© 2012 Triveni Enterprises
Vikas Nagar, Lucknow, INDIA
editor@jeb.co.in
Full paper available on: www.jeb.co.in

J. Environ. Biol.
33, 233-238 (2012)
ISSN: 0254-8704
CODEN: JEBIDP

Dose related effects of nicotine on oxidative injury in young, adult and old rats

Author Details

Anshu Jain — Division of Pharmacology and Toxicology, Defence Research and Development, Establishment, Jhansi Road, Gwalior - 474 002, India

S.J.S. Flora (*Corresponding author*) — Division of Pharmacology and Toxicology, Defence Research and Development, Establishment, Jhansi Road, Gwalior - 474 002, India
e-mail: sjsflora@hotmail.com

Publication Data

Paper received:
18 January 2011

Revised received:
26 March 2011

Accepted:
21 April 2011

Abstract

Nicotine affects a variety of cellular process ranging from induction of gene expression to secretion of hormones and modulation of enzymatic activities. The objective of the present study was to study the dose dependent toxicity of nicotine on the oxidative stress in young, adult and old rats which were administered 0.75, 3 and 6 mg kg^{-1} nicotine as nicotine hydrogen tartarate intraperitoneally for a period of seven days. No changes were observed in blood catalase (CAT) activity and level of blood reactive oxygen species (ROS) in any of the age group at the lowest dose of nicotine. However, at the highest dose (6 mg kg^{-1} nicotine) ROS level increased significantly from 1.17to 1.41µM ml^{-1} in young rats and from 1.13 to 1.40 µM ml^{-1} in old rats. However, no change was observed in blood ROS levels of adult rats. Administration of 3 mg kg^{-1} nicotine resulted in an increase in level of reduced glutathione (GSH) in rats of all the age groups. The young animals were the most sensitive as a dose of 6 mg kg^{-1} resulted in decline in the levels of reduced GSH to 0.89 mg ml^{-1} as compared to normal control (1.03 mg ml^{-1}). The antioxidant enzymes SOD and CAT were sensitive to a dose of 6 mg kg^{-1} as it resulted in decline of the enzymatic activity in all age group animals. Also, administration of nicotine at a lower dose of 3 mg kg^{-1} inhibited SOD activity from 1.48 to 1.20 units min^{-1} mg^{-1} protein in old rats. Catalase activity showed a similar trend at a dose of 3 mg kg^{-1}. Administration of nicotine also increased the blood lipid peroxidation levels at all three doses in young and old rats dose dependently. Nicotine exposure also increased ROS in brain at the doses of 3 and 6 mg kg^{-1} in all the three age groups. Brain GSH decreased significantly at high dose of nicotine (6 mg kg^{-1} b.wt.) in adult rats (4.27 mg g^{-1}) and old rats (3.68 mg g^{-1}) but in young rats level increased to 4.40 mg g^{-1} at the lower dose (0.75 mg kg^{-1} nicotine). Brain lipid peroxidation increased at all three doses of nicotine in young as well as old rats as compared to their respective normal control. The SOD activity increased significantly in young (2.88 units $min^{-1}mg^{-1}$ protein) and old rats (1.81 units $min^{-1}mg^{-1}$ protein) as compared to their respective normal at a dose of 6 mg kg^{-1}. Interestingly, the SOD activity decreased in adult rats (2.18 units $min^{-1}mg^{-1}$ protein) as compared to its normal control. Catalase activity decreased at the dose of 3 mg kg^{-1} and 6 mg kg^{-1} nicotine in young and old rats but no effect was observed in adult rats at any of the doses. Acetylcholine esterase (AchE) activity decreased in a dose dependent manner in adult and old rats. Overall, the results of the study indicate that young and old rats are more sensitive to nicotine induced oxidative stress as compared to the adult ones.

Key words

Nicotine, Oxidative stress, Glutathione, Acetyl cholinesterase, Superoxide dismulate, Catalase activity

Introduction

Among the most well characterized chemicals found in tobacco and tobacco smoke, are polycyclic aromatic hydrocarbons (PAHs) and the highly addictive alkaloid, nicotine and its metabolite (Campain, 2004). Nicotine is commonly self-administered by the inhalation of tobacco smoke and by chewing of tobacco. Nicotine has been reported to induce oxidative stress both *in vivo* and *in vitro* (Pigeolot *et al.*, 1990). The process of lipid peroxidation plays an important role in the pathogenesis of numerous human diseases. The initiation of lipid peroxidation is carried out in most cases by free

radicals such as superoxide, hydroxyl radicals etc. and other reactive oxygen species like H_2O_2 causing cellular injury. Increased lipid peroxidation levels in tissues of intraperitoneal nicotine administered rats have been reported (Helen *et al.*, 2000). Nicotine is oxidized to its main metabolite cotinine in liver and causes the formation of free radicals in tissues. The formation of these radicals along with reduction in glutathione in tissues causes oxidative damage (Gumustekin *et al.*, 2005). It has been shown that nicotine treatment at a dose of 2 mg kg^{-1} induced oxidative damage in both liver and kidney which were attenuated by the GSH supplementation (Dey and Roy, 2010).

