AMELIORATION OF ALTERED ANTIOXIDANT STATUS BY SODIUM-ORTHOVANADATE AND AZADIRACHTA INDICA LEAF EXTRACT ON CARDIAC AND SKELETAL MUSCLES ANTIOXIDANT DEFENCE SYSTEM IN STREPTOZOTOCIN INDUCED DIABETIC RATS.

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ABSTRACT
Vanadate has been reported as an effective antidiabetic agent in oral treatment of diabetes in animal models. However, vanadate exerts hypoglycemic effects at relatively high dose and several toxic effects are produced. At low doses vanadate shows no toxicity but is unable to elicit any antidiabetic effect. We used low doses of vanadate in combination with Azadirachta indica leaf extract to reduce toxicity of vanadate and evaluate antidiabetic effects. In rats, diabetes was induced by streptozotocin (55 mg/kg body weight). Streptozotocin-diabetic rats were treated separately with insulin, vanadate (0.6mg/ml), A.indica and with combined dose of vanadate (0.2mg/ml) and A.indica. At the end of the experiment, rats were sacrificed and plasma glucose levels and activities of Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx) and Glutathione reductase (GR) were determined in cytosolic fraction in heart and skeletal muscles. Diabetic rats showed hyperglycemic condition and alteration in antioxidant enzymes activities. Treatment with antidiabetic compounds resulted in restoration of enzymes activities to normal. Combined dose of vanadate and A. indica was found to be most effective in normalizing altered antioxidant enzymes system.

KEYWORDS: Sodium orthovanadate, Azadirachta indica, Streptozotocin diabetes, Oxidative stress, Antioxidant.
INTRODUCTION
Diabetes is characterized by elevated production and low utilization of glucose due to lack of insulin secretion/action. Alterations in glucose metabolism due to chronic hyperglycemia [1] in diabetes are suggested to precede the structural and functional abnormalities associated with diabetic complications such as nephropathy, retinopathy, neuropathy, and cardiac problems. [2-6]

Overproduction of oxygen free radicals and decreased efficiency of antioxidant system during diabetes results in oxidative stress. [6] The antioxidant enzymes SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), GPx (EC 1.11.1.9) and GR (EC 1.6.4.2) are some free radical scavenging enzymes in biological system. [7] Increased oxidative stress in diabetes leads to altered level of these enzymes. [8] Various biochemical processes including glucose auto-oxidation and non-enzymatic protein glycosylation result in oxidative stress. [9-11]

Azadirachta indica (Neem) is one of the most versatile medicinal plants having a wide spectrum of biological activity. There are several reports on the biological activities and pharmacological actions of Neem based on modern scientific investigation. Lower blood glucose levels and restoration of the activities of key enzymes of antioxidant system is observed on administration of A. indica to diabetic animals. [12-14] It has been demonstrated that the A. indica leaf extract has hypoglycemic, hypocholesterolemic and hyperinsulinomic effects on type 1 and type 2 diabetes mellitus patients and experimental diabetic animals. [14, 15]

Sodium orthovandate effectively controls the hyperglycemic condition in diabetes and shows remarkable insulin mimetic effect. [16, 17] Vanadate salts mimic several of metabolic and growth promoting effects of insulin [18, 19] but only at high doses. Higher dose of vanadate causes several toxic effects like diarrhea, dehydration, decreased fluid and food intake and loss in body weight. [20, 21] Lower doses of vanadate are less toxic but not effective in eliciting insulin mimetic properties. [22] To reduce vanadium compounds toxicity and enhance their hypoglycemic effect, attempts are being made to use the complex-forming capability of vanadium compounds with organic compounds. [23-25]

In this study the possibility of using low doses of sodium orthovanadate (0.2 mg/ml) with A. indica leaf extract and to evaluate their antidiabetic effect on cardiac and skeletal antioxidant enzymes in streptozotocin-diabetic rat’s cardiac and skeletal muscles was explored.
MATERIALS AND METHODS

Animals

Male albino rats of the Wistar strain weighing between 200 and 210g and age of 3-5 months were used throughout this study. Animals were kept in animal house facility of Jamia Hamdard University New Delhi, India at a constant temperature of (25˚) and relative humidity (55%). They were given standard chow and tap water ad libitum until treatment or sacrifice. All the animal experiments have been approved by the Institutional animal ethics committee of Jamia Hamdard, New Delhi, India.

