

## Protective role of silibinin in cadmium induced changes of acetylcholinesterase, ATPases and oxidative stress in brain of albino wistar rats

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### Abstract

Cadmium (Cd) a highly toxic metal is considered to be a multitarget toxicant, principally its accumulates in the liver, kidney and hardly get into the brain parenchyma by the brain-barrier system. In this study, Cd (3 mg/kg body weight (b. w.)) was subcutaneously administered to rats for 3 weeks, which shows significantly ( $P < 0.05$ ) increase acetylcholinesterase enzyme activity, with significant elevation of lipid peroxidation along with significant ( $P < 0.05$ ) decreased in the levels of antioxidants and ATPases enzymes in the brain. Oral administration of silibinin (SB) at 80 mg/kg b. w. significantly normalized the acetylcholinesterase activity and reduced the levels of lipid peroxidation and also restored the antioxidant and ATPases enzymes in the brain when compared to other doses of SB (20 and 40 mg/kg b.w.). From this study we conclude that SB improves the changes in acetylcholinesterase, ATPases and oxidative injury by Cd- induced in brain.

**Keywords:** Antioxidants, Lipid peroxidation, Brain, Cadmium

### INTRODUCTION

Cadmium (Cd) is a neurotoxic heavy metal, inorganic toxicant of great environmental and human health concern. Cd produces neurotoxicity with a complex pathology that includes neurological dysfunction in adults and children (Shukla and Singhal, 1984; Hart et al., 1989), changes in neurochemistry (Gupta et al., 1993; Gutierrez-Reyes et al., 1998; Antonio et al., 1999) and behavioral alterations in young rats, (Wong and Klaassen, 1982; Holloway and Thor, 1988). Experimental studies have reported histopathological damage with extensive haemorrhagic lesions, destruction of fibers, edema and pyknotic cells in the brain of developing rats (Wong and Klaassen, 1982; Me'ndez-Armenta et al., 2001) and young rabbits compared with adults after Cd exposure (Gabbiani et al., 1967). It has been proposed that the age of the animal plays an important role on the neurotoxic effects of Cd (Gupta et al., 1993).

Brain is highly susceptible to oxidative lipid injury (Lipid Peroxidation) (LPO), because of it having high content of peroxidizable unsaturated fatty acids, high oxygen consumption per unit weight and its poorly developed antioxidative defense mechanism and a high content of transition metals like copper and iron in several regions (Floyd, 1999; Calabuse et al., 2000). Cd can hardly get into the brain parenchyma by the brain-barrier system, i.e. the blood-brain and the blood-cerebrospinal fluid (CSF) barriers (Takeda et al., 1999).

Cd itself is unable to generate free radicals directly because it

has only one oxidation state, however, indirect generation of both radicals and non radicals such as superoxide radical, hydroxyl radical, nitric oxide and hydrogen peroxide respectively, which in turn leads to a modification in structural integrity of lipids and secondarily affects membrane bound enzymes (Acan and Tezcan, 1995; Shukla et al., 1996). Several reports shows that Cd induces the LPO and it has an inhibitory action on the antioxidant enzymes and membrane bound ATPases (Garcia and Corredor, 2003; Caragegiou et al., 2004, Pari and Murugavel, 2007). The brain tissues membrane has been susceptible to LPO, which can lead to loss of adenosine triphosphatases (ATPases) activity and cell dysfunction (Bonting, 1970; Ohinishi et al., 1982). ATPases are lipid dependent membrane bound enzymes that play a key role in the active transport of ions, maintenance of cellular homeostasis and also in neurotransmission process. Cd alters the activities of enzymes in particularly neutralizing the oxidative stress, which can interfere with brain metabolism and also contribute to the neurotoxic effect. Cd increase acetylcholinesterase activity and decrease total antioxidant status in brain of adult male rats (Caragegiou et al., 2005).

Antioxidants are very important in oxidative stress-related disorders and it act as therapeutic agents. The antioxidant possessing compound can counteract the decrease in ATPase activity and the increase in oxidative stress that are induced by cd (El-Missiry and Shalaby, 2000).

