

## NATURAL DRUGS

PROTECTIVE EFFECT OF *WITHANIA SOMNIFERA* AGAINST OXIDATIVE STRESS AND PANCREATIC  $\beta$ -CELL DAMAGE IN TYPE 2 DIABETIC RATSTARIQUE ANWER<sup>1,2\*</sup>, MANJU SHARMA<sup>1</sup>, KRISHNA KOLAPPA PILLAI<sup>1</sup> and GYAS KHAN<sup>1,2</sup><sup>1</sup>Department of Pharmacology, Faculty of Pharmacy, Jamia Hamdard, (Hamdard Nagar), New Delhi-110062, India<sup>2</sup>Department of Pharmacology, College of Pharmacy, Jazan University, P.O Box 114, Jazan, Kingdom of Saudi Arabia

**Abstract:** The aim of the present study was to investigate the effects of *Withania somnifera* (WS) on lipid peroxidation (LPO), activities of both non-enzymatic and enzymatic antioxidants and histopathological examination of pancreas in type 2 diabetic rats. Type 2 diabetes was induced by single intraperitoneal injection of STZ (100 mg/kg) to 2 days old rat pups. Oxidative stress was measured by tissue LPO levels, reduced glutathione (GSH) contents and by enzymatic activities of glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT). Administration of WS to type 2 diabetic rats caused a significant decrease in blood glucose and tissue LPO levels with significant increase in GSH contents when compared with the type 2 diabetic control rats. In addition, WS treated rats also showed a significant increase in the activities of antioxidant enzymes namely GPx, GR, GST, SOD and CAT when compared with type 2 diabetic control rats. Significant reduction in the number and size of pancreatic  $\beta$ -cells were preserved to near normal morphology by the administration of WS in type 2 diabetic rats as evident from histopathological examination. The results obtained clearly indicate that WS has shown strong free radical scavenging activity and helped in improving the non-enzymatic and enzymatic antioxidants in type 2 diabetic rats.

**Keywords:** *Withania somnifera*; antioxidant; streptozotocin; lipid peroxidation, malondialdehyde

Diabetes mellitus (DM) is a group of metabolic disorders characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both (1, 2). It is often associated with several other metabolic abnormalities such as abdominal obesity, insulin resistance, dyslipidemia and hypertension. Hyperglycemia causes generation of reactive oxygen species (ROS), ultimately leading to increased oxidative stress in a variety of tissues (3). Oxidative stress plays an important role in chronic complications of diabetes and is postulated to be associated with increased lipid peroxidation (4, 5). STZ is frequently used to induce diabetes in experimental animals through its toxic effects on pancreatic  $\beta$ -cells (6–8). The cytotoxic action of STZ is associated with the generation of reactive oxygen species causing oxidative damage (9). Disturbances of antioxidant defense systems in diabetes have been demonstrated, including alterations in the activities of antioxidant enzymes, such as GPx, GR, SOD and impaired glutathione metabolism (10). Chemicals

with antioxidant properties and free radical scavengers may help in the regeneration of  $\beta$ -cells and protect pancreatic islets against cytotoxic effects of streptozotocin (11). Decreased lipid peroxidation and improved antioxidant status may be one mechanism by which dietary treatment contributes to the prevention of diabetic complications (12).

*Withania somnifera* (WS) Dunal (Family: Solanaceae), commonly known as ashwagandha, is widely used in Ayurvedic system of medicine in India. It is an official drug and is mentioned in the Indian Pharmacopoeia (13). Several studies on this plant indicated that it possesses anti-inflammatory, antitumor, antistress, antioxidant, immunomodulatory, hemopoetic and rejuvenating properties besides its positive influence on the endocrine, cardiopulmonary and central nervous system (14–16). WS has been reported to decrease LPO in experimental animals stressed by the administration of lipopolysaccharides from *Klebsiella pneumoniae* or peptidoglycans from *Staphylococcus aureus* (17).

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Dried fruit extract of *Withania coagulans* Dunal has been shown to have hypoglycemic activity in type 1 diabetic rats (18). A WS containing Ayurvedic herbal formulation (Tarsina) was found to attenuate STZ-induced hyperglycemia and pancreatic islet superoxide dismutase activity in type 1 diabetic rats (19).

Thus, the present study was designed to investigate whether supplementation with WS has any protective effects on lipid peroxidation, activities of both non-enzymatic and enzymatic antioxidants and histopathological examination of pancreatic  $\beta$ -cell in type 2 diabetic rats.

