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Protective Potential of Apigenin against Lead Acetate Induced Alterations in Cerebellum of Rats

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ABSTRACT

Background: Lead (Pb) is a heavy metal that represents an environmental toxicant causing serious health problem. Apigenin (APG) is a plant derived flavonoid with several therapeutic activities. So, the aim of the present study was to determine the protective effect of APG against Pb induced toxicity in cerebella of treated rats.

Method: Twenty-four adult male rats (*Rattus norvegicus*) were used in the present study. They were divided into four groups of six animals per each one as follows: Group I: Rats were daily injected (*i.p.*) with normal saline as vehicle. Group II: Rats were daily injected (*i.p*) with lead acetate at a dose of 20 mg/kg b.w. Group III: Rats were daily injected (*i.p*) with lead acetate at a dose of 20 mg/kg (b.w.) followed by injection (*i.p*) with APG at dose of 20 mg/kg b.w. Group IV: Rats were daily injected (*i.p*) with APG at dose of 20 mg/kg b.w. At the end of the four-weeks experimental period, the animals were sacrificed and samples of blood and cerebellar tissue were collected.

Results: Lead induced oxidative stress, alterations in levels of studied neurotransmitters, increase in the activities of acetylcholinesterase and increase in the level of the Il-6 and TNF-α and decrease in Il-4 and Il-10 and histological alterations in cerebellum. On the other hand, treatment with APG attenuates these alterations. suggesting that APG might play a neuroprotective role in lead induced neurotoxicity.

Key words: Apigenin, Cerebellum, Lead.

INTRODUCTION

Lead can cause environmental pollution leading to serious health problem (Briffa *et al.,* 2020). Exposure to Pb can occur through consumer products such as water pipes (Duah, 2017).

Oxidative stress and inflammation have been implicated in many neurodegenerative disorders (Melzig and Wszelaki, 2013). Pb is known to alter the production of pro-inflammatory cytokines (Krocova *et al*., 2000), be a neurotoxicant (Nakata *et al*., 2017), alters the secretion of neurotransmitters (Gilbert *et al.,* 2005), causes nerve cell degeneration (Assi *et al*., 2016) and can induce histopathological changes in brain (Hossain *et al.,* 2016).

The use of some plant compounds can be useful in preventing many chronic diseases (Radad *et al*., 2014). Apigenin (APG) is one of natural flavones that is widely distributed in many shrubs, herbs, vegetables and fruits. It possesses antioxidant, anti-inflammatory and anti-apoptotic properties (Adedayo *et al*.,2017). It has been reported that APG plays a role in neurological disorders associated with oxidative stress (Salehi *et al*., 2019) and in inflammation (Tang *et al*., 2017). It was revealed that APG treatment can attenuate oxidative stress through its anti- inflammatory and anti-apoptotic activities (Li *et al.,* 2017).

So, the aim of the present study is to evaluate the protective potential of APG against lead acetate induced alterations in cerebellum of adult male rats. This was done through biochemical and histological assessments.

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MATERIALS AND METHODS

Animals

Twenty-four adult male rats (*Rattus norvegicus*) were used in the present study, each weighting 180-200 gm. They were purchased from the Animal House of Faculty of Science king Faisal University, Saudi Arabia. The rats were housed in plastic cages (6 per cage), floored with soft a wood shaving that was changed three times per week. The animals were acclimatized for 2 weeks prior the study and were maintained under a 12 h light/dark cycle at (25°C±2°C), with free access to water and rat chow. All experimental procedures were

reviewed and approved by the research ethics committee at King Faisal University (Ref. No. KFU-REC/2021-EA000332). They were housed in the animal house of College of Science, Building 9, King Faisal University during the year 2020-2021.

Experimental design

Animals were randomly divided into four groups of six animals per each one as follows:

Group I: served as the control group. Rats were daily injected (*i.p*.) with normal saline (0.9% Na Cl) as vehicle.

Group II: Served as lead acetate-treated group. Rats were daily injected (*i.p*) with lead acetate at a dose of 20 mg/kg b.w. (El Neweshy and EL Sayed, 2011).

Group III: Served as lead acetate and Apigenin-treated group. Rats were daily injected (i.p) with lead acetate at a dose of 20mg/kg (b.w.) followed by injection (*i.p*) with Apigenin (APG) at dose of 20mg/kg b.w. (Venigalla *et al.,* 2015).

Group IV: Served as Apigenin-treated group. Rats were daily injected (*i.p*) with APG at dose of 20mg/kg b.w.