Flavonolignan Silibinin (Fig,1) is a main biologically active component of the polyphenolic compound silymarin, which was the extract of milk thistle seeds from *silybum marianum*(L).Silibinin acts mainly as an effective antioxidant (Mira et al, 1994; Valenzuela and Garrido, 1994; Saller et al, 2001; Wellington and Adis, 2001) and free radical-scavenging activity (Winterbourn,2008). It also possess hepatoprotective (Ferenci et al, 1989), anticancer (Deep and Agarwal,2007), chemoprotective (Comelli et al, 2007), hypocholesterolemic (Hertog et al, 1993; Skottova et al, 1999), cardioprotective (Agoston et al, 2001; Agoston et al, 2003) and neuroactive and neuroprotective (Wang et al, 2002; Kittur et al,

Received: Oct 12, 2011; Revised: Nov 02, 2011; Accepted: Nov 24, 2011.

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2002) activities.

The protective effect of silibinin on cadmium induced neurotoxicity, however, has not been studied. We therefore investigated in wistar male rats whether silibinin therapy improves the Cd induced alterations in acetylcholinesterase, ATPases and oxidative stress in brain of experimental rats.

## MATERIALS AND METHODS

### Chemicals

Silibinin, cadmium chloride and other fine chemicals were obtained from Sigma – Aldrich, Co. (St. Louis, Mo, USA). All the other chemicals were of analytical grade obtained from a local firm (India).

### Animals and experimental design

Male wistar rats of initial body weight 180-220g were used in this experimental study. The rats were bred in the Central Animal House, Rajah Muthiah Medical College, Annamalai University (temperature  $28 \pm 2$  °C; natural light-dark cycle). The laboratory animal protocol used in this study was approved (Approval No: 610, 2009) by the Institutional Animal Ethical Committee (IAEC) at Annamalai University, Annamalai Nagar, India. In this experimental study, a total of 36 rats were used. The total rats were divided into six groups of six rats in each

Group 1	Control rats (Vehicle treated)
Group 2	Normal rats orally administrated with Silibinin (80mg/kg bw/day) dissolved in 0.1% Dimethyl Sulphoxide (DMSO) for 3 weeks.
Group 3	Normal rats were subcutaneously received Cd as cadmium chloride (3mg/kg bw/ day)(Pari and Murugavel, 2005) in isotonic saline for 3 weeks.
Group 4	Rats subcutaneously received Cd (3mg/kg bw/day) followed by oral administration of silibinin (20mg/kg bw/day) in 0.1% DMSO for 3 weeks.
Group 5	Rats subcutaneously received Cd (3mg/kg bw/day) followed by oral administration of silibinin (40mg/kg bw/day) in 0.1% DMSO for 3 weeks.
Group 6	Rats subcutaneously received Cd (3mg/kg bw/day) followed by oral administration of silibinin (80mg/kg bw/day) in 0.1% DMSO for 3 weeks.

After experimental period (21days), the treated rats were fasted overnight and sacrificed by decapitation. Blood was collected with anticoagulant and plasma were collected through centrifugation at 2000 x g for 20 min. whole brain was immediately dissected out, washed in ice-cold saline to remove the blood. The brain tissues were homogenized (10%,w/v) in appropriate buffer (pH 7.4) and centrifuged (1000 x g for 10 min) and the clear supernatant was used for various biochemical assays.

### Determination of acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was determined by using acetylthiocholine iodide as a substrate according to the method of Ellman et al (1961). AChE hydrolyzes acetylthiocholine iodide into thiocholine and butyric acid in plasma and brain tissue samples. The thiocholine reacts with 5,5' – dithiobis (2-nitrobenzoic acid) to form 5-thio-2-nitrobenzoic acid and yellow colour developed was measured

in UV-spectrophotometer at 412nm.

### Estimation of LPO and protein carbonyl contents.

LPO in brain was determined by measuring the levels of thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LOOH) by the method of Niehaus and Samuelsson (1968) and Jiang et al (1992), respectively. The level of protein carbonyl content in brain tissue homogenate was determined by the method of Levine et al (1990).

