

SEROTONIN-PROMOTED ELEVATION OF ROS LEVELS MAY LEAD TO CARDIAC PATHOLOGIES IN DIABETIC RAT

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Abstract: Patients with diabetes mellitus (DM) develop tendencies toward heart disease. Hyperglycemia induces the release of serotonin from enterochromaffin cells (EC). Serotonin was observed to elevate reactive oxygen species (ROS) and downregulate antioxidant enzymes. As a result, elevated levels of serotonin could contribute to diabetic complications, including cardiac hypertrophy. In the present study, diabetes mellitus was induced in rats by alloxan administration; this was followed by the administration of serotonin to experimental animals. ROS, catalase (CAT), superoxide dismutase (SOD), B-type natriuretic peptide (BNP) expression, and histopathological assessments were performed. Elevated ROS concentrations and decreased antioxidant enzyme activities were detected. Further, we observed an increase in cell surface area and elevated BNP expression which suggests that events associated with cardiac hypertrophy were upregulated in serotonin-administered diabetic rats. We conclude that serotonin secretion in diabetes could contribute to diabetic complications, including cardiac hypertrophy, through enhanced ROS production.

Key words: Serotonin; ROS; catalase; SOD, diabetes and cardiac hypertrophy

Received September 8, 2014; **Revised** October 3, 2014; **Accepted** October 9, 2014

INTRODUCTION

Diabetes mellitus (DM) is a complex disease characterized by imbalance in carbohydrate, lipid and protein metabolisms and is suggested to be a risk factor for heart disease. Type 1 and type 2 diabetes (T1D, T2D) differ in their clinical manifestations; however, they share some biochemical alterations, including oxidative stress and hyperglycemia. Imbalance in oxidative regulation and other signaling pathways promotes the develop-

ment of a strikingly similar set of complications (Mushtaq et al., 2013; Brownlee et al., 2005). It is believed that, in response to hyperglycemia (oral as well as luminal glucose), insulin secretion is mediated through the release of glucose-dependent insulinotropic polypeptide (GIP) as well as glucagon-like peptide (GLP-1) from gut enteroendocrine cells (EECs) (McCullough et al., 1983; Reimann et al., 2002). This glucose-sensing mechanism by different cells of the gut has been well studied in animal models as well as in culture cells (Jang et al., 2007).

It has been reported that luminal glucose also induces the release of serotonin or 5-hydroxytryptamine (5-HT), from enterochromaffin (EC) cells in the gut wall (Masuda et al., 1997; Raybould et al., 2003). Serotonin is a biogenic amine produced by numerous cell types, including enterochromaffin cells of the gut; a large amount is stored by platelets and released upon activation. Serotonin is involved in the stimulation of the cardiac sympathetic afferent nerve and causes coronary artery contraction during ischemia, supporting the role of serotonin in the regulation of myocardial structure and function via its receptors such as 5-HT_{2B}, 5-HT_{2A}, presented on the surface of many cell types (Bianchi et al., 2005). Foussal et al (2010) demonstrated that serotonin induces hypertrophy in cardiomyocytes through an intracellular signaling pathway involving monoamine oxidase (MAO)-dependent free radical generation. Growing evidence supports the role of serotonin as a stimulus of ROS-mediated cardiac hypertrophy (Bianchi et al., 2005).

It is well known that ROS generated by different sources (Cash et al., 2007) induces downregulation of the antioxidant system. The elevated oxidative stress is believed to be involved in the initiation of cardiac dysfunction and hypertrophy, leading to heart failure (Takimoto et al., 2007). Growing evidence indicates that an

inadequate antioxidant defense capacity plays an important role in cardiac hypertrophy. In cardiomyocytes, catalase is a target of numerous hypertrophic stimuli causing a decrease in catalase level and increase in ROS concentration (Murtaza et al., 2008 and 2013). Accumulating evidence supports the hypothesis that increased ROS levels in concert with downregulation of the antioxidant capacity play a critical role in the progression of cardiac hypertrophy.

The aim of this study was to evaluate potential ROS-dependent mechanisms in diabetes that are linked to cardiac hypertrophy in an animal model, and to determine the antioxidant status of serotonin-treated animals.

