A histological study on the possible ameliorating effect of selenium on chromium (VI) induced neurotoxicity in the adult male Guinea pig cerebellar cortex

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Abstract: Chromium has been considered as a potential environmental and occupational poison and increases neurobehavioral disturbances in humans and experimental animals. The antioxidant has a major role in inhibition of metal induced toxicity. Selenium (Se) has antioxidant action and is considered an essential trace element in humans. Selenium reduces oxidative stress in cerebral ischemia, Parkinson's and Alzheimer's diseases, also prevents many chronic illness as neurodegenerative diseases and specific cancers. This study was aimed to evaluate the possible protective effect of selenium against chromium induced toxicity of the cerebellar cortex of adult male Guinea pig. The guinea pigs were divided into four equal groups (10 guinea pigs each). The control group, the guinea pigs were given PBS 0.5 ml daily for three weeks intraperitoneally. Selenium treated group, the animals were administrated selenium 0.5 mg/kg/d, I.P. Chromium treated group, the guinea pigs were given potassium dichromate 60 µ/kg/d, I.P. Selenium-chromium treated group, the guinea pigs were given selenium and potassium dichromate in the same route and doses for three weeks. The four groups were subjected to histological and immunohistochemical studies. Chromium administration showed a highly significant decrease in the Purkinje cells number with prominent histological changes in the molecular, granular and Purkinje cell layers. Immunohistochemical results revealed a highly significant increase in the GFAP positive astrocytes number. Also, an apparent increase in the apoptotic cells number was observed. These histological and immunohistochemical changes were ameliorated by supplementation of selenium.

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Key words: Cerebellum, Chromium, Selenium, Caspase-3

1. Introduction

Chromium has been considered as a potential environmental and occupational poison. There are two forms of chromium, trivalent chromium Cr (III) which cross cell membrane poorly and hexavalent chromium Cr (VI) which can readily cross cell membranes. Hexavalent chromium have been classified as human carcinogens by the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) (Codd et al., 2001). Cr (VI) compounds such as chromic acid, potassium dichromate ($K_2Cr_2O_7$) and sodium chromate are widely used in plating, steel, leather, welding, paint and dye producing industries (Leonard et al., 2004).

Hexavalent chromium could cause acute and chronic toxicity, immunotoxicity, genotoxicity, neurotoxicity and dermatotoxicity (Von and Liu, 1993). It has been found that Cr (VI) induced toxicity is mainly due to excess reactive oxygen species (ROS) production. However, a detailed mechanism of tumorigenesis and malignant transformation remains unknown (Wang et al., 2011). Human is exposed to Cr (VI) through oral ingestion of contaminated water, inhalation or transdermally (Park et al., 2004).

In the cell, hexavalent chromium [Cr (VI)] can be reduced to Cr (III), which combines with macromolecules. This reduction process produces reactive oxygen species which is an important factor of hexavalent chromium induced toxicity and causes damage to many organs as kidney, liver, pancrease and cerebellum (Bagchi et al., 2002). In addition, chronic Cr (VI) exposure has been reported to increase lipid peroxidation and decrease glutathione (Thompson et al., 2011).

The antioxidant has a major role in inhibition of metal induced toxicity. Selenium has antioxidant action and is considered an essential trace element in humans (Agar et al., 2013). Dietary selenium is present in many foods like mushrooms, cereals and nuts (Barclay et al., 1995), it is present in many multivitamins and other dietary supplements including infant formula (FDA, 2015).

Selenium is important for the proper function of enzymes group called glutathione peroxidase. These enzymes play an important role in detoxification system of the body and give protection against oxidative stress (Freeth et al., 2012).

Selenium decreases oxidative stress in cerebral ischemia, Parkinson's and Alzheimer's diseases, also prevents many chronic illness as neurodegenerative diseases and specific cancers (Choi et al., 2008).

The aim of the present study is to evaluate the possible protective effect of selenium against chromium induced toxicity of the cerebellar cortex of guinea pig.

2. Material and Methods

Chemicals

- Potassium dichromate ($K_2Cr_2O_7$) was purchased from Merck (Darmstadt, Germany). It was dissolved in PBS.

- Selenium: 50µg tablets were obtained from Holland & Barrett. It was dissolved in PBS.

Animals

Forty adult male Guinea pigs of average weight 400-450 grams were used in this study. The animals were obtained from the breeding animal house, Faculty of Medicine, Menoufia University, the animals were kept in a healthy standard environmental conditions and fed with basal diet and tap water.

