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The vulnerability of diabetic rats to neurotoxin acrylamide: An interactive study

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ABSTRACT

Diabetes mellitus is a metabolic disorder with pathophysiology leading to various secondary complications such as nephropathy, neuropathy and retinopathy. The primary objective of the current study was to understand whether diabetic condition predisposes to exogenous neurotoxin insult. The neurotoxin chosen was acrylamide (ACR) because of its formation/ presence seen among various foods such as French fries, cookies and all deep fried or high heat processed carbohydrate-rich foods. Male adult Wistar rats were rendered diabetic using a single dose of streptozotocin (55mg/ kg bw, ip). After 1 week, the control and diabetic rats were sub-divided as follows: Group I: Control (received equal-volume of saline); Group II: ACR control (25 mg/ kg bw, ip, 3x/ wk); Group III – Diabetic control (received equal-volume of saline); Group IV – Diabetic administered with ACR (Diabetic + ACR; 25 mg/ kg bw, ip, 3x/ wk). Behavioural studies were carried out to check for sensory and motor functions on a weekly basis. At the end of 5 weeks, the rats were sacrificed and brain and sciatic nerve (SN) were collected for assessment of biochemical markers of oxidative mechanism and neurotransmission. Results suggest that diabetic rats were indeed predisposed to the neurotoxic effects of ACR (low dose). The development of neuropathic signs was advanced among the diabetic rats administered with ACR. The degree of oxidative impairments among diabetic rats administered with ACR was evident in SN and brain regions; the effects being more pronounced in SN. Further, an alteration in the cholinergic and dopaminergic function was also evident. Thus results obtained in this experimental design are suggestive of the vulnerability of diabetes to the neurotoxin.

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INTRODUCTION

Diabetic neuropathy (DN) is a gradual, inevitable and progressive secondary complication of untreated diabetes mellitus. With an alarming increase in diabetes around the world, a considerable fraction is showing up signs/ symptoms of some form of neuronal damage (Singh *et al.,* 2012; World Health Organization, 2017). The damage seen in DN involves both central nervous system (CNS) and peripheral nervous system (PNS). The neurons are not only damaged, but the deleterious effects of the hyperglycemic state are also seen at various levels of the nervous tissue. The main pathophysiological components are attributed to oxi-

dative stress, inflammatory response, energy depletion and altered mechanisms at the level of mitochondria and endoplasmic reticulum (Obrosova, 2009; Vincent *et al*. 2010; Prasad and Muralidhara, 2014a; Prasad *et al.,*2016). Such pathophysiology is associated with symptoms such as numbness, tingling, hyperalgesia and allodynia. However, diabetes is still a challenge for researchers to come up with a pharmacological solution to reduce the morbidity/ mortality associated with it (Yorek, 2011; World Health Organization, 2017).

Recently, studies have shown a strong relationship between type 2 diabetes and the main modifiable risk factors (excess weight, unhealthy diet, physical inactivity and tobacco use) and are similar in all regions of the world (World Health Organization, 2017). Never the less there is growing evidence that the underlying determinants of diabetes are a reflection of the major forces driving social, economic and cultural change especially in a developing country like India. Moreover, India has presumed the position of 'diabetic capital' of the world. In this view, the present study was designed to understand whether diabetic condition predisposes to exogenous neurotoxin insult. The neurotoxin chosen was acrylamide (ACR) because of its formation/ presence seen among various foods such as French fries, cookies and all deep fried or high heat processed carbohydrate-rich foods.

ACR, a well-documented human neurotoxin, is commonly used a chemical with applications in various industries such as the production of plastics, dyes, and paper, in the treatment of drinking water, wastewater and sewage (Pennisi *et al.,* 2013; Erkekoglu and Baydar, 2014). It is also used in Biological labs for gel electrophoresis. Besides ACR (monomeric form) is formed naturally during heat processing of carbohydrate-rich foods such as French fries, chips, cookies as reviewed earlier (Yener and Kalipci, 2009). Further, ACR has a high risk of contamination into surface and groundwater supplies due to its rapid solubility and mobility in water (Tepe and Cebi, 2017). ACR monomer is known to affect both central and peripheral nervous system and impacts both sensory and motor functions (Ling *et al.,* 2005; LoPachin and Gavin, 2008; Prasad and Muralidhara, 2013; Prasad and Muralidhara, 2014b). Several experiments in various models have shown the development of neuropathic signs due to ACR administration. The underlying mechanisms are believed to be the development of oxidative stress, inflammatory reactions and aberrations in neurotransmission (Tarek*et al.,* 2009; Lyn-Cook Jr *et al.,* 2011; Prasad and Muralidhara, 2013; Prasad and Muralidhara, 2014b). In the course of metabolism, ACR forms conjugates with reduced glutathione (GSH) and the resulting complex is metabolized by cytochrome P450 to form glycidamide, emphasizing the involvement of 'biotransformation' reactions (Zhu *et al.,* 2008; Ghareeb *et al.,* 2010).

