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## HESPERIDIN, AN ANTIOXIDANT FLAVONOID, PREVENTS ACRYLONITRILE-INDUCED OXIDATIVE STRESS IN RAT BRAIN

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### ABSTRACT

Acrylonitrile (ACN) is a volatile, toxic liquid used as a monomer in the manufacture of synthetic rubber, styrene plastics, acrylic fiber and adhesives. ACN is a potent neurotoxin. A role for free radical-mediated lipid peroxidation in the toxicity of ACN has been suggested. We examined the ability of hesperidin, an antioxidant flavonoid, to attenuate ACN-induced alterations in lipid peroxidation in rat brains. The daily oral administration of ACN to male albino rats in a dose of 50 mg/kg bwt for a period of 28 days produced a significant elevation in brain lipid peroxides measured as malondialdehyde (MDA) amounting to 107%, accompanied by a marked decrease in brain reduced glutathione (GSH) content reaching 63%. In addition, ACN administration resulted in significant reductions in the enzymatic antioxidant parameters of brain; superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) & glutathione-S-transferase (GST) recording 43%, 64%, 52% & 43%, respectively. On the other hand, pretreatment with hesperidin and its co-administration with ACN once daily in a dose of 200 mg/kg bwt, i.p. for 28 days ameliorated ACN-induced alterations in brain lipid peroxidation. These results suggest that hesperidin may have a beneficial role against ACN-induced oxidative stress in brain; an effect that is mainly attributed to the antioxidant property of hesperidin.

**Key words:** Acrylonitrile, Hesperidin, Lipid peroxidation, Rat brain.

### INTRODUCTION

Acrylonitrile (ACN) is a widely used monomer in the production of synthetic fibers, rubbers, plastics and as a chemical intermediate in the synthesis of a variety of products including antioxidants, pharmaceuticals and dyes (IARC, 1999; Wang *et al.*, 2002). The important use of ACN in clinical practice is in the manufacture of high permeable dialysis tubings (Ward *et al.*, 1993) and in the synthesis of artificial membrane to encapsulate Langerhans islets implants (Kessler *et al.*, 1992). ACN has been found in drinking water, occupational environments, food and cigarette smoke (Rubio *et al.*, 1990; Miller *et al.*, 1998).

Metabolism of ACN proceeds via conjugation with glutathione or epoxidation via cytochrome P4502E1 (CYP2E1) to cyanoethylene oxide (CEO). It was hypothesized that CEO metabolism via epoxide hydrolase is the primary pathway for cyanide formation (Wang *et al.*, 2002). Glutathione (GSH) conjugation has been shown to be depleted following ACN treatment *in vivo* and may decrease the

antioxidant capacity of the cells resulting in an overall increase of intracellular reactive oxygen species (ROS) and oxidative damage (Fennell *et al.*, 1991; Jiang *et al.*, 1998; Kamendulis *et al.*, 1999). Cyanide has been shown to induce oxidative stress (lipid peroxidation) in the brain of acutely treated mice and in cell lines (Guengerich *et al.*, 1981; Hogg and Guengerich, 1986). The rat brain has been shown to have a high oxidative capacity and a low antioxidant defense capacity relative to the liver. Thus, the rat brain may be more susceptible to ACN-induced oxidative stress due to an inability to efficiently combat the oxidative insult produced by ACN (Xia *et al.*, 1995; Jiang *et al.*, 1998; Kamendulis *et al.*, 1999).

Flavonoids are a group of naturally occurring compounds that are frequently present in foods of plant origin. Flavonoids have a variety of biological effects in numerous mammalian cell systems, *in vitro* as well as *in vivo*. They have been shown to exert antiinflammatory, antiallergic, antiviral, antibacterial and antitumor activities (Formica and Regelson, 1995). In fact, the pharmacological effects

of many flavonoid compounds are due to their inhibiting ability on certain enzymes and also, their antioxidant activity (Pietta, 2000). Hesperidin (HES) is one of the most abundant natural flavonoids, present in a large number of fruits and vegetables (Garg *et al.*, 2001). Some authors reported that HES prevents oxidant injury and cell death by several mechanisms, such as scavenging oxygen radicals, protecting against lipid peroxidation and chelating metal ions (Fraga *et al.*, 1987; Korkina and Afanas'ev, 1997; Miller and Rice-Evans, 1997; Jung *et al.*, 2003).