Experimental protocol

The experimental protocol were accepted by the Ethical Committee of Menoufia University. The guinea pigs were divided into four equal groups included 10 animals for each as follow.

Group I (control group): The animals of this group were given PBS 0.5 ml /d intraperitoneally for three weeks.

Group II (Selenium treated group): The guinea pigs were given selenium 0.5 mg/kg/d dissolved in 0.5 ml PBS intraperitoneally for three weeks (Hassanin *et al.*, 2013).

Group III (Chromium treated group): The guinea pigs were given potassium dichromate 60 µ /kg dissolved in 0.5 ml PBS daily, intraperitoneally, for three weeks (Qureshi and Mahmood, 2010).

Group IV (Selenium-chromium treated group): The guinea pigs were given selenium one hour before administration of potassium dichromate in the same route and doses described above**.**

The animals were scarified by cervical decapitation, 24h after the last dose of the treatment. The cerebellum was dissected out and cleaned by normal saline, from each group. Specimens of the right cerebellar cortex were excised and immersed in formal saline. Then, the cerebellar tissues were subjected to the following studies.

I. Histological study:

H&E

Paraffin sections of about 5-6 µm thickness were obtained and stained with haematoxylin and eosin (Hx&E) (Bancroft and Layton, 2010) to show the general architecture of the cerebellar cortex.

Toluidine blue

Dry slides were submerged in TB solution 0.1% at room temperature before being rinsed well with distilled water. The slides were put in the dark at room temperature and allowed to dry for 24 h. They were then dehydrated for 2 min in 100% ethanol, cleared with xylene for 5 min and covered with paramount (Fisher Scientific Co., Ottawa, Ontario, Canada) ((Bancroft and Gamble, 2013).

II-Immunohistochemical study:

Caspase-3 immunostaining:

Sections were subjected to staining with the primary rabbit polyclonal anti-caspase-3 antibody (Thermo Scientific, Lab Vision, USA) (Lee et al., 2006).

Glial fibrillary acidic protein immunostaining

Serial paraffin sections were deparaffinised and dehydrated, including positive control sections from the cerebellum. The activity of endogenous peroxidase was blocked with 0.05% hydrogen peroxide in absolute alcohol for 45 min. The slides were placed in phosphate buffered saline (PBS) for 7 min to unmask the antigenic sites, sections were put in citrate buffer for 10 min in a microwave. The slides were incubated in bovine serum albumin dissolved for 30 min in PBS to prevent nonspecific background staining. Then, the primary antibodies were applied to the sections (except for the negative control) and then incubated for 90 min at room temperature. GFAP was put to the sections. The slides were rinsed with PBS and then incubated for 60 min with anti-mouse immunoglobulin's, conjugated to a peroxidase-labelled dextran polymer (Bancroft and Gamble, 2013):

Morphometric study

Hx and eosin stained sections of the cerebellar cortex from each group were examined under light microscopy at high power field. The number of Purkinje cells was counted in 10 HPFs in each specimen. Immunostained sections were also used for counting the astrocyte cells (McGuinness, 2000).

Statistical analysis

The data were expressed as mean ±SD. The student t-test was used to evaluate the significant change in each parameter in the experimental groups when compared with the control group. The statistical analysis of data was carried out using Excel and statistical package for the social science software, version 11. The significance was set at P- value less than 0.05 (Peat and Barton, 2005).

3. Results

Histological results

H&E

The cerebellum of the control group consisted of molecular layer, Purkinje cell layer, granular layer and a central area of white matter (Fig. 1). The molecular layer was formed of few nerve cells and numerous nerve fibers. There were two types of nerve cells, the outer stellate cells and the inner basket cells (Fig. 2).

The Purkinje cell layer consisted of large pyriform cells arranged in one row along the outer margin of the granular layer. Each Purkinje cell had rounded vesicular nucleus with prominent nucleolus. The granular layer consisted mainly of closely packed deeply stained cells called granule cells which had small rounded nuclei and scanty cytoplasm (Figs. 2&3).

Examination of section from the cerebellar cortex of selenium treated group (group II) revealed an appearance more or less similar to the control group.