Because of increased incidence of diabetes (most cases diagnosed late) as well as the increased exposure of humans to ACR through consumption of various thermally processed foods, it is indeed worth studying the vulnerability of people with diabetes to ACR. The current study was designed to address the predisposition of adult rats rendered diabetic by streptozotocin (STZ) to neurotoxic effects of ACR.

MATERIALS AND METHODS

Acrylamide (99%), STZ, thiobarbituricacid (TBA), 1,1,3,3-tetra methoxypropane, 2,7-dichloro-fluorescein(DCF), 2,7-dichloro-fluorescein diacetate (DCFH-DA), 1-chloro,2,4-dinitro benzene (CDNB), ophthaladialdehyde (OPT),5,5-dithiobis-(2-nitrobenzoic acid) [DTNB], dopamine, Fura 2AM and other fine chemicals were procured from M/s Sigma Chemical Co. (St Louis, MO, USA). All other chemicals used were of analytical grade.

Animals and care

Adult (8–9 weeks old) male albino rats (CFT-Wistar strain) were drawn from the stock colony of the Central Food Technological Research Institute (a CSIR lab) Animal Facility. Animals were provided with a commercial chow diet and water *ad libitum*. Diabetes was induced by a single *IP* injection of freshly dissolved STZ (55 mg/kg body weight) in a 0.1mol/ litre citrate buffer (pH 4.5). Control rats were injected with citrate buffer alone. STZ-injected rats were provided with 5% glucose in drinking water for 48 hr. Three days after STZ injection, blood glucose levels were measured with an Accuchek Comfort Sensor Glucometer. Animals with glucose levels ≥ 350 mg/dL 72 hr after STZ injection were included in the study. After 1 wk, the control and diabetic rats were sub-divided as follows:

Group I: Control (received equal-volume of saline);

Group II: ACR control (25 mg/ kg bw, ip, 3x/ wk, for 5 wks);

Group III – Diabetic control (STZ; received equal-volume of saline);

Group IV – Diabetic administered with ACR (STZ + ACR; 25 mg/ kg bw, ip, 3x/ wk, for 5 wks)

ACR dosage was selected based on the dose standardization studies described earlier (Prasad and Muralidhara, 2013). At a dosage of 25 mg/ kg bw, *ip* 3x/ wk, for 5 wks, ACR did not cause death or induce robust sensory dysfunction or motor deficits among adult male rats. All rats were monitored

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daily for feed intake. Body weights and blood glucose check, as well as behavioural assessments, were carried out once in a week throughout the experimental period. Terminally, rats were sacrificed under mild anesthesia; brain and sciatic nerve (SN) were excised and processed on ice for biochemical analysis.

All experiments were conducted strictly in accordance with approved guidelines by the Institute Animal Ethical Committee (IECA) regulated by the Committee for Control and Supervision of Experiments on Animals(CPCSEA), Government of India, India. Handling and care of animals were strictly in compliance with the guidelines by the 'Institutional Ethics Committee' (Registration number: 49/1999/CPCSEA).

Behavioural tests

Rats from all groups were subjected to behavioural tests for the assessment of neuropathic signs (sensory and motor deficits).

Tail-immersion test for hot hyperalgesia: Thermal sensitivity was assessed among all the rats of various groups by the tail-immersion test. The tail of each rat was immersed in water maintained at a temperature of 52 ± 0.5^{o}C. The tail withdrawal latency or signs of struggle were observed and recorded. A cut-off time of 12 sec for tail flicking response was maintained among all rats (Kannan *et al.,* 1996).

Narrow beam test for the motor function: The narrow beam test was performed according to a previously described method, with minor modifications (Prasad and Muralidhara, 2013). Animals were trained to traverse a 150 cm long wooden beam, divided into three segments (1, 2, and 3) of 50 cm each from a platform at one end to the animal' home cage at the other. The beam was placed horizontally 60 cm above the floor. Each rat was tested three times. The scoring (0 to 4) was as follows- a score of 0 indicated a rat traversed the beam without falling; a score of 1 indicated a rat fell off in the third segment; a score of 2 indicated it fell in the second segment; a score of 3 indicated it fell in the first segment, and a score of 4 indicated the rat failed even to balance/sit on the beam. The average of scores for three trials per rat was taken, and mean value and SEM for the group was calculated. Higher score infers more disability with respect to motor and co-ordination function.