Oxidative stress is a major mechanism for cellular damage associated with a wide variety of neurotoxicants (Gitto *et al*., 2002; Gupta, 2004; Ohtsuki and Suzuki, 2000; Olanow and Arendash, 1994). Oxidative stress is postulated to be one of the most important mechanisms behind age-related changes in senescence. Age-related changes in sensitivity to chemicals may be the result of the alterations in their toxicokinetics. The free radical theory of aging proposes that reactive oxygen species (ROS) cause oxidative damage over the lifetime of the subject. It is the cumulative and potentially increasing amount of accumulated damage that accounts for the dysfunctions and pathologies seen in normal aging. Free radicals have previously been shown to be capable of damaging many cellular components such as DNA, proteins and lipids. The role of oxidative stress is significant especially in the brain, which consumes 20% of all inspired oxygen, contains relatively poor concentration of antioxidants and related enzymes and has abundance of polyunsaturated fatty acids that serve as a major biological target for the reactive oxygen species (Gupta, 2004).

A study has demonstrated that nicotine and chlorpyrifos elicit oxidative damage to developing neural cells both *in vitro* and *in vivo* (Qiao *et al*., 2005). The developing brain is especially sensitive because it has lower reserves of protective enzymes and antioxidants such as glutathione and compared to adult brain is deficient in glia (Gupta, 2004; James *et al*., 2005) which ordinarily protects neurons from oxidative molecules (Tanka *et al*., 1999). Another study demonstrated deleterious effects of nicotine in old rats as augmented by DNA damage, ROS concentration and lipid peroxides levels (Barros *et al*., 2007). Also, the accumulation of transition metals is higher in aged than in young brains which lead to the increased formation of hydroxyl radical, the most reactive ROS via Fenton reaction. The brain depletion of the antioxidant defense system including glutathione is significant during ageing contributing to the oxidative stress scenario.

Studies on the toxic effect of nicotine are limited and contradictory. Looking into the unavoidable and worldwide use of tobacco and its product among peoples of different age group despite of its role in various pathologies, the purpose of present investigation was to study and compare the dose dependent toxic effect of nicotine in different age group of animals.

Materials and Methods

Experimental design: Sixty male Wistar rats comprising of 20 young (60-80 g, ~5 weeks), 20 adult (180-220 g, ~20 weeks) and 20 old (450-550 g, >60 weeks) were obtained from the animal facility of Defence Research and Development Establishment (DRDE). The animals were acclimatized for 7 days prior to use. The Ethical Committee on Animal Experiments of DRDE, Gwalior, India, approved the protocols for the experiments. Throughout the experiment, animals were housed in polypropylene cages in an air–conditioned room with temperature maintained at 25 ± 2°C and 12 hr alternate day and night cycles. Rats were allowed standard pellet diet (Amrut Feeds, Pranav Agro, New Delhi, metal contents of diet, in ppm dry weight Zn 45, Cu 10, Mn 55, Fe 70, Co 5) and water *ad libitum*.

Fifteen animals from each group were exposed to nicotine as nicotine dihydrogen tartarate (Sigma Aldrich, USA) at a dose of 0.75, 3 and 6 mg kg^{-1} b.wt., n=5 for each dose intraperitoneally, and remaining five rats of each group received normal saline intraperitoneally (normal control) for one week. The selection of the nicotine doses in the present study were based on previous published studies (Erat *et al*., 2007; Abreu-Villaca *et al*., 2003; Slotkin *et al*., 2004).

After 7 days, exposure was stopped and rats from each group were sacrificed under light ether anesthesia, 24 hr after the last dosing. Blood was collected in heparinized vials. Brain was removed, washed with normal saline and all the extraneous materials were removed before studying various biochemical parameters.

Biochemical assays :

Reactive oxygen species (ROS): Amount of ROS was measured using 2', 7'-dichlrofluorescin diacetate (DCF-DA) as described by Socci *et al.* (1999). For estimation of ROS in blood, 5% RBC hemolysate and for brain 20% brain homogenate was prepared and diluted with ice–cold 40 mM tris-HCl buffer (pH 7.4). A 40 µl of 1.25 mM DCF-DA in methanol was further added. All samples were incubated for 15 min in a 37°C water bath. Fluorescence was determined at 488 nm excitation and 525 nm emission using a fluorescence plate reader (Tecan Spectra Fluor Plus).

Glutathione (GSH): Analysis of blood GSH concentration was performed by method of Ellman *et al.* (1959) slightly modified by Jollow *et al.* (1974). A 0.2 ml of whole blood was added to 1.8 ml of distilled water and incubated for 10 min at 37°C for complete hemolysis. After adding 3 ml of 4% sulphosalicylic acid, tubes were centrifuged at 2500 rpm for 15 min. To the supernatant 0.2 ml of 10 mM solution of 5,5' dithiobis- (2 nitro benzoic acid) (DTNB) was added in presence of phosphate buffer (0.1M pH 7.4). Absorbance recorded at 412 nm.