Chromium administration induced degenerative changes in the cerebellar cortex. The Purkinje cells exhibited disorganization in their arrangement and they were surrounded by spaces (Fig. 4). They exhibited reduction in number and appeared shrunken with darkly stained cytoplasm and deeply stained nuclei (Figs. 4, 5& 6). Perineuronal spaces containing degenerated cells in the molecular layer were observed (Fig. 4). The cells in the molecular layer appeared small in size and deeply stained (Fig 5). In the granular layer, the granule cells appeared small in size, deeply stained cytoplasm and became clumped together in groups (Figs. 5&6) and separated by wide spaces (Fig. 6).

Hx & E stained sections of selenium-chromium treated group revealed an appearance similar to control group (Figs. 7&8). The Purkinje cells were slightly reduced in number (Fig.7).

Toluidine blue stain

The cerebellar cortex of the control group exhibited normal viable neurons. The viable Purkinje cells appeared with rounded vesicular nuclei and prominent nucleolus, the cytoplasm contains dark Nissl's granules, the granule cells appear with lightly stained cytoplasm (Fig. 9). While, several dark pyknotic neurons stained with toluidine blue were seen in chromium treated guinea pigs. The Purkinje cells appeared with darkly stained cytoplasm. Some cells in the molecular layer were deeply stained whereas the granule cells became clumped in groups and deeply stained (Fig. 10). However, administration of selenium with chromium showed more viable neurons, nearly normal appearance of the molecular, granular and Purkinje cell layers compared with control group (Fig. 11).

Immunohistochemical results

Neuronal death detection with caspase-3

Section of the cerebellar cortex from the control group exhibited negative cytoplasmic immunoreactivity for caspase-3 in the molecular, granular and Purkinje cells layers (Fig. 12). Strong cytoplasmic immunoreactivity for caspase-3 in the Purkinje cells, some granule cells and in some cells in the molecular layer were detected in the cerebellar cortex of group III treated with chromium (Fig. 13). While section from the cerebellar cortex of the selenium-chromium treated group showed more viable neurons, nearly similar to the control group (Fig. 14).

Glial fibrillary acidic protein for detection of astrocyte

The cerebellar cortex of the control group (group I) stained with GFAP for astrocytes showed positive cytoplasmic reaction in the granular layer (Fig. 15). In the cerebellar cortex of the chromium treated group, there was marked increase in the positive cells in the granular layer, compared with the control group (Fig. 16). While, there was positive cells in the granular layer of the cerebellar cortex of the selenium-chromium treated group as control group (Fig. 17).

Morphometric study and statistical results

Table: 1 Comparison between control group and other studied groups as regards Purkinje cell and astrocyte count

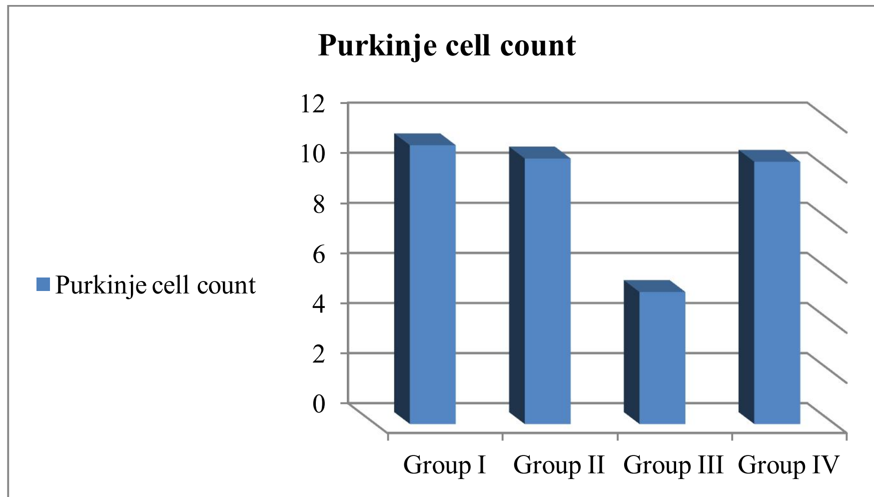
	Group I (Control group)	Group II (Selenium treated group)	Group III (Chromium treated group)	Group IV (Selenium and Chromium treated group)	t-test	P. value
Purkinje cell	11.13±0.99	10.60±1.06	5.27±0.96	10.47±0.83	122.3	0.00 P1 = 0.135 P2 = 0.00 P3 = 0.063
Astrocytes	22.73±1.49	22.33±1.50	41.20±2.24	21.50±1.65	441.3	0.00 P1 = 0.135 P2 = 0.00 P3 = 0.06

P1: Group II compared with Group I P2: Group III compared with Group I P3: Group IV compared with Group I $P<0.05$ means significant. $P<0.01$ means highly significant. $P>0.05$ means NS.