Biochemical Analysis in SN and Brain Regions

The bilateral SN was excised (about 1.5 inches in length) from the L4 segment of the spinal cord at the mid-thigh level until its branching. It was washed in ice-cold saline, freed from the adherent blood vessels and connective tissue. The SN was minced and homogenized under ice in 0.1 M Tris-HCl buffer, pH 7.4. The cytosolic fraction was obtained by centrifuging the samples at 2,000g (Muthuraman *et al.,* 2008).

Brain regions- Cortex (Ct) and Cerebellum (Cb) were dissected over ice. A 10% homogenate of brain regions was prepared in ice-cold Tris-sucrose buffer (0.25 M, pH 7.4) and centrifuged at 1,000g for 10 min at 4^{o}C to obtain the nuclear pellet. Differential centrifugation (700 and 4,500g, 10 min, 4^{o}C) was employed to isolate the cytosol from different brain regions. All samples were stored in aliquots at -20^{o}C until next use.

Measurement of ROS Generation

ROS generation was assayed using dihydro dichloro fluoresce diacetate (H_2DCFH-DA), a nonpolar compound. The intracellular esterases convert this non-polar compound to a polar derivative, which rapidly reacts with ROS to form the highly fluorescent compound DCF. Briefly, an aliquot (100 µg protein equivalent) was incubated in Locke's buffer (pH 7.4, NaCl 154 mM, KCl 5.6 mM, $NaHCO_3$ 3.6 mM, HEPES 5 mM, $CaCl_2$ 2 mM, and glucose10 mM) containing H_2DCFH-DA (5 µM, 10 µL) for 30 min at room temperature. The fluorescent product DCF was measured using a spectrofluorimeter with an excitation wavelength of 480 nm and emission of 530 nm (Chandrashekar and Muralidhara, 2008). The ROS generation was calculated from a DCF standard curve and expressed as ρmoles DCF/min/mg protein.

Measurement of Hydroperoxides (HP)

HP levels were measured according to a previously described method by using FOX 1 reagent, with minor modifications (Wolff, 1994). An aliquot of cytosolic fraction (100 µg protein equivalent) was added to 1 mL FOX reagent (100 µM xylenol orange, 250 µM ferrous ammonium sulphate, 100 µM sorbitol, 25 mM H_2SO_4) and incubated for 30 min at room temperature. The colour developed was read at 560 nm in a spectrophotometer. The concentration of HP was calculated using the molar extinction coefficient ($\varepsilon = 2.2 \times 10^5\ M^{-1}\ cm^{-1}$) and expressed as ηmol HP/mg protein.

Assessment of Lipid Peroxidation (LPO)

LPO was assessed as described previously by measuring the formation of thiobarbituric acid-reactive substances (TBARS) (Ohkawa *et al.,* 1979). Briefly, the reaction mixture contained an aliquot of cytosolic fraction of different brain regions and SN (500 µg protein equivalent), 1.5 ml acetic acid (pH 3.5, 20%), 1.5 mL of 0.8% thiobarbituric acid (0.8% w/v), and 0.2 mL SDS (8% w/v). The mixture was heated to boiling for 45 min; TBARS adducts were measured at 532 nm and quantified as

malondialdehyde (MDA)equivalents using 1,1,3,3-tetramethoxypropane as the standard.

Determination of Protein Carbonyls (PC)

PC levels in the samples were quantified by the method of Levine et al. (1990). Briefly, and an aliquot of cytosol (500 µg protein equivalent) was incubated with 2,4-dinitrophenylhydrazine for 1 hr. The protein was precipitated by adding trichloroacetic acid (20%), followed by centrifugation. The pellet was washed twice with acetone and dissolved in 2 mMTris - HCl buffer (pH 7.4, containing 200mMNaCl and 2% SDS). The optical density was measured at at370 nm and expressed as nanomoles carbonyl/mg protein (ε = 22000 $M^{-1}cm^{-1}$).

Estimation of Reduced Glutathione (GSH)

GSH content was quantified based on a fluorimetric method described previously by Mokrasch and Teschke (1984) by employing o-phthalaldehyde (OPT). Briefly, an aliquot of cytosolic fraction (100 µg protein equivalent) was added to formic acid (0.1 M) and centrifuged at 10,000 g for 10 min. An aliquot of supernatant (de-proteinized) was added to tubes containing buffered formaldehyde (1:4 (v/v) 37% formalin: 0.1 M Na_2HPO_4). Sodium phosphate buffer (0.1 M, 5 mM EDTA, pH 8.0) was added to each tube, followed by OPT (100 µg/ml). After incubation for45 min at room temperature, the fluorescence was measured at excitation and emission wavelengths of 345 and 425 nm, respectively. The concentration of GSH was calculated from a standard curve, and values were expressed as µg/mg protein.