## MATERIALS AND METHODS

### Experimental animals

Healthy albino Wistar rats were kept for breeding. The animals were maintained under controlled

condition of illumination (12 h light/12 h darkness) and temperature 20–25°C. They were housed under ideal laboratory conditions, maintained on standard pellet diet (Lipton rat feed, Ltd., Pune) and water *ad libitum* throughout the experimental period. The experimental study was approved by the Institutional Animal Ethics Committee (IAEC) of Jamia Hamdard, New Delhi, India.

### Drugs and chemicals

Standardized powdered, aqueous root extract of *Withania somnifera* (Batch No. WS/05002) was a gift sample by Natural Remedies, Bangalore, India. It contains total withanolides (3.9% w/w). Streptozotocin was purchased from Sigma Chemicals (USA). Glucose kit was procured from Span Diagnostics, Surat, India. All the other chemicals used were of analytical grade.

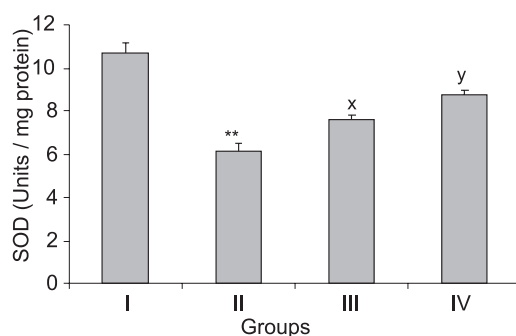


Figure 1. Effects of WS on superoxide dismutase (SOD) activity in pancreas of type 2 diabetic rats. The data are expressed as the mean  $\pm$  SE; n = 6 in each group. \*\*p < 0.001 compared with the corresponding value for citrate control animals (group I). x p < 0.01, y p < 0.001 compared with the corresponding value for type 2 diabetic control rats (group II)

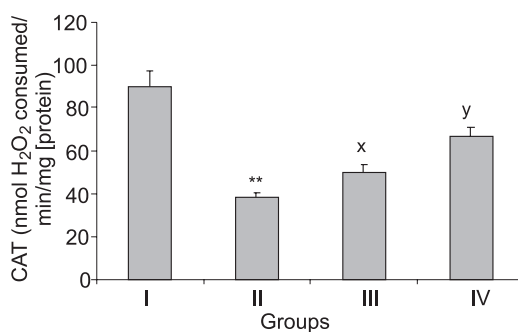


Figure 2. Effects of WS on catalase (CAT) activity in pancreas of type 2 diabetic rats. The data are expressed as the mean  $\pm$  SE; n = 6 in each group. \*\*p < 0.001 compared with the corresponding value for citrate control animals (group I). x p < 0.01, y p < 0.001 compared with the corresponding value for type 2 diabetic control rats (group II)

