INTERNATIONAL JOURNAL OF ADVANCES IN PHARMACY, BIOLOGY AND CHEMISTRY

Research Article

Total Nitric Oxide (NO) and Immunohistochemical demonstration of Nitric Oxide Synthase (NOS) in pancreatic tissue of rats with induced Diabetes mellitus by Alloxan

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ABSTRACT

Diabetes mellitus is a worldwide disease, and one of the major causes of death in human. Nitric oxide (NO), a short-lived, highly reactive free radical, influences physiological processes in virtually every organ and tissue. Free NO emerged as a fundamental signaling device to regulate critical cellular function, and is a potent mediator of cellular damage in some conditions. The aim study is to investigate the effect of induced diabetes mellitus of rats on the level of total NO and immunohistochemical demonstration of nitric oxide synthase (NOS) of pancreatic tissue. 30 male Wister rats were conducted in this study were divided randomized into control group two Diabetic groups were undergo for diabetes mellitus induction by IP injection of alloxan (Sigma) with 100 mg/kg B.W, The animals injected once per week for 4 weeks, blood and tissue samples were taken after finish the injections and after 10 days, samples used for measurement of total NO and immunohistochemistry of NOS. Results of total NO showed increase levels after injection of alloxan induced DM 21.06±0.52, the rats of group after 10 days of DM induction showed more increase of total NO 32.59±0.47 compare with normal rats which records 14.95±0.9. this difference was significant $P<0.01$. theimmunohistochemical results of NOS detection approve the total NO results that illustrate expression in few cells within islet of langerhans, after induction DM showed increase expression of NOS, after 10 days the results revealed a high expression appeared to be filled the whole cells in the islet of langerhans of diabetic rats. In conclusion, Hyperglycemia and diabetes mellitus is associated with an increased NO biosynthesis. Increased oxidative stress and free radical may contribute to the high NO levels in diabetes. Furthermore, the high expression of NOS progress associated with increase the complication of diabetes.

Key words: Nitric oxide (NO), nitric oxide synthase (NOS), Diabetes Mellitus, Alloxan, Immunohistochemistry.

1. INTRODUCTION

Diabetes mellitus is a group of metabolic disease characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Diabetes is characterized by progressive β cell loss in pancreas and, it is widely accepted that reactive oxygen species (ROS) contribute to pancreatic cell or tissue damage and dysfunction both in type 1 and 2 diabetes, even though the underlying mechanisms differ[1,2]. DM is a disorder characterized by hyperglycemia, it is a heterogeneous primary disorder of charbohydrate metabolism with varies aetiology culminating in absolute relative insulin deficiency or insulin resistance or both[3]. There is a reservoir of basic information that suggest the involvement of oxidative stress in the pathogenesis of diabetes mellitus[4].

Alloxan, a b-cytotoxic toxic glucose analogue, is commonly used for the development of animal model of Type-I Diabetes Mellitus[5]. Alloxan is rapidly taken up by the pancreatic b-cells through GLUT2

receptors [6, 7]. NO, a short-lived, highly reactive free radical, influence physiological processes in virtually every organ and tissue, Muriel[8] and Sydow *et al.*[9] mentioned that free radical NO emerged as a fundamental signaling device to regulate some critical cellular function. Furthermore, this case considers a potent mediator of cellular damage in several conditions. Ghosh and Darzi[10] reported that free radical NO exhibits a remarkably broad spectrum of functions, including neurotransmission and memory formation, regulation of blood pressure, mediation of the bactericidal and tumoricidal activity, and liver regeneration. In addition, free radical NO is produced from the amino acid L-arginine through reaction catalyzed by the enzymes nitric oxide synthase (NOS).

The present study aims to investigate the effect of induced diabetes mellitus of rats on the level of total NO and immunohistochemical demonstration of NOS of pancreatic tissue.

2. MATERIAL AND METHODS

2.1. Study design and animals groups

The study was carried out in laboratories of college of medicine – Wasit University, Iraq, from the period of December 2015 – April 2016. 30 male Wister rats were conducted in this study purchased from Institute of fertility and embryology research, Al-Nahrain University, and maintained in cages (3 rats/cage) under controlled temperature (20 – 22°C) and lighting conditions. The animals were fed a standard pellet diet and water ad libitum. Experimental protocols met the "Guidelines of Animal Experimentation, After 7 days of acclimatization, the animals were divided randomized into two experimental groups and third control group (n = 10 of each) which received saline IP, and two Diabetic groups were undergo for diabetes mellitus induction by IP injection of alloxan (Sigma) with 100 mg/kg B.W. The animals injected once per week for 4 weeks. After induction of diabetes mellitus (FSB > 200 mg/dl consider to be a diabetic) the animals of one of diabetes group were euthanized immediately , animals of other group were euthanized after 10 days of DM induction, Blood and pancreas tissue samples collected for total NO and immunohistochemistry of NOS detection respectively. The blood sugar of rats was estimated by Glucometer (Accua check glucometer, USA) using commercially available glucostix reagent strips.

