

Original Research

Increase in oxidative stress and mitochondrial impairment in hypothalamus of streptozotocin treated diabetic rat: Antioxidative effect of *Withania somnifera*

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Abstract: Hypothalamus, the primary brain region for glucose sensing, is severely affected by oxidative stress in diabetes mellitus. Oxidative stress in this region of brain may cause severe impairment in neuronal metabolic functions. Mitochondria are prominent targets of oxidative stress and the combination of increased oxidative stress and mitochondrial dysfunctions may further decline hypothalamic neuronal functions. In the present study we examined the oxidative damage response, antioxidative responses and mitochondrial membrane permeability transition in hypothalamus of streptozotocin-treated diabetic rats. Our results show that streptozotocin significantly increases hypothalamic lipid peroxidation, protein carbonyl content while glutathione peroxidase and reduced glutathione were declined. Mitochondrial impairment marked by an increase in mitochondrial membrane permeabilization was seen following streptozotocin treatment in the hypothalamus. The oral administration of *Withania somnifera* root extract stabilized mitochondrial functions and prevented oxidative damage in the hypothalamus of diabetic rat. These findings suggest an increase in the oxidative stress and decline in antioxidative responses in the hypothalamus of streptozotocin treated diabetic rats. *Withania somnifera* root extract was found useful in reducing oxidative stress and mitochondrial impairment in hypothalamus of diabetic rat.

Key words: Hypothalamus, mitochondria, oxidative stress, *Withania somnifera*, streptozotocin.

Introduction

The hypothalamus in the central nervous system regulates the energy homeostasis and provides the hub for integrating the nutritional information (1, 2). Any changes in the energy status are reflected by appropriate metabolic responses in hypothalamus. In recent years, various studies showed the pivotal role of reactive oxygen species (ROS) in the regulation of energy homeostasis by the hypothalamus (3, 4). However, increased ROS may severely impair the energy homeostasis. ROS are mostly produced by electron leakage during mitochondrial metabolism (5, 6, 7). However ROS production can be increased during different conditions, and if not rapidly neutralized, it results in oxidative stress that significantly damage DNA, proteins, and lipids and eventually contributes to apoptosis (8). The mitochondria are not only the main organelles producing cellular energy but also are linked to the production of ROS. Thus any alterations in mitochondrial functions may cause detrimental effect in cell functioning and it may be the central cause for various metabolic diseases including diabetes.

Diabetes mellitus is a common metabolic disorder which is associated with various chronic complications including those which are related with brain. Streptozotocin (STZ; 2-deoxy-2(3-methyl-3-nitrosoureido)-D-glucopyranose) is a broad-spectrum antibiotic having diabetogenic properties (9, 10) and is widely used to induce diabetes in experimental animals. STZ mediates its diabetogenic properties by selective destruction of pancreatic beta cells via generation of ROS. Various biochemical studies have shown the beneficial effects of herbs or their extracts in the treatment of diabetes mellitus (11). *Withania somnifera* (WS), commonly known as Ashwagandha is one of the herbs which is widely

used in Indian traditional medicine, the Ayurveda for the treatment of diabetes mellitus. The pharmacological effects of WS is due to the withanolides steroidal lactones that exhibit antidiabetic, antihyperlipidaemic, antioxidant, antibacterial, antifungal, antitumor, antistress, immunomodulatory, haemopoetic, and rejuvenating properties (12, 13, 14, 15, 16, 17, 18). However, whether WS extract reduce oxidative stress in STZ induced oxidative responses in the brain, especially in the hypothalamus is not well understood. In the present study we investigated the effects of STZ on balance between oxidative stress and the cellular antioxidative responses in the hypothalamus. We further evaluated the effect of WS extract on the oxidative stress responses in the hypothalamus of STZ treated rats.

Materials and Methods

Chemicals

Hydrochloric acid (HCl), ethyl acetate, ethanol, sodium hydroxide (NaOH) were purchased from HiMedia Laboratories (Mumbai, India). Potassium dihydrogen orthophosphate (KH_2PO_4), dipotassium hydrogen orthophosphate (K_2HPO_4), metaphosphoric acid, sodium chloride and ethylene diamine tetra acetate (EDTA) were obtained from Merck, India. Streptomycin sulphate, GA, trichloroacetic acid (TCA), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferro-

Received November 18, 2015; Accepted January 16, 2016; Published January 22, 2016

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zine), sodium acetate, ascorbic acid, 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) and di-nitrophenyl-hydrazine (DNPH) were obtained from Sigma Chemical Company Inc. (St. Louis, MO, USA).

