Effect of L-arginine on the Expression of Endothelial Nitric Oxide Synthase (eNOS) in Heart Tissues of Diabetic Rats

Ahmed Mohamed M. Okasha[1] and Hend Mohamed Abdel Ghany[1*]

[1]*Department of Biochemistry, Faculty of Medicine, Minia University, 61511 Egypt.*

Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aims: This study aimed to determine the role of L-arginine on eNOS expression and its role on the level of some antioxidants as reduced glutathione and catalase in alloxan-induced diabetic rats.
Study Design: Ninety adult male albino rats were divided into three (3) groups of thirty rats (30) each: Group (I): Control group, Group (II*)*: Diabetic control group, Group (III): L-arginine treatment group. The second and the third groups were made diabetic by intraperitoneal administration of 80 mg/kg alloxan monohydrate, while L-arginine (100 mg/kg in sterile 0.9% Nacl) was given orally to rats in group III for one week before alloxan injection, and a further four weeks after induction of diabetes. The animals were sacrificed and blood collected for the determination of biochemical and 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.

_____

Corresponding author: E-mail: mostafa.gedan@gmail.com;

1. INTRODUCTION

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

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].

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 E 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].

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Diagnostic kits for catalase, reduced glutathione, cholesterol, triglycerides, HDL, LDL and malondialdehyde were obtained from Biodiagnostic (Egypt), Glucose kit 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.

2.2 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 manufacturers' instruction. Total RNA was extracted by Ribozol reagent from heart tissues for determination of expression of eNOS gene by RT- PCR.

2.3 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:

Nitrate/nitrite concentration =

$$\boxed{\frac{\text{Abs sample} - \text{blank Abs}}{\text{Slope of Std curve}}}\ (\mu L \text{ of sample})$$

2.4 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 -80ºC 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 follow: 5 min, at $95^{o}C$ (initial denaturation); 94ºC for 20 sec; $55^{o}C$ for 30 sec; 72ºC for 40 sec (30 cycles); and 72ºC for 5 min (final extension). The $MgCl_2$ concentration used for 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 54ºC, in a thermal cycler (Progene, Techne (Cambridge) LTD., UK) [7].

2.5 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.

2.6 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.

3. 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 56.9 nmol/ml in diabetic rats to 30.3 nmol/ml in treated group (Table 1). The cardiac NO level in diabetic rats was a significantly decrease from (25±6 nmol/mg protein) to (21±9 nmol/mg protein) in the treated and diabetic rat respectively.

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

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

4. 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.
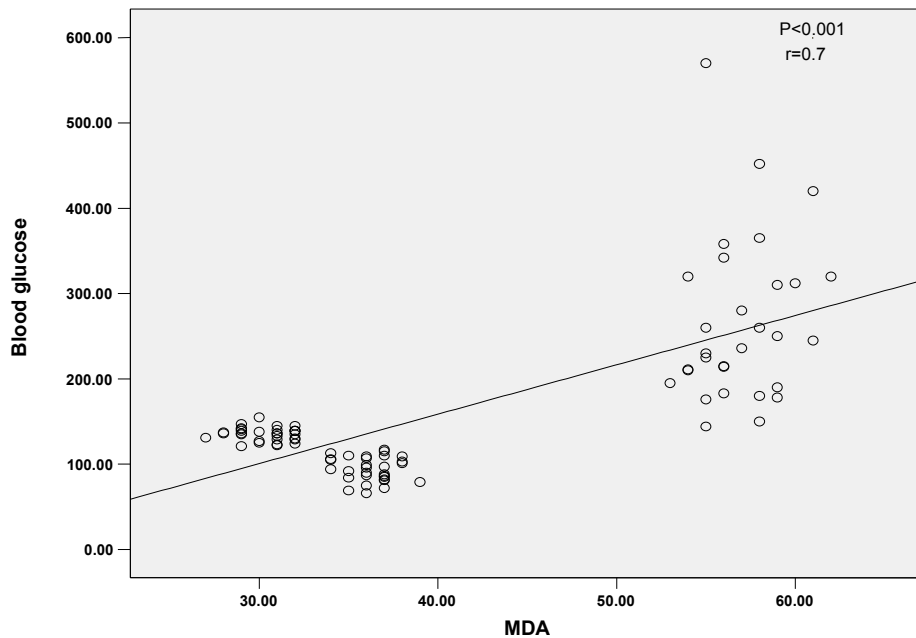