### Determination of antioxidant activities.

The levels of reduced glutathione (GSH) in the brain was estimated by spectrophotometric method based on the reaction with Ellman's reagent (19.8mg DTNB in 100ml of 0.1% sodium citrate) according to Moron et al (1979). The total sulphdryl groups (TSH) levels was determined after the reaction with 5,5'-dithiobis (2-nitrobenzoic acid) using the method of Ellman (1959). The activities of superoxide dismutase (SOD)(Kakkar et al., 1984), Catalase (CAT)(Sinta, 1972), glutathione peroxidase (GPx)(Rotuck et al., 1973) and glutathione-S-transferase (GST)(Habig et al., 1974) were also measured in brain tissue homogenate by spectrophotometrically. The protein concentrations were also measured in brain tissue homogenate by the method of Lowry et al (1951).

### Determination of ATPases activities

Total ATPase activity in brain tissue homogenate was measured by the method of Evans (1969). The ATPase activity in 0.1ml aliquot of the tissue homogenates were measured in a final volume of 2ml, which containing 0.1ml of 0.1M Tris-HCl (pH 7.4), 0.1ml of 0.1M NaCl, 0.1ml of 0.1M MgCl<sub>2</sub>, 1.5ml of 0.1M KCl, 0.1ml of 1mM EDTA and 0.1ml of 0.01M ATP. The reaction was arrested after 20min by the addition of 1ml of 10% TCA and then centrifuged (1500 x g for 10 min), and the liberated inorganic phosphorus (Pi) was estimated in the protein free supernatant. The liberated Pi was estimated according to the method of Fiske and Subbarow (1925).

The activity of Na<sup>+</sup>/K<sup>+</sup>-dependent ATPase was determined by the method of Bonting, (1970). In this assay, 0.2ml of brain tissue homogenate was added to the mixture containing 1ml of 184mM Tris-HCl buffer (pH 7.5), 0.2ml of 50mM MgSO<sub>4</sub>, 0.2ml of 50mM KCL, 2ml of 600mM NaCl, 0.2ml of 1mM EDTA and 0.2ml of 10mM ATP and incubated for 15min at 37°C. The reaction was stopped by the addition of 1ml of ice-cold 10%TCA. Then the amount of Pi liberated was estimated in protein-free supernatant at 412nm. The activity of Ca<sup>2+</sup>-ATPase was assayed according to the method of Hjertan and Pan (1983). In brief, 0.1 ml of tissue homogenate was added to mixture containing 0.1 ml of 125 mM Tris-HCl buffer (pH 8), 0.1 ml of 50 mM CaCl<sub>2</sub> and 0.1 ml of 10mM ATP. The contents were incubated at 37°C for 15 minute. The reaction was then arrested by the addition of 0.5 ml of ice cold 10% TCA and centrifuged. The amount of Pi liberated was estimated in supernatant. The activity of Mg<sup>2+</sup> - ATPase was assayed by the method of Ohinishi et al. (1982). The incubation mixture contained 0.1 ml of 375 mM Tris-HCl buffer (pH 7.6), 0.1 ml of 25 mM MgCl<sub>2</sub>, 0.1 ml of 10 mM ATP, 0.1 ml of 10 mM ATP, 0.1 ml water and 0.1 ml of tissue homogenate. The contents were incubated for 15 minute at 37°C and the reaction was arrested by adding 0.5 ml of 10 % TCA. The liberated Pi was then estimated in protein free supernatant. The activities of these ATPase

enzymes in tissue homogenate were expressed as  $\mu\text{g Pi}$  liberated/min/mg protein.

### Statistical analysis

All data's are expressed as mean  $\pm$  SD of number of experiments ( $n=6$ ). The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 11.5 (SPSS, Cary, NC, USA), and the individual comparison were obtained by Duncans' Multiple Range Test (DMRT). Values were considered statistically significant when  $P<0.05$  (Duncan, 1957).

## RESULTS

To determine whether the Silibinin (SB) has beneficial effect in Cd induced oxidative injury in brain, we performed this study with a fixed dose of SB (80 mg/kg body weight).

### Levels acetylcholinesterase activities

Fig. 2 indicates the activity of AchE in plasma and brain of control and experimental rats. The activities of AchE in brain and plasma was significantly ( $P<0.05$ ) decreased in Cd intoxicated rats whereas in the oral administration of SB (80 mg/kg body weight) significantly ( $P<0.05$ ) reversed to near normal.