Experimental Animal

Adult male Wistar rats (*Rattus norvegicus*) weighing 250-300 g were obtained from College of Veterinary Science and Animal Husbandry, Mhow, India. All animals were housed at $25 \pm 1^\circ\text{C}$ with 12 hr light / dark cycle with access to food and water ad libitum. All studies and experimental protocol using these rats were approved by the research committee of Vikram University in accordance with the International guidelines for the care and use of laboratory animals.

Experimental Design

The experiment was performed in four groups. Forty rats were randomly divided in these four groups of 10 animals in each. Group-I was served as control whereby animals received no treatment. Group-II was administered orally once a day with WS extract (35 mg/kg body weight) throughout the experiment. In Group-III, rats were treated intraperitoneally with STZ (60 mg/kg body weight). Group-IV was treated with STZ similar to Group-III but administered with extract of WS as in Group-II. Dose of the plant extract was adjusted based upon the mean of total antioxidant activity of the extract determined by the FRAP and Ferrozine assays as described below. After 30 days treatment the blood glucose level was measured and the animals were euthanized by decapitation. Hypothalamus were rapidly excised and placed into ice cold isolation medium as described (19). Tissues were weighed, finely minced and homogenized in isolation medium (10% w/v) using glass homogenizer and utilized for biochemical measurements.

Plant materials and fractionation of extract

Powdered roots of WS were subjected to acetone extraction at room temperature to obtain a brown solid extract upon solvent evaporation. The extract was further fractionated using methanol: hexane (1:1) liquid-liquid extraction (20, 21). Evaluation of total antioxidant activity was done for each fraction as described below. Since the main antioxidant activity was found in the methanolic fraction it was dried and reconstituted in 12% (v/v) aqueous methanol. The alcohol was completely evaporated and the reconstituted compound was dissolved in distilled water prior to oral administration to the rats.

Total antioxidant activity

Total antioxidant activity of plant extract was assayed by using two well-established assays:

I) Ferric (Fe^{3+}) reducing antioxidant power (FRAP) was assayed as described by Benzie and Strain, 1996 (22) which is based on the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion at low pH. This reduction causes a formation of blue colored ferrous tripyridyl triazine (Fe^{2+} -TPTZ) complex which can be measured at 593 nm. Working FRAP reagent (3.0 ml) was mixed with diluted plant extract (100 μl) or ascorbic acid. The mixture was vortex mixed and absorbance at 593 nm was obtained against the blank. Freshly prepared aqueous

ascorbic acid solutions of 100, 500, and 1000 μM (equivalent to 200, 1000, and 2000 μM FRAP) were used as standard. Absorbance values were expressed as mM ascorbic acid equivalent (AAE) per 100 g of fresh weight (FW) of the root (mM AAE/100 g FW).

II) Ferrozine assay: The chelating activity of the extract was estimated by the method of Dinis *et al.*, (23). To 1 mg dried plant extract dissolved in 1ml distilled water (1mg/ml), 0.05 ml of FeCl_2 (2 mM) was added. After 30 seconds 0.1 ml ferrozine (5 mM) was added. The mixture was shaken vigorously and left to stand at room temperature and the absorbance was observed at 562 nm after 10 min. Control samples lacks the extract. Absorbance values at 562 nm represent the amounts of iron reduced by the extract were converted to mM AAE/100 g FW.

Total protein assay

Total protein content of hypothalamus tissue homogenates was measured by Folin-phenol reaction as described by Lowry *et al.*, (24). A standard curve of BSA was prepared in each assay to measure the extent of derivatization.

Lipid Peroxidation (LPO)

LPO was determined by measuring thiobarbituric acid reactive substance (TBARS) in terms of malonaldehyde equivalent (MDA) using the molar extinction coefficient of $1.56 \times 10^5 \text{ min}^{-1} \cdot \text{cm}^{-1}$ as described by Ohkawa *et al.*, (25). Hypothalamus tissue was homogenized in 50 mM phosphate buffer (pH 7.7), centrifuged at $3,000 \times g$ for 15 min, and the supernatant was used for the assay. Samples of 0.1 ml supernatant were mixed with 0.2 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml 20% glacial acetic acid, and 1.5 ml of 0.8% thiobarbituric acid (TBA). Then test tubes containing reaction mixture were shaken and heated at 95°C for 60 min in a water bath, and then cooled under tap water before mixing with 1 ml distilled water and 5 ml mixture of n-butanol and pyridine (15: 1). The reaction mixture was centrifuged at $2,200 \times g$ for 10 min. The upper organic layer was isolated for the measurement of TBARS value based on absorbance at 532 nm in a Perkin-Elmer UV-Spectrophotometer (19). The results were expressed as nM TBARS/mg protein.