At the end of the four-weeks experimental period, the animals were dissected under light anaesthesia by exsanguination the combination of 10 mg/kg xylazine and 100 mg/kg ketamine HCl (Sontakke *et al.,* 2007). Trunk blood samples and cerebellum tissue were collected for analysis.

Serum preparation

At the end of the experiment, blood sample was collected from rats in clean dry centrifuge tubes, containing no anticoagulation factors were allowed to clot for a minimum of 30 min at 37°C before centrifuged to obtain serum (1500 rpm for 15 min). Then serum was stored at -20°C until the analysis.

Preparation of tissue homogenate

cerebellum tissues were immediately removed and washed using chilled saline solution. These tissues were minced and separately homogenized (10% w/v) in ice-cold sodium-potassium phosphate buffer (0.01 M, pH 7.4) containing 1.15% KCl using a homogenizer (Potter-Elvehjem). The homogenate was centrifuged at 10,000 xg for 20 min at 4°C and the resultant supernatant was used for studied analysis.

Estimation of studied parameters

a) Evaluation of lead level: The concertation of lead was estimated in blood and cerebellum tissue using Atomic Absorption Spectroscopy according to the described method by Khan *et al*. (2006).

b) Biochemical parameters.

Oxidative stress markers

Activities of CAT, SOD, GPx were determined in cerebellar tissues using their relevant commercial kits. The level of MDA as an index of lipid peroxidation was determined through its reaction with thiobarbituric acid (TBA) using the Lipid Peroxide Kit according to the instructions of the supplier. TBARS values were calculated as nmol MDA per g tissue

Inflammatory markers

The cerebellum TNF-α (Human TNF α ELISA Kit, OriGene Technologies Inc., Rockville), IL-6 (Rat Interleukin 6 ELISA Kit, MyBioSource, Inc., San Diego, USA), IL-4 (Rat Interleukin 4 (IL-4) ELISA Kit, MyBioSource, Inc., San Diego, USA) and IL-10 (Rat Interleukin 10 (IL-10) ELISA Kit , MyBioSource, Inc., San Diego, USA) levels were measured using standard kits according to the provided scheme accompanied with the kit.

Neurotransmitters levels

GABA, serotonin (5-HT) and dopamine (DA) levels were determined as described in commercial kits.

The level of GABA was determined as described in the manual of Rat Gamma-aminobutyric acid, GABA ELISA Kit. In principle, the provided microtiter plate has been pre-coated with an antibody specific to GABA. Standards or samples were then added to the microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for GABA. Next, avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. Then a TMB substrate solution was added to each well. Only those wells that contain GABA, biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of GABA in the samples is then determined by comparing the optical density (O.D.) of the samples to the standard curve. GABA level was calculated as mg/ml/g tissue.

Determination of AchE activity

Determination of AchE activity was estimated according to the manufacturer's protocol.

The activity of acetylcholinesterase enzyme was performed using AmpliteTM Colorimetric Acetylcholinesterase Assay Kit according to the instructions of the supplier. Briefly, 20 µL of 50 units/mL acetylcholinesterase standard stock solution was added to 980 µ L of assay buffer to generate 1000 mU/mL standard. 200 µ L was taken of 1000 mU/mL standard to perform 1:3 serial dilutions to get 300, 100, 30, 10, 3, 1 and 0 mU/mL standard acetylcholinesterase solutions. Acetylcholinesterase standards and acetylcholinesterase-containing test samples were added into a 96-well white/clear bottom microplate. 50 µL of acetylthiocholine reaction mixture, containing 250 µL from 20 × DTNB (5.5'-dithio-bis-(2-nitrobenzoic acid), was added to each well of the acetylcholinesterase standard, blank control and test samples to make the total acetylcholinesterase assay volume of 100 µL/ well. The reaction was incubated for 30 min at room temperature and protected from light. The increase in absorbance was monitored at 405 nm with an absorbance microplate reader. The absorbance in blank wells was used as a control and subtracted from the values for those wells with the

acetylcholinesterase reactions. An acetylcholinesterase standard curve was drawn. The acetylcholinesterase activity was reported as mU/mg protein.

Microscopic examination

The cerebellar samples were fixed in 10% neutral formalin solution for 24 h, dehydrated in ascending series of ethanol, cleared in xylene then embedded in paraffin wax. Sections were stained with conventional haematoxylin and eosin (H&E) dye and examined using light microscope (Bancraft and Gamble, 2002).

Statistical analysis

SPSS program was used for the statistical analysis of data with one –way ANOVA to compare the experimental groups. All variables were compared using one-way analysis of variance (ANOVA) followed by LSD multiple range test. Differences at $P<0.05$ were considered as statically significant.