MATERIALS AND METHODS

Reagents

N,N-diethyl-para-phenylenediamine DEPPD (Fluka Analytical), 0.1M sodium acetate buffer pH 4.8 (VWR International Ltd), ferrous sulfate (Sigma Aldrich), phosphate buffer (pH 5.5) and H₂O₂ were used. All chemicals were of analytical grade and commercially available.

Table 1. General characteristics of experimental animals treated with alloxan and serotonin

Groups	body weight (bw) in g		glucose level (mg/dl)		
	initial bw	final bw	at start	mid	at end
Alloxan + Serotonin	174	169	92	295	188
Serotonin	169	192	82	77	74
Alloxan	202	107	78	415	521
Saline	173	177	90	99	107

Animal body weights at the start and end of the experiment, and glucose concentrations before treatment, at the start of the experiment, at mid point and at the end of the experiment, were measured.

Experimental design

The high levels of serotonin in diabetic patients and its role in diabetic complications have been previously reported. To determine the effects of serotonin in diabetes, endogenous serotonin levels were increased in diabetic rats. Sprague-Dawley rats were obtained from the Pakistan National Institute of Health and divided into four groups ($n = 5$ each group, each rat weighing between 160-200 g). Group I was administrated with a single injection of alloxan; after induction of diabetes, the rats were given daily intraperi-

toneal (i.p) injections of serotonin (60 mg/kg). Group II was administrated with daily injections of serotonin for 10 days. Group III (diabetic control) received a single dose of alloxan (120 mg/kg). Group IV (normal control) received daily injections of normal saline (1.2 ml).

All experimental animal handling was as prescribed by the Institutional Experimental Model Ethical Committee and EC guidelines for animal experiments. Animals were fed regularly and maintained well at the animal house facility of Quaid-i-Azam University.