Protein carbonyl content

Protein carbonyl content of hypothalamus homogenates were evaluated according to Levine *et al.* (26) with some modifications. Briefly, hypothalamus was homogenized in 100 mM phosphate buffer (pH 7.4) containing 0.1% digitonin. Now 0.5 ml sample is mixed with streptomycin sulphate solution (10% w/v) up to a final concentration of 1% to precipitate DNA. The solution was mixed and left to stand for 15 min at room temperature, and then it was centrifuged at $2,800 \times g$ for 10 min. The supernatant was collected and 0.2 ml was divided equally between two test tubes. Both the tubes were incubated with DNPH (1.6 ml, 10 mM in 2 M HCl) and 1.6 ml of 2M HCl respectively for 60 minutes. Subsequently, the proteins were precipitated using 20% trichloroacetic acid (TCA) and centrifugation at $3,400 \times g$ for 10 min at room temperature. The pellet was resuspended in phosphate buffer and washed thrice

with ethyl acetate: ethanol (1:1 v/v) to remove excess DNPH. The final protein pellet was dissolved in 1.5 ml of 6 M guanidine hydrochloride. The carbonyl content was evaluated by detecting the absorbance at 370 nm in a Perkin-Elmer UV-Spectrophotometer. A standard curve was obtained using bovine serum albumin (BSA) and included in each assay to determine linearity and the extent of derivatization. Results were expressed in nM carbonyl group/mg protein.

Reduced glutathione (GSH)

The GSH content of hypothalamus tissue homogenates was quantitated according to Jollow et al. (27) that involves the spectrophotometric assessment of the 5-thio-2-nitrobenzoate in the presence of NADPH and glutathione reductase. Briefly, hypothalamus tissue was homogenized in 0.5 M ice cold metaphosphoric acid and centrifuged for 15 min at 16,000 x g at 4 °C. The 0.5 ml supernatant was mixed with 4 ml of ice cold 0.1 mM solution of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) in 0.1 M phosphate buffer (pH 8.0) and the optical density was obtained at 412 nm in a Perkin-Elmer UV-Spectrophotometer. A standard calibration curve was prepared using GSH.

Glutathione peroxidase (GPx) activity

GPx activity was measured by a coupled enzyme reaction that monitors oxidation of NADPH to NADP⁺ accompanied by a decrease in absorbance at 340 nm at 27 °C, as described by Lawrence and Burk (28). Briefly, the tissue samples were homogenized in 250 mM potassium phosphate buffer (pH 7.0) and centrifuged at 4°C at 500 x g for 15 min. The resulting supernatants were re-centrifuged at 10,000 x g for 20 min and supernatant was collected for measurement of GPx activity. Now the 0.1 ml of the sample is mixed with the reaction mixture containing 50 mM phosphate buffer pH 7.4, 4 mM NADPH, 1 unit glutathione reductase, and 0.7 mM H₂O₂ as the substrate. GPx activity was measured from the decrease of optical density at 340 nm due to NADPH oxidation between 2 and 4 min after the start of the reaction. GPx activity was expressed as nmol NADPH oxidized/mg protein.

Mitochondrial preparation

The animals were sacrificed, hypothalamus were rapidly excised and immediately placed into ice cold isolation medium of 0.25 M sucrose. Tissue was homogenized (10% w/v) by using glass homogenizer in an isolation buffer containing 0.25 M sucrose, 1 mM EDTA and 10 mM Tris- HCl (pH 7.4). Mitochondria were isolated from hypothalamus by differential centrifugation method as described in Jat et al. (29) and Parihar et al. (30). The experiments for membrane permeability transition were performed immediately after purification of the mitochondria.