*A.indica* leaf extract (aqueous)

One kg of freshly collected, shade dried, powdered leaves of *A. indica* were allowed to soak overnight in 4 liters of distilled water at room temperature. The suspension was then centrifuged at 5000 rpm for 20 min and filtered through Whatman No.1 filter paper. The filtrate was lyophilized to yield 12.9 gm of dry powder and stored at -20˚C. A measured amount of the *A.indica* leaf extract (AILE) was dissolved in distilled water at suitable concentration prior to experiment.

Induction of diabetes and experimental design

A group of rats was starved for 12 h and diabetes was induced by a single intraperitoneal injection of streptozotocin dissolve in normal saline (pH 7.5), at a dose of 55 mg /kg body weight. Control animals were given only the vehicle. Animals with fasting blood glucose (FBG) concentration > 250 mg/dl were included into the study. The animals were then grouped into Control (C), diabetic (D), insulin treated diabetic (I), vanadate treated diabetic (V), *Azadrachta indica* treated diabetic (A) and diabetic treated with vanadate and *Azadirachta indica* (V+A).

The insulin treated diabetic group (I) received IP injection of 2 units of protamine-zine insulin for 3 weeks and were given the normal pellet diet and tap water *ad libitum* until the date of the experiment. The vanadate-treated diabetic group (V) rats were given 0.6 mg/ml of sodium orthovanadate dissolved in drinking water. In order to minimize the mortality due to vanadate toxicity the vanadate solution was prepared in 0.5% of sodium chloride as done by Heyliger et al. The *A.indica* group (A) was given aqueous extract of *A.indica* leaves orally at a dose of 500 mg/ kg of body weight. The treatment was continued until the day of sacrifice. Tap water was given together with the food *ad libitum*. V+A grouped animals were...
given aqueous extract of neem leaves orally at a dose of 500 mg/ Kg of body weight and vanadate in drinking water(0.2mg/ml) containing 0.5% NaCl.

**Preparation of tissue homogenate**

Rats were sacrificed by cervical dislocation. Heart and skeletal muscles were rapidly excised and washed with chilled normal saline. The tissues were then blotted dry and weighed. 10% (w/v) tissue homogenates were prepared in 0.25 M Sucrose, 0.02M Triethanolamine hydrochloride buffer of pH 7.4 containing 12 M Dithiothreitol (DTT). Homogenates were then centrifuged at 1000 × g for 10 min to remove nuclei and cell debris. The supernatant was again centrifuged at 100,000 × g for 30 min to obtain cytosolic fraction. All the procedures were carried out at 4˚C. The supernatants were then used for determination of enzymes activity.

**Determination of enzyme activities**

The assay of superoxide dismutase (SOD) was performed by following the method of Marklund et al. [27] This assay is based on the ability of SOD to inhibit the autoxidation of pyrogallol by 50%. The assay mixture of 1 ml contained in final concentration, 50 mmol/l sodium phosphate buffer, 0.1mmol/l EDTA, 0.48mmol/l pyrogallol and appropriate amount of tissue extracts containing 7-10 µg of protein. The change in absorbance was monitored at 420 nm for 3 min at 25˚C against blank. One unit of enzyme activity is defined as the amount of enzyme that causes 50% inhibition of pyrogallol autoxidation. The activity of catalase (CAT) was measured by the method of Aebi. [28] The assay mixture of 1 ml in final concentration contained 50 mmol/l sodium phosphate buffer pH 7.0 and 10 mmol/l hydrogen peroxide. The reaction was started by addition of cytosolic fraction containing 2-3µg protein. Change in absorbance was monitored at 240 nm at 25˚C. One unit of enzyme is define as the amount of enzyme required to break down 1 µmol of H$_2$O$_2$.

