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Role of Hesperidin on Nictotine Toxicity

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Abstract: In the present study we have assessed the protective effect of hesperidin a flavonoid against nicotine induced chronic lung damage. The effect on nicotine is assessed by the elevation of marker enzymes, antioxidants and lipid peroxidation in nicotine treated rats. Lung damage was induced by subcutaneous injection of nicotine at a dose of 2.5 mg kg^{-1} body weight. Hesperidin was administered orally at different doses (25, 50, 75, 100 and 150 mg kg^{-1} body weight). The level of marker enzymes in plasma and antioxidants in lung, kidney and liver were estimated. Supplementation of hesperidin decreased the levels of all the marker enzymes; it was significant in 25 mg kg^{-1}. The result shows that hesperidin is protective against the lung damage caused by nicotine at 25 mg kg^{-1}.

Key words: Antioxidants, hesperidin, lipidperoxidation, nicotine

INTRODUCTION

Nicotine, the major component of cigarette smoke plays an important role in the development of cardiovascular diseases and lung cancer (Czernin and Waldherr, 2003). It has been reported that it induces oxidative stress in both *in vitro* and *in vivo* (Church and Pryor, 1958). Oxidative stress is a disturbance in the pro-oxidant-antioxidant system leading to potential damage. This imbalance results in decrease in antioxidant levels or increase in reactive oxygen species (Mary *et al*., 2002). The resulting reactive oxygen species, which includes hydroxyl radicals, superoxide and hydrogen peroxide play an integral role in modulation of several physiological function but can also be destructive if produced in excessive amounts (Doelman and Blast, 1990). This can damage major cellular components including membrane lipids, proteins, carbohydrates and DNA thus resulting in tissue damage (Helen *et al*., 2003). To minimize the oxidant damage to biological molecules, there are several antioxidant defense mechanisms that detoxify reactive products or convert them to products that are quenched by other antioxidants (Fridovich, 1978). Previous investigations from our laboratory have shown, enhanced lipid peroxidation and depletion of antioxidants in tissues during nicotine induced lung toxicity (Kalpana and Menon, 2004).

Hesperidin, a flavanone glycoside is present predominantly in citrus fruits (Garg *et al*., 2000). Flavonoids are a important group of polyphenolic compounds which play a protective role by inhibition of enzymes of oxygen-reduction pathways and sequestration of transient metal cations and also quenching free radicals (Naik, 2003). A number of studies have examined the antioxidant activity, anticancer and free radical scavenging properties of hesperidin (Miller and Rice, 1997). Since nicotine toxicity to the lungs is due to the formation of oxygen free radicals, we thought that hesperidin a flavonoid with antioxidant property may protect the lungs from nicotine induced oxygen free radicals. The present study was undertaken to test this hypothesis.

MATERIALS AND METHODS

Animals: Colony in-bred strains of adult male albino Wistar rats weighing 160-220 g obtained from the Central Animal House, Department of Medicine, Raja Muthiah Medical College of Health Science, Annamalai University, Annamalai nagar, Tamilnadu, India were used in the study. The animals were housed in polypropylene cages at a constant temperature of 22±1°C, relative humidity 55±5% with 12 h dark-light cycle. They were fed with standard pellet (Agro Corparation Private Ltd., Banglore, India) and water *ad libitum*. The animals were maintained according to the guidelines of National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India and this research was approved by the ethical committee of Annamalai University.

Chemicals: Nicotine, hesperidin and other fine chemicals were obtained from Sigma Chemicals Company, St.Louis

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USA. All other chemicals and reagents used were of analytical grade.

Experimental groups and treatment regimen: The rats were randomly distributed into seven different groups with six animals each under identical conditions and were given dose schedule as given below.

Group-I: Control animals given subcutaneous injection of physiological saline (0.5 mL kg^{-1}).

Group-II: Animals received nicotine 2.5 mg kg^{-1} in 0.5 mL physiological saline subcutaneously for 5 days.

Group-III: Animals received nicotine 2.5 mg kg^{-1} in 0.5 mL physiological saline subcutaneously for 5 days, with Hesperidin 25 mg kg^{-1} in water orally.

Group-IV: Animals received nicotine 2.5 mg kg^{-1} in 0.5 mL physiological saline subcutaneously for 5 days, with Hesperidin 50 mg kg^{-1} in water orally.

Group-V: Animals received nicotine 2.5 mg kg^{-1} in 0.5 mL physiological saline subcutaneously for 5 days, with Hesperidin 75 mg kg^{-1} in water orally.