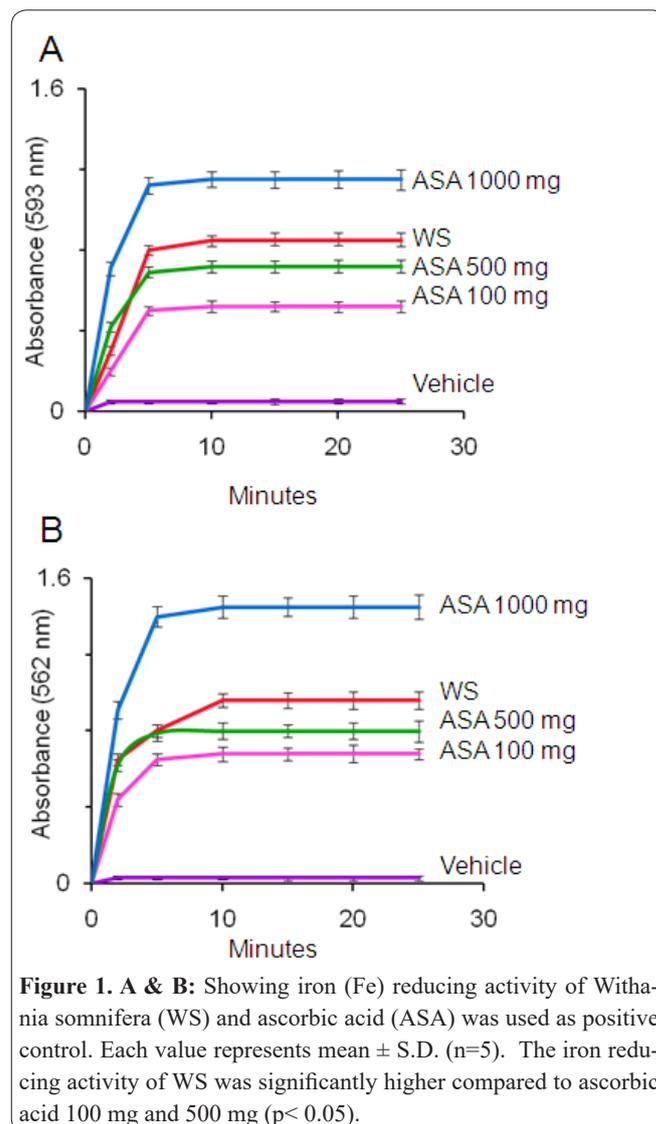
Determination of membrane permeability transition (MPT)

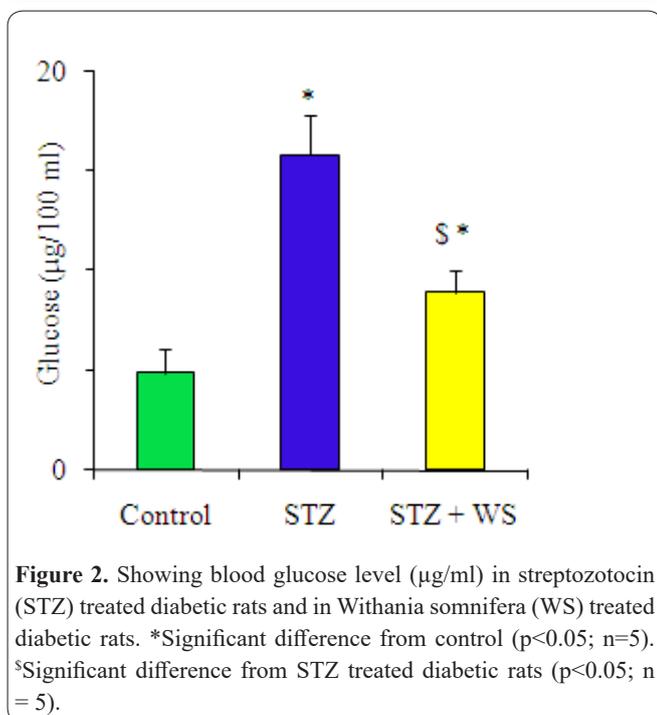
Isolated mitochondria from the hypothalamus of control and STZ treated mice (0.5 mg protein) were re-suspended in 2 ml Hank's balanced salt solution (HBSS; 5.4 mM KCl, 0.44 mM KH₂PO₄, 0.34 mM Na₂HPO₄, 0.49 mM MgCl₂, 0.41 mM MgSO₄, 132 mM NaCl, 10

mM HEPES, (pH 7.3), and pyruvic acid (10 mM)/malate (5 mM). MPT was induced in the isolated mitochondria by adding 3 μM CaCl₂. Experiments were performed on isolated mitochondria from different groups in different test tubes at room temperature. Mitochondrial swelling was estimated spectrophotometrically from the changes of light scattering at 540 nm measured in mitochondrial suspensions (0.5 mg of protein in 2 ml) as described (31).