Reduced glutathione (GSH) content was measured as described by Hissin and Hilf (1973). Briefly, 0.25 g of tissue sample was homogenized on ice with 3.75 ml of phosphate-EDTA buffer and 1 ml of 25% HPO_3. The total homogenate was centrifuged at 100,000 g for 30 min at 4°C. A 0.5 ml supernatant and 4.5 ml phosphate buffer (pH 8.0) were mixed. The final assay mixture (2.0 ml) contained 100 ml supernatant, 1.8 ml phosphate-EDTA

buffer and 100 µl O-phthaldehyde (OPT; 1000 ml ml^{-1} in absolute methanol, prepared fresh). Samples were incubated at room temperature for 15 min and fluorescence was measured at 350 nm (E_x)/420 nm (E_m) with Tecan Spectra Fluor Plus (Germany).

Superoxide dismutase (SOD): The SOD activity was assayed by the method of Kakkar *et al.* (1983). Reaction mixture contained 1.2 ml of (0.052 mM) sodium pyrophosphate buffer, 0.1 ml of (186 mM) phenazine methosulphate, 0.3 ml of 300 mM nitro blue tetrazolium. A 0.2 ml of the supernatant obtained after centrifugation (1500 xg, 10 min followed by 10000xg, 15min) of 5% RBC hemolysate/tissue homogenate was added to the reaction mixture. Enzymatic reaction was initiated by adding 0.2 ml of NADH (780 mM) and stopped by adding 1 ml of glacial acetic acid. Colour intensity of the chromogen was measured at 560 nm. Activity was expresse as units $min^{-1}mg^{-1}$ of protein.

Catalase (CAT): Catalase activity in tissue and blood was assayed following the procedure of Sinha *et al.* (1972). Briefly, 0.1 ml of 5% tissue homogenate or 5% RBC hemolysate was incubated with 0.5 ml of H_2O_2 (0.2 M) at 37°C for 90 sec in the presence of 0.01 M phosphate buffer (pH 7.4). Reaction was stopped by adding 5% dichromate solution. Further, samples were incubated at 100°C for 15 min in boiling water bath. Amount of H_2O_2 consumed was determined by recording absorbance at 570 nm.

Thiobarbituric acid reactive substances (TBARS): Measurement of lipid peroxidation was done by the method described by Ohkawa *et al.* (1979). Tissue lipid peroxidation was measured in whole-brain homogenate [5% homogenate (w/v) in 150 mM KCl] for 30 min at 37°C. The incubation was interrupted by adding 0.1 ml of 10% trichloroacetic acid. After centrifugation (1 ml) supernatant was then mixed with 1 ml of 0.65% thiobarbituric acid. The mixture was then kept in a boiling water bath for 15 min. The malondialdehyde formation was determined by reading absorbance at 535 nm. The amount of TBARS was calculated using a molar extinction coefficient of $1.56x10^5 M^{-1}cm^{-1}$.

For blood TBARS estimation, 0.1 ml of 5% RBC hemolysate was added to 0.2 ml of 8.1% SDS (w/v) and incubated for 10 min, To this 1.5 ml of 20% acetic acid was added followed by adding 1.5 ml of 0.8% thiobarbituric acid (w/v) and 0.7 ml distilled water and incubated for 1 hr in boiling water bath. One ml of distilled water was added to the solution after cooling and centrifuged at 6000 rpm for 15 min. The absorbance of supernatant was read at 532 nm and the values were expressed as moles of MDA ml^{-1} of blood.

Brain acetylcholinesterase (AchE) activity: A 10% brain homogenate (w/v) was prepared in 0.25 M sucrose. Activity of AchE in brain was carried out according to the method of Ellman *et al.* (1961) using acetylthiocholine as substrate. The activity of AchE was measured at 412 nm and its unit is expressed as nmol $min^{-1}mg^{-1}$ protein.

Statistical analysis: Experimental results were expressed as the mean ± SE and accompanied by a number of observations. Data are assessed by the method of one-way analysis of variance (ANOVA). If this analysis indicated significant difference among the group mean, then unexposed and exposed group (with or without treatment) was compared by Student's t-test. Values with matching symbol notation in each column were not significant at 5% level of probability.