Glutathione peroxidase (GPx) activity was measured using a coupled enzyme assay as described by Lawrence and Burk. [29] The assay mixture of 1ml contained in final concentration, 10 mmol/l potassium phosphate buffer pH 7.0, 25 mmol/l EDTA, 0.5 mmol/l Glutathione (GSH) , 2 mmol/l sodium azide, 1.5 IU Glutathione reductase (GR) , 0.1mmol/l NADPH and cytosolic fraction containing 50 µg protein. The reaction was started by addition of t-butyl hydroperoxide and the decrease in absorbance was monitored at 25˚C at 340 nm. One unit of enzyme activity is defined as 1 µmol of nicotinamide adenine dinucleotide phosphate (NADPH) oxidized/min/mg protein. The activity of Glutathione reductase (GR)
was determine in the soluble tissue extracts by method of Erden and Bor. \[30\] The reaction mixture of 1ml contained the following in the final concentration: 4.1mmol/l Tris-HCL pH 7.5, 15mmol/l MgCl₂, 5.7 mmol/l EDTA, 60 mmol/1KCl, 2.6 Glutathione disulfide (GSSG) and 0.1 mmol/l of NADPH. The reaction was started by addition of cytosolic fraction containing 100µg protein. The decrease in absorbance was monitored at 25°C at 340 nm. One unit enzyme activity is defined as 1µmol of NADPH oxidized/min/mg protein.

**Lipid peroxidation**

Lipid peroxidation was assessed by measuring the malondialdehyde (MDA) formed, an end product of fatty acid peroxidation, by using thiobarbituric acid reactive substance (TBARS) method. \[31\] The 10% tissue homogenate was centrifuged at 1000xg for 10 min and deproteinized with half volume of 20% trichloroacetic acid (TCA). Precipitated protein was removed by centrifugation. The supernatant in 10mM potassium phosphate buffer (pH 7.4) was incubated at 80 °C for 15 min in water bath with 0.53% thiobarbituric acid in glacial acetic acid and centrifuged. The concentration of MDA-TBA complex was determined spectrophotometrically at 532 nm against blank and results are expressed as nmol MDA formed /mg protein. Soluble protein was determined by method of Bradford using bovine serum albumin as standard. \[32\]

**RESULTS**

**Effect on antioxidant enzymes**

Increased tissue oxidative stress in diabetic condition leads to change in antioxidant defense system. Therefore the activities of some major antioxidant enzymes were measured in control, diabetic and treated rats. As evident from fig. 1, SOD activity shows increase in diabetic cardiac muscle and decrease in skeletal muscles. CAT activity increased in cardiac muscles and skeletal muscles as shown in fig. 2. Activity of GPx increased in cardiac muscle and decreased in skeletal muscles in diabetic state as shown in fig. 3. Fig. 4 shows GR activity to be increased in cardiac muscle and decreased in skeletal muscle of diabetic rats. The enzyme activities are expressed as per milligram protein and therefore, represent true change under these conditions. Treatment with insulin, vanadate, *A. indica* and vanadate and *A. indica* in combination rectify the disturbed activity of antioxidant enzymes to normal. Combined treatment with vanadate and *A. indica* is more effective in correcting altered enzyme activity.
Fig. 1 Change in activity of SOD in Cardiac and skeletal muscles of control, diabetic and diabetic rats after 21 days of treatment. Each value is a mean ±SEM

Fig. 2 Change in activity of CAT in Cardiac and skeletal muscles of control, diabetic and diabetic rats after 21 days of treatment. Each value is a mean ±SEM

Fig. 3 Change in activity of GPx in Cardiac and skeletal muscles of control, diabetic and diabetic rats after 21 days of treatment. Each value is a mean ±SEM
Fig. 4 Change in activity of GR in Cardiac and skeletal muscles of control, diabetic and diabetic rats after 21 days of treatment. Each value is a mean ±SEM.