2.2. Measurement of Total NO

Total NO was measured by the Griess reagent method using a kit purchased from Promega (USA) and according to the manufacturer's instructions. Briefly, standard dilutions were prepared to establish a standard curve and allowed the sulfanilamide solution and N-(1-naphthyl) ethylene diamine dihydrochloride (NED) solution to equilibrate to room temperature (15–30 min). There was 50µL of serum added from each sample to duplicate wells of a 96-well plate. 50 µL of the sulfanilamide was dispensed to each well, incubate the plate for 10 min at room temperature, protected from the light. There was 50 µL of the NED solution dispensed, then incubated again at room temperature for 10 min, protected from the light. The solution changed to a purple/magenta color. The absorbance of each well was determined in a plate reader at 535 nm and calculated the nitrite concentrations of the samples after constructing a standard reference curve.

2.3. Immunohistochemistry

NO synthase primary antibody (Sigma, USA), Streptavidin-biotin-peroxidase compound(SABC), 3,3_-diaminobenzidine (DAB), biotinylatedgoatanti rabbit IgG and bovine serum albumin (BSA) were purchased from BosterBio (CA). Pancreatic tissues samples were fixed in 10% formalin, dehydrated in ethanol, embedded in paraffin wax and sectioned at 5 µm thick. After dewaxing, rehydration, inactivation of endogenous enzymes with 3% H_2O_2 for 30 min, washing in D.W. then we added 5% BSA (confining liquid) and incubated at room temperature for 20 min. The sections were incubated in primary antibodies against NO for 14 hrs at 4◦C and washed in PBS. Biotinylated goat anti-rabbit IgG (secondary antibody) incubation for 30 min at 20–37◦C, after that washing and incubated in SABC 37◦C for 30 min. After a final wash, tissues were stained with DAB for 30 min, counter-stained with Harris hematoxylin, and mounting.

2.4. Statistical Analysis

All values were expressed as the mean ± SEM. The data were analyzed by the Student's *t*-test. Numbers per group were as indicated. *P* value less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Fasting Blood glucose

Table 1 were summarized the results of fasting blood sugar of the rats of control group and diabetic groups before starting and after induction of diabetes mellitus with alloxan injection. In the control group the results of blood sugar were 89.31± 0.95 mg/dl and 91.46±0.82 mg/dl, at starting and after finish the experiment respectively, while in the animals of diabetic group recoded 93.44±1.06 and 495.52±0.75 before and after alloxan injection respectively, the

results of fasting blood sugar on 10 days after DM induction were 524.4±0.54, the statistical analysis of blood sugar concentrations explained a highly significant $P<0.01$compared with normal and control records.

3.2. Total Nitric Oxide (NO)

The results of total nitric oxide were summarized in (Table 2) represented as mean±SEM , briefly the results of control animals showed similar records 14.95±0.9, after alloxan injection and induced DM, NO increased than record of control group, the results were 21.06±0.52, this difference compare with control statistically was significant $P<0.01$, after 10 days of DM induction the total nitric oxide revealed more increase in concentrations 32.59±0.47, this decrease was significantly compared with control and the step after injection $P<0.01$.

3.3. Immunohistochemistry results.

The biochemical results of total nitric oxide approved by immunohistochemistry demonstration of nitric oxide synthase of the diabetic animals, the results of control animals a few cells revealed expression of NOS in islet of Langerhans of the pancreatic tissue as shown in (Figure 1), after injection of alloxan and induced DM the immunohistochemical results revealed a markedly increase of NOS expression in the cells in islet of Langerhans of those animals which illustrated in (Figure 2- A,B), after 10 days of injection the NOS expression showed a more increased expression of NOS than at time of DM induction revealed to fill the whole cells in islet of Langerhans attributed to adverse effect of diabetes millets on the expression and production of nitric oxide in the pancreatic tissue especially in islets of Langerhans as shown in (Figure -3 (A,B).