Results

STZ induces diabetes in experimental animals by increasing the oxidative stress. The WS has been shown to possess strong antioxidant activity that can restore the antioxidative power in the cell and reduce the oxidative stress mediated cell damage. Our data showed a significant difference in the markers of oxidative stress between diabetic mice and WS treated diabetic mice. In the present study first we tested the antioxidant activity of WS using two different assays. As shown in Fig. 1A and Fig. 1B the iron reducing activity of WS caused increase in the absorbance at 593 and 562 nm. Ascorbic acid was used as positive control for the estimation of reducing power of WS. Fig.2 shows the blood glucose level in control, STZ treated and STZ + WS treated rats. The blood glucose level was significantly increased from 4.9 ± 1.17 μg/ml in control to 15.84 ± 2.02 μg/ml





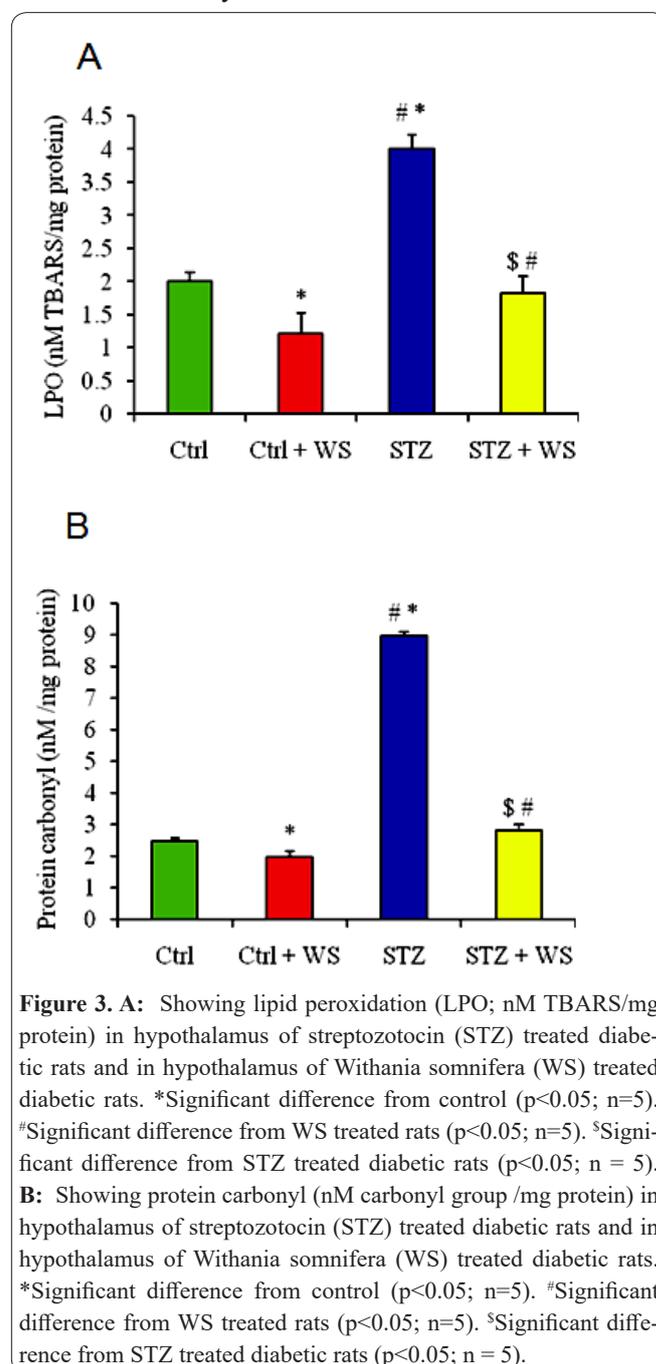
in hypothalamus of the rats treated with STZ. The blood glucose level was significantly decreased in hypothalamus of the rats treated with STZ along with the oral administration of WS extract. The LPO was decreased from $15.84 \pm 2.02 \mu\text{g}/\text{ml}$ in hypothalamus of the rats treated with STZ to $8.96 \pm 1.1 \mu\text{g}/\text{ml}$ in hypothalamus of the rats treated with STZ along with the oral administration of WS extract.