Fig. 1. Correlation between malondialdhyde (MDA) and blood glucose level in Alloxan-induced diabetic rats

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

	Group I (control) Mean ± SE	Group II (Diabetic) Mean ± SE	Group III (Treated) 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
Glutatione (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<0.001
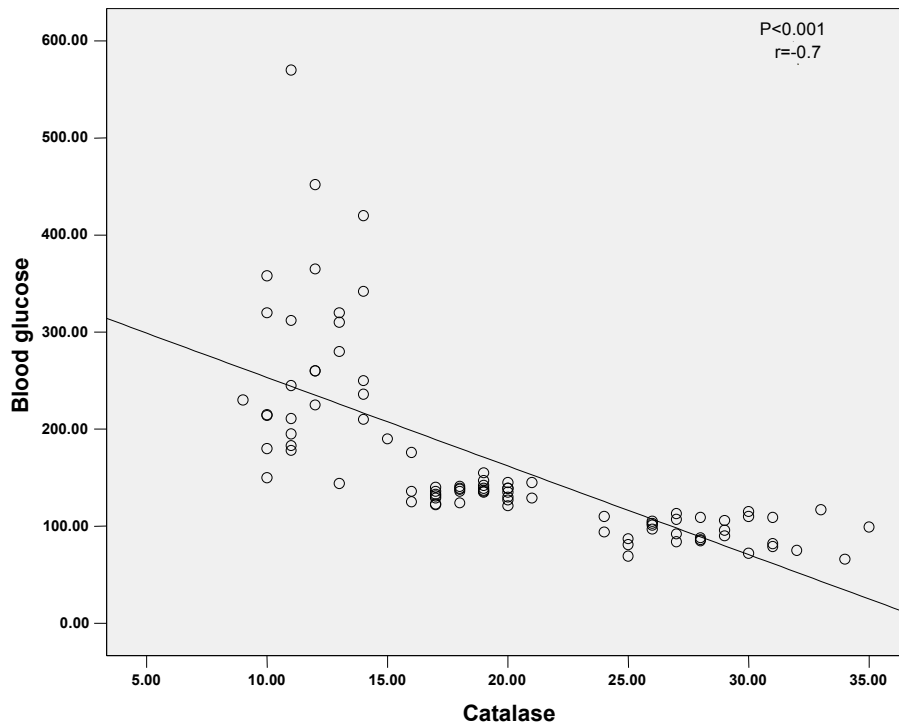

Fig. 2. Correlation between catalase and blood glucose level in alloxan-induced diabetic rats

The significant reduction in level of MDA indicates a decrease in lipid peroxidation and the 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 18.5 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 affect vascular reactivity. Atherosclerotic cardiovascular disease that affect patients with diabetes mellitus may be resulted from the decrease of eNOS protein in endothelial cells [13].
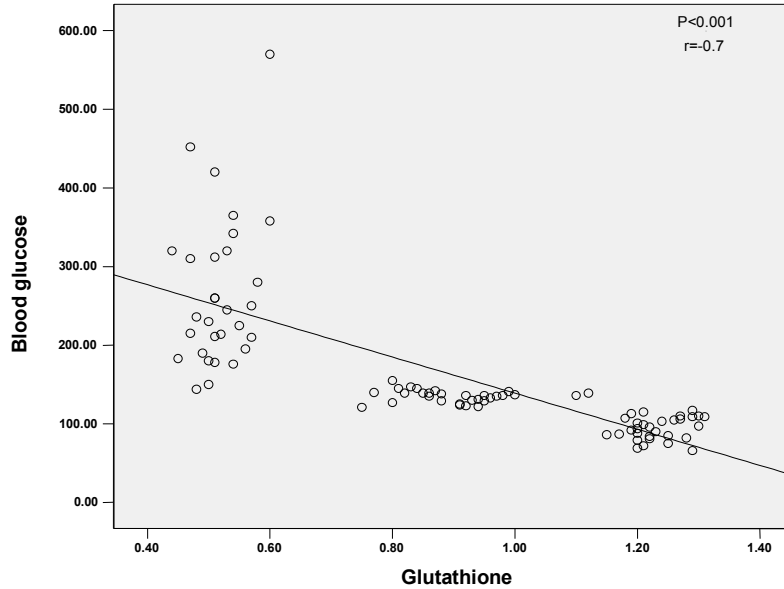