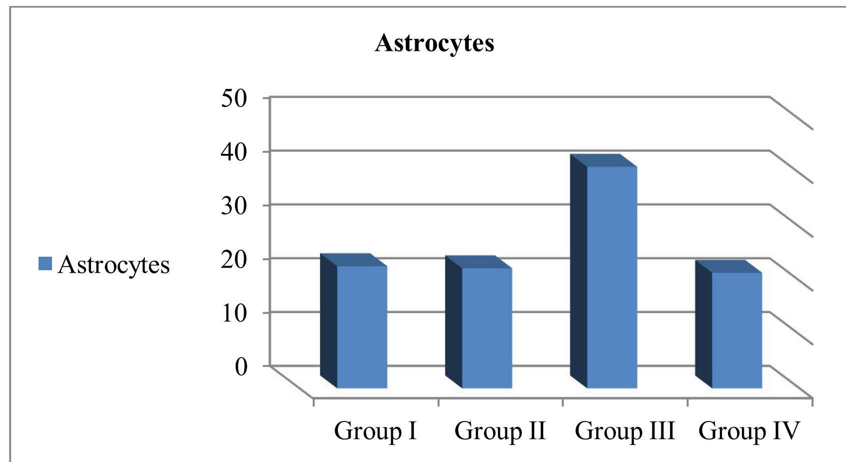
There was no significant difference in the mean number of Purkinje cells between selenium treated group (group II) and control group. In chromium treated group, the number of purkinje cells showed highly significant decrease. In selenium-chromium treated group, the mean number increased and showed no significant difference compared with the control group (Table 1 and Histogram 1).

GFAP positive astrocytes number showed highly significant increase in chromium treated group compared with the control group. Guinea pigs treated with selenium or with selenium and chromium

revealed no significant differences compared with the control group (Table 1 and Histogram 2).



Histogram 1. Mean number of Purkinje cell in different groups. HPF, high power field.



Histogram 2. Mean number of astrocytes in different groups. HPF, high power field.

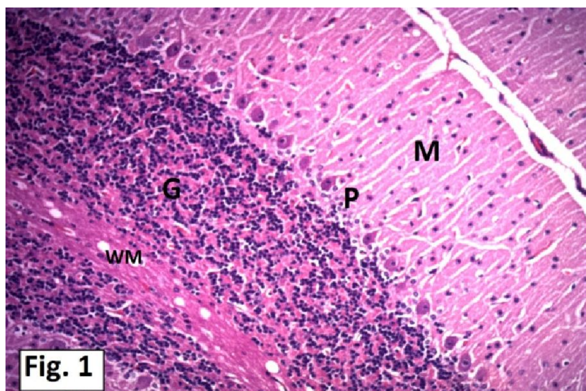


Fig. 1: A photomicrograph of the cerebellum of control group (group I) showing a molecular layer (M), Purkinje cell layer (P), granular layer (G) and a central area of white matter (WM). Hx&E X 200

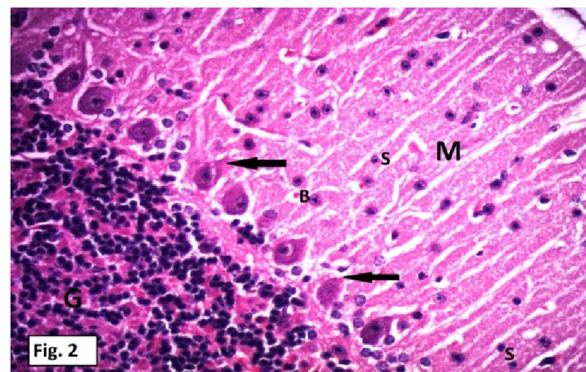


Fig. 2: A photomicrograph of the cerebellar cortex of control group (group I) showing the outer molecular layer (M) which contains stellate cells (S), basket cells (B) and numerous nerve fibers. Middle Purkinje cell layer (P) which consists of one layer of large pyriform cells (arrows) and inner granular layer (G) which is composed of tightly packed small cells with darkly stained nuclei. Hx&E X400

