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Neuroprotective role of vitamin E, green tea extract, and *spirulina* in rats treated with cadmium chloride

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ABSTRACT

Background & Aim: Spirulina (*Spirulina plantesis*) and Tea (*Camellia sinensis*) has a long history of use in folk medicine. Recently, They have been widely studied for their potential antioxidant properties. The present study investigated neuroprotective effect of Spirulina and green tea extract against Cadmium [Cd]-induced brain lesions and to compare these effects with neuroprotective effect of Vitamin E.

Experimental: The rats were randomly divided into 5 groups and were treated with the following treatment for a period of 4 weeks. Control, $CdCl_2$ alone, Vitamin E with $CdCl_2$, Green tea extract with $CdCl_2$, Spirulina solution with $CdCl_2$. On the 29th day, animals were sacrificed humanely and their brains were placed in formalin. Hippocampus, caudate putamen, corpus callosum, cerebellum, and cerebrum were studied by light microscopy.

Results: Neuronophagia, satellitosis of oligodendrocytes, hyperemia, hemorrhage, neuronal necrosis, central chromatolysis, neuronal atrophy, laminar necrosis, and status spongiosus were the observed changes. Thickness of granular layer and molecular layer of cerebellum and size/diametr of purkenje cells reduced when cadmium used alone at $P<0.05$. A thicker and different molecular layer and almost normal purkenje cells were observed when Spirulina was combined with cadmium at $P<0.05$. Likewise,spirulina significantly decreased the number of cadmium-induced necrotic cells in hippocampus and cerebral cortex but not in caudate putamen at $P<0.05$. Spirulina showed the most protective effect on brain tissue in comparison with vitamin E and green tea extract. Green tea extract had the weakest effect. The hippocampus was the most affected area of the brain among the others in this survey.

Recommended applications/industries: The results shows Spirullina as excellent antioxidant source indicating its use possibly in combination with vitamin E and other antioxidant agent in order to prevent adverse effects of free radicals.

1. Introduction

Cadmium (Cd) is one of the potent environmental and occupational metallic toxicants, widely dispersed in the environment (Méndez-Armenta and Ríos, 2007). Cd toxicity as an industrial pollutant, a food contaminant, and as one of the major components in cigarette smoke has been proven (Manca *et al*., 1991). In nonsmokers, food is the most important route of Cd entrance into the body (Donma and Metin Donma, 2005). Because of its great involvement in cancer, Cd has been classified as a category 1 carcinogen by the International Agency for Research on Cancer and the USA's National Toxicology Program (Waisberg et al., 2003). It damages a variety of organs, including the liver, kidney**,** lung and testis (El-Refaiy and Eissa, 2012), as well as brain (Mendez-Armenta *et al*., 2001; Nishimura *et al*., 2011). Acute exposure to Cd produces hepatic, pulmonary, and testicular injury, while chronic exposure results in renal and bone injury and cancer (Klaassen *et al*., 1999). Cadmium is able to enter the brain parenchyma and cause neurological alterations (Nishimura *et al*., 2011). It is more toxic to young animals due to the immaturity of the blood brain barrier (Wong and Klaassen, 1982).

A well-known effect of Cd both *in vitro* and *in vivo* is the generation of free radicals in cells (Méndez-Armenta and Ríos, 2007), followed by oxidative stress (El-Refaiy and Eissa, 2012). It induces lipid peroxidation (LPO) in all tissues, mainly in the lung and brain (Manca *et al*., 1991). Brain tissue is highly susceptible to LPO due to its high rate of oxygen utilization, its abundant supply of polyunsaturated fatty acids, and a high content of transition metals like copper and iron in several regions (Calabrese *et al*., 2000). Another mechanism through which cadmium induces oxidative stress is by inhibiting enzymatic and non-enzymatic antioxidants in various tissues (Shagirtha *et al*., 2011).

Spirulina [Spirulina platensis], is a microscopic and filamentous blue-green alga used as food it is considered as a rich source of some macro- and micronutrients including amino acids, chlorophyll, gamma-linolenic acid, carotenoids, and vitamins (Belay, 2002). It has an antioxidant effect that might be associated with phycocyanin, a phycobiliprotein found at high concentration in *Spirulina* (Pinero Estrada *et al*., 2011). *Spirulina* protects the liver from the toxic effects caused by Cd (Amin *et al*., 2006).