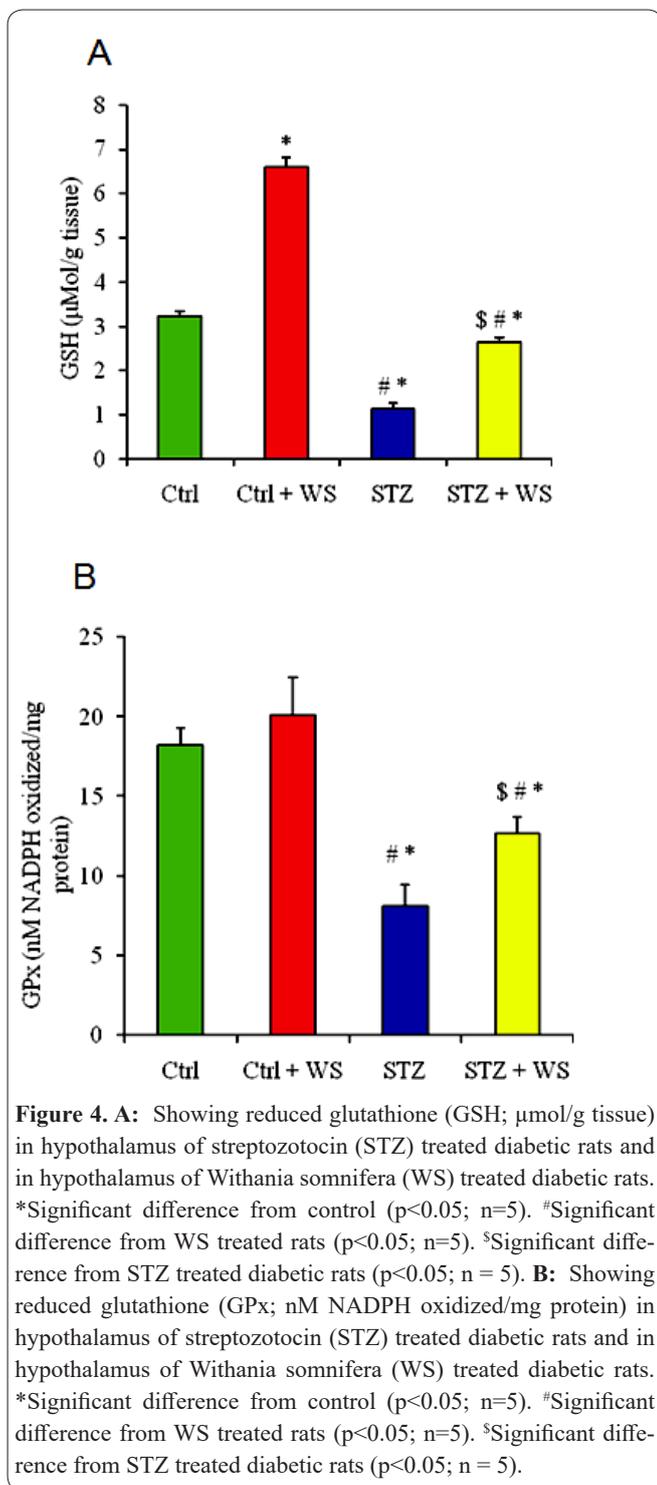
Membrane lipids are susceptible to oxidation thus peroxidation products can serve as potential oxidative stress biomarkers (32). STZ induces diabetes by increasing ROS levels. As increase in ROS increases LPO therefore in the present study we tested the effect of STZ on LPO in hypothalamus of diabetic rats. Fig. 3A showed a significant increase in LPO in hypothalamus of the rats treated with STZ as compared to control. The level of LPO was increased from $2.008 \pm 0.13 \text{ nM TBARS}/\text{mg}$ proteins in control to $4.016 \pm 0.20 \text{ nM TBARS}/\text{mg}$ proteins in hypothalamus of the rats treated with STZ. The LPO was significantly decreased in hypothalamus of the rats treated with STZ along with the oral administration of WS extract. The LPO was decreased from $4.016 \pm 0.20 \text{ nM TBARS}/\text{mg}$ proteins in hypothalamus of the rats treated with STZ to $1.834 \pm 0.25 \text{ nM TBARS}/\text{mg}$ proteins in hypothalamus of the rats treated with STZ along with the oral administration of WS extract. To observe the effect of WS on endogenous level of LPO we also supplemented WS extract to normal rats (without STZ treated). Our findings showed that WS supplementation reduced the endogenous level of LPO from $2.008 \pm 0.13 \text{ nM TBARS}/\text{mg}$ proteins (control) to $1.21 \pm 0.32 \text{ nM TBARS}/\text{mg}$ proteins in the hypothalamus. These results suggest that WS extract is effective in reducing both endogenous level and STZ-induced LPO in hypothalamus.