4. DISCUSSION

The present study was conducted to investigate the total NO and in situ demonstration in rats with induced DM, the study showed increase the level of NO and NOS expression in the animal with diabetes mellitus compare with normal rats this changes come parallel with progression with DM prognosis, we attempted to identify the hypothesis the effect of DM on NO levels. Several lines of evidence have shown that diabetes mellitus and complication therefore from the disorder can arise due to oxidative stress, a fewer publishes reported previously concerning with effects of diabetes mellitus on the nitric oxide and nitric oxide synthase , diabetes mellitus in rats induced by alloxan administration attributable to a specific irreversible toxic effect of alloxan on beta cell of pancreas. Alloxan is rapidly reduced in the body forming dialuric acid that undergoes auto-oxidation yielding detectable amounts of hydrogen peroxide, superoxide anion, and hydroxyl free radicals. Mir and Darzi[11] observed thatthese reduced species of oxygen, particularly the extremely reactive OH radical, are believed to initiate alloxan-based attack on beta cells. The deleterious effects of alloxan causing hyperglycaemia, might be due to rapid inhibition of insulin secretary mechanism. Once Nitric oxide represent a free radical molecules which focuses the effect of hyperglycemia of product NO and NOS, Nitric oxide is likely to be involved in the defective insulin-mediated stimulation of blood flow in type 2 diabetes [12] as well as in the pathogenesis of diabetic nephropathy [13]. Zierath *et al.*[14] reported that the stimulation of NOS activity is downstream effect of Akt activation by insulin. Therefore, insulin resistance may be leading to reduce nitric oxide production in type 2 diabetes.

A previous study revealed the site of NOx production is likely the endothelial cells, which express the bulk of constitutive NOS activity responsible for NO release into the bloodstream[15]. Whether the observed defects in NOx metabolism are due to diabetes itself, to the accompanying nephropathy or to insulin resistance, possibly amplified by hypertension[14].

On the other hand insulin, besides its stimulatory effect on NOx production, also increased NOx removal. It has recently been demonstrated that the prolonged exposure of endothelial cells to high glucose increases both NO and superoxide anion production [16]. The increase in NO is due to increased expression of NOS mRNA and NOS protein expression. Although NO metabolites may be increased in diabetes, bioactive NO is reduced which might be explained by increase in superoxide production which overrules the increased NO and decreases its bioavailability by reacting with it[17]. That approved our results detecting the effect of diabetes on NO and the increase of NO and NOS levels come due to the diabetes mellitus.

Ramakrishna and Rama[18] demonstrated that due to alloxan is an effective pro-oxidant to ß cells of the pancreatic islets of Langerhans cause single stranded breaks in the islets cell DNA. It is now recognized that sustained hyperglycemia in diabetic patients, causes protein glycation and genertates free radicals through auto oxidation and polyol pathway [19] high levels of free radicals with concurrent decline of antioxidant defence mechanism may lead to the damage of cellular organelles and enzymes [20] this can culminate in increased lipid peroxidation and development of insulin resistance, which may consequently promote the development of complications of diabetes mellitus.[21]. NOS from

which NO is derived, is a pH dependent enzyme. Tessari *et al.*[21] mentioned that NOS is active slightly in alkaline conditions, but is suppressed in acidic conditions. In diabetes, glycolysis and ketoacidosis force pH toward acidic conditions.

Asymmetrical dimethyl arginine (ADMA) normally, does not accumulate in the blood because it is rapidly eliminated in the urine through normal kidney function, may prevent the elimination of the major NOS inhibitor, ADMA, thereby limiting the production of NO [22].

CONCLUSION

Hyperglycemia and diabetes mellitus is associated with an increased NO biosynthesis. Increased oxidative stress and free radical may contribute to the high NO levels in diabetes. Furthermore, the high expression of NOS progress associated with increase the complication of diabetes.

ACKNOWLEDGMENTS

We are grateful to the staff of animal house unit/ College of Science / Wasit University, especially Dr. Zina. In addition, we thankful for staff Microbiology and Histology laboratory departments / College of Medicine / Wasit University for providing requirements and support this work. Furthermore, we are grateful to Dr. Shaima for outstanding efforts.

Table 1

The concentrations of fasting blood sugar of animals groups (mg/dl).

Animal group	Normal state	After $CdCl_2$ injection	7 days after injection
Control group	89.31± 0.95	93.44±0.86	92.56±0.82
Diabetic group *	91.46±1.06	495.52±0.75	524.4±0.54

(*) difference between the diabetic conditions and control records significant (P<0.01)

Table 2

The concentrations of total nitric oxide (NO) of animals groups (µmol/L).