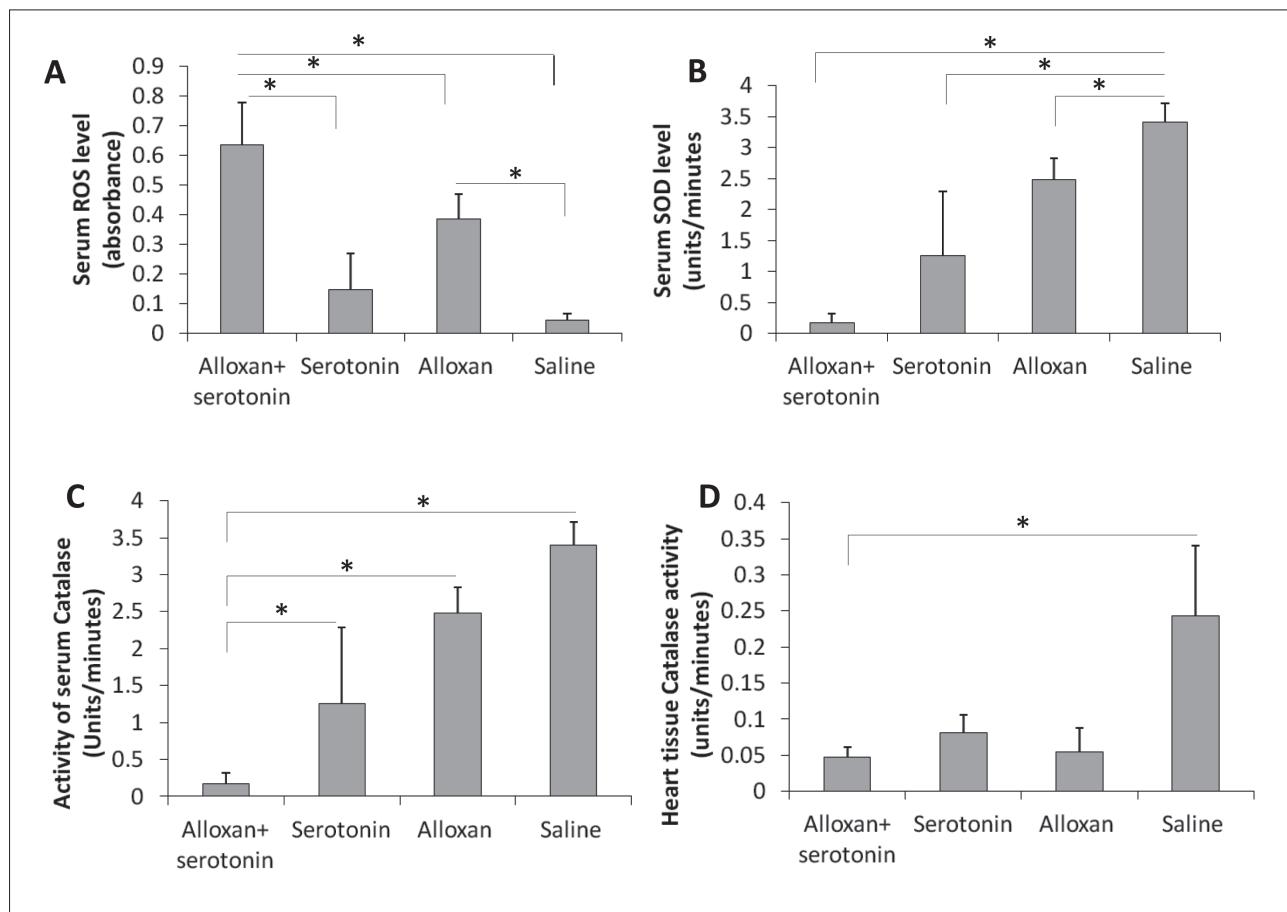


Fig. 1. Serotonin downregulates antioxidant enzymes via ROS generation in diabetes. ROS and antioxidant analysis in experimental groups (alloxan + serotonin, serotonin, alloxan and saline). **A.** Quantification of serum free radicals. Graphs show the average ROS level (absorbance) in different experimental groups. **B.** Antioxidant quantification of serum SOD activities (U/min). **C.** Serum catalase activities (U/min) in different experimental groups. **D.** Quantification of catalase levels in heart tissues. Data are expressed as mean \pm S.D. Graphs are representative of at least three experiments performed in triplicate; * P value is significant when less than 0.05.

Animal procedures

Rat body weights (Table 1) and morning fasting blood glucose concentrations were measured before the treatments and at the end of the treatment. After 10 days, all rats were killed, the blood was collected and heart tissues were excised and stored either in liquid nitrogen at -80°C or in formalin until further experimental analysis.

Serum ROS measurement

The serum free radical status was analyzed according to the previously developed method (Hayashi et al., 2007).

Catalase activity in serum and heart tissues

Serum catalase level was measured using H₂O₂ as a substrate (Aebi et al., 1974). The difference in the level of absorbance at 240 nm was used to calculate the enzymatic activity of catalase in units/min. One unit is described as a difference of 0.01 in O.D. taken at an interval of one min.

SOD activity of serum samples

Serum SOD activity was measured as described previously (Wayne et al., 1987).

Histopathology of heart tissue

Formalin-fixed heart specimens were embedded in paraffin, 3-μm sections were prepared and were subjected to hematoxylin-eosin staining for morphological evaluation. Sections were then observed under polarized light and bright field microscope of different magnifications.

Cell surface area measurement

To determine the cell arrangement and size of heart, tissue slides were developed and observed under light microscope at 40x magnification. Cell surface areas were measured by SPOT 4.0.

Detection of stress marker (BNP) by immunoblotting

Serum samples were used for detection of BNP transcription factors by Western blotting. Samples were subjected to SDS-PAGE and transferred onto nitrocellulose membranes (pore size 0.45 μm) (Santa Cruz Biotechnology) according to the manufacturer's instructions. After blocking with non-fat milk, the membranes were incubated with primary antibody (anti-BNP; FL-134 a rabbit polyclonal IgG, Santa Cruz Biotechnology, California, U.S) (1:500 dilution), overnight at 4°C, and then with goat anti-rabbit antibody IgG-AP (Santa Cruz Biotechnology) (1:2000 dilution) for 1 h at room temperature. The membranes were stained for 30 min in alkaline phosphatase (AP) development solution containing 1 ml of 1X AP reaction buffer with each 50 μl of NBT and BCIP solutions (Tiangen, Beijing, China).

Statistical analysis

Statistical analysis was performed with SPSS 16; p<0.05 was considered significant.

RESULTS

Glucose concentrations and heart weights of experimental model

Elevated blood glucose is a hallmark of both type 1 and type 2 diabetes. Glucose levels are presented in Table 1. After the administrations of alloxan, the glu-

cose level was increased compared to normal rats. After administration of serotonin, the glucose concentration decreased when compared to the diabetic group. The heart weight/tibia length ratio was increased in serotonin-treated diabetic rats (Fig. 2 B).