No previous studies are available on the effect of HES against ACN-induced toxicity in rat brains. Therefore, the aim of this study was to explore the effect of HES on the lipid peroxidation status and the activities of enzymatic antioxidants: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione-S-transferase (GST) and on the non-enzymatic antioxidant; reduced glutathione (GSH) in the brains of rats treated with ACN.

## MATERIALS AND METHODS

### Chemicals:

ACN was obtained from Aldrich Chemical Company (Millwaukee, WI, USA) and was given by oral gavage as an aqueous solution in a dose of 50 mg/kg bwt, daily, for 28 days (Fechter *et al.*, 2003; Mahalakshmi *et al.*, 2003).

HES was obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA), dissolved in a mixture of dimethylsulphoxide and water (1: 9, v/v) and injected i.p. in a dose of 200 mg/kg bwt, daily, for 28 days (Tirkey *et al.*, 2005).

5,5-Dithio-bis (2-nitrobenzoic acid), pyrogallol, 2-thiobarbituric acid (TBA), 1, 1', 3, 3'-tetramethoxypropane, 1- chloro -2,6- dinitrobenzene, nicotinamide adenine dinucleotide phosphate reduced form (NADPH), crystalline bovine serum albumin, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide dismutase (SOD) and catalase (CAT) were all purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

All other chemicals were of the highest available commercial grade.

### Animals:

Male Swiss albino rats weighing 200-225 g were obtained from our animal facility (Al-Azhar University, Cairo, Egypt). The animals were maintained under standard laboratory conditions of relative humidity (55 ± 5%), temperature (25 ± 2 °C) and light (12 h light / 12 h dark). They were fed

standard diet pellets (El-Nasr Chemical Company, Abou-Zaabal, Cairo, Egypt) and water was provided *ad libitum*.

### Experimental design:

Thirty-two rats were classified into four groups (8 rats each) and subjected to treatments as follows:

Group I: received 1 ml distilled water per 100 g bwt per day by oral gavage for 28 days and served as a control group.

Group II: received an aqueous solution of ACN by oral gavage in a dose of 50 mg/kg bwt, daily, for 28 day.

Group III: received HES i.p. in a dose of 200 mg/kg bwt, daily, for 28 days.

Group IV: received HES i.p. in a dose of 200 mg/kg bwt 24 h before starting ACN treatment as well as concomitantly with ACN, once daily for 28 days.

### Tissue preparation:

Twenty-four h after administration of the last dose, the animals were anaesthetized using ether and sacrificed by cervical decapitation. Brain tissues were cleared of adhering fat, weighed accurately and cut into small pieces and then homogenized in ice-cold homogenization buffer [10 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.4); 20 mM EDTA; 30 mM KCl] (Abdel-Wahab, 2005) to give 10% homogenate. The homogenate was then made into aliquots and used for determination of brain contents of GSH and MDA and enzymatic activities of SOD, CAT, GSH-Px and GST.

### Biochemical analysis:

Brain GSH content was determined spectrophotometrically following the method of Sedlak and Lindsay (1968). Lipid peroxidation was determined colorimetrically using the method of Uchiyama and Mihara (1978) by determining the tissue MDA content in the form of thiobarbituric acid reactive substances (TBARS) using 1,1',3,3'-tetramethoxypropane as a standard. SOD activity was determined by assessing the inhibition of pyrogallol autooxidation (Marklund, 1985). GST activity was estimated by the method of Habig *et al.* (1974) using 1-chloro-2,4-dinitrobenzene as a substrate in presence of GSH. GSH-Px activity was determined following NADPH oxidation at 340 nm in the presence of excess glutathione reductase, GSH and hydrogen peroxide (Paglia and Valentine, 1967). The activity of CAT was assayed by the method of Clairborne (1985). Protein content in the brain tissue was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

### Histopathological examination of the brain:

Two rats from each group were sacrificed under light ether anesthesia (24 h after the last dosing), and brain samples of all groups were preserved in 10% neutral buffered formalin as described by Luna (1968). Brain sections were cut at 5  $\mu$ m thickness using a rotary microtome and stained with haematoxylin and eosin. Sections were examined and photographed under light microscope.

### Statistical analysis:

The InStat version 2.0 (GraphPad, ISI Software, Philadelphia, PA, USA, 1993) computer program was used to compute statistical data. Data were expressed as means  $\pm$  SEM. Multiple comparisons were done using one-way ANOVA followed by Tukey-Kramer as a post-ANOVA test. Probability level  $\leq 0.05$  was used as the criterion for significance.

dose of 200 mg/kg bwt) 24 h prior to ACN administration as well as concomitantly with ACN once daily for 28 days resulted in a marked reduction in brain MDA content (55%) and a significant increase in brain GSH content (183%) in comparison with ACN-treated group.