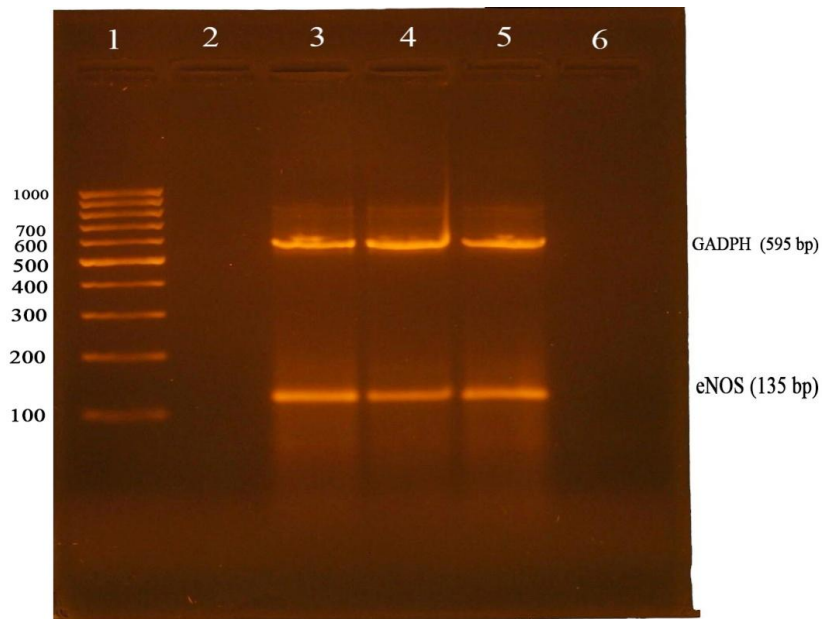**Fig. 3. Correlation between reduced glutathione and blood glucose level in alloxan-induced diabetic rats**

Fig. 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
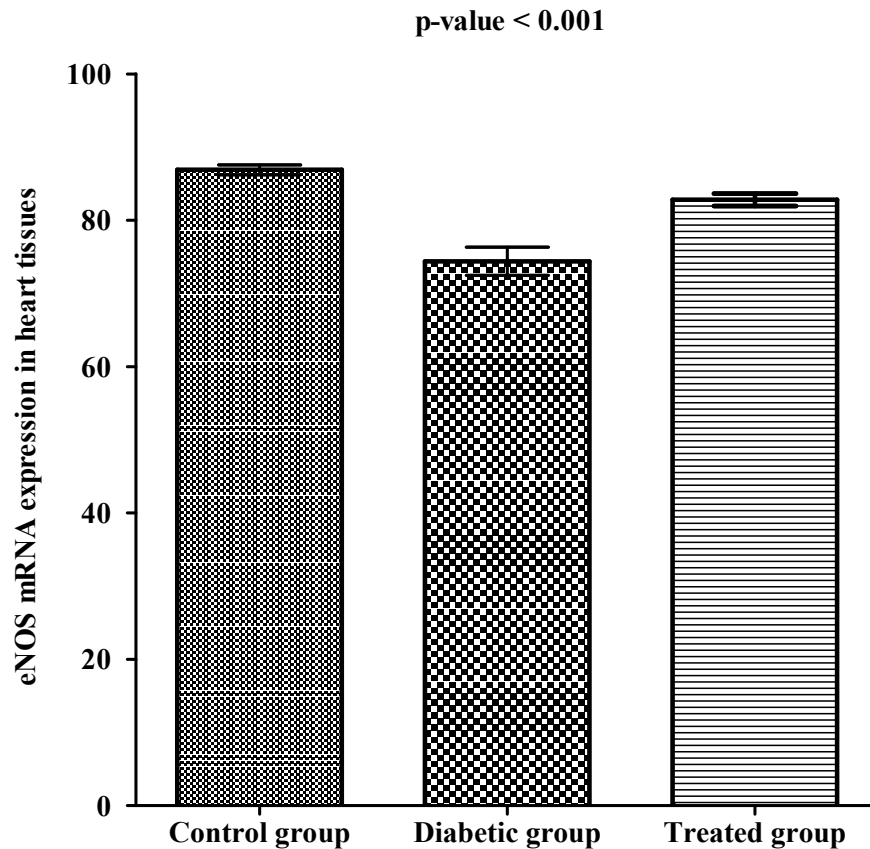
p-value < 0.001

Fig. 5. 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

Our results show that cardiac NO level was significantly decrease 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 diabetic group at P<0.001.