Serotonin increases free radical generation in diabetic rats

Significantly increased ROS levels were observed in the sera of diabetic and serotonin-treated diabetic rats as compared to normal saline-treated rats

(Fig. 1A). In addition, a non-significant increase in ROS level in the serotonin-treated group when compared to the normal saline-treated group was observed. These findings indicate that there may exist a correlation between serotonin and free radicals that further precipitates disease progression.

Downregulation of antioxidant enzymes (CAT and SOD) in serotonin-treated groups

Examination of the effect of serotonin on anti-oxidant enzymes in alloxan-induced diabetic rats

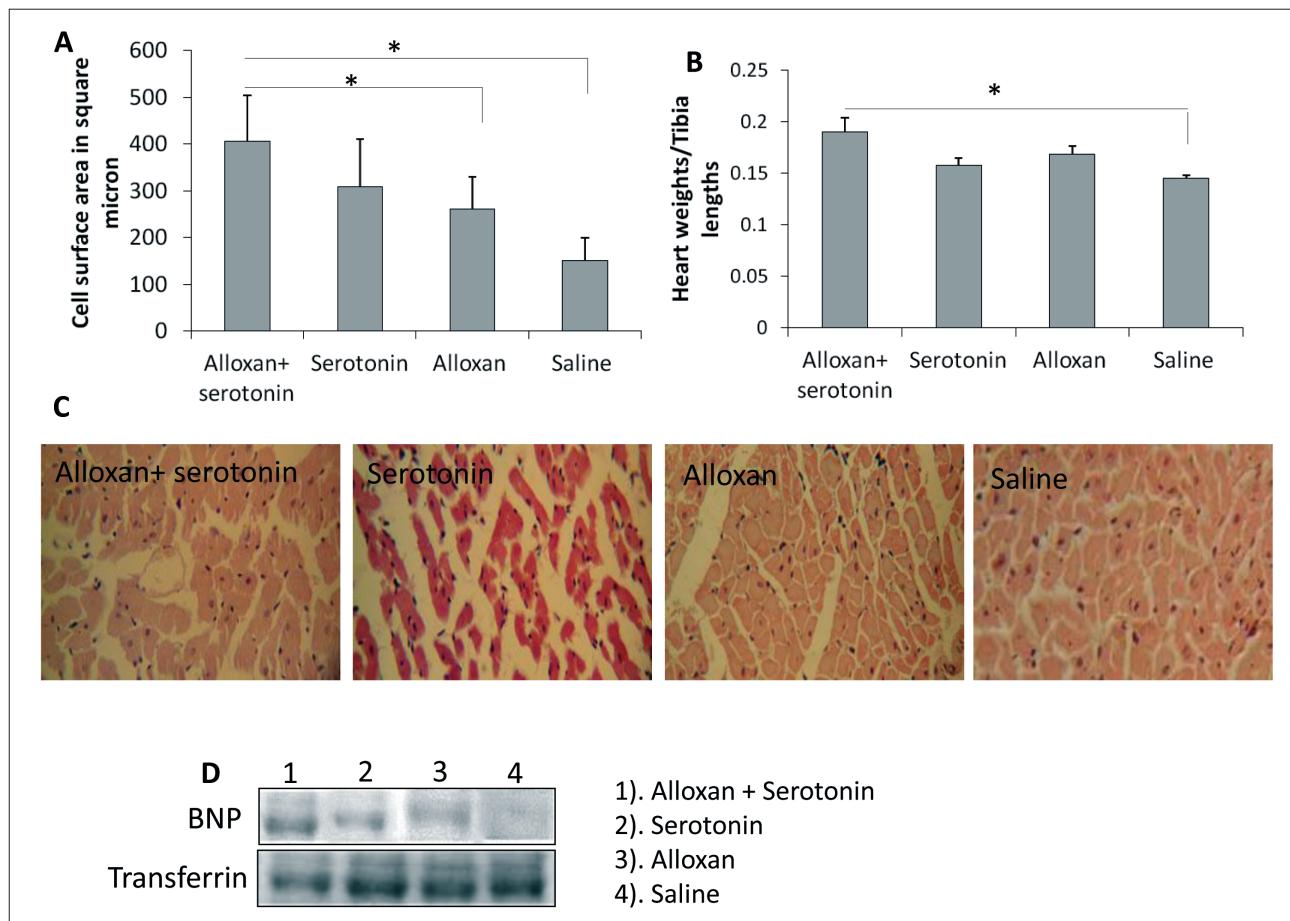


Fig. 2. Enhanced serotonin level in diabetes participates in cardiac pathologies. Cardiac hypertrophy analysis in experimental groups (alloxan + serotonin, serotonin, alloxan and saline). **A.** Cell surface areas (μ^2) analysis as a marker of cardiomyocyte hypertrophy. **B.** Experimental group heart weights/tibia length ratio analysis. **C.** Hematoxylin-eosin staining of heart tissue. **D.** Examination of the expression of the marker of cardiac hypertrophy, BNP, by immunoblotting (transferrin served as a positive loading control). All data are representative of at least three experiments performed in triplicate; * P value is significant when less than 0.05.

revealed a significant decrease in SOD level in the alloxan + serotonin as compared to the saline group (Fig. 1B). Similarly, downregulation in SOD levels in the serotonin-treated group was observed when compared with the normal saline-treated group. Catalase levels were significantly decreased in the alloxan + serotonin- and serotonin-treated as compared to the normal saline-treated group, suggesting that serotonin affects the catalase activity (Fig. 1C).

Effect of serotonin on histology and cell surface area in heart tissue

A significant increase in cell surface and disorder in cardiac tissues was observed in the alloxan + serotonin group when compared to other groups, including the control saline-treated group (Figs. 2A and C). Along with other histological changes, elevations in heart weight and tibia length ratio were observed (Fig. 2B). These findings suggest a possible link between diabetes and cardiac hypertrophy via enhanced serotonin production.

Expression of B-type natriuretic peptide (BNP) in heart tissue

BNP is considered a hallmark of cardiac disorders as it has a beneficial effect on the failing heart. Serum BNP expression was observed by standard immunoblotting; transferrin was served as a positive loading control (Fig. 2 D).

DISCUSSION