Moreover, administration of ACN induced significant decreases in the enzymatic antioxidant parameters in the brain; SOD, CAT, GSH-Px and GST (43%, 64%, 52%, & 43%, respectively in comparison with the control group). In contrast, pretreatment with HES and its co-administration with ACN ameliorated the reductions in these parameters resulting in marked elevations in SOD, CAT, GSH-Px & GST, by 73%, 169%, 197% & 71%, respectively, as compared to ACN-treated group (Table 2).

## RESULTS

Table (1) revealed that daily oral administration of ACN to male albino rats in a dose of 50 mg/kg bwt for 28 days produced a significant elevation in brain lipid peroxides represented as MDA amounting to 107%, accompanied by a marked decrease in brain GSH content (63%) as compared with the control group. On the other hand, i.p. injection of HES (in a

Histopathological examination of brain sections of normal (control group) and HES-treated rats showed normal histological structure of neuronal cells (Fig. 1). On the other hand, administration of ACN to rats revealed damage to neuronal cells of the brain manifested by oedema and interstitial neuronal atrophy with perineuronal vacuolation (Fig. 2). Pretreatment of rats with HES nearly normalized the histopathological changes induced by ACN (Fig. 3).

**Table (1):** Effect of ACN and/or HES on brain contents of MDA and GSH

Parameters	Control	ACN (% change <sup>a</sup> )	HES	ACN + HES (% change <sup>b</sup> )
MDA (nmol/mg protein)	1.82 $\pm$ 0.11	3.77 $\pm$ 0.2 <sup>a</sup> (+ 107 %)	1.7 $\pm$ 0.8 <sup>b</sup>	1.7 $\pm$ 0.15 <sup>b</sup> (- 55 %)
GSH ( $\mu$ g/mg protein)	5.82 $\pm$ 0.48	2.16 $\pm$ 0.29 <sup>a</sup> (- 63 %)	5.64 $\pm$ 0.35 <sup>b</sup>	6.12 $\pm$ 0.51 <sup>b</sup> (+ 183 %)

Data are expressed as means  $\pm$  SEM of six rats per group.

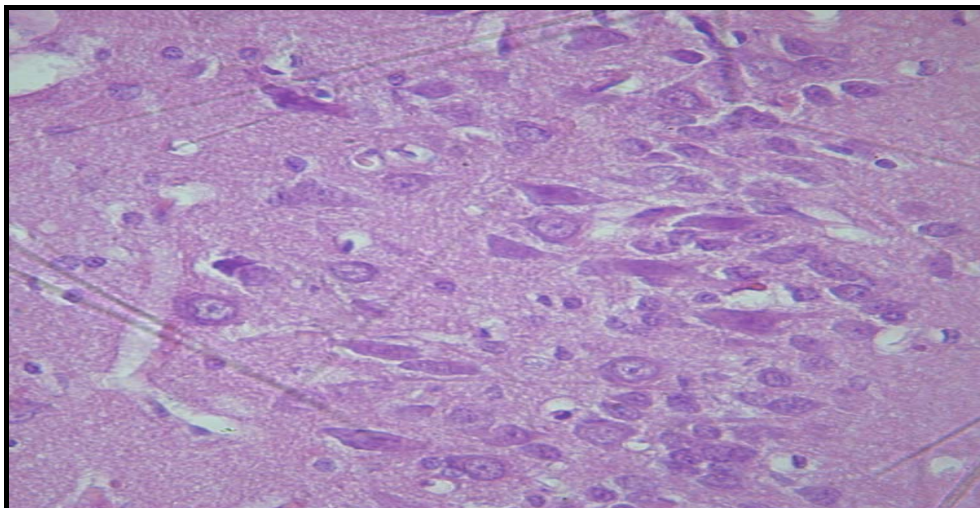
a: Significantly different from control group; b: Significantly different from ACN-treated group using one way ANOVA followed by Tukey-Kramer for multiple comparison test at  $P \leq 0.05$ .