Group-VI: Animals received nicotine 2.5 mg kg^{-1} in 0.5 mL physiological saline subcutaneously for 5 days, with Hesperidin 100 mg kg^{-1} in water orally.

Group-VII: Animals received nicotine 2.5 mg kg^{-1} in 0.5 mL physiological saline subcutaneously for 5 days, with Hesperidin 150 mg kg^{-1} in water orally.

At the end of the experimental period (22nd week) all the animals were sacrificed by cervical dislocation after an overnight fast. Blood was collected in heparinised tubes and centrifuged at 5000 rpm for 10 min. Plasma was separated by aspiration, transferred into effendorfs tubes and stored at -20°C for analysis. The liver kidney and lungs were removed, cleared off blood and transferred immediately to ice cold container containing 0.9% sodium chloride for various estimations.

Biochemical estimations: The activity of alkaline phosphatase was estimated by the method of King and Armstrong (1998). Aspartate transaminase and alanine transaminase by method (Reitman and Frankel, 1957). The activity of lactate dehydrogenase was assayed by the method (Young, 1975). Lipid peroxidation by the method (Ohkawa *et al*., 1979). Hydroperoxides was estimated by the method (Jiang *e al*., 1992). Total reduced glutathione (GSH) by the method (Ellman, 1959). Glutathione peroxidase (GPx) activity was assayed by the method (Rotruck *et al*., 1973). Superoxide Dismutase (SOD) was assayed by the method (Kakkar *et al*., 1942) and Catalase (CAT) by the method (Sinha, 1972). Tissue ascorbic acid was determined by the method (Roe and Kuether, 1942) and Vitamin-E level was measured by the method (Baker *et al*., 1980). Protein was determined by the method (Lowry *et al*., 1951).

Statistical analysis: The data were analyzed using Analysis of Variance (ANOVA) and the group means were compared by Duncan's Multiple Range Test (DMRT). The difference was considered to be significant when $p<0.05$.

RESULTS

In nicotine treated rats, the activities of ALP, ALT, AST and LDH were significantly increased when compared to the control. Administration of hesperidin to nicotine treated rats at different doses significantly decreased the activities of these enzymes when compared with the nicotine treated animals and hesperidin at the dose of 25 mg kg^{-1} body weight found to be more effective (Table 1).

The levels of TBARS and hydroperoxides were significantly increased in the liver, lung and kidney of nicotine treated animals. Administration of hesperidin at various doses significantly decreased the levels of TBARS and hydroperoxides and at the dose of 25 mg kg^{-1} body weight hesperidin found to be more significantly effective (Table 2). The Table 3 shows that the levels of GSH and GPx in liver, kidney and lung of experimental animals were decreased significantly in the nicotine treated group of animals. Significant protection was seen in the hesperidin supplemented nicotine treated animals but at 25 mg kg^{-1} body weight it was found to be more very effective than the other doses.

Both SOD and CAT were significantly decreased in the nicotine treated group of animals. The activities of SOD and CAT were significantly elevated in the liver, kidney and lungs of experimental animals on supplementation of hesperidin to the nicotine treated animals and the dose at 25 mg kg^{-1} body weight was found to be most effective when compared to the other doses (Table 4).

The levels of vitamin-C and vitamin-E were significantly reduced in the nicotine treated animals when compared to the control group of animals. Administration of hesperidin to nicotine treated rats at different doses elevated the levels of vitamin-C and vitamin-E significantly and the dose of 25 mg kg^{-1} body weight was found to be more effective when compared to respective groups (Table 5).

Table 1: Activities of marker enzymes in the plasma (mean±SD; n = 6)

Groups	ALT (IU/L) (U^A)	AST (IU/L) (U^A)	ALP (IU/L) (U^A)	LDH (IU/L) (U^A)
Control	42.24±2.68^a	80.03±5.65^a	85.13±4.69^a	125.53±7.31^a
Nicotine	83.05±4.98^b	143.33±12.32^b	172.48±15.31^b	176.90±9.54^b
N + H25	46.63±3.29^c	90.50±5.77^c	97.07±9.57^c	131.48±6.59^c
N + H50	50.65±3.15^d	96.16±5.94^d	102.66±6.32^d	141.66±6.17^d
N + H75	55.26±2.97^e	100.86±6.11^e	106.43±7.17^e	145.90±6.44^e
N + H100	58.23±2.84^f	106.45±7.29^f	116.83±6.98^f	151.25±5.97^f
N + H150	61.08±3.03^g	113.09±8.32^g	123.96±6.39^g	160.90±5.15^g