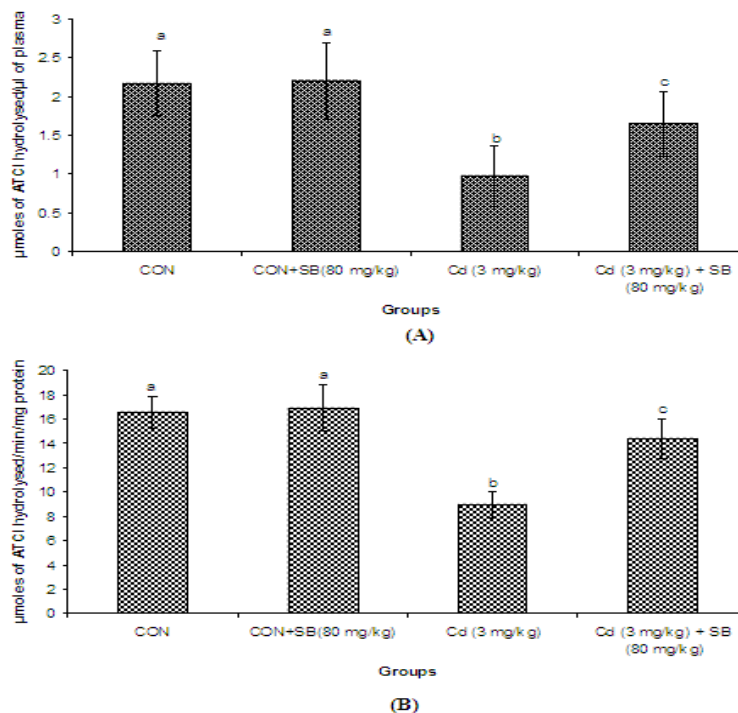


Fig 2. Changes in the activities of acetylcholinesterase (AChE) in plasma (A) and brain (B) of control and experimental rats. ATCl: acetyl thiocholine iodide; CON: control; SB: silibinin; Cd: cadmium. Values are mean  $\pm$  SD for 6 rats in each group. <sup>a-c</sup>In each row, means with different superscript letter differ significantly at  $p<0.05$  (DMRT).

### Levels of LPO, protein carbonyl contents and GSH

The levels of LPO products (TBARS and LOOH) and protein carbonyl contents were significantly ( $P<0.05$ ) increased, while the level of GSH is significantly ( $P<0.05$ ) decreased in brain of control

and experimental rats (Table. 1). After oral administration of SB (80 mg/kg body weight) to Cd intoxicated rats, the elevated levels of TBARS, LOOH and protein carbonyl contents were significantly ( $P<0.05$ ) decreased with significant ( $P<0.05$ ) increase in the level of GSH when compared with Cd alone treated group.

Table 1. Changes in the levels of LPO markers, protein carbonyl contents and GSH in brain of control and experimental rats

Parameters	Control	Normal + SB (80 mg/kg)	Normal + Cd (3 mg/kg)	Cd (3 mg/kg) + SB (80 mg/kg)
TBARS ( $\mu\text{mol/g tissue}$ )	13.05 $\pm$ 0.96 <sup>a</sup>	12.02 $\pm$ 0.93 <sup>a</sup>	21.48 $\pm$ 3.06 <sup>b</sup>	15.96 $\pm$ 1.59 <sup>c</sup>
LOOH (mmol/g tissue)	1.02 $\pm$ 0.14 <sup>a</sup>	0.96 $\pm$ 0.05 <sup>a</sup>	1.95 $\pm$ 0.34 <sup>b</sup>	1.26 $\pm$ 0.07 <sup>c</sup>
Protein Carbonyl Contents (nmol/mg protein)	3.97 $\pm$ 0.54 <sup>a</sup>	3.41 $\pm$ 0.56 <sup>a</sup>	8.39 $\pm$ 0.84 <sup>b</sup>	4.68 $\pm$ 0.07 <sup>c</sup>
GSH ( $\mu\text{g/mg protein}$ )	3.76 $\pm$ 0.23 <sup>a</sup>	3.82 $\pm$ 0.21 <sup>a</sup>	1.33 $\pm$ 0.18 <sup>b</sup>	2.94 $\pm$ 0.43 <sup>c</sup>