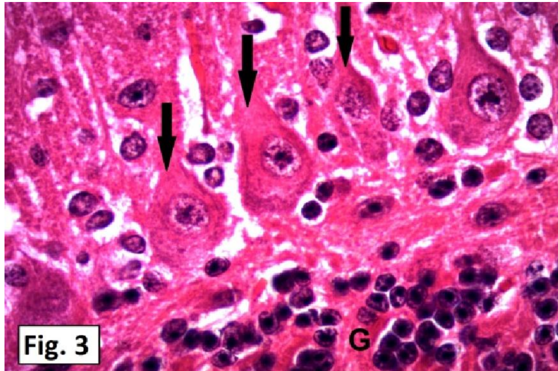


Fig. 3: A photomicrograph of the cerebellar cortex of control group (group I) showing purkinje cells (arrows) having rounded vesicular nuclei with prominent deeply stained nucleolus. The granular layer (G) consists mainly of numerous small granule cells with deeply stained nuclei and scanty cytoplasm. Hx&E X100

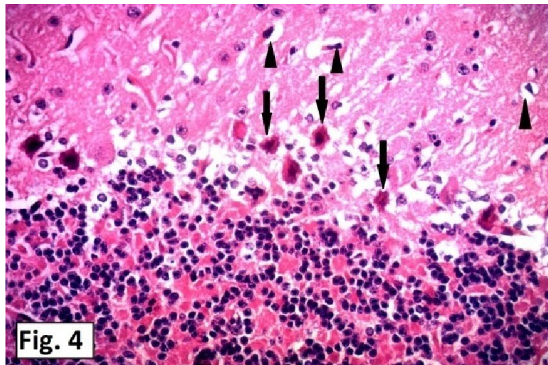


Fig.4: A photomicrograph of the cerebellar cortex of chromium treated group (group III) displaying small shrinked purkinje cells (arrows) which are disorganized in arrangement and are surrounded by spaces. Perineuronal spaces containing degenerated cells are also seen in the molecular layer (arrow heads). Hx&E X400

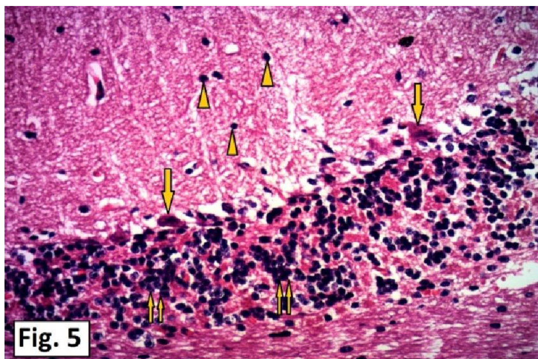


Fig. 5: A photomicrograph of the cerebellar cortex of chromium treated group (group III) showing purkinje cells which appear shrunken with deeply stained nuclei (arrows) and apparent decrease in number. The granule cells appear small in size and deeply stained and become clumped together in groups (double arrows). The cells in the molecular layer appear small in size and deeply stained (arrow heads). Hx&E X 400

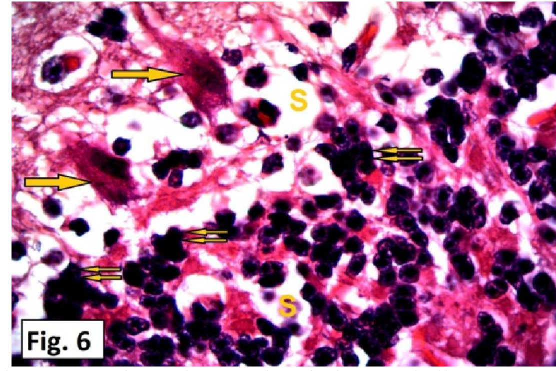


Fig. 6: A photomicrograph of the cerebellar cortex of chromium treated group (group III) showing purkinje cells (arrows) with darkly stained nuclei and darkly stained cytoplasm. The granule cells appear with deeply stained nuclei and become clumped in groups (double arrows). Wide spaces between cells are observed (S). Hx&E X 1000