Results and Discussion

Effect on blood: The dose dependent and age related effect of nicotine on blood oxidative stress variables is shown in Table 1. In young rats, at low dose of nicotine (0.75 mg kg^{-1} b.wt.) none of the blood oxidative stress variables were found altered except blood

Table - 1: Dose dependent effect of nicotine on blood biochemical variables in different age rats

Age	Nicotine treatments (mg kg^{-1} b.wt.)	ROS	GSH	TBARS	SOD	CAT
Young	Normal animals	1.17 ± 0.05*	1.03 ± 0.04*	166.7 ± 3.6*	1.15 ± 0.07*	31.57 ± 1.43*
	0.75	1.24 ± 0.03*	1.05 ± 0.02*	351.3 ± 23.3†	1.11 ± 0.03*	31.09 ± 0.99*
	3	1.40 ± 0.04†	1.23 ± 0.03†	391.8 ± 5.7†	1.00 ± 0.04*	28.15 ± 1.38*
	6	1.41 ± 0.02†	0.89 ± 0.02†	400.8 ± 16.0†	0.77 ± 0.03†	26.47 ± 0.76†
Adult	Normal animals	0.95 ± 0.04*	1.12 ± 0.05*	277.4 ± 13.0*	2.26 ± 0.04*	39.73 ± 1.23*
	0.75	0.95 ± 0.04*	1.14 ± 0.01*	322.3 ± 23.6*	1.83 ± 0.05†	38.44 ± 1.54*
	3	1.01 ± 0.03*	1.29 ± 0.03†	322.5 ± 9.9†	1.58 ± 0.11†	35.27 ± 1.35†
	6	0.96 ± 0.06*	1.19 ± 0.02*	384.0 ± 20.3†	1.48 ± 0.10†	32.68 ± 0.91†
Old	Normal animals	1.13 ± 0.01*	0.98 ± 0.06*	276.6 ± 16.3*	1.48 ± 0.09*	35.35 ± 1.50*
	0.75	1.16 ± 0.03*	1.33 ± 0.08†	368.7 ± 11.2†	1.33 ± 0.07*	33.84 ± 0.49*
	3	1.40 ± 0.02†	1.63 ± 0.04†	377.6 ± 6.0†	1.20 ± 0.03†	31.11 ± 0.76†
	6	1.40 ± 0.03†	0.88 ± 0.03*	392.8 ± 5.7†	1.12 ± 0.03†	26.36 ± 0.80†

Reactive Oxygen Species (ROS) = µM ml^{-1} blood; Reduced glutathione (GSH) = mg ml^{-1} blood, TBARS = nmoles MDA produced ml^{-1} blood, Superoxide dismutase (SOD) = units $min^{-1}mg^{-1}$ protein and Catalase (CAT) = µmoles H_2O_2 produced $min^{-1}mg^{-1}$ protein; Values are mean of five replicate±SE. Values with matching symbol notation in each column are not significant at 5% level

