortality rates. The lifestyle consumption of a high calorie diet, obesity and sedentary life led to a rapid increase in the number of diabetic's worldwide [1]

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It has been found that alloxan and streptozotocin selectively damage pancreatic β -cells and exhibit the most potent diabetogenicity. They have been widely used for induction of experimental diabetes mellitus type I. The mechanism that has been accepted is the fragmentation of nuclear DNA of pancreatic β -cells, leading to the development of diabetes and accumulation of superoxide or hydroxyl radicals in the case of alloxan [2].

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There is association between oxidative stress and uncontrolled diabetes in humans and animals. The decreased level of nonenzymatic antioxidants levels, such as vitamin C and in diabetes mellitus, suggesting that oxidative stress in diabetes is partly due to ineffective antioxidant system lead to impaired activities of catalase and reduced glutathione [3].

L-Arginine is an α -amino acid, first isolated in 1886. In mammals, arginine is classified as a semi-essential or conditionally essential amino acid, depending on the developmental stage and health status of the individual [4].

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. In the presence of cardiovascular risk factors, endothelial dysfunction is frequently encountered. Several molecular defects could account for reductions in endothelium dependent vascular relaxation. Endothelial dysfunction could be due to decreased eNOS expression. However, several studies have shown that cardiovascular risk factors are associated with an increase rather than a decrease in eNOS expression. The increased expression of eNOS in vascular disease is likely to be a consequence of an excess production of H_2O_2 [5].

It was found that L-arginine in the presence of oxygen and NADPH-d in a reaction catalyzed by nitric oxide synthase (NOS) can produce NO [6]. Mammalian cells contain different NOS isotypes. These isotypes are: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). The eNOS can be detected in, endothelial cells, ventricular myocytes, endocardial cells and other myocardial cells, and need Ca^{2+} and calmodulin to be active. Glucometabolic and peroxidative stress may be the cause of endothelial injury in diabetics. The endothelium- dependent relaxation induced by acetylcholine is greatly reduced in the aorta of diabetic rats as suggested by experimental studies. [6]

Materials and Methods

Chemicals and Reagents

Diagnostic kits for catalase, reduced glutathione, cholesterol, triglycerides, HDL, 72

LDL and malondialdehyde were obtained from Biodiagnostic (Egypt), Glucose kit 73

from BioMed Diagnostic (Egypt), Alloxan (Biotechnology Co., Egypt), and L-Arginine from Sigma-Aldrich (Egypt). Nitric oxide colorimetric assay kit was obtained from Bio Vision (USA). All other reagents were of analytical grade.

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Experimental animals:

Ninety adult apparently healthy male albino rats (200 – 220 gm) were obtained from the National Research Center, Giza, Egypt. Animals were given standard rat diet (El-Nasr, Cairo, Egypt) containing 18 % protein, 2.7 % lipid, 10.06 % fibers and 2600 K cal energy and tap water *ad libitum*.

The experiment was conducted according to the ethical norm approved by Institutional Animal Ethics Committee (IAEC). They were kept at the constant environmental condition for acclimatization; food and water were provided *ad libitum* for one week before the experiment.

Animals were divided into three groups of thirty (30) rats each:

1. Group I: Animals served as control group
2. Group II (Diabetic group): Animals served as positive control
3. Group III (Treated group): Animals were given L-arginine orally for one week at 100 mg/kg before induction of diabetes then L-arginine was given for another four weeks.

Rats in the second group and the third group were made diabetic by a single intraperitoneal injection of alloxan monohydrate (80 mg/kg in sterile 0.9% NaCl).

After 72 hours 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 sacrificed and the blood was collected and centrifuged to obtain serum 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 to manufacturers' instruction. Total RNA was extracted by RiboZol reagent from heart tissues for determination of expression of eNOS gene by RT-PCR.

Detection of the level of NO

Nitric oxide was detected by Nitric Oxide colorimetric assay kit (BioVision, USA) according to manufacturers' instructions: nitrate reductase was used to convert nitrate to nitrite utilizing. Then the Griess reagents was used to convert nitrite to a deep purple azo compound. The amount of the azochromophore reflects nitric oxide amount in samples accurately. The detection limit of the reaction is a 0.1 nmol nitrite/well.

Absorbance was measured at 540 nm as a function of nitrate and/or nitrite concentration.