RESULTS AND DISCUSSION

Effect of APG on lead levels in lead treated rats

There was a significant increase in lead level in cerebellar tissues of rats treated with lead indicating the bioaccumulation of lead in these tissues. Treatment with APG caused decrease in lead level (Table 1). These results were also reported (Niu *et al.,* 2017). The ability of APG to reduce Pb level may be due to its ability to chelate metals (Rice-Evans *et al*., 1996).

Effect of APG on oxidative stress in lead treated rats

Cerebellar tissues of rats treated with lead acetate showed significant decrease in studied antioxidant enzyme activities and increase in the MDA levels (Table 2). This was previously reported (Abubakar *et al.,* 2019) and could be one of the direct consequences of Pb toxicity.

These changes were attenuated by the treatment with APG which was previously reported in diabetic rats (El Barky *et al*., 2014). The bioactivity of APG is related to its chemical structure: the hydroxyl groups can combine with free radicals and the C_2-C_3 can stabilize structure (Niu *et al*., 2017).

Effect of APG on inflammatory markers inlead treated rats

Exposure of rats to lead acetate caused increase in the serum levels of proinflammatory cytokines and decrease in levels of anti-inflammatory cytokines. These results agree with that of Adedayo *et al.* (2017).

On the other hand, APG improved these alterations (Table 3). This was previously reported (Zhao *et al*., 2019).

It is well known that chronic neuroinflammation can cause many neurodegenerative diseases explaining the ability of APG as neuroprotective through its anti-inflammatory effect.

Effect of APG on some neurotransmitter levels and acetylcholinesterase in lead treated rats

There was a significant increase in acetylcholinesterase and GABA level but a decrease in the concentrations of serotonin and dopamine (Table 4) in cerebellar tissues of rats treated with lead acetate.

It has been reported that the mechanism of lead effect on neurotransmitter metabolism involves alteration in neurotransmitter storage and release (Lidsky and Schneider *et al.,* 2003). In addition, lead can interfere with the release of neurotransmitters by penetration of the endothelial cells at the blood brain barrier and substitute for calcium ions and be taken up by calcium-ATPase pumps (Toro *et al*., 2008). Also, lead disrupts the activity of a protein localized in the synaptic terminal that appear to be important for transmitter release (Bovton *et al.,* 2001). These changes were markedly attenuated by the treatment with APG. These results are in agreement with previous reports (Yi *et al*., 2008). It was reported that treatment with APG can attenuate these changes.

Effect of APG on cerebellar histology in in lead treated rats

In GI and GIV groups, normal cerebellar cortex appeared (Fig 1A,1B,4A,4B), granular layer contained small neurons, followed by Purkinje layer and molecular layer (Fig 1C,4C).

Table 1: Effect of APG on the lead level in sera and cerebellar tissues of rats treated with lead.

Groups	Serum (µg/ml)	Cerebellum (µg/g tissue)
I	0.14^{c}±0.01	0.16^{c}±0.01
II	5.4^{a}±0.08	4.2^{a}±0.04
III	1.5^{b}±0.06	1.6^{b}±0.01
IV	0.12^{c}±0.01	0.14^{c}±0.01
F (p)	2410.410^{**} (<0.001)	7910.469^{**}(<0.001)

Data are presented as means s±SE. N= six experimental animals per group. Mean values with similar subscripts in the same column are insignificant. **Statistically significant at P≤0.01.

Table 2: Effect of APG on oxidative stress markers in cerebellar tissues of rats treated with lead.

Groups	CAT (U/mg protein)	SOD (U/mg protein)	GP_X (mU/mg protein)	MDA (nmol/g tissue)
I	45.80^{a}±0.25	67.30^{a}±0.08	57.30^{a}±0.08	5.35^{c}±0.12
II	14.39^{c}±0.13	26.45^{c}±0.13	20.45^{c}±0.13	32.39^{a}±0.11
III	35.80^{b}±0.25	47.32^{b}±0.14	37.40^{b}±0.11	13.42^{c}±0.15
IV	47.41^{a}±0.13	63.29^{a}±0.09	53.45^{a}±0.13	5.32^{b}±0.14
F (p)	5733.836**(<0.001)	32099.744**(<0.001)	26157.885**(<0.001)	9248.414**(<0.001)

Data are presented as means s±SE. N= six experimental animals per group. Mean values with similar subscripts in the same column are insignificant. **Statistically significant at P≤0.01

Table 3: Effect of APG on inflammatory response in in cerebellar tissues of rats treated with lead.