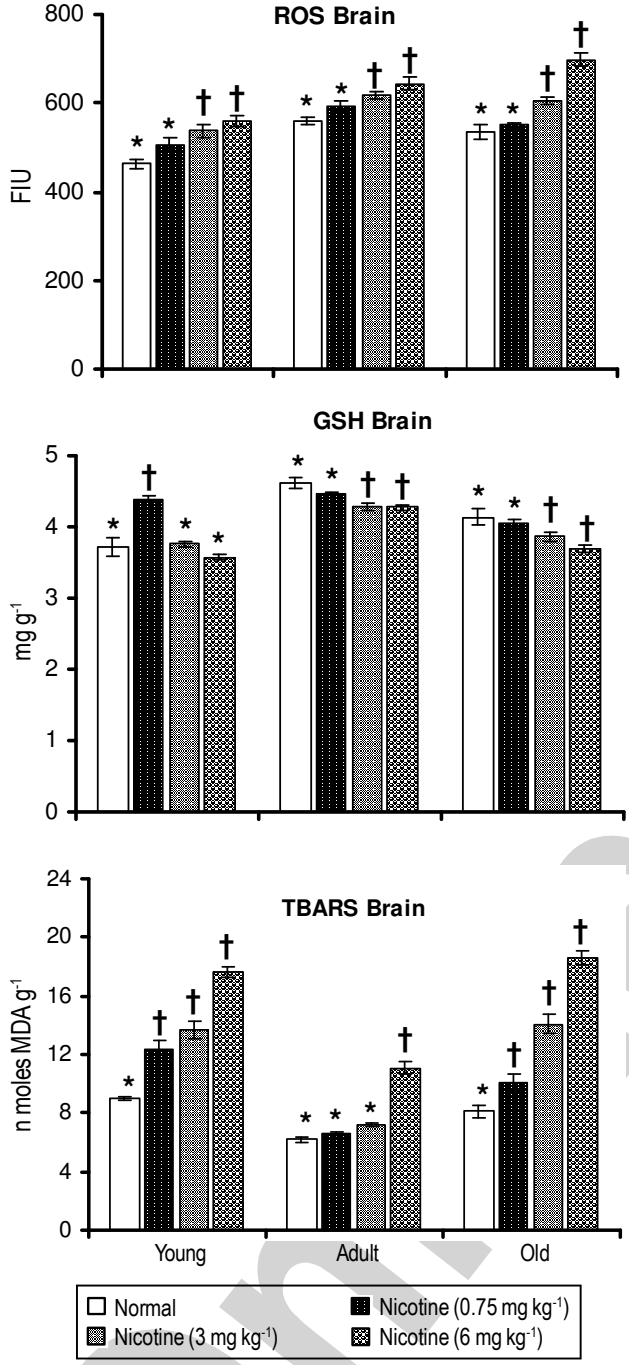

Fig. 1: Dose dependent effect of nicotine on brain oxidative stress in different age rats; Reactive oxygen species (ROS) - Fluorescence intensity unit (FIU); Reduced glutathione (GSH) = mg g^{-1} and TBARS = n moles MDA g^{-1}. Values are mean of five replicates±SE. Values with matching symbol notation in each column are not significant at 5% level

lipid peroxidation which showed significant elevation ($p<0.001$) as compared to normal control. On the other hand, at high dose of nicotine (6 mg kg^{-1} b.wt.) there was a significant increase ($p<0.05$) in blood ROS, lipid peroxidation and a corresponding decrease in blood GSH, SOD and CAT in young rats ($p<0.5$) (Table 1). The median dose of nicotine (3 mg kg^{-1}) was also effective in inducing oxidative stress as the levels of GSH and blood TBARS were found elevated on nicotine exposure.

In adult rats, at low dose (0.75 mg kg^{-1} b.wt.) of nicotine exposure there were no alterations in any of the oxidative stress variables except a significant decline in blood SOD activity ($p<0.05$) (Table 1). A significant increase ($p<0.05$) in blood GSH and TBARS level and decline in blood SOD and CAT activity was observed on 3 mg kg^{-1} of nicotine exposure (Table 1). Interestingly, there was no significant difference in blood ROS and GSH levels at the highest dose of nicotine (6 mg kg^{-1} b.wt.), although significant increase in blood lipid peroxidation ($p<0.01$) and significant decline in SOD and CAT activity ($p<0.05$) was observed (Table 1).

In old rats, at low dose of nicotine exposure there was a significant elevation observed in blood GSH ($p<0.05$) and lipid peroxidation ($p<0.001$). However, significant increase in blood ROS and TBARS, and significant decline in SOD and CAT activity was observed at high dose of nicotine. The dose of 3 mg kg^{-1} also produced similar effects on oxidative stress variables as the higher dose as significant increase in blood ROS and TBARS levels ($p<0.05$) and a significant decrease in SOD and catalase activity ($p<0.05$) was observed (Table 1)

Effect on brain: Effect of different doses of nicotine in brain oxidative stress variables in rats of all age groups are shown in Fig. 1 and Fig. 2. In young rats, on exposure to low dose of nicotine (0.75 mg kg^{-1} b. wt.) there was a significant alterations observed in GSH and TBARS level (Fig. 1) and SOD activity (Fig. 2), which increased significantly. At high and medium dose of nicotine administration a significant increase in brain ROS, TBARS and SOD activity and a decrease in CAT (Fig. 2) and AchE activity was found (Fig. 2).

In adult rats, brain ROS increased significantly and a corresponding decline ($p<0.05$) in GSH (Fig. 1) and AchE activity (Fig. 2) was also observed at all the three doses of nicotine administration. However, brain lipid peroxidation level (Fig. 1) increased ($p<0.001$) only at the highest dose of nicotine. Brain SOD activity (Fig. 2) showed marked depletion ($p<0.05$) at 3 and 6 mg kg^{-1} of nicotine, however no change was observed in CAT activity (Fig. 2).

In old rats, brain ROS and TBARS level (Fig, 1) increased and AchE activity (Fig, 2) decreased ($p<0.05$) at all 3 doses of nicotine. Furthermore, nicotine administration with 3 and 6 mg kg^{-1} for the period of 7days resulted in a significant decrease ($p<0.05$) in GSH level (Fig. 1) and CAT activity (Fig. 2). The SOD activity (Fig. 2) was significantly ($p<0.05$) elevated at high dose of nicotine exposure.

Previous studies suggested that nicotine metabolism requires oxygen which is mediated by monooxygenases of the cytochrome P-450 type (Nakayama, 1988; Cashman *et al.*, 1992). Interestingly, one of the most important consequences of the metabolism of toxic chemicals by mixed function oxidases is the

