LOWER DOSES OF VANADATE IN Combination WITH AZADIRACHTA INDICA LEAF EXTRACT RESTORE ALTERED ANTIOXIDANT STATUS IN THE BRAIN OF STREPTOZOTOCIN INDUCED DIABETIC RATS

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ABSTRACT

Azadirachta indica leaf extract has been reported to have hypoglycemic effects. Sodium orthovanadate also exerts antidiabetic effects but at relatively high doses with several toxic effects. So, in the present study we investigate the effects of oral administration of Azadirachta indica leaf extract and vanadate, separately and in combination, on the activities of antioxidant enzymes in streptozotocin induced diabetic rats. Diabetes was induced by a single intraperitoneal injection of streptozotocin (55mg/kg body wt). Animals were divided into six groups namely non diabetic control, diabetic control, diabetic treated with insulin (2U), diabetic treated with leaf extract (500mg/kg body weight), diabetic treated with vanadate (0.6mg/ml) and diabetic treated with both vanadate (0.2mg/ml) and Azadirachta indica leaf extract (500mg/kg body weight). After treatment, the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) were determine in cytosolic fraction of brain tissue. Treatment with different antidiabetic compounds restored the above enzymes activity to normal. Combined dose of Azadirachta indica and vanadate was found to be the most effective in normalizing these Enzyme activities.

KEYWORDS: Sodium orthovanadate, Azadirachta indica, Streptozotocin diabetes, Oxidative stress, Antioxidant.
INTRODUCTION
Diabetes is a metabolic disorder characterized by increase in blood glucose level. Hyperglycemic condition is either due to decrease in insulin secretion or/and irresponsiveness to insulin. Increase in the blood glucose leads to oxidative stress by production of reactive oxygen species (ROS). Oxidative Stress is a characteristic feature in hyperglycemic condition\(^1\) and prolonged oxidative stress leads to various diabetic complications such as nephropathy, retinopathy, neuropathy, and cardiac problems.\(^2\) Increased oxidative stress disturbs the activities of various antioxidant enzymes\(^3\), such as superoxide dismutase (EC 1.15.1.1), catalase (EC 1.11.1.6), glutathione peroxidase (EC 1.11.1.9) and glutathione reductase (EC 1.6.4.2) which prevent the accumulation of toxic product by scavenging free radicals.\(^4\) Various organs including brain are adversely affected due the increased oxidative stress.\(^3, 5\)

Brain consumes 20% of the oxygen in the body and is more vulnerable to oxidative stress than other organs because it has a low content of antioxidants and high content of unsaturated fatty acids and catecholamines that are easily oxidized.\(^6\) Oxidative stress in brain can cause microvascular cerebral diseases, e.g. stroke, cerebral haemorrhage, and brain infarction.\(^7, 8\) The alterations in the activities of antioxidant enzymes in brain during diabetes condition have been reported.\(^3\) In diabetes increased oxidative stress also cause increased lipid peroxidation.\(^9\)

Azadirachta indica is an effective antidibetic plant and various reports have demonstrated that its leaf extract has hypoglycemic, hypocholesterolemic and hyperinsulinomic effects in diabetic condition.\(^10, 11, 12\) Sodium orthovanadate is a remarkable antidiabetic compound and mimic insulin like effect.\(^3, 14\) Vanadium and its various complexes have been found effective in normalizing diabetic complications and reducing blood glucose level. Doses of vanadate effective in normalizing the increased blood glucose level shows various toxic effects.\(^15\) such as diarrhoea, dehydration, decreased fluid and food intake and loss in body weight.\(^16-18\) At lower doses antidiabetic potential of vanadate is reduced and it is less effective in normalizing blood glucose level. To reduce Sodium orthovanadate toxicity and to increase its hypoglycemic effect, its complex-forming capability with organic compounds is utilized.\(^19-21\) Vanadium also improves the altered glucose and lipid Homeostasis.\(^15, 22\)

In the present study, attempts have been made to reduce the toxicity of vanadate without compromising its antidiabetic effects by reducing the dose of vanadate and combining it with
Azadirachta indica leave extract. The activities of antioxidant enzymes such as Superoxide dismutase (SOD), Catalase (CAT), Gluthathione peroxidase (GPx) and Gluthathione reductase (GR) are evaluated in brain tissue.