Nitrate/nitrite concentration was calculated according to this equation:

$$\text{Nitrate/nitrite concentration} = \frac{\text{Abs sample} - \text{blank Abs}}{\text{Slope of Std curve}} \left(\mu\text{L of sample} \right)$$

mRNA analysis (rt-PCR).

Total RNA was extracted from homogenates of heart tissue using RiboZol solution (Ambio, Solon, USA) according to the manufacturer's instructions. RNA samples were stored at -80°C until analysis. RT-PCR was performed according to manufacturer 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, Omniscript and Sensiscript reverse transcriptases). Polymerase chain reaction (PCR) amplification was performed as follow: 5 min, at 95 °C (initial denaturation); 94 °C for 20 sec; 55 °C for 30 sec; 72 °C for 40 sec (30 cycles); and 72 °C for 5 min (final extension). The MgCl₂ concentration used for cDNA amplification was 1.2 mM. The following primer pairs were used: 5'-CGA GAT ATC TTC AGT CCC AG C-3' (sense) and 5'-GTG GAT TTG CTG CTC TCT AGG-3' (antisense) for eNOS. PCR was performed with rat GAPDH as an internal standard (Thermo scientific) USA .Sense 5'-CAGTGCCAGCCTCGTCTCAT-3', antisense 5'-GGCCCATCCACA GTCTTC-3', sample for rat GAPDH has same condition for rat eNOS except the annealing temperature was 54°C, in a thermal cycler (Progene, Tech (Cambridge) LTD., UK) [7].

Detection of PCR product

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.

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Statistical analysis

The data were expressed with SPSS version 13 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 software (Scio Cooperation, Fredrick, Maryland) and expressed as mean ± SEM

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Results

The result shows that L-arginine reduces blood glucose level by 49% from 266.7 mg/dl in alloxan group to 135.1 mg/dl in treated group (Table 1). Also, L-arginine reduced the triglyceride level by 25.5% from 200.8 mg/dl in group I to 149.6 mg/dl in treated group and LDL level by 22% from 210.2 mg/dl in group II to 120.4 mg/dl in treated group. Administration of L-arginine increased HDL level by 97% from 17.5 mg/dl in group I to 34.5 mg/dl in treated group (Table 1) and decreased 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%.

Reduced glutathione level increased by 80% from 0.5 mg/dl in diabetic control group to 0.9 mg/dl in treated group (Table 1 and Fig 1), while MDA level reduced by 47% from 62.9 nmol/ml in diabetic rats to 30.3 nmol/ml in treated group (Table 1). The card NO level in diabetic rats was a significantly decrease from $(25 \pm 6 \text{ nmol/mg protein})$ to $(21 \pm 9 \text{ nmol/mg protein})$ in the treated and diabetic rat respectively.

There was a negative correlation between catalase and blood glucose (Figure 2) and reduced glutathione and blood glucose (Figure 3).

Figure 4 shows the mRNA eNOS RT-PCR analysis. The band of mRNA for eNOS in the part of diabetic rats (Group II) appears significantly decreased when compared to that the control lane (with GAPDH as internal standard). Also there was significant increase in expression in treated group (III) than group I.

Table 1: Effect of L-arginine on blood glucose, lipoprotein, and some antioxidant parameters in alloxan-induced diabetic rats.

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

Values are mean±SEM.

Values with different superscripts within the same row are significantly different at P<0.05.