**Effect on lipid peroxidation**

Fig. 5 shows an increase in lipid peroxidation in both heart and skeletal muscles of diabetic rats. Treatment with insulin, vanadate, *A. indica* and vanadate and *A. indica* in combination reversed the above altered parameter to normal values. Combined treatment with vanadate and *A. indica* is more effective in correcting altered enzyme activity.

Fig. 5 Change in the malondialdehyde levels in Cardiac and skeletal muscles of control, diabetic and diabetic rats after 21 days of treatment. Each value is a mean ±SEM

**DISCUSSION**

The present work is an attempt to study the effect of 3 weeks treatment with insulin, vanadate, *A. indica* and vanadate and *A. indica* on streptozotocin induced diabetic rats. The persistence hyperglycemic condition in diabetes reported to cause increased production of
oxygen free radicals through autooxidation and nonenzymatic glycation. Generation of the oxygen free radical generation under normal physiological condition is controlled by antioxidant defense system and there is critical balance between the generation of oxygen free radical and the antioxidant defense system used by organisms to deactivate and protect themselves from free radical toxicity. \[33\] The oxidative stress may be amplified by a continuing cycle of metabolic stress, tissue damage, and cell death, leading to increased free radical production and compromised free radical inhibitory and scavenger systems, which further exacerbate the oxidative stress. \[34\] Free radicals may react with a variety of biomolecules including lipids, carbohydrates, proteins, nucleic acids and macromolecules of connective tissue thereby interfering with cell function. Several studies have shown that oxidative stress is a feature of various chronic diseases. \[35-37\] An alteration of antioxidant enzymes in diabetic condition is reported by various studies. We have studied four major antioxidant enzymes SOD, CAT, GPx and GR to evaluate the antioxidant status in heart and skeletal muscles using vanadate and \textit{A. indica} in combination. Antioxidant enzyme CAT showed a significant increase in heart and skeletal muscles, whereas SOD, GPx and GR increased in heart and decreased in skeletal muscles. The results are in arrangement with previous studies. \[31, 38-39\]

Increased oxidative stress in diabetes lead to autoxidation of glucose, formation of advanced glycated end product, and increased polyol pathways with concomitant increase in cellular lipid peroxidation and damage of membrane in diabetes. \[22, 40\] In present study the formation of TBARS, a product of lipid peroxidation, was significantly increased in diabetic heart and skeletal muscles. The results are in agreement with previous studies. \[41\] This increased peroxide formation during diabetes disturbs the anatomical integrity of the membrane leading to inhibition of several membrane bound enzymes. \[42\] The results indicate a sharp decreased level of TBARS in diabetic rats treated with insulin, vanadate, \textit{A. indica} and combined treatment with vanadate and \textit{A. indica}. The combine treatment is more effective in bringing back the values to normal.

Thus treatment with insulin, vanadate, \textit{A. indica} and combined treatment with vanadate and \textit{A. indica} corrected the altered level of SOD, CAT, GPx and GR in heart and skeletal muscles. \textit{A.indica} treatment restored the altered enzyme activities. The combined treatment is more effective in correcting the disturbed level of antioxidant enzymes.
CONCLUSION
In conclusion we can say that apart from increased glucose levels, diabetes has an adverse effect on various important enzymes of the cardiac and skeletal muscles, like SOD, catalase, Glutathione reductase and glutathione peroxidase. It also increases lipid peroxidation in these muscles. Treatment with aqueous extracts of *Azadirachta indica*, with and without vanadate, reverses the activity of these enzymes to normal. The combined treatment is more effective in correcting the disturbed level of antioxidant enzymes, with reduction in the toxicity of vanadate, and alleviating the adverse effects of diabetes.

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REFERENCES


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