**Antioxidant and ATPases activities**

The Fig 3 demonstrates the changes in the antioxidant enzymes activity in brain of control and experimental rats. A significant ( $P<0.05$ ) decrease in the activities of SOD, CAT, GPx and GST were observed in Cd intoxicated rats on comparison with control rats. Oral administration of SB (80 mg/kg body weight)

significantly ( $P<0.05$ ) reverse the antioxidant activities to near normal. The data in table 2 demonstrate the activity of ATPase enzymes ( $\text{Na}^+/\text{K}^+$ -ATPase,  $\text{Ca}^{2+}$ -ATPase and  $\text{Mg}^{2+}$ -ATPase) in brain of control and experimental rats. Treatment with SB (80 mg/kg body weight) significantly ( $P<0.05$ ) increased the activities of ATPases enzymes in brain when compared to Cd alone treated rats.

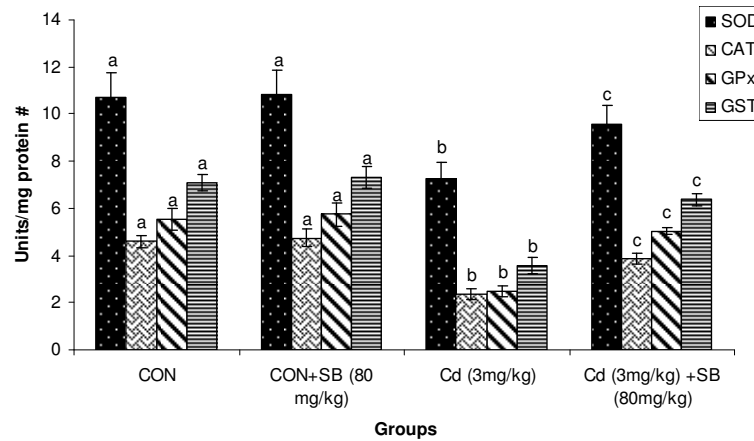


Fig 3. Changes in the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) in brain of control and experimental rats

CON: control; SB: silibinin; Cd: cadmium. The activities of the enzymes are expressed as follows: SOD - One unit of enzyme activity was taken as the enzyme reaction, which gave 50% inhibition of nitroblue tetrazolium reduction in one minute/mg protein; CAT -  $\mu\text{moles}$  of  $\text{H}_2\text{O}_2$  consumed/min/mg protein; GPx -  $\mu\text{g}$  of glutathione consumed/min/mg protein; GST-  $\mu\text{moles}$  of 1-chloro-2,4-dinitrobenzene- GSH conjugate formed/min/mg protein. Values are mean  $\pm$  SD for 6 rats in each group. a-c In each row, means with different superscript letter differ significantly at  $p<0.05$  (DMRT).

Table 2. Changes in the activities of adenosine triphosphatase (ATPase) enzymes in brain of control and experimental rats

Groups	Control	Normal +SB (80 mg/kg)	Normal + Cd (3 mg/kg)	Cd (3 mg/kg) + SB (80 mg/kg)
Total ATPases ( $\mu\text{g Pi}$ liberated/min/mg protein)	1.55 $\pm$ 0.14 <sup>a</sup>	1.59 $\pm$ 0.15 <sup>a</sup>	0.98 $\pm$ 0.07 <sup>b</sup>	1.38 $\pm$ 0.15 <sup>c</sup>
$\text{Na}^+/\text{K}^+$ - ATPase ( $\mu\text{g Pi}$ liberated/min/mg protein)	0.40 $\pm$ 0.03 <sup>a</sup>	0.42 $\pm$ 0.01 <sup>a</sup>	0.23 $\pm$ 0.01 <sup>b</sup>	0.35 $\pm$ 0.04 <sup>c</sup>
$\text{Ca}^{2+}$ - ATPase ( $\mu\text{g Pi}$ liberated/min/mg protein)	0.55 $\pm$ 0.09 <sup>a</sup>	0.58 $\pm$ 0.05 <sup>a</sup>	0.29 $\pm$ 0.03 <sup>b</sup>	0.48 $\pm$ 0.04 <sup>c</sup>
$\text{Mg}^{2+}$ - ATPase ( $\mu\text{g Pi}$ liberated/min/mg protein)	0.43 $\pm$ 0.04 <sup>a</sup>	0.44 $\pm$ 0.04 <sup>a</sup>	0.21 $\pm$ 0.01 <sup>b</sup>	0.35 $\pm$ 0.05 <sup>c</sup>