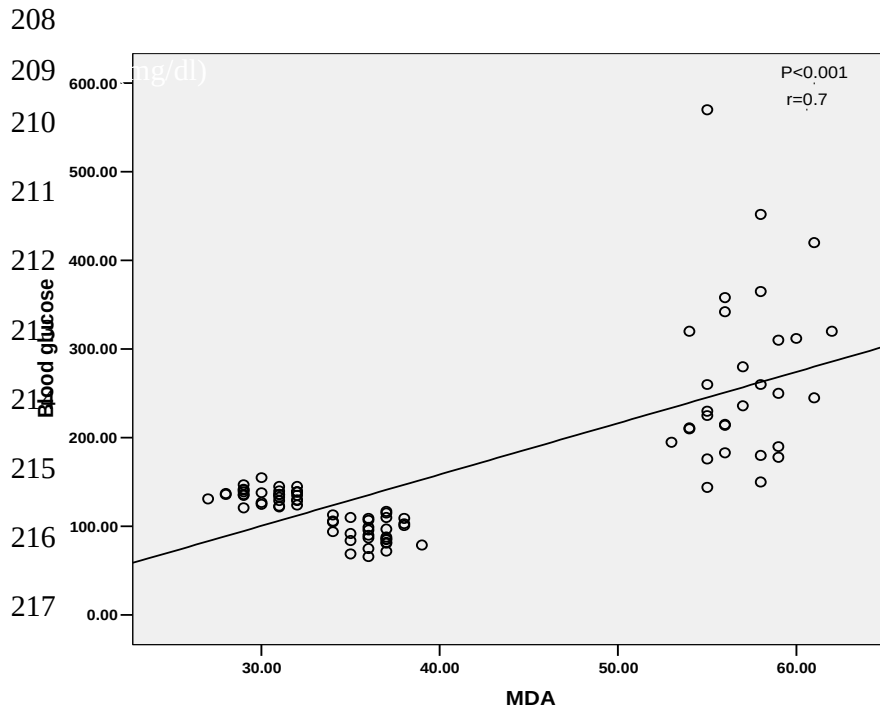


Figure 1: Correlation between malondialdehyde (MDA) and blood glucose level in Alloxan-induced diabetic rats.

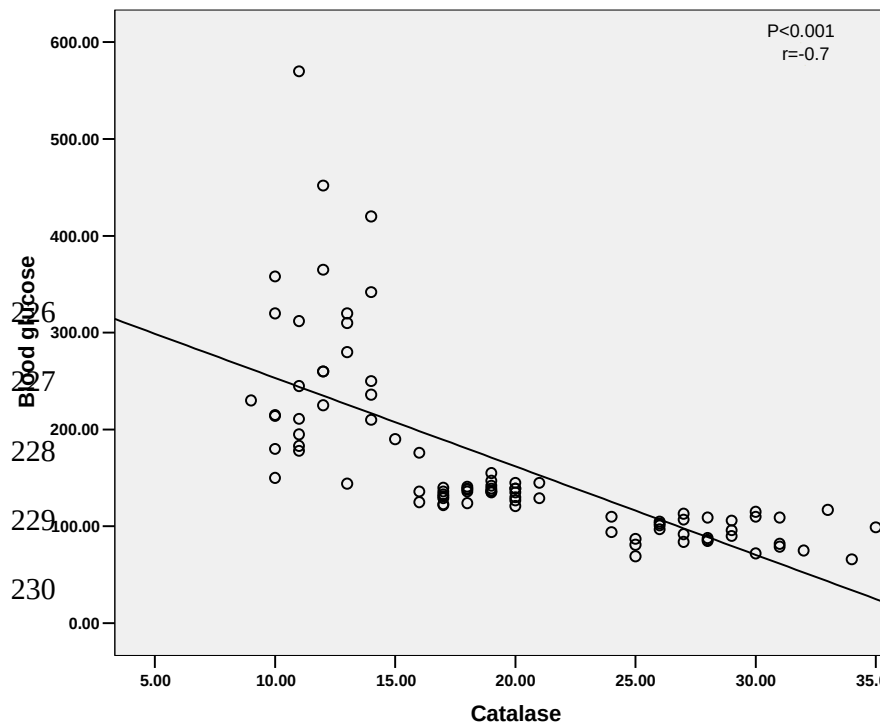


Figure 2: Correlation between catalase and blood glucose level in alloxan-induced diabetic rats.