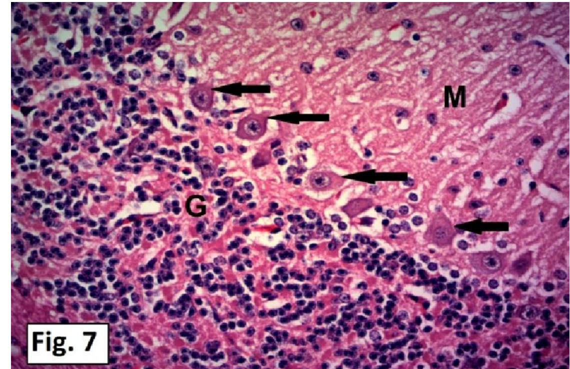


Fig. 7: A photomicrograph of the cerebellar cortex of selenium-chromium treated group (group IV) showing purkinje cells (arrows) which remain regularly spaced, retain normal size and morphology with apparent slight decrease in number compared with the control group.. The molecular (M) and granular (G) layers appear nearly normal. Hx&E X 400

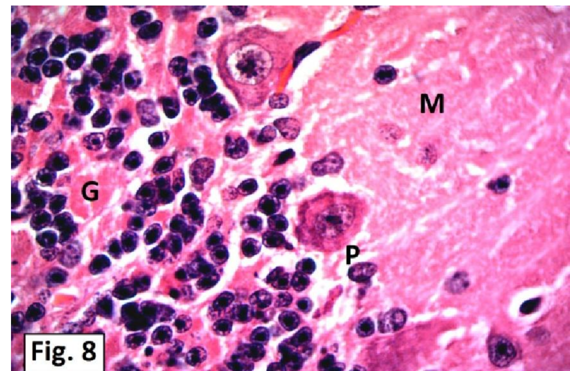


Fig. 8: A photomicrograph of the cerebellar cortex of selenium-chromium treated group (group IV) showing molecular (M), granular (G) and Purkinje cell layers (p) having an appearance similar to control group. Hx&E X 1000

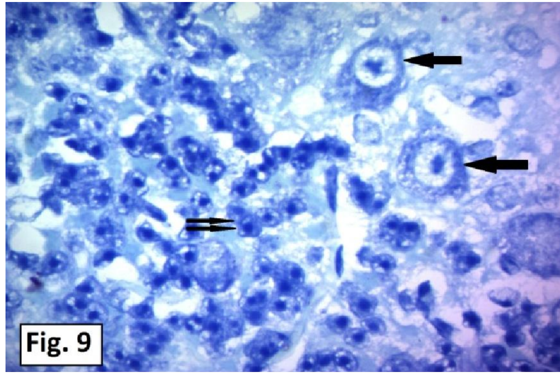


Fig. 9: A photomicrograph of the cerebellar cortex of control group (group I) showing viable purkinje cells (arrows) with rounded vesicular nuclei and prominent nucleolus. The cytoplasm contains Nissl's granules. The granule cells appear with lightly stained cytoplasm (double arrows). **Toluidine blue X 1000**

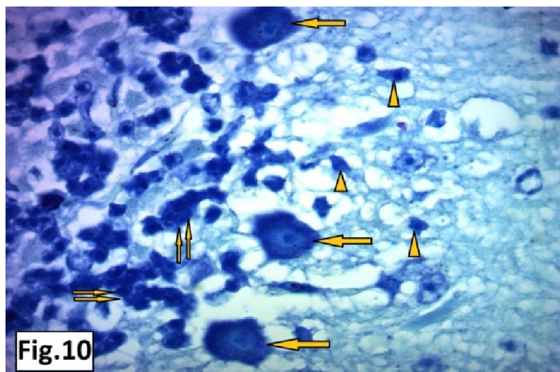


Fig.10: A photomicrograph of the cerebellar cortex of chromium treated group (group III) showing Shrinked Purkinje cells with darkly stained cytoplasm (arrows). Some cells in the molecular layer are deeply stained (arrowheads) whereas the granule cells become clumped in groups and deeply stained (double arrows). Toluidine blue X 1000

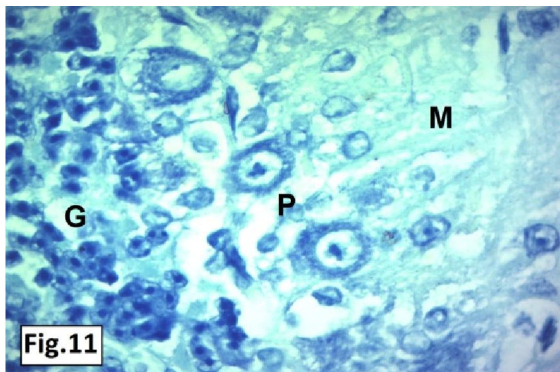


Fig.11: A photomicrograph of the cerebellar cortex of selenium-chromium treated group (group IV) showing nearly normal appearance of the molecular (M), granular (G) and Purkinje cell layers (P) compared with the control group. Toluidine blue X 1000