Protein carbonylation is a type of protein oxidation caused by ROS. We tested the effect of STZ induced diabetes on the level of protein carbonyl content in the hypothalamus. As shown in Fig. 3B, the levels of protein carbonyls were significantly increased in hypothalamus treated with STZ as compared with control. The levels

of protein carbonyl significantly increased from $2.48 \pm 0.08 \text{ nM carbonyl group}/\text{mg}$ proteins in control to $8.95 \pm 0.14 \text{ nM carbonyl group}/\text{mg}$ proteins in hypothalamus treated with STZ. The protein carbonyl levels were decreased from $8.95 \pm 0.14 \text{ nM carbonyl group}/\text{mg}$ proteins in hypothalamus treated with STZ to $2.828 \pm 0.19 \text{ nM carbonyl group}/\text{mg}$ proteins in hypothalamus of the rats treated with STZ along with oral administration of WS extract. To observe the effect of WS on endogenous level of protein carbonyl content we also supplemented WS extract to normal rats (without STZ treated). Our findings showed that WS supplementation reduced the endogenous level of protein carbonyl content from $2.48 \pm 0.08 \text{ nM carbonyl group}/\text{mg}$ proteins (control) to $1.975 \pm 0.18 \text{ nM carbonyl group}/\text{mg}$ proteins in the hypothalamus. These results suggest that WS extract is effective in reducing both endogenous level and STZ-induced protein carbonyl in hypothalamus.

Reduced glutathione (GSH) is the most abundant antioxidant that acts as a free radical scavenger (33). GSH is utilized by tissue in order to reduce the oxida-





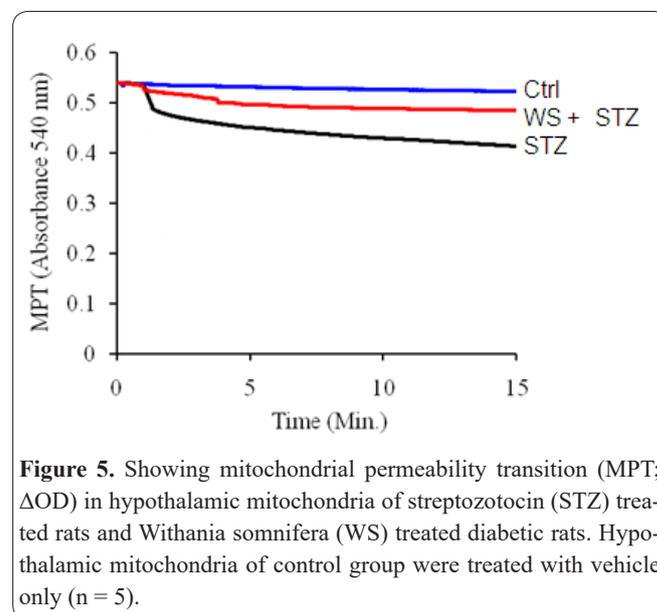
Stress. We tested the effects of STZ-induced oxidative stress on the levels of GSH in hypothalamus. As shown in Fig. 4A, STZ treatment caused a significant ($p < 0.05$) decrease in the GSH (1.14 ± 0.13 µmol/g) in hypothalamus as compared to control (3.23 ± 0.12 µmol/g). Oral administration of WS extract to STZ treated rats significantly increased the level of GSH up to 2.65 ± 0.11 µmol/g compared with STZ treated group (1.14 ± 0.13 µmol/g). To observe the effect of WS on endogenous level of GSH we also supplemented WS extract to normal rats (without STZ treated). Our findings showed that WS supplementation increased the endogenous level of GSH (6.6 ± 0.22 µmol/g) in the hypothalamus. These results suggest that WS extract is effective in increasing endogenous level of GSH as well as preventing the decline in GSH level in hypothalamus of STZ treated rats.

Glutathione peroxidase (GPx) is a part of cellular antioxidant defense systems that detoxify peroxides and protects the cell from the free radical induced oxidative damage. Fig. 4B showed decrease in the activity of GPx (8.14 ± 1.29 nM NADPH oxidized/mg protein) in the hypothalamus of STZ treated rats as compared to control (18.21 ± 1.12 nM NADPH oxidized/mg protein). Oral administration of WS extract to rats previously treated with STZ increased the activity of GPx up to 12.67 ± 1.02 nM NADPH oxidized/mg protein. To observe the effect of WS on endogenous level of GPx activity we also supplemented WS extract to normal rats (without STZ treated). Our findings showed no significant change in the GPx activity in the hypothalamus of WS extract treated rats as compared to control. These results suggest that WS extract is effective in preventing the decline in GPx activity in the hypothalamus of STZ treated rats.