Activities of Selected Enzymes

Catalase (CAT) activity was determined according to a previously described method (Aebi, 1984). The reaction was initiated by adding an aliquot (equivalent to 50 µg protein) to 1 mL reaction mixture containing 8.8 mM H_2O_2 (3%), 0.1 mM sodium phosphate buffer, pH 7.0 The decrease in H_2O_2 was monitored for 3 min at 240 nm and expressed as nmol H_2O_2decomposed/min/mg protein (ε = 43.6 $mM^{-1}cm^{-1}$).

Superoxide dismutase (SOD) activity was determined by monitoring the inhibition of quercetin auto-oxidation. To a volume of 1 mL reaction mixture containing 3–5 µg protein, 0.016 M sodium phosphate buffer (pH 7.8), 8 mM N,N,N,N-tetramethylethylenediamine, and0.08 mM ethylenediamine tetraacetic acid (EDTA), quercetin (1.5 mg in 1 mL dimethylformamide) is added to initiate the reaction. Oxidation of quercetin was monitored for 3 min at 406 nm and expressed as the amount of protein required to inhibit 50% of quercetin auto-oxidation (Kostyuk and Potapovich, 1989).

Glutathione-S-transferase (GST) activity was quantified by monitoring the conjugation of glutathione to CDNB at 340 nm as described previously (Guthenberg *et al.,* 1985). The reaction was started by adding a cytosolic aliquot (0.01 mg protein equivalent) to phosphate buffer containing CDNB (0.1 M, pH 6.5 containing 0.5 mM EDTA, 0.075 mMCDNB, 0.05mM GSH). The increase in the optical density at340 nm was recorded over 3 min, and the activity expressed as nmol conjugate formed/min/mg protein (ε = 9.6 $mM^{-1}cm^{-1}$).

Glutathione reductase (GR) activity was measured (Carlberg *et al.,* 1984) by the addition of cytosolic aliquot (0.2 mg protein equivalent) to a reaction mixture of phosphate buffer (0.2 M, pH7.0) containing 2 mM EDTA, 20 mM oxidized glutathione, and 2 mM NADPH. The decrease in the absorbance at 340 nm resulting from oxidation of NADPH was monitored for 3 min and the activity expressed as nmol NADPH oxidized/min/mg protein (ε = 6.22 $mM^{-1}cm^{-1}$).

Thioredoxin reductase (TRR) activity in the test sample was measured as described previously by monitoring the reduction of DTNB at 412 nm in a potassium phosphate buffer (0.1M, pH 7.0) containing 10 mM EDTA, 0.2 mM NADPH (Luthman and Holmgen, 1982). The activity was expressed as nmol substrate reduced/min/mg protein (ε = 13.6 $mM^{-1}cm^{-1}$).

Cytosolic Calcium Levels

Intracellular calcium levels were measured in the SN and the brain regions by using Fura-2AM (Aoshima *et al.,* 1997). The cytosol equivalent to 50 µg protein was incubated in Tris HCl buffer (0.1 M, pH 7.8) for 30 min at 37°C. The fluorescence (excitation: 488 nm and emission: 525 nm) was measured and the amount of calcium was calculated using a standard curve and expressed as ng/mg protein.

The activity of Acetylcholinesterase (AChE)

AChE activity was determined according to the method of Ellmann *et al.,* 1961; by taking a reaction mixture containing phosphate buffer (0.1 M, pH 8.0), 10 mM 5,5-dithiobis 2-nitrobenzoic acid (DTNB), an aliquot of cytosol (100g protein equivalent), and acetylthiocholine iodide (150 mM); change in absorbance was monitored at 412 nm for 3 min. The enzyme activity was expressed as ηmoles of substrate hydrolyzed/min/mg protein (ε = 13.6 $mM^{-1}cm^{-1}$).

Determination of Dopamine (DA)

DA levels in the cytosol of SN and brain regions were analyzed by previously described by Dalpiaz *et al.,* 2007. Injecting the sample into an HPLC column (Discovery C-18, 25 cm x 4.6 mm, 5 mm;

Supelco Sigma-Aldrich) equipped with a UV detector set at 280 nm. The mobile phase consisted of 0.2% aqueous trifluoroacetic acid in methanol (70:30 v/v) and a flow rate of 1mL/min was maintained. DA levels were calculated by running a standard and expressed as µg DA/g tissue.