Values not sharing a common superscript letter (a, b, c, d, e, f and g) differ significantly at $p<0.05$ (Duncan's multiple range test)

Table: 2: Levels of TBARS and hydroperoxides in the plasma, lung, liver and kidney (mean±SD; n = 6)

	Thiobarbituric acid reactive substances				Hydroperoxides Plasma			
Groups	Plasma (mM/dL)	Lung (mg/100 tissue)	Liver mM/100 g tissue)	(Kidney (mM/100 g tissue)	Plasma (x10^{-5} mM/dL)	Lung (mM/100 g tissue)	Liver (mM/100 g tissue)	Kidney (mM/100 g tissue)
Control	3.28±0.27^a	0.73±0.11^a	0.73±0.13^a	0.46±0.09^a	9.43±0.97^a	53.00±3.97^a	41.75±2.84^a	34.21±2.55^a
Nicotine	6.75±0.42^b	1.60±0.32^b	1.25±0.19^b	0.63±0.14^b	18.36±1.57^b	74.11±5.44^b	65.80±4.22^b	47.40±3.09^b
N + H25	3.33±0.22^c	0.80±0.27^c	0.88±0.12^c	0.55±0.12^c	12.56±1.22^c	57.06±4.04^c	46.91±3.04^c	36.53±2.78^c
N + H50	4.66±0.35^d	0.90±0.23^d	0.950±0.15^d	0.58±0.11^d	15.06±1.34^d	62.53±4.97^d	55.40±4.01^d	41.50±3.01^d
N + H75	5.10±0.41^e	0.75±0.25^e	1.55±0.24^e	0.68±0.16^e	15.46±1.72^d	64.13±5.02^{de}	57.56±3.99^e	43.05±3.22^e
N + H100	5.20±0.39^f	1.06±0.27^f	1.13±0.31^f	0.64±0.17^f	16.55±1.42^e	65.43±4.97^{ef}	58.96±3.77^f	44.63±3.54^f
N + H150	5.616±0.37^g	1.166±0.19^g	1.178±0.35^g	0.66±0.18^g	17.43±1.66^f	66.40±5.11^f	60.83±3.22^g	44.76±3.42^f

Values not sharing a common superscript letter (a,b,c,d,e,f and g) differ significantly at $p<0.05$ (Duncan's multiple range test)

Table 3: Levels of GSH and activities of GPx in the plasma, lung, liver and kidney (mean±SD; n = 6)

	GSHGPx				Plasma			
Groups	Plasma (mg/dl)	Lung mg/100 g tissue	Liver mg/100 g tissue	Kidney mg/100 g tissue	Haemoly sate Units*/ mg Hb	Lung Units*/mg proteins	Liver Units*/mg proteins	Kidney Units*/mg proteins
Control	34.38±3.14^a	127.66±8.42^a	138.70±9.31^a	106.36±7.32^a	25.26±2.31^a	13.26±1.42^a	11.43±0.87^a	9.58±0.82^a
Nicotine	18.53±2.12^b	58.83±4.7^f	71.95±4.47^h	57.53±4.02^h	15.44±1.85^b	8.33±1.12^b	8.56±0.71^b	6.78±0.65^b
N + H25	32.66±5.07^c	117.66±9.72^b	128.01±8.32^c	95.82±5.05^c	22.66±1.98^c	12.18±1.23^c	10.86±1.15^c	8.53±0.73^c
N + H50	25.63±2.79^d	100.50±7.50^e	94.00±5.97^d	90.11±5.11^d	20.41±2.15^d	11.33±0.94^d	10.45±1.07^d	7.46±0.65^d
N + H75	23.63±2.17^e	97.36±5.50^e	86.16±4.66^e	86.63±4.92^e	19.88±1.34^e	11.06±0.78^c	10.30±1.12^e	7.36±0.59^e
N+H100	21.06±1.97^f	81.83±4.63^d	80.35±4.32^f	77.88±3.97^f	19.65±1.57^f	10.85±0.97^f	9.94±0.83^f	7.50±0.43^f
N+H150	20.46±1.84^f	72.90±3.12^e	78.05±4.14^g	73.53±3.81^g	19.30±1.72^g	10.61±0.81^g	9.81±0.92^g	7.11±0.34^g

Values not sharing a common superscript letter (a, b, c, d, e, f and g) differ significantly at $p<0.05$ (Duncan's multiple range test), * Unit-μ moles of glutathione utilized/min

Table 4: Activities of SOD and CAT in plasma, lung, liver and kidney (mean±SD; n = 6)