MPT is a Ca^{2+} dependent increase of permeability of mitochondrial membrane that leads to mitochondrial swelling and finally cell death. MPT is considered as the consequence of oxidative damage to pre-existing membrane proteins or lipids via protein carbonylation or LPO (34). Therefore, we measured the MPT in hypothalamic mitochondria of control, STZ treated rats and STZ treated rats having oral administration of WS extract. Our results illustrated an increase in the MPT marked by decrease in the optical density at 540 nm in hypothalamic mitochondria of STZ treated rats as compared to control (Fig. 5). Oral administration of WS resulted in significant decrease in MPT in the hypothalamus of STZ treated rats marked by an increase in optical density at 540 nm.

Discussion

The hypothalamus is the key cerebral area involved in the regulation of glucose homeostasis (35, 36). Deregulation in hypothalamic metabolism results in various chronic diseases such as diabetes, obesity, dyslipidemia etc. (37). ROS produced in diabetic subjects can damage the hypothalamus thus deregulating the energy homeostasis. Our study shows that STZ increases oxidative stress and decreases the endogenous antioxidants such



as GPx and GSH in the hypothalamus. Mitochondria of hypothalamus of STZ treated rats showed increase in MPT. The diabetic rat supplemented with WS extract resulted in reduction in oxidative stress marked by reduction in LPO and protein carbonyl and increase in antioxidants such as GSH and GPX in hypothalamus. Hypothalamic mitochondria showed improvement in MPT by supplementation of WS extract. The medicinal properties of WS is due to the presence of various polyphenols such as gallic acid, syringic, benzoic, p-coumaric and vanillic acids and various flavonoids such as catechin, kaempferol, and naringenin (38). These polyphenols especially flavonoids are capable of increasing the antioxidative capacity, therefore, limits the risk of various degenerative diseases associated with oxidative stress.

LPO is the irreversible oxidative damage of the membranes caused by intense ROS production. The present study showed an increase in LPO significantly in hypothalamus of STZ treated rats while oral administration of WS caused its decrease. The effectiveness of WS extract in reducing oxidative stress is in concordance with our previous studies (39). Several other authors have also reported decrease in the level of LPO in different parts of brain and pancreatic tissue by the oral administration of WS extract (40, 41).

Increase in oxidative stress also increase protein carbonylation. Hence the measurement of level of protein carbonylation has various advantages over other oxidative stress markers. In the present study our results have shown a significant increase in protein carbonylation in hypothalamus of STZ treated rats while oral administration of WS to STZ treated rats caused significantly decrease in the level of protein carbonylation. Attenuating effect of WS against protein carbonylation was also observed in hippocampus and cortex tissue (39) and in lens of STZ treated rats (42).

GSH is intracellular nonprotein thiol which serves as the first line of defense against ROS. STZ caused a significant reduction in the level of GSH in hypothalamus. GSH is significantly increased by oral administration of WS extract. Previous studies also showed that WS improve the level of GSH in hippocampus, cerebral cortex, liver, kidney and heart (40, 43).

GPx is a selenium dependent antioxidant enzyme responsible for the reduction of hydrogen peroxide (44). Increased oxidative stress causes decrease in the activity of GPx. Our observation shows a significant reduction in the activity of GPx in hypothalamus of STZ treated rats. The GPx activity was significantly increased by oral administration of WS. Our results are clearly consistent with other studies in which remarkable increase in GPx activity was found on oral administration of WS in hippocampus, cerebral cortex, liver, kidney, heart, pancreatic tissue and eye lens (40, 41, 42, 43).

MPT is the consequence of increased oxidative stress which is prevented by wide variety of antioxidants. Plant polyphenols are generally involved in defense against oxidative stress, and thus may play an important role in protecting mitochondria against MPT. In the present study MPT was increased in hypothalamic tissue of STZ treated rats while oral supplementation of WS extract significantly decreased the MPT. Thus WS extract can be used to prevent the mitochondrial impairment against STZ induced oxidative stress.

We conclude that STZ increases oxidative stress and decrease the antioxidative responses in hypothalamus of rat. STZ also increases MPT while WS significantly ameliorates the oxidative stress and mitochondrial impairment caused by STZ. WS is an indigenous medicinal plant used in ayurveda from ancient times in India. As shown in the present study the WS significantly prevents hypothalamus from STZ-induced oxidative stress. However, the mechanism by which WS exerts multi targeted effects on different regions of brain is unknown. In future it will be promising to examine the molecular mechanism by which WS extract and its active component protects tissue from oxidative damage.

Acknowledgements

Authors gratefully acknowledge the financial support from Department of Science and Technology, New Delhi (INSPIRE-DST to Priyanka Parihar).

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