Animal group	Normal state	After $CdCl_2$ injection	7 days after injection
Control group	14.95±0.9	14.58±0.69	13.92±0.55
Diabetic group **	14.86±0.5	21.06±0.52	32.59±0.47

(**) difference between the diabetic conditions and control records significant (P<0.01)

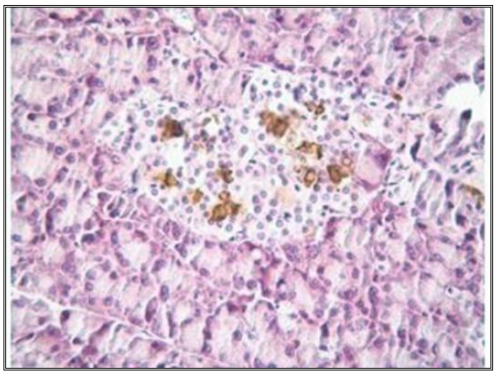

Figure 1

Immunohistochemistry of NOS detection in cells islet of Langerhans of pancreas of rat of control group, only few cells showed the expression (arrows) of NOS. (X400)

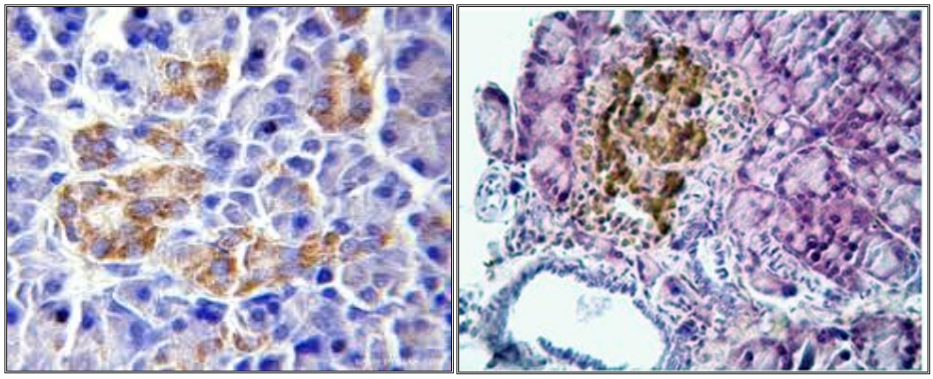

Figure 2

immunohistochemistry of NOS detection in cells islet of Langerhans of pancreas of rat of diabetic group after DM induction showed higher expression of NOS in the cells as appeared in brown color (arrows). X400

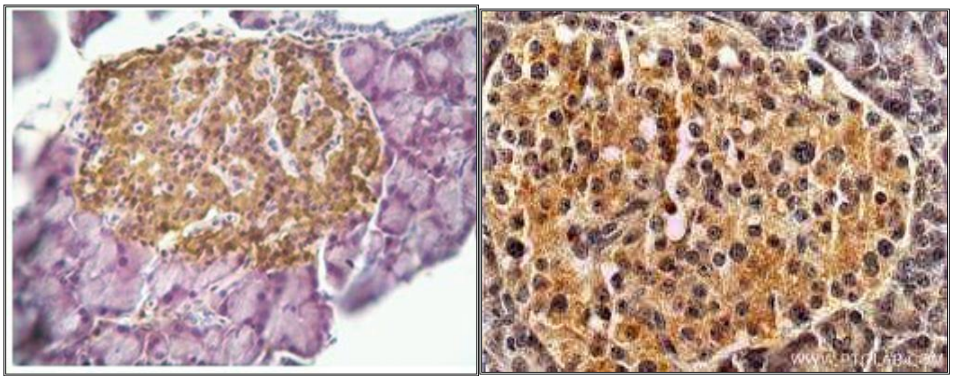

Figure 3

immunohistochemistry of NOS detection in cells islet of Langerhans of pancreas of rat of diabetic group after 10 days of DM induction showed extensive expression of NOS in whole cells within the islets as appeared in brown color (arrows). X400

