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Protective role of extracts of neem seeds in diabetes caused by streptozotocin in rats

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Abstract

Effect of petroleum ether extracts of kernel (NSK) and husk (NSH) of neem (*Azadirachta indica* A. Juss, Meliaceae) seeds on the prevention of oxidative stress caused by streptozotocin (STZ) was investigated. Diabetes mellitus was induced in adult male Wistar rats after administration of STZ (55 mg/kg b.wt., i.p., tail vein).The effect of NSK (2 gm/kg, b.wt.) and NSH (0.9 gm/kg, b.wt.) orally for 28 days was investigated in diabetic rats. Insulin-treated diabetic rats (6 U/kg, i.p., 28 days.) served as positive control. Diabetic rats given normal saline served as diabetic control. Rats that neither received STZ nor drugs served as normal control. Serum creatine phosphokinase (CPK) increased in diabetic rats was significantly decreased on insulin, NSK, and NSH treatments. The decrease in activities of superoxide dismutase (SOD) and catalase (CAT) and increase in lipid peroxidation (LPO) of erythrocytes as observed in diabetes was regained after insulin, NSH, and NSK treatments. However, there was insignificant improvement in SOD, CAT, and LPO of kidney on NSK and NSH treatment. In spite of increased CAT and SOD activities in liver and heart, LPO was also increased in diabetic rats. Insulin, NSH, and NSK treatments significantly protected animals from cardiac damage but not hepatic. Results suggest that NSH and NSK prevent oxidative stress caused by STZ in heart and erythrocytes. However, no such preventive effect was observed on renal and hepatic toxicity.

Keywords: *Azadirachta indica*; Streptozotocin; Diabetes; Cardioprotective effect; Antioxidant

1. Introduction

Neem (*Azadirachta indica* A. Juss, Meliaceae) is an indigenous tree grown all over India and Burma and is attributed to have many medicinal properties. Despite considerable progress in the management of diabetes mellitus by synthetic drugs, the search for indigenous antidiabetic agents still continues. Antihyperglycemic/hypoglycemic activity of neem leaves in dogs (Murty and Rao, 1978) and neem seed oil (Dixit et al., 1986) was reported. Neem seed oil is also reported to have spermicidal activity (Sinha et al., 1984). Previously, biochemical and hematological changes in rats and effect on cattle tick *Boophilus microplus* have been reported (Gupta et al., 2000, 2001a,b).

Hyperglycemia can cause oxidative stress, which in turn may result in cellular tissue damage. The harmful influence of diabetes on metabolism of tissues and organs is well known. Likewise, uncontrolled hyperglycemia can lead to disturbances in the structure and functions of organs (Kuyvenhoven and Meinders, 1999; West, 2000).

Streptozotocin (STZ) causes diabetes mellitus. Diabetes is associated with the generation of reactive oxygen species (ROS) causing oxidative damage particularly to heart and kidney (Mohamed et al., 1999). Glucose level increased the production of free radicals cell damage markers, such as malonaldehyde and conjugated dienes (Cuncio et al., 1995).

The present investigation was undertaken to evaluate the protective effect of petroleum ether extracts of neem (*Azadirachta indica*) seed husk and kernel on oxidative damage induced by STZ in tissues.

2. Material and methods

2.1. Animal

Male adult albino rats (150–200 g) were procured from the Laboratory Animal Resource Section of the Indian

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Veterinary Research Institute, Izatnagar. The animals were maintained on standard ration and provided with clean drinking water ad lib. The animals were kept in air-conditioned room (temperature $20 \pm 2\,^{\circ}C$) and acclimatized for a period of 7 days.

2.2. Plant material

Seeds of neem (*Azadirachta indica*) were procured from the local market and were dried in shade. From the seeds, husks, and kernel were separated. Petroleum ether extracts (60–80 °C) were obtained using Soxhlet apparatus.

2.3. Induction of diabetes

To induce diabetes, STZ (Sigma), prepared freshly in citrate buffer, pH 4.5, was immediately injected intravenously (55 mg/kg) through tail vein (Tomlinson et al., 1992). The rats were monitored for plasma glucose levels at weekly intervals. The rats with fasting glucose value of >250 mg/dl were considered diabetic. Blood samples were drawn by retro-orbital venepuncture technique. Plasma was separated by centrifugation at 2000 rpm for 15 min. Glucose levels were measured by *o*-toludine method using standard kits from Qualigens India Ltd., India. Based on the plasma glucose levels, uniformly diabetic rats were selected on day 30 after the injection of STZ.