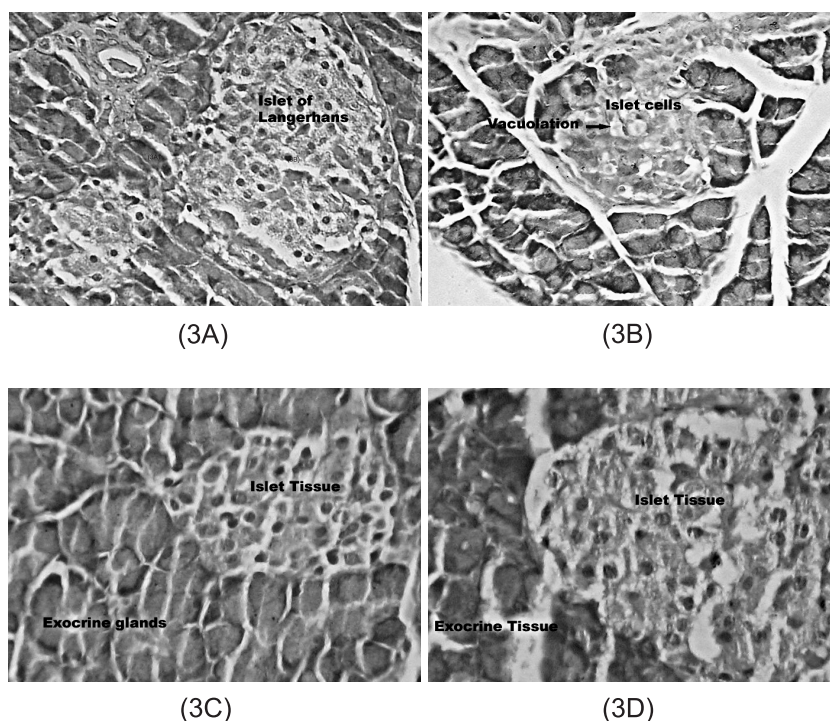


Figure 3. Hematoxylin and eosin-stained sections of rat pancreas: (3A) Represents citrate control rat showing normal lobules of pancreatic acini  $\beta$ -cells. (3B) Represents type 2 diabetic control rat showing lobules of pancreatic acini with areas of fibrosis and depletion of  $\beta$ -cells. (3C) Represents pancreatic section of type 2 diabetic + WS (200 mg/kg) treated rat showing mild fibrosis of pancreatic acini with regeneration of  $\beta$ -cells. (3D) Represents pancreatic section of type 2 diabetic + WS (400 mg/kg) treated rat showing normal lobules of pancreatic acini and  $\beta$ -cells.

### Induction of diabetes

To induce type 2 diabetes, STZ (100 mg/kg) in citrate buffer (pH 4.5) was administered intraperitoneally to 2 days old rat pups (20). Another group of pups received only citrate buffer. Ninety days after STZ treatment, development of diabetes was confirmed by measuring blood glucose levels. Rats with fasting blood glucose levels of 200 mg/dL or higher were considered to be diabetic.

### Experimental design

The rats were divided into four groups comprising of six animals in each group as follows:

- Group I: Citrate control, received citrate buffer (pH 4.5)
- Group II: Type 2 diabetic control, received STZ in single dose (100 mg/kg, *i.p.*)
- Group III: Type 2 diabetic treated, received WS (200 mg/kg, *p.o.*)
- Group IV: Type 2 diabetic treated, received WS (400 mg/kg, *p.o.*)

WS (200 mg/kg and 400 mg/kg) was dissolved in water and given until the end of the study (5 weeks) to group III and IV animals.

### Biochemical estimations

On the last day of experiment, blood samples were collected for biochemical estimations. Blood glucose was determined by glucose oxidase (21) method using a commercial diagnostic kit from Span Diagnostics Ltd., Surat, India. Later, the animals were sacrificed and pancreas was removed, cleaned and washed in ice-cold normal saline for biochemical study. LPO was measured by thiobarbituric acid (TBA) reaction with malondialdehyde (MDA), a product formed due to the peroxidation of membrane lipids (22), whereas GSH was determined by the method of Jollow et al., (23) based on the development of a stable color complex with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). GPx and GR activities were measured by the oxidation of NADPH (24). GST activity was estimated according

to the method of Habig et al., (25) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. SOD activity was expressed as unit's per mg protein and one unit of enzyme is defined as the enzyme activity that inhibits autoxidation of pyrogallol by 50% (26). CAT was estimated following the method of Claiborne (27) based on a decrease in absorbance at 240 nm due to consumption of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>).

### Histopathological examination of pancreas

The pancreas was isolated immediately after sacrificing the animal and washed with ice-cold saline. It was then fixed in 10% neutral buffered formalin solution. Sections of 3–5 µm thickness were stained with hematoxylin and eosin (H.E.) for histopathological examination.

### Statistical analysis

Data were expressed as the mean ± standard error (SE) of the mean. For a statistical analysis of the data, group means were compared by one-way

analysis of variance (ANOVA) with *post-hoc* analysis. The Tukey-Karmer *post-hoc* test was applied to identify significance among groups;  $p < 0.05$  was considered to be statistically significant.

## RESULTS

### Effect of WS on hyperglycemia

Table 1 shows the effect of WS on the blood glucose level. Significant ( $p < 0.001$ ) increase in blood glucose level was observed in type 2 diabetic control rats when compared with citrate control rats. Oral administration of WS at two doses (200 and 400 mg/kg) reduced the blood glucose levels significantly ( $p < 0.001$ ) in a dose-dependent manner.

### Effect of WS on LPO levels

Table 1 shows the levels of malondialdehyde (MDA), a secondary product of LPO in pancreatic tissue homogenate. STZ treatment resulted in a significant ( $p < 0.001$ ) increase in MDA levels in type 2 diabetic control rats when compared with citrate

Table 1. Effects of *Withania somnifera* on blood glucose, LPO and reduced glutathione (GSH) contents in pancreas of type 2 diabetic rats.