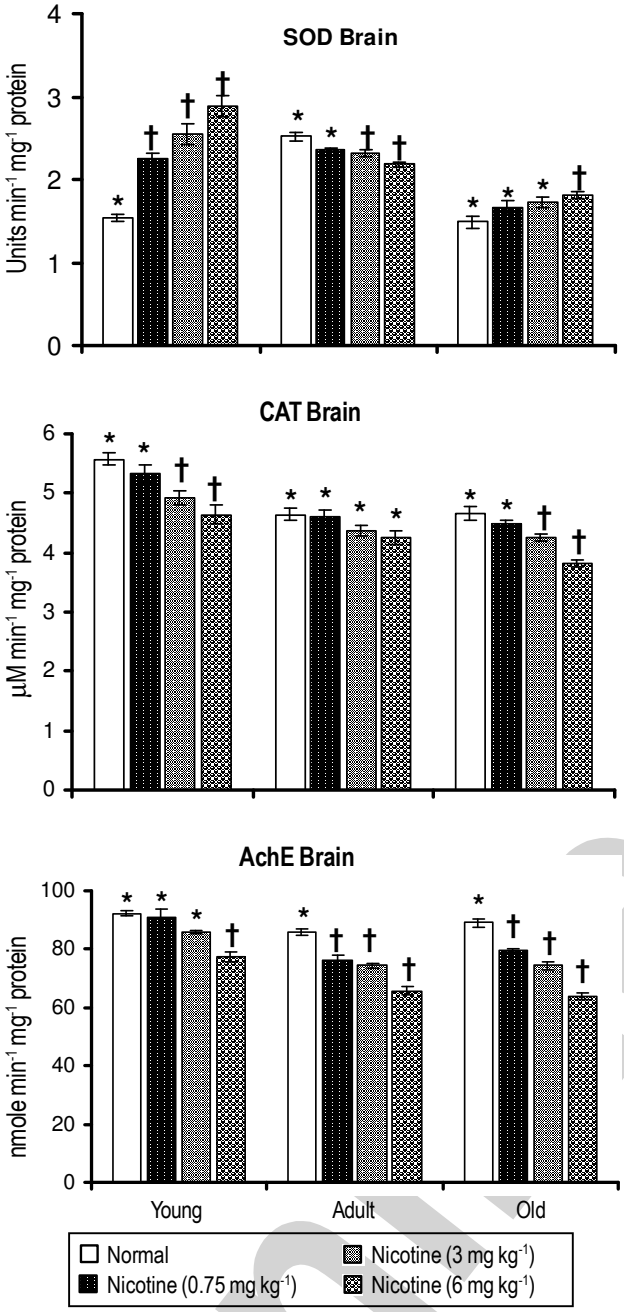

Fig. 2: Dose dependent effect of nicotine on brain antioxidant levels and acetylcholine esterase activity in different age rats; Superoxide dismutase (SOD) = units $min^{-1}mg^{-1}$ protein; Catalase (CAT) = µmoles H_2O_2 produced min^{-1} mg^{-1} protein and Acetyl choline esterase (AchE) = n moles $min^{-1}mg^{-1}$ protein. Values are mean of five replicates±SE. Values with matching symbol notation in each column are not significant at 5% level

formation of free radicals (Meister, 1981). Therefore, free radicals may be generated through the increased activity of the cytochrome P-450 enzymes during the intracellular metabolism of nicotine enantiomers (Yildiz *et al.*, 1998). Nicotine has been recognized to result in oxidative stress by inducing the generation of reactive oxygen species (ROS) by various mechanisms (Yildiz *et al.*, 1998). Nicotine induces oxidative stress which may play an important role in the development of cardiovascular disease and lung cancer in smokers (El-Sokkary *et al.*, 2007). Highest dose of nicotine (6 mg kg^{-1} body wt.) induced higher ROS concentration in old rats (Barros *et al.*, 2007). Our present finding also indicate the increase level of blood ROS in young and old rat upon treatment with high dose (6 mg kg^{-1} b.wt.) of nicotine. Our results also showed elevated blood TBARS level in nicotine-administered rats which agree with studies showing increased MDA levels as index of blood lipid peroxidation (Suleyman *et al.*, 2002). Increased concentration of lipid peroxidation products observed in nicotine treated rats is also associated with decreased activity of scavenging enzymes such as SOD and CAT (Helen *et al.*, 2000). A decrease in the activities of these enzymes can lead to the excessive availabilities of superoxides and peroxy radicals, which in turn generate hydroxyl radicals resulting in the initiation and propogation of LPO (Sacks *et al.*, 1978). Current study reveals the fact that increased level of lipid peroxidation is associated with decreased blood SOD and CAT activity on high dose of nicotine exposure, however the same were not observed in case of low and medium dose of nicotine in young rats and adult rats, and at low dose in old rats.

Brain is one of the most susceptible organs to oxidative impairment as there are lot of evidence indicating nicotine induced increase in brain ROS, lipid peroxidation and decrease in level of non enzymatic antioxidant that is GSH (Gumustekin *et al.*, 2003), which is in agreement with our present finding in different age rats brain except for young rats in which level of GSH increased on 3 mg kg^{-1} of nicotine administration. Proposed mechanism that nicotine produces oxidative stress include disruption of the mitochondrial respiratory chain leading to leakage from electron transport in cardiomyocytes of rabbits (Gvozdjakova *et al.*, 1992), decrease in Chinese hamster ovary cells (Yildiz *et al.*,1999), decreased activities of CAT and SOD in various tissues in rats (Helen *et al.*, 2000).