Tea (*Camellia sinensis*) is one of the most common drinks around the world (Graham, 1992). In recent years, green tea has been widely studied for its contribution in preventing hypercholesterolemia, cancer, atherosclerosis, coronary artery diseases and inflammation (Sharangi, 2009). Green tea is an excellent source of polyphenols known as green tea catechins. The important catechins of green tea are (−)-epicatechin, (−)-epicatechin-3-gallate, (−)-epigallocatechin, and (−)-epigallocatechin- 3-gallate (Babu et al., 2006). They are able to enter the brain (Abd El Mohsen *et al*., 2002), contributing to antioxidant effect by scavengering free radicals (Sharangi, 2011). Green tea extract intake in rats increased total antioxidant statue index and decreased lipid peroxidation products in the liver (Skrzydlewska *et al*., 2002).

Present study is designed to investigate the neuroprotective effects of Spirulina and green tea extract against Cd, and to compare these effects with those of vitamin E, as a major non-enzymatic antioxidant present in the lipid structures of cells (Wagner *et al*., 1996).

2. Materials and Methods

2.1. Chemicals and reagents

Vitamin E in the form of α-tocopherol (95.5%), cadmium in the form of cadmium chloride hydrate (98%) ($CdCl_2$), and *Spirulina* (algal lyophilized cells) were purchased from Sigma-Aldrich Corporation, Milwaukee,WI, USA. Green tea was purchased from a traditional herbal shop.

2.2. Animals

Thirty healthy weanling albino Wistar rats, each weighing 40-50 g, were obtained from the animal house at the Faculty of Veterinary Medicine, University of Tabriz. They were kept under constant temperature of 23 °C with a 12h light/dark cycle, and fed a standard pellet diet and water ad libitum. After one week of acclimatization, the animals were divided into five groups of six and were treated with the following treatment for a period of 4 weeks.

Group I: Untreated control rats**, GroupII**: $CdCl_2$ [50 ppm mixed with diet]**, Group III**: Vitamin E [intraperitoneal, 100 mg/kg body weight [b.w.] weekly]

+ $CdCl_2$ [50 ppm mixed with diet]**, Group IV**: Spirulina solution [500 mg/kg b.w., in 5 cc water by gavage weekly] + $CdCl_2$ [50 ppm mixed with diet]**, Group V**: Green tea alcoholic extract [1.5% w/v] [dissolved in drinking water] + $CdCl_2$ [50 ppm mixed with diet]

2.3. Preparations of green tea extract

In order to prepare decaffeinated tea extract, green tea leaves were first soaked in hot distilled water [1:5 w/v] for 15 min. The supernatant was then removed from the hot water by ethyl acetate in equal volume. Then, by maceration method, the remaining part from tea leaves was extracted with 70% ethanol. The extract was filtered and the solvent was evaporated in a rotary evaporator under reduced pressure at 40°C. The resulting extract was frozen at -20ºC until use.

2.4. Histopathology

Experiments were performed according to the guidelines established in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. At the end of exposure [29th day], the animals were sacrificed by ether anesthesia. Each rat's entire brain was removed and fixed immediately in 10% neutral buffered formalin. To reach the cortex, corpus callosum, and caudate putamen, one slice was taken involving these regions. A similar action was performed for excision of the hippocampus. Half of the cerebellum was removed by a sagittal incision (Fig1).

All the landmarks were chosen from the book, "The Rat Brain in Stereotaxic Coordinates" (Paxinos and Watson, 2007). After routine histological procedures, paraffin blocks were sectioned into 5-µm thick sections, stained with hematoxylin and eosin and examined under light microscope. For histopathological and histomorphometric studies, the following parameters were examined.

I. Thickness of the cerebellar cortex molecular layer [ML] and granular layer [GL] using a microscope with a graticule attached to the eyepiece.