2.4. Treatment of animal

Animals were divided into five groups of six animals and treated as follows:

Group-I was given citrate buffer and served as control (without STZ). STZ induced diabetic rats were divided in four groups (Groups II–V). Group-II diabetic control. Group-III positive control (insulin 6 U/kg, i.p.). Group-IV NSK (2 gm/kg, b.wt., $LD_{50} < 18$ mg/kg). Group-V NSH (0.9 gm/kg, b.wt., 1/20 LD_{50}) (Gupta et al., 2001a,b). Treatments were given orally for 28 days.

2.5. Collection of samples

The blood samples were collected at the start and 7th, 14th, 21st, and 28th day of experiment in two aliquots from retro-orbital plexus using micro-capillary technique (Sorg and Buckner, 1964). In one of the aliquot, no anticoagulant was used. Serum was separated. In the second aliquots, heparin was used as anticoagulant. Plasma was separated. The erythrocytes from heparinized blood were employed for membrane preparation by the method of Hanahan and Ekhlom (1974) using 1 mM EDTA in hypotonic buffer. The erythrocytes were washed thrice with ice-cold saline and suspended in 50% (v/v) of saline. After blood collection, all the rats were sacrificed by euthanasia. The organs such as liver, heart, and kidney were excised immediately and kept on ice, homogenized, and homogenates were centrifuged at $10{,}000 \times g$ for 10 min in Sorvall refrigerated centrifuge. Supernatants were collected and stored at 4 °C.

2.6. Estimation of antioxidant enzymes

2.6.1. Superoxide dismutase

Superoxide dismutase (SOD) activity was determined in the homogenates and erythrocytes according to Madesh and Balasubramanium (1998). A colorimetric assay involving generation of superoxide by pyrogallol auto-oxidation and the inhibition of superoxide-dependent reduction of the tetrazolium dye, MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) to its formazan by SOD was measured at 570 nm. Amount of MTT formazan was calculated by using molar extinction coefficient E_{570} of $17{,}000\,M^{-1}\,cm^{-1}$. One unit of SOD was defined as the amount of protein required to inhibit the MTT reduction by 50%.

2.6.2. Catalase

Catalase (CAT) activity was measured in homogenates and erythrocyte by the method of Maehly and Chance (1954). The utilization of H_2O_2 by CAT in the samples was measured spectrophotometrically as decrease in optical density at 254 nm. The substrate (H_2O_2) concentration was 20 mM for erythrocyte and cardiac tissue CAT measurement, while 2 mM for the renal CAT.

2.6.3. Lipid peroxidation

Lipid peroxidation (LPO) in tissue homogenates and erythrocytes was studied by measuring thiobarbituric acid reactive substances (TBARS) according to Ohkawa et al. (1979). The results are expressed as nmol malonaldehyde (MDA) formed per ml packed erythrocyte and nmol MDA formed per g tissue per 30 min in heart, liver, and kidney homogenates.

2.6.4. Serum creatine phosphokinase

Serum from animals was evaluated for creatine phosphokinase (CPK) activity using the kit obtained from Qualigens India Ltd.

2.7. Statistical analysis

Statistical analysis of data was done using Student' '*t*'-test (Snecdecor and Cochran, 1976).

3. Results

3.1. Protection against cardiac injury by petroleum ether extract of neem seed kernel and husk

Serum CPK activity is increased significantly in STZ-induced diabetic rats as compared to normal rats (Table 1). Treatment with insulin (6 U/kg, i.p.), petroleum ether extract

Table 1
Effect of NSK and NSH given orally daily for 28 days on serum creatine phosphokinase of STZ-induced diabetic rats

Treatment	Creatine phosphokinase (IU/l)
Control (citrate buffer)	57.77 ± 9.19
Diabetic control	184.89 ± 56.19
Insulin (6 U/kg, i.p.)	79.23 ± 27.01**
NSK (0.9 g/kg)	120.51 ± 36.45*
NSH (2.0 g/kg)	94.92 ± 22.86**

Values are mean±S.E. of six animals. * $P < 0.05$, ** $P < 0.01$ as compared to diabetic control.

of kernel (NSK, 2.0 g/kg) and husk (NSH, 0.09 g/kg) resulted in a significant reduction of serum phosphokinase activity of diabetic rats. The efficiency of decrease in CPK was in order of insulin > NSK > NSH.