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

Some studies show that diabetes stimulate alterations of calcium handling and decrease 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 impairement of the NO pathway may activste atherogenesis, exogenous NO activate endothelium downregulates pro-inflammatory activity, such as cytokines secretion, and functional activity, such as transendothelial migration of granulocytes [16].

5. CONCLUSION

The study shows that administration of L-arginine to diabetic rats increase 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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Wild S, Roglic G, Green A, Sicree R, King H, Global prevalence of diabetes: Estimates for the year 2000 and projections for 2030. Diabetes Care. 2004;27(5):1047–53.
2. Renold AE. Streptozotocin and alloxan in experimental diabetes: Comparison of the two models in rats Diabetes Annu. 1988;4:592-608.
3. Maritim AC, sanders RA, Watkins JB. Diabetes, oxidative stress and antioxidant: A review. J Biochem Mol Toxicol. 2003;17:24–38.
4. Tapiero. L-Arginine. Biomedicine and Pharmacotherapy. 2002;56(9):439–445.
5. Förstermann U, Münzel T. Endothelial nitric oxide synthase in vascular disease: Circulation. 2006;113:1708-1714.
6. Mari F, Alfredo G, Maria A, Antonia P, Nicole L. Endothelial Nitric Oxide Synthase (ENOS) expression and localization in healthy and diabetic rat hearts. Annals of Clinical and Laboratory Science. 2001;31:179-186.
7. Innis M, Gelfand D, Sninsky J. PCR Protocols, a guide to methods and applications. Academic Press, San Diego. 1990;1-482.
8. Balligand JL, Cannon PJ. (Nitric oxide synthases and cardiac muscle. Autocrine and paracrine influences. Arterioscler Thromb Vasc Biol. 1997;17:1846-1858.
9. Sikorski BW, Hodgson WC, King RG. Effect of haemoglobin and N-nitro-L-arginine on constrictor and dilator response of aortic rings from streptozotocin diabetic rats. Eur J Pharmacol. 1993;242:275-282.
10. Broca C, Breil V, Cruciani GC. Aminoacid Support in the prevention of diabetes and diabetic complications: Am. J. physiol. Endocrinol. Metab. 2004;287:463.
11. Felaco M, Grilli A, Gorbunov N, Di Napoli P, De Lutiis MA, Di Giulio C, Conti P. Endothelial NOS expression and ischemia-reperfusion in isolated working rat heart from hypoxic and hyperoxic conditions. Biochim Biophyis Acta. 2000;1524:203-211.
12. Ritz E, Fliser D, Nowicki M. Hypertension and vascular disease as complications of diabetes. laragh JV, Bernner BM eds. New York, NY: Raven press Ltd 1995;2321–2334.
13. Caballero F, Gerez E, Batlle A, Vasquez. Preventive aspirin treatment of streptozotocin induced diabetes: Blockage of oxidative status and prevention of heme enzymes inhibition. Chem biol Interact 2000;126:215-225.
14. Mohan P, Brutsaert DL, Sys SU. Myocardial performance is modulated by interaction of cardiac endothelium derived nitric oxide and prostaglandins. Cardiovasc. Res. 1995:29:637-640.
15. Meininger CJ, Marinos RS, Hatakeiama K, Martinez-Zagauilan RD, Rojas J, Kelly KA, Wu G. Impaired nitric oxide production in coronary endothelial cells of the spontaneously diabetic BB rat is due to tetrahydro-biopterin deficiency. Biochem J 2000;349:353-356.
16. Fowler AA III, Fisher BJ, Sweeney LB, Wallace TJ, Natarajan R, Ghosh SS, Ghosh S. Nitric oxide regulates interleukin 8-gene expression in activated endothelium by inhibiting NF-kappa B binding to DNA: Effects on endothelial function. Biochem Cell Biol. 1999;77:201-208.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=845&id=3&aid=7411