II. Mean diameter / size of the Purkinje cell using the same microscope.

III. Number of necrotic neurons in cerebral cortex, hippocampus and caudate putamen.

IV. Histopathological changes.

Necrotic neurons were identified microscopically by chromatolysis, atrophied, pyknotic nuclei, and loss of nucleus. The means and the standard deviation of each of the parameters measured were determined. All data are reported as the mean ± S.E.M. To compare the values acquired after treatment among all groups, one-way analysis of variance [ANOVA] was used followed by Tukey post hoc test using SPSS software version 11.5. Differences were considered significant at $p < 0.05$.

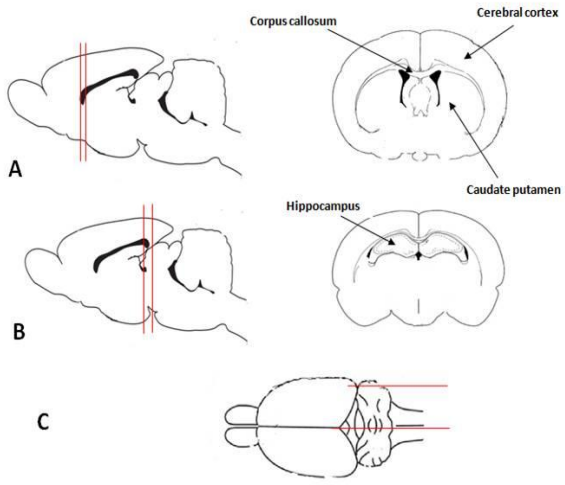

Fig 1. Incision lines and cross sections of the rat brain for specimens taking. **(A)**. Incision lines and cross section of the rat brain for receiving to Caudate putamen;Corpus callosum and cerebral cortex. (**B)**. Incision lines and cross section of the rat brain for receiving to hippocampus. (**C)**. Incision lines on the rat brain for receiving to cerebellar nuclei and cortex.

3. Results and discussion

No death was observed throughout the 4-week period of the experiment. Under light microscopy, the control group showed no lesions. In other groups, lesions with varying severity were observed. The hippocampus was found the most vulnerable region to Cd toxicity with major neurodegenerative changes. The other regions were the cerebral cortex, cerebellar nuclei, caudate putamen, cerebellar cortex, and corpus callosum respectively. In the Cd treated group changes in the cerebellum consisted of scattered necrosis in the Purkinje layer with hyperemia in the granular layer. Cerebellar nuclei showed neuronal necrosis with neuronophagia, satellitosis of oligodendrocytes, and hyperemia. In the hippocampus, observed lesions were neuronal necrosis with predominant pyknotic nuclei and loss of nucleus in some neurons, central chromatolysis, oligodendrocyte satellitosis, capillary hyperemia, hyperchromatic neurons, and atrophied

neurons with detachment from surrounding neuropil, neuronophagia, and vacuolization. Infiltration of macrophages and lymphocytes to perivascular spaces was observed with lymphocytes predominating. Caudate putamen showed mild hemorrhage in Virchow-Robin spaces [VRS]. Neuronal necrosis, gemistocytic astrocytes, neuronophagia, and hyperemia were obvious too. In the corpus callosum, axonal swelling, hyperemia, and mild hemorrhage were observed. In some regions, status spongiosus was evident as well. Oligodendrocyte satellitosis, gemistocytes, macrophages and lymphocytes infiltration to perivascular spaces, distinct laminar necrosis of the neurons in external pyramidal layer, severe hyperemia, gliosis, and neuronal vacuolization were observed in cerebral cortex. White matter showed axonal degeneration, interstitial edema, and edema in VRS (Fig 2).