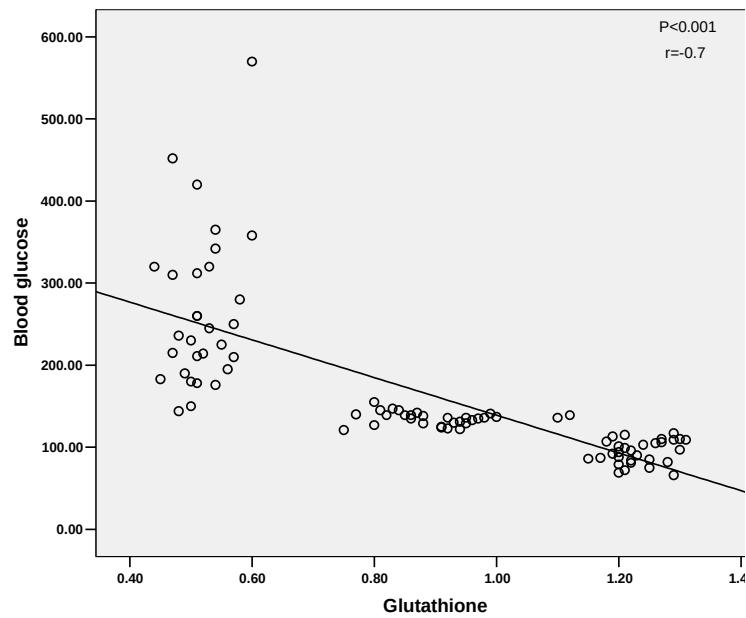


Figure 3: Correlation between reduced glutathione and blood glucose level in alloxan-induced diabetic rats.

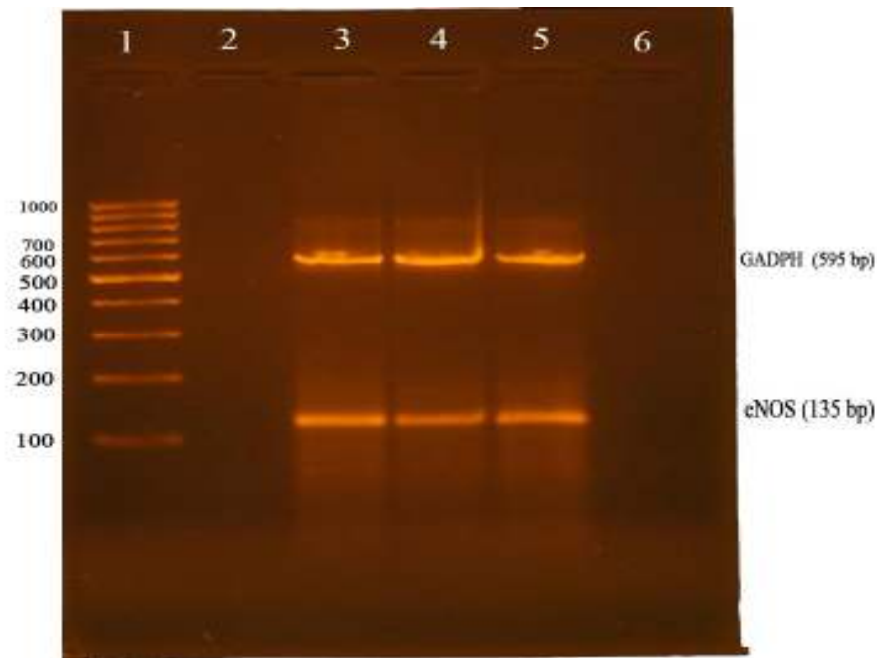


Figure 4: Agarose gel electrophoresis of RT-PCR product showing rat eNOS expression (135 bp) in heart tissue of different groups.

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

It has been established that poorly controlled long standing metabolic disorders, such as diabetes mellitus frequently results in other complications, including cardiovascular diseases [8]. This study reveals a number of important findings on the relationship between diabetes and the consumption of L-arginine. Studies have shown that L-arginine administration reduces lipid peroxide levels in diabetic patients, due to direct

scavenging of superoxide anions. It also enhances relaxation by increasing NO release, which in turn scavenges superoxide anions known to be elevated in diabetic arteries [9].

The results obtained from the present study which showed that treatment of diabetic rats with the L-arginine leads to significant reduction in blood glucose level is in agreement with reports by Broca et al. [10], and can be attributed to its effect on enzymes that digest carbohydrates leading to a decrease in glucose absorption in the intestine.

The significant reduction in level of MDA indicates a decrease in lipid peroxidation and antioxidant enzymes, catalase and reduced glutathione 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 [7].

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 reports by Fu et al. [11].

The antioxidant enzymes, reduced glutathione and catalase increased significantly ($P < 0.001$) after treatment with L-arginine from 0.5 mg/dl in diabetic group to 0.9 mg/dl in treated group, showing treatment with L-arginine increased reduced glutathione by 80%, and increased catalase by 55.5% from 11.9 U/L in diabetic group to 18.7 U/L in treated group. This data is in line with studies by Li et al. [12].