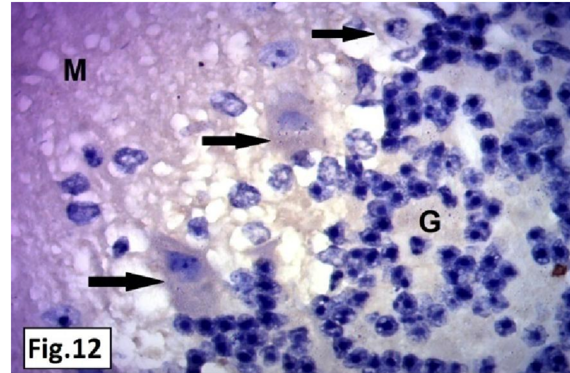


Fig.12: A photomicrograph of the cerebellar cortex of control group (group I) showing negative cytoplasmic immunoreactivity for caspase-3 the purkinje cells (arrows), molecular layer (M) and granular layers (G). Caspase-3 X1000

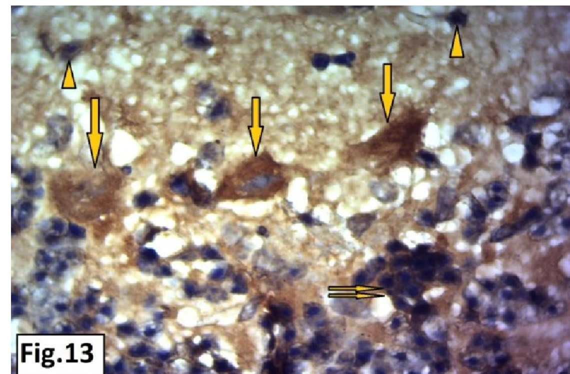


Fig.13: A photomicrograph of the cerebellar cortex of chromium treated group (group III) showing strong cytoplasmic immunoreactivity for caspase-3 in the Purkinje cells (arrows) as well as some granule cells (double arrows) in the granular layer and some cells in the molecular layer (arrow heads). Caspas-3 X1000

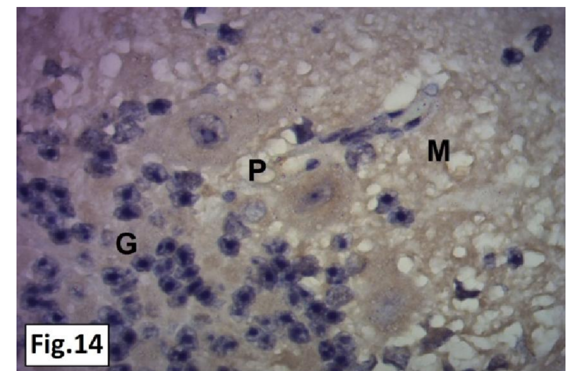


Fig. 14: A photomicrograph of the cerebellar cortex of selenium-chromium treated group (group IV) showing negative cytoplasmic immunoreactivity for caspase 3 in the molecular (M), granular (G) and purkinje cell layers (P) Caspase-3 X1000

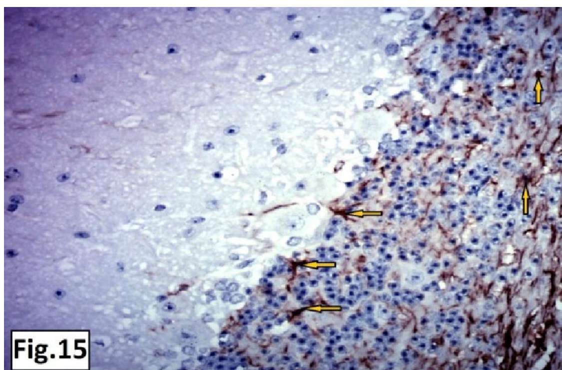


Fig. 15: A photomicrograph of the cerebellar cortex of control group (group I) showing positive astrocyte (arrows) reactivity in the granular layer on using GFAP immunostaining. Glial fibrillary acidic protein immunostaining X400