Previously it was reported that both luminal and oral glucose levels induce the release of serotonin from enterochromaffin cells of the gut, and that receptors for serotonin present on the surface of many types of gut cells are responsible for the ac-

tivities of serotonin (Bianchi et al., 2005; Foussal et al., 2010). Hara et al (2011) reported increased serotonin concentrations in patients with DM. Similarly, Bainchi et al (2005) determined the role of serotonin in cardiac myocyte hypertrophy via ROS generation. Based on this evidence, we hypothesized a serotonin-based link of diabetes to diabetic complications, including cardiac hypertrophy. The observed decreased glucose levels in serotonin-treated diabetic rats confirm the effect of serotonin (Yamada et al., 1989) in an experimental model of diabetes.

We demonstrated in our study that free radical generation in diabetes is accelerated through serotonin elevation, which contributes further to the pathological condition in diabetes, including an imbalance in the body's antioxidant system. As serotonin induces free radicals, which cause different abnormalities, the elevation in serotonin secretion in diabetes links diabetes to diabetic complications such as diabetic cardiac hypertrophy. D'Autreux et al. (2007) reported that free radicals play an important role in human physiological and pathophysiological processes. ROS react with proteins, lipids, carbohydrates and nucleic acids, inducing irreversible functional alterations or complete destruction of cells (Krause et al., 2007). ROS is balanced by the body's natural defense system consisting of antioxidant enzymes such as CAT and SOD (Brieger et al., 2012). The increased cell surface area (Murtaza et al., 2011) and heart weights, along with BNP expression in serotonin-treated alloxan-induced diabetic rats further support the suggested serotonin-based link between diabetes and cardiac hypertrophy which is associated with perturbations in cardiac myocyte physiology and morphology (Liu F et al., 2014).

In conclusion, increased glucose levels in diabetes are responsible for serotonin secretion from enterochromaffin cells of the gut that subsequently

accelerate ROS production. ROS induce downregulation of the antioxidant enzymes such as CAT and SOD which play important roles in diabetic complications, including cardiac hypertrophy. The use of serotonin-releasing agents as recreational and psychoactive drugs must be addressed very strictly since in diabetes these agents augment the risk of cardiac pathologies.

Acknowledgments: This study was funded by the Higher Education Commission (HEC) of Pakistan through a PhD scholarship to Tahir Ali.

Conflict of interest disclosure: None.

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