**Table (2):** Effects of ACN and/or HES on the levels of enzymatic antioxidants in rat brain

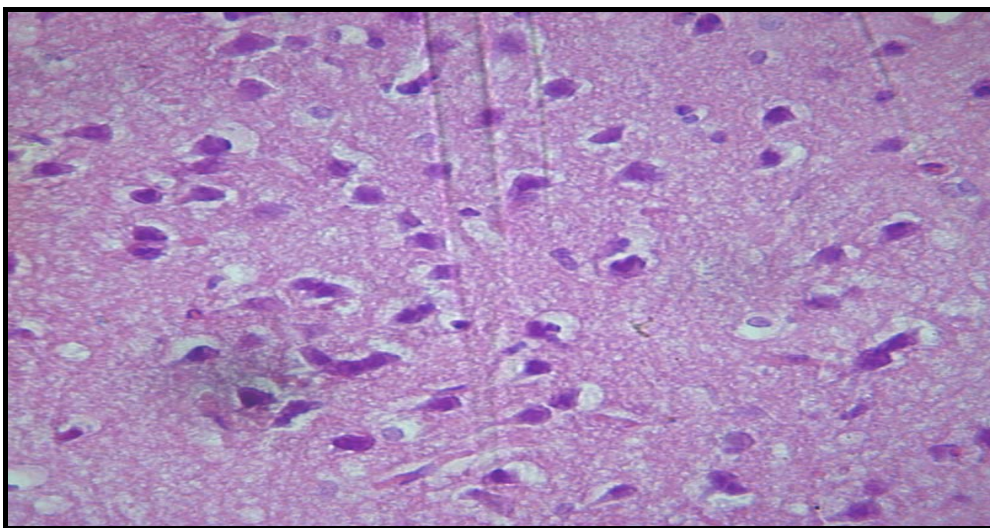
Parameters	Control	ACN (% change <sup>a</sup> )	HES	ACN + HES (% change <sup>b</sup> )
SOD (U/mg protein)	7.22 $\pm$ 0.19	4.11 $\pm$ 0.13 <sup>a</sup> (- 43 %)	7.91 $\pm$ 0.17 <sup>a,b</sup>	7.1 $\pm$ 0.09 <sup>b</sup> (+ 73 %)
CAT (U/mg protein)	4.35 $\pm$ 0.1	1.56 $\pm$ 0.07 <sup>a</sup> (- 64 %)	3.7 $\pm$ 0.08 <sup>a,b</sup>	4.2 $\pm$ 0.14 <sup>b</sup> (+ 169 %)
GSH-Px (nmol/min/mg protein)	2.87 $\pm$ 0.13	1.38 $\pm$ 0.06 <sup>a</sup> (- 52 %)	3.95 $\pm$ 0.18 <sup>a,b</sup>	4.1 $\pm$ 0.15 <sup>a,b</sup> (+ 197 %)
GST (nmol/min/mg protein)	5.59 $\pm$ 0.45	3.18 $\pm$ 0.21 <sup>a</sup> (- 43 %)	4.62 $\pm$ 0.17 <sup>b</sup>	5.43 $\pm$ 0.1 <sup>b</sup> (+ 71 %)

Data are expressed as means  $\pm$  SEM of six rats per group.

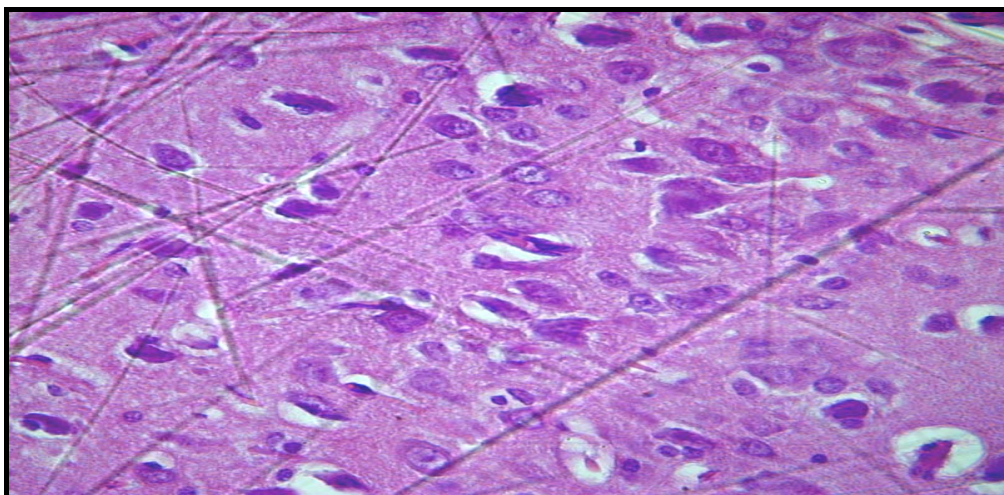
a: Significantly different from control group; b: Significantly different from ACN-treated group using one way ANOVA followed by Tukey-Kramer for multiple comparison test at  $P \leq 0.05$ .