Determination of Protein

Protein concentrations in all test samples were determined by the method of Lowry *et al.,* 1951. Briefly, an aliquot of the sample was incubated with Folin–Ciocalteau'sphenol in an alkaline medium (30 min) followed by measurement of OD at 750 nm using a UV-visible spectrophotometer. The amount of protein was quantified with bovine serum albumin as the standard.

Statistical Analysis

Results are represented as the group mean of 6 +/- SEM for each experimental group. The data were analyzed by one-way ANOVA followed by a post hoc Tukey test to compare the control and treatment groups; P≤ 0.05 was considered statistically significant. All statistical analysis was performed in SPSS17.0.

RESULTS AND DISCUSSION

The primary objective of this interactive model was to understand whether diabetic condition predisposes to exogenous neurotoxin insult. In line with this view, the following data was obtained with respect to various relevant parameters to DN.

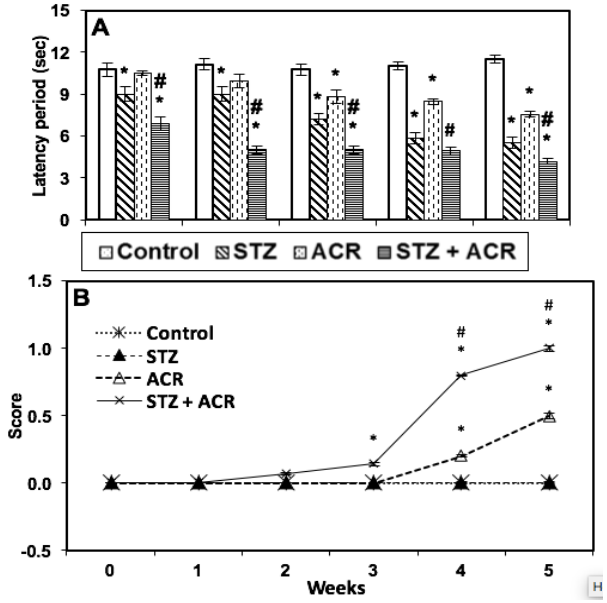

Figure 1: Effect of acrylamide intoxication on sensory function- hot hyperalgesia (A) and motor function- narrow beam test (B) among diabetic rats

(Values are mean ± SE (n=6). Data analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test for comparison of means. * significant against control; # against diabetic, at p ≤ 0.05).

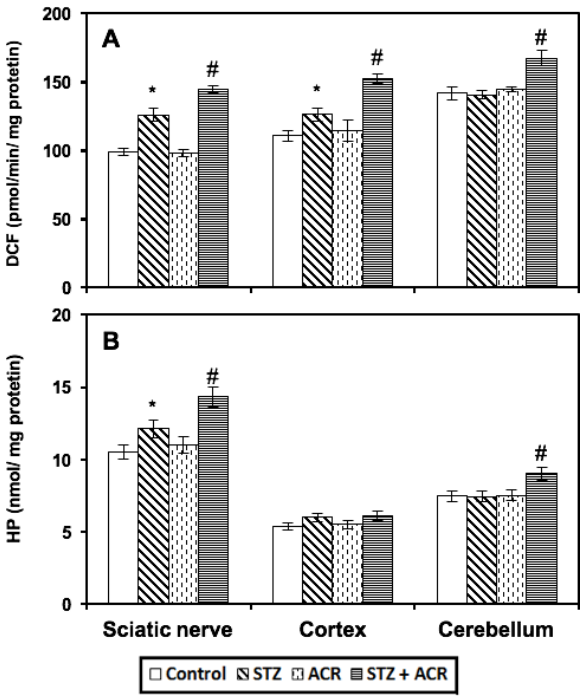

Figure 2: Effect of acrylamide intoxication on the levels of ROS (A) and HP (B) in sciatic nerve and brain regions of diabetic rats

(Values are mean ± SE (n=6). Data analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test for comparison of means. * significant against control; # against diabetic, at p ≤ 0.05).