3.2. Antioxidant activity

STZ administration resulted in a significant elevation of cardiac SOD, CAT, and LPO. With repeated administration of insulin, NSK, and NSH for 28 days, the cardiac LPO was significantly reduced comparable to normal control rats (Table 2).

Erythrocyte LPO was significantly increased in diabetic control and after repeated administration of NSK and NSH, the LPO was significantly reduced comparable to the normal control rats. Erythrocyte CAT and SOD was decreased in diabetic rats as compared to normal control which was regained to some extent by insulin and NSH (Table 3).

STZ administration resulted in a significant elevation in hepatic SOD, CAT, and LPO in diabetic control as compared to normal control (Table 4). In spite of the rise in the antioxidant enzyme activity in hepatic tissue, there was significant elevation of LPO. Repeated administration of NSK and NSH were ineffective in inhibiting the hepatic LPO. Hepatic CAT activity after NSH treatment was significantly inhibited as compared to diabetic control and comparable to control.

SOD and CAT enzyme activities were significantly decreased in kidney as compared control (Table 5) along with the significant elevation of LPO. With repeated administration of NSK and NSH, the activity was regained to some extent but not comparable to control.

Table 2
Effect of NSK and NSH orally for 28 days on SOD, CAT, and LPO of heart in STZ-induced diabetic rats

Treatment	CAT (Ua×10^3)	SOD, Ub	LPO, c
Control (citrate buffer)	4.06 ± 2.56	15.04 ± 2.24	12.91 ± 7.53
Diabetic control	7.50 ± 3.17	20.73 ± 5.31	39.42 ± 9.71
Insulin (6 U/kg)	4.46 ± 1.87*	12.24 ± 3.75*	17.29 ± 6.44
NSK (0.9 g/kg)	6.37 ± 2.97	17.00 ± 8.58	25.77 ± 10.44*
NSH (2.0 g/kg)	5.29 ± 1.56	15.46 ± 11.24	24.29 ± 6.75*

Values are mean±S.E. of six animals. * $P < 0.05$, ** $P < 0.01$ as compared to diabetic control. Ua: velocity constant per second, Ub: amount of hemoglobin (mg) inhibiting MTT by 50%, c: nM of MDA produced/g tissue per minute.

Table 3
Effect of NSK and NSH orally for 28 days on SOD, CAT, and LPO of erythrocytes in STZ-induced diabetic rats

Treatment	CAT (Ua×10^3)	SOD, Ub	LPO, c
Control (citrate buffer)	6.06 ± 2.80	81.00 ± 17.44	5.86 ± 14
Diabetic control	3.46 ± 1.19	52.01 ± 33.15	9.43 ± 1.85
Insulin (6 U/kg)	4.90 ± 2.97	73.51± 40.43	5.61 ± 1.24*
NSK (0.9 g/kg)	1.50 ± 0.32*	86.80 ± 31.41	5.39 ± 1.29**
NSH (2.0 g/kg)	4.75 ± 3.63	83.17 ± 30.91	5.01 ± 1.12**

Values are mean±S.E. of six animals. * $P < 0.05$, ** $P < 0.01$ as compared to diabetic control. Ua: velocity constant per second, Ub: amount of hemoglobin (mg) inhibiting MTT by 50%, c: nM of MDA produced/g tissue per minute.

Table 4
Effect of NSK and NSH orally for 28 days on SOD, CAT, and LPO of liver in STZ-induced diabetic rats

Treatment	CAT (Ua×10^3)	SOD, Ub	LPO, c
Control (citrate buffer)	58.72 ± 19.05	24.21 ± 5.41	24.28 ± 11.7
Diabetic control	94.64 ± 17.49	30.18 ± 3.31	47.14 ± 4.02
Insulin (6 U/kg)	64.91 ± 33.50	21.78 ± 5.58	23.96 ± 8.24
NSK (0.9 g/kg)	60.37 ± 21.66	32.19 ± 10.71	39.09 ± 11.80
NSH (2.0 g/kg)	74.48 ±20.69	33.66 ± 9.68	49.55 ± 8.14

Values are mean±S.E. of six animals. * $P < 0.05$, ** $P < 0.01$ as compared to diabetic control. Ua: velocity constant per second, Ub: amount of hemoglobin (mg) inhibiting MTT by 50%, c: nM of MDA produced/g tissue per minute.