**Fig 1: Brain section of a control or HES-treated rat showing normal structure of neuronal cells (H&E stain; X 300)**



**Fig 2: Brain section of rat treated with ACN for 28 days showing oedema and interstitial neuronal atrophy with perineuronal vacuolation (H&E stain; X 300)**



**Fig 3: Brain section of rat treated with ACN and HES. Neuronal degeneration was reduced and the brain morphology was close to normal (H&E stain; X 300)**

## DISCUSSION

Increased levels of lipid peroxidation in the rat brain induced by ACN administration (represented by increased MDA) were observed in the present study on rats after 28 days of treatment with ACN. This is in agreement with the findings of Jiang *et al.* (1998) and Esmat *et al.* (2007). Several studies have indicated the role of oxidative stress in the toxicity of ACN. For instance, the major pathway of ACN elimination is its conjugation with GSH to form mercapturic acid through the GST activity (Fennell *et al.*, 1991). By depleting GSH, ACN may decrease the antioxidant levels of the cells leading to an overall increase in intracellular reactive oxygen species (ROS) and oxidative damage. Metabolism of ACN results in the production of cyanide. Cyanide has been shown to induce oxidative stress (lipid peroxidation) in the brain of acutely treated mice and cell lines by inhibiting mitochondrial respiratory chain, CAT and GSH-Px (Mills *et al.*, 1996; Kanthasamy *et al.*, 1997).

The results of Our study show that both enzymatic (SOD, CAT, GSH-Px & GST) and non-enzymatic (GSH) antioxidant defense systems are impaired in ACN-treated rats. The effects of ACN on the suppression of activities of SOD and CAT may be related to the toxicity of ACN. GSH-Px and GST are glutathione-dependent intracellular enzymatic antioxidants. GSH-Px is responsible for the removal of ROS, such as peroxides, while GST is essential for conjugation. ACN may decrease the activities of these enzymes by defective synthesis or inactivation by binding (Nerland *et al.*, 2001; Mahalakshmi *et al.*, 2003).

The non-enzymatic free radical scavengers, GSH, vitamin E and vitamin C exist in their interconvertible forms and participate in the detoxification of ROS. GSH participates in enzymatic reduction of membrane hydroperoxy-phospholipids and prevents the formation of secondary alkoxyl radicals when organic peroxides are homolyzed (Reed, 1990; Sen and Hanninen, 1994). Binding of ACN to these antioxidants, especially GSH, results in the induction of oxidative stress and impaired regeneration of other antioxidants (Esmat *et al.*, 2007). Studies with  $^{14}\text{C}$  ACN have shown that ACN covalently binds with sulfhydryl groups of protein (Ahmed *et al.*, 1982) and to tissue macromolecules and nucleic acids (Pilon *et al.*, 1988). This explains the reduction in GSH content of tissues. Depletion of GSH in cells increases their susceptibility to oxidative damage.

The present findings show that HES treatment attenuated lipid peroxidation in the rat brain induced by ACN as manifested by decreased MDA level, accompanied by increased GSH content and enhanced activities of CAT, SOD GSH-Px & GST

enzymes. These results could be attributed to the potential antioxidant effect of HES (Tirkey *et al.*, 2005; Balakrishnan and Menon, 2007) and are in agreement with those obtained by Miyake *et al.* (1998) who demonstrated that HES treatment improved GSH levels in livers and kidneys of diabetic rats. Moreover, these findings are consistent with those of Kaur *et al.* (2006) who demonstrated that HES attenuates lipopolysaccharide-induced hepatotoxicity in rats possibly by preventing cytotoxic effects of NO and oxygen free radicals. On the other hand, Our results are against the findings obtained by Cho (2006) who suggested that HES exerts minimal or no protective effects on the oxidative neuronal damage induced by  $\text{H}_2\text{O}_2$ , xanthine & xanthine oxidase or excess glutamate in the cortical cultures of rat brains.

The histopathological findings demonstrated that administration of ACN induced various degenerative changes in neuronal cells which confirmed the biochemical evidence of oxidative stress. Treatment with HES obviously mitigated the histopathological changes induced by ACN.

In summary, the present data indicate that ACN-induced brain damage might be related to oxidative stress. Co-administration of HES lessened the negative effects of ACN on the brain by inhibiting free radical mediated process; an effect that could be attributed to the antioxidant property of HES.

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