Groups	Treatment	Blood glucose (mg/ dL)	MDA (nmol/mg protein)	GSH (nmol/g tissue)
I	Citrate control	97.18 ± 3.02	0.864 ± 0.025	0.594 ± 0.015
II	STZ (100 mg/kg, <i>i.p.</i> )	324.66 ± 10.87**	1.866 ± 0.034**	0.233 ± 0.011**
III	Type 2 diabetic + WS (200 mg/kg, <i>p.o.</i> )	151.01 ± 4.08 <sup>γ</sup>	1.484 ± 0.019 <sup>χ</sup>	0.338 ± 0.011 <sup>χ</sup>
IV	Type 2 diabetic + WS (400 mg/kg, <i>p.o.</i> )	121.28 ± 1.80 <sup>γ</sup>	1.168 ± 0.017 <sup>γ</sup>	0.467 ± 0.016 <sup>γ</sup>

The data are expressed as the mean ± SE; n = 6 in each group. \*\* $p < 0.001$  compared with the corresponding value for citrate control rats (group I). <sup>χ</sup> $p < 0.01$ , <sup>γ</sup> $p < 0.001$  compared with the corresponding value for type 2 diabetic control rats (group II).

Table 2. Effect of *Withania somnifera* on glutathione metabolizing enzymes (GPx, GR and GST) activities in pancreas of type 2 diabetic rats.

Groups	Treatment	GPx	GR	GST
I	Citrate control	162.50 ± 3.36	317.08 ± 5.99	302.85 ± 8.23
II	STZ (100 mg/kg, <i>i.p.</i> )	98.55 ± 4.12**	220.80 ± 5.88**	210.73 ± 7.12**
III	Type 2 diabetic + WS (200 mg/kg, <i>p.o.</i> )	119.58 ± 4.83 <sup>χ</sup>	246.94 ± 7.95 <sup>χ</sup>	233.45 ± 3.99 <sup>χ</sup>
IV	Type 2 diabetic + WS (400 mg/kg, <i>p.o.</i> )	143.13 ± 5.46 <sup>γ</sup>	286.94 ± 5.26 <sup>γ</sup>	265.48 ± 4.75 <sup>γ</sup>

GPx (nmol NADPH oxidized/min/mg protein); GR (nmol NADPH oxidized/min/mg protein); GST (nmol CDNB conjugates formed/min/mg protein). The data are expressed as the mean ± SE; n = 6 in each group. \*\* $p < 0.001$  compared with the corresponding value for citrate control rats (group I). <sup>χ</sup> $p < 0.01$ , <sup>γ</sup> $p < 0.001$  compared with the corresponding value for type 2 diabetic control rats (group II).

control rats. The levels of MDA were significantly ( $p < 0.01$  and  $< 0.001$ ) decreased in WS (200 mg/kg and 400 mg/kg) treated type 2 diabetic rats when compared with type 2 diabetic control rats (group II).

#### Effect of WS on GSH contents

Table 1 shows the contents of GSH in type 2 diabetic control rats which was significantly ( $p < 0.001$ ) decreased in pancreatic tissue homogenate when compared to the citrate control rats (group I). WS (200 and 400 mg/kg) treatment significantly ( $p < 0.01$  and  $< 0.001$ ) increased the contents of GSH in type 2 diabetic rats when compared with type 2 diabetic control rats (group II).

#### Effect of WS on antioxidant enzymes (AOE) levels

Figures 1–3 demonstrate the activities of GPx, GR, GST, SOD and CAT in pancreas of citrate control, type 2 diabetic control and type 2 diabetic treated rats. A significant ( $p < 0.001$ ) decrease in the activities of GPx, GR, GST, SOD and CAT was a notable manifestation of STZ toxicity. Administration of WS (200 and 400 mg/kg) significantly ( $p < 0.01$  and  $p < 0.001$ ) improved the activities of these enzymes in type 2 diabetic rats when compared with type 2 diabetic control rats (group II). The lower dose of WS (200 mg/kg) produced statistical significance  $p < 0.01$ , while the higher dose (400 mg/kg) produced statistical significance  $p < 0.001$ .

#### Effect of WS on histological changes

Section of rat pancreas from citrate control group showed, normal pancreatic acini and  $\beta$ -cells of islets of Langerhans (Fig. 3A). Type 2 diabetic control rats showed degenerative and lytic changes in the islets of Langerhans of pancreas. There was depletion of  $\beta$ -cells and the dimension of the islet was also considerably reduced and shrunken (Fig. 3B). The histological picture of pancreas was improved in type 2 diabetic rats treated with WS (Fig. 3C and 3D), as evidenced by the increase in size and number of  $\beta$ -cells.