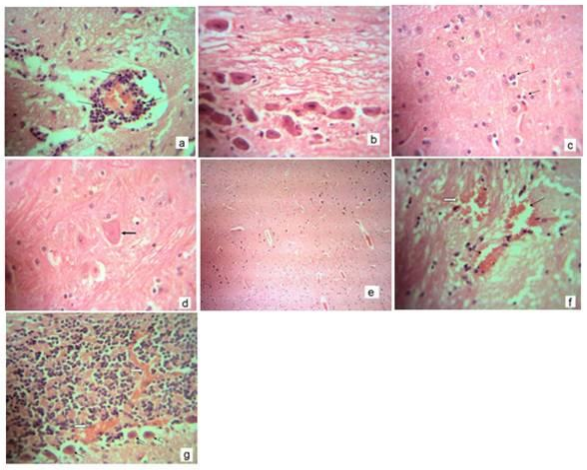

Fig 2. Histopathological findings of brain tissue. **(a).** Perivascular cuffing with mononuclears in hippocampus.H&E staining.×800. **(b).** Neuronal necrosis in one cerebellar nucleus.H&E staining.×800. **(c).** Oligodendriocytessatellitosis in cerebral cortex.H&E staining.×800. **(d).** Central chromatolysis and vacuolation in a neuron of caudate putamen.H&E staining. ×800. **(e).** Marked laminar necrosis in external pyramidal layer of cerebral cortex.H&E staining. ×200. **(f).** Hyperemia and mild hemorrhage in corpus callosum.H&E staining. ×800. **(g).** Necrosis in purkinje and hyperemia in granular layer of cerebellum.H&E staining. ×800.

Co-administration of *Spirulina* with Cd reduced the degenerative changes to a remarkable level in comparison with the Cd treated group. Hemorrhage and laminar necrosis were absent, although scattered neural necrosis did exist. Furthermore, other changes like edema, satellitosis, neuronal necrosis, and slight gliosis were present. In simultaneous administration of vitamin E with Cd, histopathological lesions were slighter than those observed in the group treated with Cd alone. The cortex showed only scattered neural necrosis. The group that received green tea extract with Cd showed slighter lesions compared with the Cd treated group. The kind of the lesions was similar with the other groups. Laminar necrosis with hemorrhage observed in the cerebral cortex. Changes were more severe than in the vitamin E treated group.

Histomorphometric studies showed that Cd reduced the thickness of GL and ML of cerebellum [148.0±2.5 µm and 173.0±8.0 µm, respectively] as compared with control group [166.0±4.30 µm and 215.0±5.3 µm, respectively]. A thicker and markedly different ML was observed when *Spirulina* combined with Cd. The mean diameter of the Purkinje cell was significantly reduced in the cadmium as well as in the cadmium and vitamin E and cadmium and green tea extract groups compared with the control group. These were statically significant at $p<0.05$. Spirullina significantly impeded this effect at $p<0.05$ (Table 1). Likewise, number of cadmium-induced necrotic cells in cortex, hippocampus significantly reduced when *Spirulina* combined with Cd (Table 2). The difference between green tea extract and Vitamin E groups with Cd group alone was not significant at $p<0.05$.

The most prominent change detected from light microscopy of the five brain regions was neuronal degeneration and necrosis, characterized mainly by chromatolysis, neuronal atrophy, pyknotic nuclei, and finally loss of nucleus. It can be attributed mainly to the oxidative stress caused by Cd. Cadmium exposure increases reactive oxygen species, leading to lipid and protein peroxidation, changes to intercellular stability, damage to deoxyribonucleic acid (DNA) and membranes and consequently inducing cell death (Stohs *et al*., 2001; Waisberg *et al*., 1999). Likewise, Cd is able to reduce the activity and level of antioxidant enzymes like superoxide dismutase (SOD) and concentration of reduced glutathione (GSH) (El-Sokkary and Awadalla, 2011). SOD and GSH are potent antioxidant agents and their depletion indicates oxidative stress (Manca *et al.*, 1991). There are relatively low levels of antioxidants such as GSH, vitamin E, catalase and SOD in the brain (Calabrese *et*

al., 2000). Cd causes morphological changes in neurons by sustained increase in intracellular Ca^{2+} concentratin (Yoshida, 2001).

Table 1. Mean thickness of cellebelar layers and mean diameter of purkenje cells (mean ±SEM) in micrometer (µm).