Adult rats exposed to 0.3 mg kg^{-1} amounts of nicotine for 7 continuous days had decreased activities of SOD and GPX and increased MDA and ROS level in brain (Hritcu *et al.*, 2009). Our result also showed decreased activity of SOD activity in adult brain and also decreased CAT activity in young and old brain. Previously, it has been reported that superoxide anions and hydrogen peroxide are the main source of nicotine-induced free radical production depleting the cellular glutathione level (Suleyman *et al.*, 2002). Other studies have reported inhibition of kidney and testes SOD and activation of liver SOD in nicotine-treated rats. The study also reported inhibition of liver CAT and activation of kidney, lung and testes catalase in nicotine-treated rats (USEPA, 1992).

However, an increase in SOD activity in young and old brain at all three doses needs further investigation. Nicotine is known to have agonist properties to acetylcholine receptor which results in the release of acetylcholine at higher concentration of nicotine. This excessive concentration of acetylcholine may lead to depletion of

acetylcholine esterase activity which can be seen in the present study on nicotine treatment in different age rats. Our findings thus illustrate that young and old rats are more sensitive to nicotine induced oxidative stress.

Acknowledgments

Authors thank Dr. R. Vijayaraghavan, Director of DRDE for his support and encouragement and AJ thanks Defence Research and Development Organization for the award of Senior Research Fellowship (SRF).

References

Abreu-Villaca, Y., F.J. Seidler, C.A. Tate and T.A. Slotkin: Nicotine is a neurotoxin in the adolescent brain: Critical periods, patterns of exposure, regional selectivity and dose thresholds for macromolecular alterations. *Brain Res*., **979**, 114-128 (2003).

Barros, D.M., F.G. Galhardi, J.L. Ribas Ferriera, L.B. Guterres, O. Dickel, L.A. Geracitano, I. Izquierdo and J.M. Monserrat: The benefits and drawbacks of nicotine exposure in the cortex and hippocampus of old rats. *Neurotoxicol.,* **28**, 562-568 (2007).

Campain, J.A.: Nicotine: Potentially a multifunctional carcinogen. *Toxicol. Sci*., **79**, 1-3 (2004).

Cashman, J.R., S.B. Park, Z.C. Yang, S.A. Wrighton, P. Jacob and N.L. Benowitz: Metabolism of nicotine by human liver microsomes: Stereoselective formation of trans-nicotine N'-oxide. *Chem. Res. Toxicol.*, **5**, 639-646 (1992).

Dey, S.K. and S. Roy: Role of reduced glutathione in the amelioration of nicotine-induced oxidative stress. *Bull. Environ. Contam Toxicol*., **84**, 385-389 (2010).

Ellman, G.L.: Tissue sulphydryl groups. *Arch Biochem*., **82**, 70-77 (1959).

Ellman, G.L., K.D. Courtney, V. Andres and R.M. Featherstone: A new and rapid colorimetric determination of acetyl cholinesterase activity. *Biochem. Pharmacol.,* **7**, 88-95 (1961).

El-Sokkary, G.H., S. Cuzzocrea and R.J. Reiter: Effect of chronic nicotine administration on the rat lung and liver: Beneficial role of melatonin. *Toxicol.*, **239**, 60-67 (2007)

Erat, M., M. Ciftci, K. Gumustekin and M. Gul: Effects of nicotine and vitamin E on glutathione reductase activity in some rat tissues *in vivo* and *in vitro*. *Eur. J. Pharmacol.*, **554**, 92-97 (2007).

Gitto, E., R.J. Reiter, M. Karbownik, D.X. Tan, P. Gitto, S. Barberi and I. Barberi: Causes of oxidative stress in the pre and perinatal period. *Biol. Neonate*, **81**, 146-157 (2002).

Gumustekin, K., K. Altinkaynak, H. Timur, S. Taysi, N. Oztasan, M.F. Polat, F. Ackay, H. Suleyman, S. Dane and M. Gul: Vitamin E but not H*ippophea rhamnoides* L. prevented nicotine-induced oxidative stress in rat brain. *Hum. Exp. Toxicol*., **22**, 425-431 (2003).

Gumustekin, K., M. Ciftci, A. Coban, S. Altikat, O. Aktas, M. Gul, H. Timur and S. Dane: Effects of nicotine and vitamin E on glucose-6-phosphate dehydrogenase activity in some rat tissues *in vivo* and *in vitro*. *J. Enzym. Inhibition Med. Chem*., **20**, 497-502 (2005).

Gupta, P.C. and C.S. Ray: Epidemiology of betel quid usage. *Ann. Acad. Med*., **33**, 31-36 (2004).

Gvozdjakova, A., J. Kucharská and J. Gvozdjak: Effect of smoking on the oxidative processes of cardiomyocytes. *Cardiol*., **81**, 81-84 (1992).