## DISCUSSION

DM is a significant growing health problem in developed and developing countries with excess morbidity and mortality. It has been postulated that oxidative stress is involved in the etiology of diabetic complications that arise from chronic hyperglycemia and increased lipogenesis (28). Oxidative

stress is characterized by increased LPO and/or altered non-enzymatic and enzymatic antioxidant systems (29). For many years it has been suggested that patients with diabetes undergo chronic oxidative stress which results from a disturbance in the balance between the formation of free radicals in the body and scavenging of these radicals (30). This can be appreciated by evaluation of markers for oxidative stress such as LPO, GSH, GPx, GR, GST, SOD and CAT in such patients. Our previous study also demonstrated that LPO increases in STZ-induced diabetic rats (31).

Type 2 diabetic control rats exhibited persistent hyperglycemia. WS treated rats, however, have significantly reduced the blood glucose levels thereby showing its antihyperglycemic activity (Table 1). Recently we have reported that WS normalizes hyperglycemia in NIDDM rats by improving insulin sensitivity (32).

The elevated levels of LPO are attributed to the enhanced production of reactive oxygen species (ROS) (superoxide radicals, hydrogen-peroxide and hydroxyl radicals). Since LPO affects the cellular integrity only when antioxidant mechanisms are no longer able to cope with the free radical generation, supplementation of an antioxidant could gear up the detoxification machinery. In the present study, we observed a significant increase in the levels of MDA, a secondary product of LPO, in pancreas of type 2 diabetic rats. The WS treatment to such rats has shown significant decrease in the levels of LPO suggesting its possible role in scavenging hydroxyl and peroxy radicals (Table 1). The LPO lowering effect could be attributed to withanolide one of its main ingredients which is known for its antioxidant activity (33).

GSH is known to protect the cellular system against the toxic effects of lipid peroxidation. It has been proposed that antioxidants that maintain the concentration of GSH may restore the cellular defense mechanisms, block lipid peroxidation and thus protect the tissue against oxidative damages (34). A marked decrease in the GSH contents of pancreas was observed in type 2 diabetic control rats (Table 1). Furthermore, WS treatment showed a significant increase in GSH contents in the pancreas of type 2 diabetic rats.

STZ treatment resulted in a significant decrease in the activities of enzymatic antioxidants viz., GPx, GR, GST, SOD and CAT. This finding is in agreement with the results of our previous study (31). GPx has been reported to reduce hydroperoxides to water by using GSH as a hydrogen donor (35), whereas GR is able to regenerate its GSH from

oxidized glutathione (GSSG). The decreased content of GSH would lead to an increase in the production of ROS and a decrease in the GST-mediated elimination of electrophilic xenobiotics and some of the end products of LPO. GPx by virtue of its ability to catabolize both H<sub>2</sub>O<sub>2</sub> and LPO is uniquely positioned to protect tissues from ROS. The level of this antioxidant in  $\beta$ -cells is extremely low and overexpression of GPx in islets provides enhanced protection against oxidative stress. In the present study, we showed that WS increases the activities of GSH dependent enzymes such as GPx, GR and GST in type 2 diabetic rats (Table 2).

The decreased activities of SOD and CAT in tissue is due to excess availability of superoxide (O<sub>2</sub><sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in the biological systems, which in turn generate hydroxyl and peroxy radicals, resulting in the initiation and propagation of lipid peroxidation. WS treatment showed a significant augmentation in the activities of SOD as well as CAT in pancreatic tissue of type 2 diabetic rats (Fig. 1 and 2).

Protective effect of WS is probably due to the counteraction of free radicals by its antioxidant nature and is also evident from the histological observation of pancreas which shows similar morphology as compared to the citrate control rats. Type 2 diabetic control rats showed significant reduction in the number and size of pancreatic  $\beta$ -cells. The higher dose of WS treated rats showed almost normal sized islets in the pancreas but the low dose treated rats did not show much significant improvement in islets number and size. However, the precise molecular mechanism by which WS exerts its protective effect against oxidative stress and  $\beta$ -cell damage remains to be established. In conclusion, the results of the present study indicate that WS supplementation reduces lipid peroxidation, possibly by scavenging free radicals, and increasing the defense status of antioxidants and associated enzymes. Further clinical studies are necessary to validate these effects.

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*Received: 08. 11. 2010*