Table 5
Effect of NSK and NSH orally for 28 days on SOD, CAT, and LPO of kidney in STZ-induced diabetic rats

Treatment	CAT (Ua×10^3)	SOD, Ub	LPO, c
Control (citrate buffer)	42.63 ± 4.05	23.92 ± 1.68	28.92 ± 4.95
Diabetic control	18.99 ± 6.66	17.85 ± 4.27	52.61 ± 9.88
Insulin (6 U/kg)	21.62 ± 5.61	24.51 ± 7.17	6.04 ± 7.27*
NSK (0.9 g/kg)	26.80 ± 7.95*	18.54 ± 4.41	54.07 ± 9.90
NSH (2.0 g/kg)	25.52 ± 13.31	19.70 ± 3.51	58.24 ± 4.29

Values are mean±S.E. of six animals. * $P < 0.05$, ** $P < 0.01$ as compared to diabetic control. Ua: velocity constant per second, Ub: amount of hemoglobin (mg) inhibiting MTT by 50%, c: nM of MDA produced/g tissue per minute.

4. Discussion

STZ is a commonly employed compound for induction of type-1 diabetes (Tomlinson et al., 1992). STZ causes diabetes by rapid depletion of β-cells which leads to reduction

in insulin release. Hyperglycemia causes oxidative damage by generation of ROS (Mohamed et al., 1999) and development of diabetic complications (Donnini et al., 1996; Baynes and Thorpe, 1999). Further, the STZ diabetic animals may exhibit most of the diabetic complications, namely, myocardial cardiovascular, gastrointestinal, nervous, vas deferens, kidney, and urinary bladder dysfunctions (Ozturk et al., 1996).

Increased serum CPK level in diabetic rats indicate cardiac muscular damage. Elevated concentration of CPK were recovered by treatment with insulin, NSK, or NSH suggesting their cardioprotective effect. Insulin was more effective as compared to NSK and NSH.

SOD and CAT are considered primary antioxidant enzymes, since they are involved in direct elimination of ROMs (Halliwell and Cutteridge, 1985). Effect of STZ in LPO, CAT and SOD activities was found to be tissue dependent. In spite of increased cardiac CAT and SOD activities in diabetic rats, increase in LPO was observed. Higher LPO and low SOD and CAT activity indicates an oxidative stress condition. The effect on LPO, CAT, and SOD was reversed by insulin, NSK, and NSH treatments. The observation suggests that in order to overcome the oxidative damage in heart, some other compensatory mechanisms exist in heart in addition to antioxidant enzymes. Reversal of increased enzymes and inhibition of LPO appears to be due to free radical scavenger activity of petroleum ether extract of neem seed kernel (NSK) and husk (NSH) in heart. The erythrocyte LPO was significantly increased in diabetic controls with reduction in antioxidant enzyme activities of SOD and CAT. Treatment with insulin and NSK stimulated SOD and CAT to reverse oxidative damage to erythrocyte membrane.

The study revealed that CAT and SOD activities were significantly inhibited along with an elevation of LPO in kidney of STZ-treated diabetic animals which is not reversed by different treatments. The results are in agreement with earlier data (Bryzewska et al., 1995; Parthiban et al., 1995). Significant increase in LPO, SOD, and CAT in pancreas, heart, and blood, and increase in glutathione peroxidase in kidney and pancreas in diabetes was reported by Kakar et al. (1995). Increase in heart LPO in diabetic rats was observed by Krishna Kumar et al. (1999).

The experimental results indicated that LPO played a role in tissue injury in STZ-induced diabetic rats. Petroleum ether extract of neem seed kernel (NSK) and husk (NSH) reduced the LPO in heart and erythrocytes, thus effectively protected cell functions and structure. STZ-induced diabetic oxidative changes of cardiac and erythrocyte toxicity as observed was reversed by the significant stimulation of antioxidant defense mechanism in erythrocytes or compensatory elevation of antioxidant defense mechanism in cardiac tissue by NSK and NSH. In STZ-induced diabetes, however, renal and hepatic toxicity was not prevented by NSK and NSH.

The results indicate that petroleum ether extract of neem seed kernel (NSK) and husk (NSH) showed significant protection against the oxidative damage induced by STZ in heart and erythrocytes of rats. NSK and NSH may act as cardioprotective and free radical scavenger agent.

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