	SOD				CAT			
Groups	Heamoly state unit# /mg Hb	Lung Unit #/mg proteins	Liver Unit #/mg proteins	Kidney Unit #/mg proteins	Haemolysate Units*/ mg Hb	Lung Units*/mg protein	Liver Units*/mg protein	Kidney Units*/mg protein
Control	3.85±0.32^a	12.35±0.77^a	10.00±0.77^a	8.10±0.72^a	3.15±0.29^a	45.40±3.29^a	75.28±5.67^a	20.46±2.03^a
Nicotine	2.35±0.24^b	6.35±0.37^b	5.83±0.35^b	5.01±0.34^b	1.56±0.11^b	28.15±1.09^b	55.23±4.02^b	11.35±1.25^b
N + H25	3.75±0.29^c	12.36±0.89^a	9.65±0.42^c	7.60±0.44^c	2.57±0.22^c	43.26±2.95^c	72.50±5.01^c	18.08±1.72^c
N + H50	3.78±0.37^d	11.15±0.79^a	8.56±0.39^d	6.53±0.45^d	2.40±0.25^d	39.33±3.11^d	68.40±5.22^d	17.58±1.56^d
N + H75	3.63±0.27^e	10.15±0.88^c	7.46±0.49^e	6.25±0.39^e	2.36±0.19^e	37.20±2.85^e	67.13±4.88^e	16.65±1.44^e
N+ H100	3.51±0.29^f	9.05±0.70^g	7.25±0.52^f	6.10±0.36^f	2.23±0.17^e	35.00±2.72^f	66.40±4.70^f	15.36±1.52^f
N+ H150	3.41±0.30^g	8.83±0.66^g	7.00±0.51^g	6.01±0.42^g	2.03±0.18^g	34.16±2.15^g	65.36±4.15^g	14.28±1.32^g

Values not sharing a common superscript letter (a, b, c, d, e, f and g) differ significantly at $p<0.05$ (Duncan's multiple range test), # Unit-Enzyme reaction which gives 50% inhibition of NBT reduction/min, *Unit-μ moles of H_2O_2 liberated/min

Table 5: Levels of Vitamin-C and Vitamin-E in plasma and liver (mean±SD; n = 6)

	Vitamin E		Vitamin C	
Groups	plasma (mg/dl)	Liver μM/mg tissue	Plasma (mg/dl)	Liver (mg/100g tissue)
Control	1.58±0.46^a	0.95±0.23^a	1.93±0.37^a	1.40±0.25^a
Nicotine	0.78±0.26^b	0.54±0.15^b	0.68±0.46^b	0.68±0.16^b
N + H25	1.41±0.34^c	0.71±0.09^c	1.43±0.19^c	1.33±0.11^d
N + H50	1.23±0.41^e	0.60±0.11^d	0.96±0.12^d	1.218±0.12^c
N + H75	1.83±0.31^d	0.60±0.13^d	0.85±0.15^d	1.038±0.15^{fg}
N + H100	1.03±0.21^f	0.65±0.12^d	0.76±0.12^{bd}	0.983±0.17^{eg}
N + H150	0.98±0.17^g	0.65±0.17^d	0.73±0.16^{bd}	0.96±0.09^{ef}

Values not sharing a common superscript letter (a, b, c, d, e, f and g) differ significantly at $p<0.05$ (Duncan's multiple range test)

DISCUSSION

Nicotine the major component of cigarette smoke plays an important role in the development of lung cancer and lung related complications (Benowitz, 1986). The metabolites of nicotine causes a significant increase in DNA strand breakage (Weitberg and Corvese, 1997). Nicotine also causes oxidative damage which is prominent in the lungs, kidney, brain and liver (Deniz, 2004). This leads to increase in the levels of reactive oxygen species and lipid peroxidation (Ames and Shigenaga, 1993). Lipid peroxidation and generation of free radicals are the process associated with the pathogenesis of many diseases (Ames and Shigenaga, 1993). The damage to the tissues by nicotine is evidenced by the elevation of bio marker enzymes in the plasma and antioxidants in lung, kidney and liver (Deniz, 2004). The marker enzymes namely ALP, ALT, AST and LDH were elevated in plasma of nicotine treated rats. These marker enzymes are the important index for the diagnosis of lung diseases and it indicates the damage of cells, cellular leakage and loss of functional integrity of cell membrane in lung and liver (Matusiewicz *et al*., 1993). ALP exists in pulmonary surfactant particles (Harada *et al*., 2002). ALP is expressed with high catalytic activity in the lungs and exists in pulmonary surfactant particles which are secreted from type II pneumocytes. It is decreased in conditions like pulmonary fibrosis and hepatitis (Cobben *et al*., 1999). AST and ALT are enzymes found in the cytoplasm which appear in cell and is elevated during cellular necrosis (Kenneth, 2001). LDH is a cytoplasmic enzyme, which is used to detect cell damage or cell death which takes place during an influx of polymorphonuclear neutrophils and activation of alveolar macrophages (Turna, 2004). The neutrophil has been implicated as an important contributor to the lung injury incurred during the damage. A high LDH level indicates interstitial fibrosis of the lung following alveolar damage and it has been proposed as a marker of pulmonary fibrosis (Hagadorn *et al*., 1971).