**Discussion**

Numerous studies demonstrate that changes in the antioxidant system and impairment of sodium and potassium transport are essential factors involved in the brain tissue disturbance in various kinds of oxidative injury in the central nervous system (Jenner, 1998; Slyshenkov et al., 2002; Pari and Murugavel, 2007). The potency of Cd as a neurotoxin has been demonstrated in both invitro and in vivo studies (Antonio et al., 2003; Mendez-Armenta et al., 2003; Monroe and Halvorsen, 2006; Pari and Murugavel, 2007).

Oxidative stress affects numerous cellular components, such as DNA, lipids and proteins, through oxidation reactions. Recent

studies have shown that cadmium produce reactive oxygen species, resulting in an increased lipid peroxidation depletion of sulfhydryls, altered calcium homeostasis and finally DNA damage (Mendez-Armenta et al., 2003; Pari and Murugavel, 2007).

Oxidative stress refers to the cytopathological consequences of an imbalance between the production of free radicals in the brain and the ability of the cell to defend against them (Zhu et al., 2006). Cd can penetrate the blood brain barrier and accumulate into the brain which is easily susceptible to oxidative lipid injuries, named lipid peroxidation (LPO) (Gutierrez-Reyes et al., 1998). In this study, the elevation of LPO and increased formation of protein carbonyls with reduced levels of TSH in Cd intoxicated rat brain might be due to over production of free radicals and LPO end products, where

leads to oxidative modification of proteins.

## CONCLUSION

"leads to oxidative modification of proteins." Chelation of Cd reduces the oxidative stress, reverses the antioxidant level and glutathione metabolizing enzymes activities due to the presence of hydroxyl groups in SB and it react with free radicals (Sun and Oberley, 1996).

Changes in the activities of membrane bound ATPases are estimated to exert a significant impact on the physiological and metabolic functions of cellular membrane. Previous studies showed that Cd decreased the membrane fluidity and increased LPO by binding to phospholipids (Shaikh et al., 1999), and target various intracellular proteins and membrane transporters at their cytoplasmic side by binding to their reactive SH or COO groups (Stacey and Klaassen, 1999).

The maintenance of the cation gradient by the ATPase enzymes is essential and fundamental for the regulation of cell growth and differentiation, which are critical for the normal functioning of the cells. The changes in ionic concentrations can bring about diverse types of cell injury, which ultimately lead to cell death (Kane, 1996). Our data indicate a remarkable diminution in the activities of membrane bound ATPases in the tissues. These findings corroborate with the previous studies in which Cd has been shown to inhibit the activities of Na<sup>+</sup>/K<sup>+</sup> ATPase, Mg<sup>2+</sup> ATPase and Ca<sup>2+</sup> ATPase (Torre et al., 2000; Garcia and Corredor, 2003; Carageorgiou et al., 2004).

Administration of SB depleted the elevation of LPO in the tissues and sustained the activities of membrane bound enzymes. The preservation of cellular membrane integrity by SB depends on their antioxidant properties that neutralize the oxidative reactions. The lipophilic nature of SB favours its passage through the membrane, and can accommodate in the lipid bilayer, thereby protecting the radical attack and maintaining the normal physiology of the membrane. In addition, SB reduced the accumulation of Cd and improved the levels of endogenous antioxidants involved in membrane protection (Van Acker et al., 1995), which in turn reduced the Cd induced alterations in membrane bound enzymes as well as ionic gradients within the cell.

In conclusion, our studies shows that the SB may improve the changes in membrane bound enzymes such as acetylcholinesterase, ATPases and oxidative injuries induced by the Cd in brain. Its action may be due to the presence of hydroxyl groups in SB and it react with free radicals. Further studies are also required to study of the SB in Cd intoxicated rats.

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