A defect in nitric oxide function develops very early in diabetes mellitus and affects vascular reactivity. Atherosclerotic cardiovascular disease that affects patients with diabetes mellitus may be resulted from the decrease of eNOS protein in endothelial cells [13].

Our results show that cardiac NO level was significantly decreased in diabetic rats (21 ± 9 nmol/mg protein) as compared with control group (43 ± 18 nmol/mg protein), and significantly increased in treated group (25 ± 6 nmol/mg protein) as compared with control group at $P < 0.001$.

Nitric oxide has important effects on cardiovascular system and affects cardiac function. Cardiac muscle contracts in response to chemical transmitters, oxygen tensions and physical factors by modulating transmembrane calcium currents, intracellular calcium level, and the contractile proteins [14].

Some studies show that diabetes stimulates alterations of calcium handling and decreases nitric oxide production in coronary endothelial cells, due to tetrahydrobiopterin deficiency, and in the contractile proteins that impair myocardial function independent of altered metabolism [15]. Although impairment of the NO pathway may activate atherogenesis, exogenous NO activates endothelium and downregulates pro-inflammatory activity, such as cytokine secretion, and functional activity, such as transendothelial migration of granulocytes [16].

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Conclusion

The study shows that administration of L-arginine to diabetic rats increases HDL, reduced glutathione and catalase, while it decreases LDL, TAG, Cholesterol and MDA. It also increased the expression of eNOS in heart tissues of diabetic rat.

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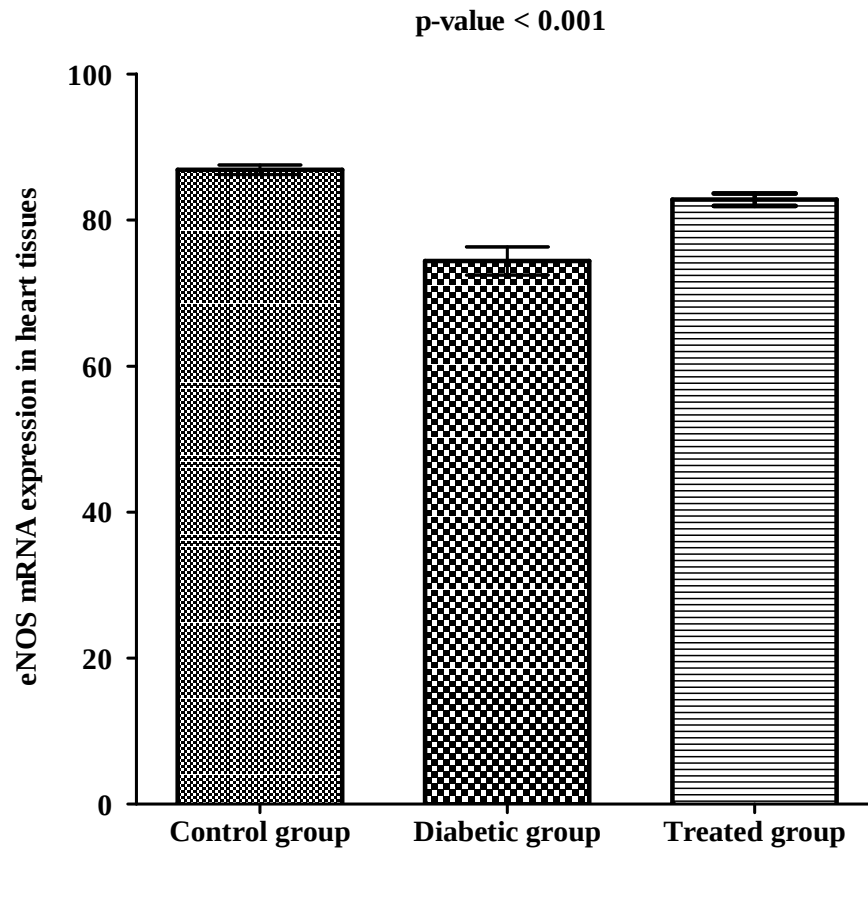


Figure 2: eNOS expression in heart tissues in different group (show significant decrease in diabetic group as compared to control group at P value <0.001, and significant increase in treated group as compared with diabetic group at P value <0.001).

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