Lipid peroxidation is enhanced in the nicotine induced tissue damage. It occurs as a consequence of oxidant induced injury (Jason, 2003). Monitoring the extent of lipid peroxidation in the lung may aid in detection of lung disorder were involvement of active oxygen species is implicated. The lipid peroxidation is enhanced in the liver, lung and kidney of the nicotine treated rats. Nicotine has been reported to generate lipid peroxidation in tissues (Janet, 1990). Thus the damage to the tissues in the nicotine treated animals may be due to the excessive generation of free radicals.

GSH associated enzymes present in the epithelial lining fluid of the lower respiratory tract may be the first line of defense against lung damage. Sustained oxidative challenge to the lung results in depletion of GSH and other antioxidants from the lungs (Meister, 1994).

GSH is a major non-protein thiol in living organisms which plays a central role in coordinating the body's antioxidant defense process (Deneke and Fanburg, 1989). Perturbation of GSH status of a biological system has been reported to lead to serious consequences. GSH depletion is due to the destruction of free radicals (Shyamala *et al*., 2003). The decreased levels of tissue GSH in nicotine treated rats may be due to the enhanced utilization during detoxification of nicotine.

SOD, CAT and GPx constitutes a mutually supportive team of defense against reactive oxygen species which have been found decreased in nicotine treated rats. SOD is a ubiquitous enzyme with an essential function in protecting aerobic cells against oxidative stress. Its primarily a mitochondrial enzyme usually found in the plasma membrane (McCord and Fridovich, 1983). Catalase is a tetrameric hemoprotein that undergoes alternate divalent oxidation and reduction at its active site in the presence of H_2O_2 and catalyzes the dismutation reaction (Deisseroth and Dounce, 1999). GPx is a seleno enzyme two third of which is present in cytosol and one third in mitochondria (Ren *et al*., 1995). It catalyses the reaction of hydroperoxides with reduced glutathione to form glutathione disulphide (GSSG) and the reduction product of the hydroperoxide (Meister and Anderson, 1983). Depletion of the activities of SOD, CAT and GPx in the liver, kidney and lungs of nicotine treated rats may be due to the increased utilization of these antioxidants to counter lipid peroxidation. Vitamin E and vitamin C was found to be significantly decreased in nicotine treated rats. α-tocopherol, the major constituent in the membrane is viewed as a last line defense against membrane lipid peroxidation (Gester, 2002).

Thus its protection is by terminating the lipid peroxidation side chain rather than scavenging extracellular non-lipid radicals that initiate lipid peroxidation. Vitamin C is a naturally occurring free radical scavenger which decreases free radical ability and lipid peroxidation sequence. It regenerates membrane bound α-tocopherol radical and removes the radical from the lipid to the aqueous phase. It also protects tissues from lipid peroxidation both *in vivo* and *in vitro* (Choi *et al*., 2004). Increased lipid peroxidation in the liver of nicotine treated rats was associated with the decreased vitamin C and E levels and this can, therefore, be related to the insufficient antioxidant potential.

Hesperidin administration reversed the changes induced by nicotine in rats. It enhanced the antioxidant status in the lungs in nicotine treated rats. Hesperidin is

a flavanone glycoside and studies have shown that flavonoids are effective antioxidant (Adriana *et al*., 2003). In this study we have observed that their flavonoids protects the lungs from nicotine toxicity. Earlier studies from our lab has shown that feurulic acid a monophenolic compound. Also reduced the nicotine toxicity. Number of studies of shown that hesperidin an antioxidant decreased the oxidative stress and prevents CCl_4 under liver toxicity (Naveen *et al*., 2005). Present study also shows that the most effective dose of hesperidin is 25 mg kg^{-1}.

Thus the study shows the potent effect of hesperidin as a very good antioxidant. Present study also shows that this flavonoid can decrease the intensity of lung complication in nicotine treated animals and hence gives protection to the lung tissue.

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