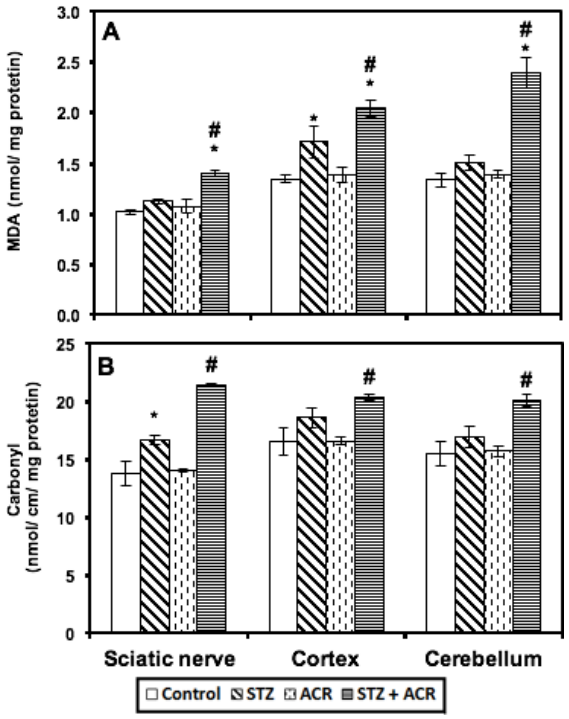

Figure 3: Effect of acrylamide intoxication on the levels of MDA (A) and PC (B) in sciatic nerve and brain regions of diabetic rats

(Values are mean ± SE (n=6). Data analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test for comparison of means. * significant against control; # against diabetic, at p ≤ 0.05).

Table 1: Effect of acrylamide (ACR) intoxication on the activities of antioxidant enzymes in sciatic nerve and brain regions of diabetic rats

Parameters/ Group	Control	STZ	ACR	STZ + ACR
Sciatic nerve				
GST[1]	19.5 ± 1.4	19.2 ± 2.4	22.9 ± 1.4	21.8 ± 2.5
SOD[2]	4.50 ± 0.2	5.87 ± 0.4*	4.80 ± 0.2	6.90 ± 0.2 *#
CAT[3]	2.72 ± 0.2	3.25 ± 0.3	2.65 ± 0.4	4.05 ± 0.4 *#
Cortex				
GST[1]	40.6 ± 1.6	35.4 ± 1.8	47.0 ± 1.7*	20.6 ± 3.1*#
SOD[2]	72.6 ± 2.3	89.8 ± 6.5	66.0 ± 3.6	126.0 ± 4.0*#
CAT[3]	5.91 ± 0.3	8.51 ± 0.2*	7.08 ± 0.2	10.43 ± 0.6 *#
Cerebellum				
GST[1]	38.9 ± 2.1	39.2 ± 4.1	43.5 ± 1.7*	24.3 ± 1.5 *#
SOD[2]	70.6 ± 2.5	68.7 ± 1.1	55.0 ± 1.1*	71.1 ± 2.9
CAT[3]	6.11 ± 0.3	4.48 ± 0.3*	6.88 ± 0.3	4.33 ± 0.5 *

[1]–Glutathione-S-transferase, µmol/ min / mg protein; [2]–Superoxide dismutase, U/ mg protein; [3]–Catalase, nmol of hydrogen peroxide decomposed/ min/ mg protein.
(Values are mean ± SE (n=6). Data analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test for comparison of means. * significant against control; # against diabetic, at $p \leq 0.05$).

Table 2: Effect of acrylamide (ACR) intoxication on GSH levels and the activities of reductases in sciatic nerve and brain regions of diabetic rats

Parameters/ Group	Control	STZ	ACR	STZ + ACR
Sciatic nerve				
GSH[1]	4.58 ± 0.12	4.10 ± 0.10	4.69 ± 0.4	4.22 ± 0.03 *
GR[2]	16.5 ± 1.7	19.5 ± 0.7*	17.5 ± 0.5	7.88 ± 0.12 *#
TRR[3]	4.66 ± 0.23	3.37 ± 0.31*	4.65 ± 0.4	4.48 ± 0.18 #
Cortex				
GSH[1]	4.82 ± 0.2	4.38 ± 0.2	4.91 ± 0.4	5.08 ± 0.1
GR[2]	25.3 ± 3.1	27.5 ± 4.2	26.2 ± 3.6	22.6 ± 1.5 #
TRR[3]	16.4 ± 2.0	22.9 ± 0.4*	17.1 ± 0.2	10.8 ± 1.2 *#
Cerebellum				
GSH[1]	5.03 ± 0.04	4.72 ± 0.26	5.15 ± 0.1	4.76 ± 0.13
GR[2]	24.6 ± 3.1	32.2 ± 4.9	25.3 ± 1.1	11.1 ± 0.07 *#
TRR[3]	17.7 ± 1.2	19.4 ± 0.3	16.2 ± 1.2	5.5 ± 0.15 *#