Groups	Pro-Inflammatory		Anti-Inflammatory	
	IL-6	TNF-α	IL-4	IL-10
I	58.25^{c}±0.10	51.29^{c}±0.11	43.26^{c}±0.13	40.41^{c}±0.14
II	236.4^{a}±0.17	332.4^{a}±0.12	29.3^{a}±0.11	241.4^{a}±0.13
III	156.06^{b}±0.11	126.26^{b}±0.13	33.28^{b}±0.14	129.06^{c}±0.11
IV	58.3^{c}±0.13	51.5^{c}± 0.18	43.4^{c}±0.13	39.5^{b}±0.13
F (p)	1892432.584**(<0.001)	926443.380**(<0.001)	1434303.680**(<0.001)	1254277.086**(<0.001)

Data are presented as means s±SE. N= six experimental animals per group.
Mean values with similar subscripts in the same column are insignificant. **Statistically significant at P≤0.01.
The inflammatory cytokines are expressed as pg/ mg protein.

Table 4: Effect of APG on some biochemical parameters in cerebellar tissues of rats treated with lead.

Groups	AchE (U/mg protein)	GABA (ng/mg protein)	5-HT (µg/g tissue)	DA (µg/g tissue)
I	33.44^{c}±0.15	32.53^{b}±0.08	231.0^{a}±0.35	231.7^{a}±0.26
II	276.4^{a}±0.12	62.52^{a}±0.10	59.33^{d}±0.12	91.82^{c}±0.25
III	122.5^{b}± 0.28	43.12^{c}±0.30	229.4^{a}±0.20	117.3^{a}±0.12
IV	31.4^{c}± 0.11	33.51^{b}±0.13	96.22^{c}±0.10	232.4^{b}±0.11
F (p)	418872.575**(<0.001)	6530.831**(<0.001)	174485.406**(<0.001)	138824.877**(<0.001)

Data are presented as means s±SE. N= six experimental animals per group.
Mean values with similar subscripts in the same column are insignificant. **Statistically significant at P≤0.01.
AchE; acetylcholine esterase, GABA; γ-amino butyric acid, 5-HT; serotonin, DA; Dopamine.

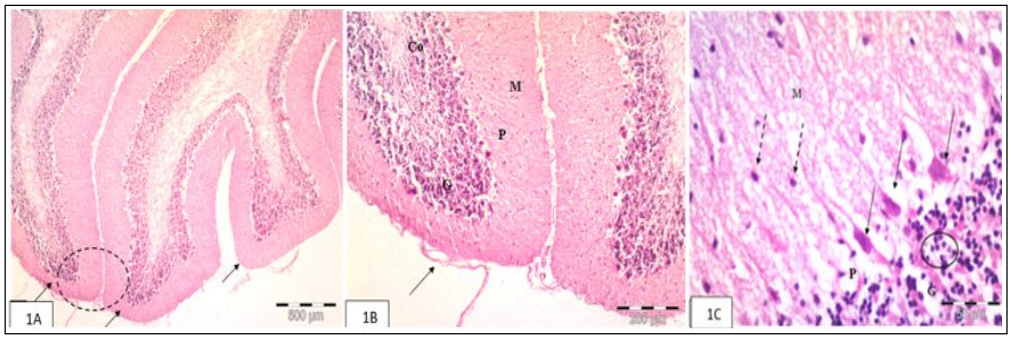

Fig (1a-1c): Light micrographs of control cerebellum cortex section (H&E) illustrating: Fig (1a): folium (arrows) X100. Fig (1b): Enlarged part from the previous figure (dashed-circle) illustrating: central core (Co), granular layer (G) Purkinje layer (P), molecular layer (M) and meninges (arrow)X400. Fig (1c): granular layer (G) with small neurons (circle), Purkinje cell layer (P) with single row of large neurons have single axon (black-arrows) and molecular layer with primarily glial cells (dashed-arrows) X1000.

Fig (2a-2c): Light micrographs of lead – cerebellum cortex section (H&E) illustrating: Fig (2a): narrow folium (arrows) forming continuous thin layer of tissue (arrows) X100. Fig (2b): Enlarged part from the previous figure (dashed-circle) illustrating: central core (Co) followed by wide separation (dashed-circle) between granular layer (G), Purkinje cell layer and molecular layer (M) with high glial cells population X400. Fig (2c): necrotic Purkinje cells with ill-defined arborization (arrows), vacuolated glial cells (dashed-arrows) in lytic molecular layer (circle). Note noticeable space between granular layer and Purkinje cell layer (star) X1000.