REFERENCES

1. Cnop M, Welsh N, Jonas JC, Jorns A, Lenzen S, Eizirik DL. Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. Diabetes, 2005; 54 (2): S97–S107.
2. Kikumoto Y, Sugiyama H, Inoue T, Morinaga H, Takiue K, Kitagawa M, Fukuoka N, Saeki M, Maeshima Y, Wang D, Ogino K, Masuoka N, Makino H. Sensitization to alloxan-induced diabetes and pancreatic cell apoptosis in acatalasemic mice. Bioch. etBioph. Acta. 2010; 1802(2): 240–246
3. International expert committee report on the role of the A1C assay in the diagnosis of diabetes . Diabetes care. 2009. 32:1327 – 1334.
4. Gwarzo MY, Nwachukui VA, Lateef AO. Prevention of alloxan induced dianbetes mellitus in rat by vitamin a dietary supplementation. Asian J Anim. Sci. 2010; 4(4): 190 – 196.
5. Szkudelski T. The mechanism of alloxan and streptozotocin action in b-cells of the rat pancreas. Physiol. Res. 2001, 50(6):536–46.
6. Elsner M, Tiedge M, Guldbakke B, Munday R, Lenzen S. Importance of the GLUT2 glucose transporter for pancreatic beta cell toxicity of alloxan. Diabet. 2002; 45(11): 45:1542–9.
7. Mir MS, Darzi MM, Khan HM, Kamil SA, Sofi AH, Wani SA. Pathomorphological effects of Alloxan induced acute hypoglycaemia in rabbits. Alexandria J of Med. 2013; 49(4): 343–353.
8. Muriel P. regulation of nitric oxide synthesis in the liver. J Appl. Toxicol. 2000, 20(3): 189 – 95.
9. Sydow K, Mondon CE, Cooke JP. Insulin resistance: Potential role of the endogenous

nitric oxide synthase inhibitor ADMA. Vasc Med. 2005; 10:S35–43.
10. Ghosh A, Sherpa ML, Bhutia Y, Pal R, Dahal S. Serum nitric oxide status in patients with type 2 diabetes mellitus in Sikkim. Int J Appl Basic Med Res. 2011; 1(1): 31–35.
11. Mir SH, Darzi MM. Histopathological abnormalities of prolonged alloxan-induced diabetes mellitus in rabbits. Int. J. Exp. Path. 2009; 90(1): 66–73.
12. Tessari P, Cecchet D, Cosma A, Vettore M, Coracina A, Millioni R, Iori E, Puricelli L, Avogaro A, Vedovato M. Nitric Oxide Synthesis Is Reduced in Subjects With Type 2 Diabetes and Nephropathy. *Diab.* 2010; 59(9): 2152–2159.
13. Vincent MA, Montagnani M, Quon MJ. Molecular and physiologic actions of insulin related to production of nitric oxide in vascular endothelium. Curr. Diab. Rep. 2003; 3(4): 279-88.
14. Zierath JR, He L, Guma A, Odegoard Wahlstrom E, Klip A, Wallberg-Henriksson H. Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NIDDM. Diabetol.1996; 39(10):1180-1189.
15. Chu S, Bohlen HG. High concentration of glucose inhibits glomerular endothelial eNOS through a PKC mechanism. Am. J Physio.l. Ren. 2004; 287(3): F384–F392.
16. Maejima K, Nakano S, Himeno M, Tsuda S, Makiishi H, Ito T, Nakagawa A, Kigoshi T, Ishibashi T, Nishio M, Uchida K. Increased basal levels of plasma nitric oxide in type 2 diabetic subjects. Relationship to microvascular complications. J Diab. Complic. 2001; 15(3):135–143.
17. Zahedi AS, Ghasemi A, Azizi F. Serum nitric oxide metabolites in subjects with metabolic syndrome. Clini. Biochem. 2008; 41(16-17):1342–1347.
18. Ramakrishna V and J Rama. Evaluation of oxidative stress in insulin dependent diabetes mellitus (IDDM) patients. Diagn.Pathol. 2007, 2:22-22.
19. Ottaviano FG, Handu DE and Loscalzo J. Redox regulation in the extracellular environment. Circ. J. 2008; 72(1): 1-16.
20. Demozay D, Mas JD, Rocchi S and Van obberghen E. FALDH reverses the deleterious action of oxidative stress induced by lipid peroxidation product 4-hydroxynonenal on insulin signaling in 3T30L1 adipocytes. Diab. 2008; 57(5): 1216-1226.
21. Muniyappa R, Quon MJ. Insulin action and insulin resistance in vascular endothelium. Curr. Opin. Clin. Nutr. Metab. Care. 2007; 10(4): 523–530.
22. Tessari P, Coracina A, Puricelli L, Vettore M, Cosma A, Millioni R, Cecchet D, Avogaro A, Tiengo A, Kiwanuka E. Acute effect of insulin on nitric oxide synthesis in humans: a precursor–product isotopic study. Am. J Phys. Endoc. Metab. 2007; 293(3):E776–E782.