[1]–Reduced Glutathione, ug/ mg protein µmol/ min / mg protein; [2]–Glutathione Reductase, nmol; NADPH oxidized/ min/ mg protein; [3]–Thioredoxin Reductase, nmol of substrate/ min/ mg protein
(Values are mean ± SE (n=6). Data analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test for comparison of means. * significant against control; # against diabetic, at $p \leq 0.05$)

Feed intake, growth and Blood glucose levels

Significant reduction in body weights by the end of the first week was evident among diabetic rats (with or without ACR administration). While the feed intake was higher (20- 50%) among diabetic rats from the second week onwards, the marginal reduction was evident among STZ + ACR rats (Data not shown). However, no reduction in body weight gain was evident among control and ACR rats. Terminal body weights (g, Mean ± SE) of various groups were as follows- Control: 278 ± 10; ACR: 265 ± 20; STZ: 184 ± 20; STZ + ACR: 145 ± 7. Further, 10% mortality ensued among STZ + ACR group at the end of 5 weeks. The blood glucose levels among both control and ACR rats were in the normal range. ACR at the administered dosage did not affect the blood glucose levels among the diabetic rats (Data not shown). Terminally, the mean blood glucose levels (mg/ dL) among various groups were as follows, Control: 98 ± 3; ACR: 95 ± 5; STZ: 507 ± 40; STZ + ACR: 511 ± 34. These results indicate that ACR (low dose) *per se* did not interfere with the feed intake or blood glucose levels and its action is at cell/ tissue physiology only.

Effect on sensory and motor function

Among diabetic rats, a marginal decrease in the latency period with respect to hyperalgesia test was evident at the end of the first week, while ACR administered diabetic rats displayed a marked reduction (36%) in the latency period. At the end of five weeks, the *per*cent reduction in the latency period was: -STZ: 50%; ACR: 34%; STZ+ ACR: 64% (Fig.1A). Interestingly Diabetic rats did not develop motor deficits throughout the experimental period (Fig. 1B). Clinically significant motor impairment among people with diabetes is reported

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only in advanced cases of DN (Anderson, 2012). Hence in the present study, a period of 5 weeks of untreated diabetes did not exhibit motor deficits although sensory impairment was evident (Hyperalgesia). The small nerve fibres that transmit pain (hyperalgesia) and are liable to be oxidatively damaged faster than other nerve fibres that are involved in non-noxious stimuli transmission. It is only later that damage to the motor neurons takes place under hyperglycemic state (Pradat *et al.,* 2001; Zochodne *et al.,* 2008). However, diabetic rats administered with ACR displayed robust signs of motor dysfunction as early as three weeks and was progressive, while ACR *per se* group developed marginal motor deficits only by the end of four weeks (Fig. 1B).

Biochemical markers in SN and brain regions

Effect on oxidative stress markers

While the ROS levels were enhanced only in SN (28%) and Ct of diabetic rats, no alteration was evident among ACR administered rats (Fig. 2A). However, ACR administered diabetic rats showed significantly enhanced ROS levels in SN (46%) and brain regions. A similar trend was evident in HP levels in SN (Fig. 2B). A marked increase in MDA levels was apparent in SN (38%) and brain regions (Ct: 52%; Cb: 80%) of Diabetic + ACR group (Fig. 3A). While a significant increase in PC levels was evident only in SN of diabetic rats, a moderate elevation in brain regions occurred among STZ + ACR group (Fig. 3B). This indicates that ACR is potentiating the oxidative stress mechanisms under the diabetic condition as reported earlier (Obrosova, 2009; Prasad and Muralidhara, 2014a).

Effects of ACR on the activities of antioxidant/ detoxifying enzymes and GSH levels

Perturbations in the activities of GST, SOD and CAT in SN and brain regions of various groups are presented in Table 1. SN of diabetic rats showed a significant increase only in the activity of SOD and CAT among the ACR administered diabetic rats (SOD: ~50%; CAT: ~40%). The activity of GST was reduced in the brain regions with no alteration in SN. While the GSH levels showed no significant change in the brain regions, it was reduced in the SN among the STZ + ACR group (Table 2).

Further the activity of TRR was found to be reduced drastically in brain regions of diabetic rats and the activity of GR was reduced in both brain regions and SN with ACR (Table 2). In general, it appears that the diabetic rats are defending against hyperglycemia-induced stress in the nervous tissue in 5-6 weeks of onset of diabetes, as there is no drastic alteration in the antioxidant system. However, with ACR administration, diabetic rats show-up significant changes in the antioxidant system to combat the higher levels of oxidative markers such as ROS, HP, LPO and PC. Never the less, the degree of oxidative impairments among diabetic rats administered with ACR was evident in SN and brain regions; the effects being more pronounced in SN. However, the defence / antioxidant mechanisms are perhaps insufficient since the nervous tissue is oxidatively damaged (evident by high levels of oxidative markers) and hence contributes to the deficits observed in behavioural assessments.