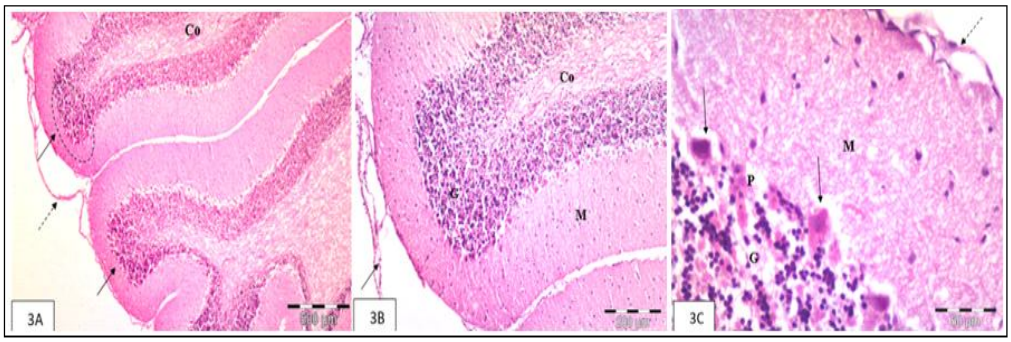

Fig (3a-3c): Light micrographs of lead+Apigenin cerebellum cortex section (HandE) illustrating: Fig (3a): nearly normal structure with broad series of deeply convoluted folds (arrows) supported by branching central core (Co). Meninges (dashed-arrow) X100. Fig (3b): Enlarged part from the previous figure (dashed-circle) illustrating: improvement in both granular layer (G) and the molecular layer (M). Note, meninges (arrow) and central core (Co) X400. Fig (3c): Light micrograph of lead acetate+Apigenin cerebellum cortex section illustrating: granular layer (G) with no separation from Purkinje layer (P), Purkinje cell layer with fairly pear-shaped cell bodies and degenerated dendrites (arrows), molecular layer (M) with few glial cells. Not, manning with simple squamous epithelium (dashed-arrow) X1000.

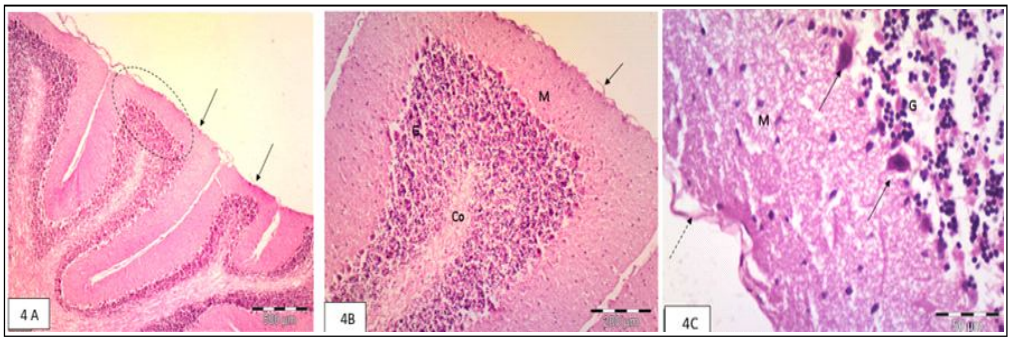

Fig (4a-4c): Light micrographs of Apigenin treated cerebellum section(HandE) illustrating: Fig (4a): folium (arrows) X100. Fig. (4b): Enlarged part from the previous figure (dashed-circle) illustrating: central core (Co), granular layer (G), molecular layer (M) and meninges (arrow) X400. Fig (4c): normal granular layer (G) packed small neurons followed by Purkinje cells with dendrites extended into the molecular layer (arrow) and meninges (dashed-arrow) X1000.

Cerebellum tissue of GII revealed disrupted cerebellar cortex (Fig 2A) with degenerative changes appeared (Fig 2B) and necrotic Purkinje cells (Fig 2C).

GIII demonstrated improvement in some areas in cerebellar cortex (Fig 3A). Improvement in both granular layer and the molecular layer was clear (Fig 3B) but less improvement was recorded in Purkinje layer (Fig 3C). These findings agree with other studies (Isaac *et al*., 2020).

CONCLUSION

The results of this study revealed that lead induced biochemical and structural alterations in cerebellar tissues of intoxicated rats. On the other hand, treatment of these rats with Apigenin attenuates these alterations So, this study suggested that Apigenin might play a neuroprotective role in lead induced neurotoxicity.

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Ethical statement

The experimental protocol of this investigation was approved by Institutional Animal Care and Use Committee (IACUC) at the King Faisal University with Research Ethics Committee number: KFU-REC/2021-03-05.

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Conflict of interest: None.

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