	Group I	Group II	Group III	Group IV	Group V
Granular layer	166.0±4.30	148.0±2.5*	158.0±4.6	160.0±3.5	148.0±2.5*
Molecular layer	215.0±5.3	173.0±8.0*	195.0±5.0	210.0±3.7§	181.0±6.7*†
Purkenje cells	25.6±1.5*	18.8±1.2*	20.4±1.02*	25.0±1.3§	19.4±0.6 *†

*: statically significant difference from control group, §: statically significant difference from group II, †: statically significant difference from group III, ($p<0.05$)

Table 2. Mean number of cadmium- induced necrotic cells (mean±SEM)

	Group I	Group II	Group III	Group IV	Group V
Cortex	0.0±0.0	1.8±0.3*	1.2±0.3	0.2±0.2§	1.6±0.2*†
Hippocampus	0.0±0.0	2.4±0.4*	1.8±0.3*	0.6±0.2§	1.8±0.3*
Caudate putamen	0.2±0.2	1.8±0.3*	1.4±0.2	0.8±0.3	1.6±0.4*

*: statically significant difference from control group, §: statically significant difference from group II, †: statically significant difference from group III, ($p<0.05$).

Our findings are in agreement with a previous study which reported morphological changes such as hyperchromatic cells, necrosis, interstitial edema, and alteration of Purkinje cells in the brain of rats exposed to Cd (Mendez-Armenta *et al*., 2001). In young rats, Cd produced slight damage to the endothelium of some blood vessels in a necrotic area with hemorrhage and infiltration of macrophages and lymphocytes into the perivascular space (Wong and Klaassen, 1982). Infiltration of macrophages and lymphocytes and hemorrhage were observed in our examination.

In order to prevent long-term Cd exposure toxicity, both endogenous antioxidants synthesized in tissues and exogenous antioxidants in the diet are needed. In the current study, introducing different co-treatment supplementations such as vitamin E, *Spirulina*, and green tea extract into the diet resulted in reduction of histopathological changes in the brain. Vitamin E, as an effective liposoluble antioxidant, is proven to interact with free radicals and prevents lipid peroxidation (Silva *et al.*, 2005). In the vitamin E treated group, histopathological changes were significantly reduced. Laminar necrosis in the cortex was diminished to scattered necrosis in some regions. It is speculated that vitamin E impedes oxidative stress initiated by free radicals in the cells, which lead to cell necrosis. It is speculated that vitamin E impedes oxidative stress initiated by free radicals in the cells, which lead to cell necrosis.

We also observed that green tea extract slightly mitigated histopathological changes in several brain regions. This can be attributed to the antioxidants that exist in green tea extract. Green tea co-administered with lead increased the activity of SOD and concentration of GSH and ameliorated histopathological changes induced by lead in the three regions of brain in treated rats (Khalaf *et al*., 2012).

Besides phycocaine, *Spirulina* is found to be a good source of flavonoids, vitamin C, vitamin E and GSH (Jeyaprakash and Chinnaswamy, 2005). Furthermore, Zn^{2+} found in *Spirulina* is a potent antagonist against Cd neurotoxicity (Valko *et al*., 2005). In rats exposed to lead, *Spirulina* was effective against oxidative damage caused by lead (Upasani and Balaraman, 2001).

Spirulina protected brain tissue better than the other materials of our research. Green tea extract was least effective among the others tested. This can be attributed to the several antioxidant components that exist in *Spirulina*. In addition, metals like Cu and Zn in *Spirulina* can serve as cofactors for the SOD enzyme and consequently antioxidant status will increase. In one study, an alcohol extract of *Spirulina* displayed better antioxidant property in the inhibition of LPO (65%) in comparison with α-tocopherol (35%) and β-carotene (48%) (Belay, 2002). Hippocampus lesions were more intense than those of other regions. It implies that Cd exerts its toxicity mainly in hippocampus. In a recent study of rats exposed to Cd, the greatest concentration of Cd was observed in the hippocampus region (Mendez-Armenta *et al*., 2001). Another study revealed that the hippocampus is the region most affected after cadmium intoxication (Kaoud *et al*., 2010).

4. Conclusion

Spirulina protected brain tissue better than the other materials of our research. Green tea extract was least effective among the others tested. The hippocampus was the most affected area of the brain among the others. Those other regions were the cerebral cortex, cerebellar nuclei, caudate putamen, cerebellar cortex, and corpus callosum respectively.

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