Helen, A., K. Krishnakumar, P.L. Vijayammal and K.T. Augusti. Antioxidant effect of onion oil (*Allium cepa*. Linn) on the damages induced by nicotine in rats as compared to alpha-tocopherol. *Toxicol. Lett*., **116**, 61-68 (2000).

Hissin, P.J. and R.A. Hilf: A fluorometric method for the determination of oxidized and reduced glutathione in tissue. *Anal. Biochem*., **74**, 214-226 (1973).

Hritcu, L., A. Ciobica and L. Gorgan: Nicotine-induced memory impairment by increasing brain oxidative stress. *Cent. Eur. J. Biol.,* **4**, 335-342 (2009).

James, S.J., W. Slickker, S. Melnyk, E. New, M. Pogribna and S. Jernigan: Thimerosal neurotoxicity is associated with glutathione depletion: Protection with glutathione precursors. *Neurotoxicol*., **26**, 1-8 (2005).

Jollow, D.J., J.R. Mitchell, Z. Zamppaglione and J.R. Gillette: Bromobenzene induced liver necrosis. Protective role of glutathione and evidence for 3, 4-bromobenzene oxide as the hepatotoxic metabolites. *Pharmacol.,* **11**, 151-155 (1974).

Kakkar, P., B. Das and P.N. Viswanathan: A modified spectrophotometric assay of superoxide dismutase. *Ind. J. Biochem. Biophy*., **21**, 130-132 (1983).

Meister, A.: Metabolism and functions of glutathione. *Trends Biochem. Sci*., **6**, 231-234 (1981).

Nakayama, H.: Nicotine metabolism in mammals. *Drug Metabol. Drug Interact*, **6**, 95-122 (1988).

Ohkawa, H., N. Ohishi and K. Yagi: Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem*., **95**, 351-358 (1979).

Ohtsuki, K. and T. Suzuki. Roles of molecular chaperons in the nervous system. *Brain Res. Bull*., **53**, 141-146 (2000).

Olanow, C.W. and G.W. Arendash: Metals and free radicals in neurodegeneration. *Curr. Opin Neurol*., **7**, 548-58 (1994).

Pigeolet, E., P. Corbisier, A. Houbion, D. Lambert, C. Michiels, M. Raes, M.D. Zachary and J. Remacle: Glutathione peroxidase, superoxide dismutase and catalase inactivation by peroxides and oxygen derived free radicals. *Mech. Ageing Dev*., **51**, 283-97 (1990).

Qiao, D., F.J. Seidler and T.A. Slotkin. Oxidative mechanisms contributing to the developmental neurotoxicity of nicotine and chlorpyrifos. *Toxicol. Appl. Pharmacol*., **206**, 17-26 (2005)

Sacks, T., C.F. Moldow, P.R. Craddock, T.K. Bowers and H.S. Jacob. Oxygen radicals mediate endothelial cell damage by complement-stimulated granulocytes. An *in vitro* model of immune vascular damage. *J. Clin. Invest*., **61**, 1161-1167 (1978).

Sinha, A.K.: Colorimetric assay of catalase. *Anal Biochem*., **47**, 389-394 (1972).

Slotkin, T.A., M.M. Cousins and F.J. Seidler: Administration of nicotine to adolescent rats evokes regionally selective upregulation of CNS 17 nicotinic acetylcholine receptors. *Brain Res*., **1030**, 159-163 (2004).

Socci, D.J., K.B. Bjugstad, H.C. Jones, J.V. Pattisapu and G.W. Arendash: Evidence that the H-Tx rat model. *Exp. Neurol*., **155**, 109-117 (1999).

Suleyman, H., K. Gumustekin, S. Taysi, S. Keles, N. Oztasan, O. Aktas, K. Altinkaynak, H. Timur, F. Akcay, S. Akar, S. Dane and M. Gul: Beneficial effects of *Hippophae rhamnoides* L. on nicotine induced oxidative stress in rat blood compared with vitamin E. *Biol. Pharm. Bull*., **9**, 1133-1136 (2002).

Tanaka, J., K. Toku, B. Zhang, K. Isihara, M. Sakanakaa and N. Maeda: Astrocytes prevent neuronal cell death induced by reactive oxygen and nitrogen species. *Glia.,* **28**, 85-96 (1999).

US Environmental Protection Agency (USEPA): Respiratory health effects of passive smoking, lung cancer and other disorders. Washington, DC: US Environmental Protection Agency (US EPA Publication 600, 6-90/006F), (1992).

Yildiz, D., N. Ercal and D.W. Armstrong: Nicotine enantiomers and oxidative stress. *Toxicol.,* **130**, 155-165 (1998).

Yildiz, D., Y.S. Liu, N. Ercal and D.W. Armstrong: Comparison of pure nicotine- and smokeless tobacco extract-induced toxicities and oxidative stress. *Arch. Environ. Contam. Toxicol.,* **37**, 434-439 (1999).