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.[23] Control animals were given only the vehicle. Animals with fasting blood glucose (FBG) concentration > 250 mg/dl were included in 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. Brain 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. 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. 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 H2O2.

Glutathione peroxidase (GPx) activity was measured using a coupled enzyme assay as described by Lawrence and Burk. 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. 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/l KCl, 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. The 10% tissue homogenate was centrifuged at 1000×g 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.

**RESULTS**

**Effect on antioxidant enzymes**

Oxidative stress in diabetic condition leads to alterations in antioxidant defense system. Therefore the activities of some major antioxidant enzymes were measured in control, diabetic and treated rats. Fig 1 shows change in the weight of brain and the values are almost similar in each group. As shown in fig. 2, SOD activity shows decrease in diabetic brain. CAT activity decrease in brain as shown in fig. 3. As evident from fig 4 activity of GPx increased in diabetic brain. Fig. 5 shows GR activity to be increased in brain 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 brain weight of control, diabetic and diabetic rats after 21 days of treatment. Each value is a mean ±SEM

Fig. 2 Change in activity of SOD in Brain of control, diabetic and diabetic rats after 21 days of treatment. Each value is a mean ±SEM

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

Fig. 5 Change in activity of GR in Brain of control, diabetic and diabetic rats after 21 days of treatment. Each value is a mean ±SEM.

Fig. 6 Change in the malondialdehyde levels in brain of control, diabetic and diabetic rats after 21 days of treatment. Each value is a mean ±SEM.
Effect on lipid peroxidation

As shown in Fig. 6 lipid peroxidation increased in brain of diabetic rat. 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.

DISCUSSION

The present study is undertaken to explore the effect of experimental diabetes on different parameters like SOD, CAT, GPx, GR and lipid peroxidation in rat brains. The toxicity of vanadate is reduced by combining it with the Azadirachta indica leaf extract. Hyperglycemia leads to oxidative stress that is disturbance in the production and removal of reactive oxygen species (ROS). Oxidative stress over a long period of time leads to various diabetic complications including attenuation of ROS scavenging capacity of antioxidant enzyme system. Various studies have shown the change in the antioxidant enzymes during diabetic condition. The antioxidative defense system like SOD and CAT showed lower activities in brain during diabetes and the results agree well with the earlier studies. The activity of enzymes decrease due to their decreased expression in diabetic condition. Production of high ROS such as H$_2$O$_2$ and hydroxyl radicals partially inactivates these enzymes. GPx activity increased in diabetic rat brains. The increased GPx activity represents a compensatory mechanism to degrade H$_2$O$_2$, which is produced in excess during the metabolism of catecholamines. The result agrees well with previous studies. GR activity also increases in the diabetic brain. Treatment of the diabetic animals with insulin, vanadate, Azadirachta indica and combined treatment of A. indica with lower dose of vanadate restored the altered activities of SOD, catalase, GPx and GR. Combined treatment is more effective in bringing back altered values to normal.

Prolong hyperglycemic condition leads various diabetic complications by increased production of ROS. Increase production of free radicals from auto-oxidation of glucose, formation of advance glycatedend products (AGEs) and increased polyol pathway, with concomitant increase in cellular lipid peroxidation is seen. The lipid peroxidation degenerative processes can result in enzyme activity changes. In the present study the formation of TBARS, a product of lipid peroxidation reaction, was significantly increased in diabetic brain tissues, as reported in earlier studies. The results indicate a sharp decreased level of TBARS in diabetic rats treated with insulin, vanadate, A. indica and combined
treatment with vanadate and A. indica. The combine treatment is more effective in bringing back the values to normal.

Thus treatment with insulin, vanadate, A. indica and combined treatment with vanadate and A. indica corrected the altered level of SOD, CAT, GPx and GR in brain. The combined treatment is more effective in correcting the disturbed level of antioxidant enzymes.

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