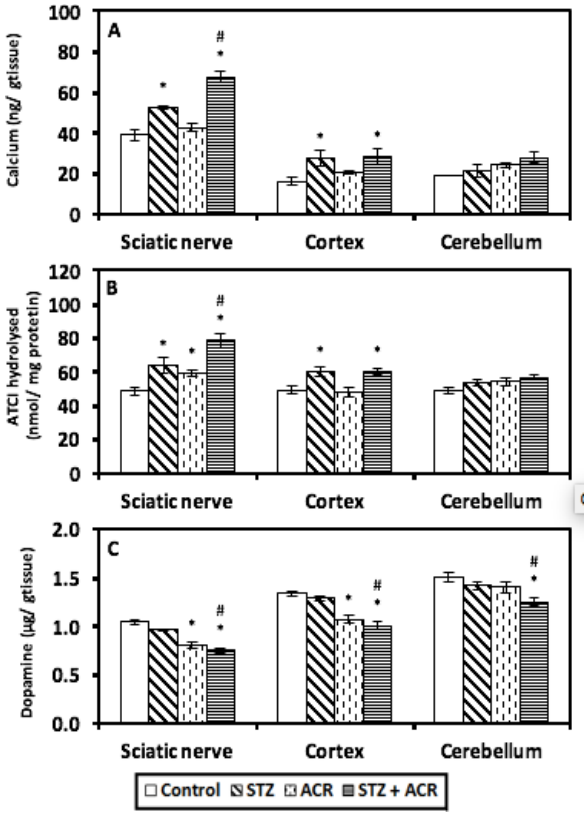

Figure 4: Effect of acrylamide intoxication on the levels of calcium (A), acetylcholinesterase activity (B) and dopamine levels (C) in sciatic nerve and brain regions of diabetic rats
(Values are mean ± SE (n=6). Data analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test for comparison of means. * significant against control; # against diabetic, at $p \leq 0.05$).

Effect of ACR on cytosolic calcium levels and neurochemical markers

Among diabetic rats, a robust elevation (44%)in the cytosolic calcium levels was evident only in SN (Fig. 4A). To address the effect of ACR on neurotransmission the cholinergic and dopaminergic function was assessed in terms of the activity of AChE and levels of DA respectively. The activity level of AChE was significantly elevated among diabetic rats in SN and Ct; however, it was further enhanced in the STZ + ACR group only in SN (Fig. 4B). Ambiguously, in Cb the AChE activity exhibited no significant change among all the groups.

Further, the diabetic rats did not exhibit depletion in DA levels either in SN or brain regions (Fig. 4C). A significant depletion (about 20%) ensued among ACR administered rats only in SN and Cb, however, among diabetic rats administered with ACR showed significant depletion in DA levels in SN as well as brain regions (Fig. 4C). These factors probably contribute to the development of neuropathic signs earlier than diabetic rats or ACR administered control rats. Further, these data emphasize that SN be more prone to damage by ACR among diabetic rats and hence exhibits the associated phenotypic changes observed in the behavioural tests.

Although the alterations in the several biochemical markers examined in the present study have been differential in the brain (part of CNS) and SN (part of PNS), there exists a complementarity with respect to sensory/ motor function between them. As for the differential response, it may be due to the existence of inherent differences in the detoxification process in different regions of the nervous system.

CONCLUSION

In the interactive model, diabetic rats exhibited relatively higher susceptibility to the neurotoxin ACR, as evidenced by advancement in the development of sensory and motor dysfunctions. The degree of oxidative impairments among diabetic rats administered with ACR was more pronounced in both SN and brain regions. Dopaminergic function appeared more vulnerable as evidenced by higher depletion in the levels of DA in SN and brain regions of ACR administered diabetic rats. The elevated activity of AChE in SN among diabetic rats with ACR administration emphasizes the cholinergic dysfunction.

In summary, this study is one of the primary studies towards understanding the need for right dietary choices among people with diabetes because of DN associated morbidity. Further, it suggests that ACR may profoundly affect people with metabolic disease such as diabetes perhaps by lowering the TDI (40 µg/kg bw/d) of ACR (Tardiff *et al.,* 2010). Although no clinical studies so far refer to the neurotoxicity of dietary ACR, it is important to be considerate of the growing population with metabolic disorders.

Conflict of Interest

The authors declare no conflict of interest

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