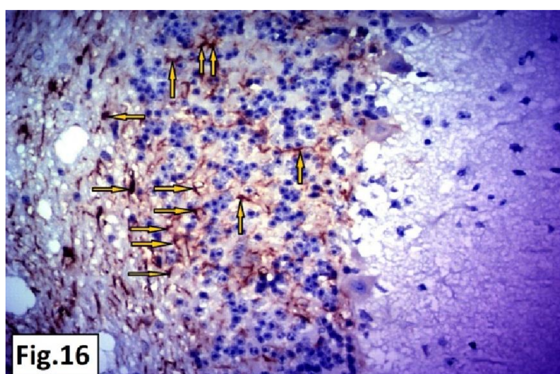


Fig. 16: A photomicrograph of the cerebellar cortex of chromium treated group (group III) showing strong positive astrocyte (arrows) reactivity and increase in astrocyte number in the granular layer on GFAP immunostaining. Glial fibrillary acidic protein immunostaining X400

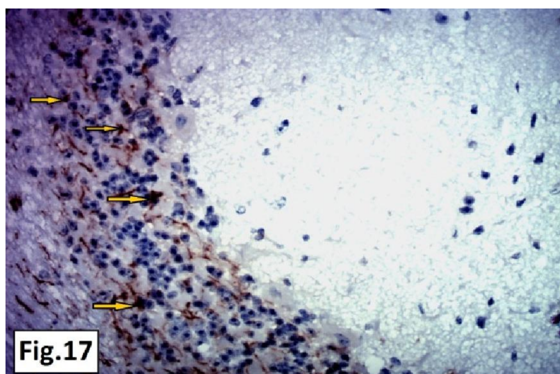


Fig. 17: A photomicrograph of the cerebellar cortex of selenium-chromium treated group (group IV) showing positive astrocyte (arrows) reactivity in the granular layer on using GFAP immunostaining. Glial fibrillary acidic protein immunostaining X400

4. Discussion

Chromium has been considered as a potential environmental and occupational poison and increases neurobehavioral disturbances in experimental animals and humans (Codd et al., 2001). In the present study, chromium supplementation led to histological alterations in the cerebellar cortex. There was a highly significant decrease in the Purkinje cells number, it appeared with dark stained nuclei and dark stained cytoplasm. There was disturbance in the arrangement of the Purkinje cells. Similar results have been reported by others after treatment with metals (El-Neweshy and El-Sayed, 2010). Soudani et al., 2012 reported that, the most common damage in the cerebellar cortex in animals treated with potassium dichromate was located in the Purkinje cell layer, there were a reduction of Purkinje cell number and disorganization in their arrangement, there was also oedema around Purkinje cell. The brain consumes 20% of the body's oxygen and has a relatively poor antioxidant defense system. So the brain is susceptible to lipid peroxidation and free radical attack as reported by previous studies (Bagchi et al., 2001). Chromium induced cerebellar toxicity by increasing cellular oxidative stress and decreasing the activity of antioxidants (Thompson et al., 2011). Previous studies have reported that chromium cytotoxicity is due to induction of oxidative stress by enhanced ROS production [22]. Once Cr (VI) enters the body, it can efficiently penetrate cellular membranes through anionic channels. In the cell, it can be reduced by cellular reductant like glutathione to produce intermediates such as Cr V and Cr IV and Cr III (Travacio et al., 2000). The intracellular reduction of hexavalent chromium produces ROS which could interact with cellular component and cause cell damage.

The conversion of Cr (VI) to Cr (III) occurs through glutathione which act as an electron donor. The sulfhydryl group of cysteine of glutathione has a high affinity for metals. Also, GSH may be oxidized due to the interaction with the free radicals induced by K2Cr2O2. These several pathways have been proposed to explain the GSH level depletion in chromium induced toxicity (Hojo and Satomi 1991). It was reported that glutathione depleted cells are resistant to Cr (VI) toxicity (Pourahmad and O'Brien, 2001).

In the present study, prominent perineuronal spaces around basket and stellate cells were observed. These cells appear with dark cytoplasm and dark nucleus. These results found by Buttermore et al., 2012 who reported that basket axon collaterals synapse with the Purkinje soma and form specialized structure called the pinceau, which are responsible for cerebellar function. Loss of purkinje neurons leads to disorganization of the pinceau morphology, this may explain vacuolation around basket cells. Excessive

production of ROS leads to oxidative stress, which contributes to tissue damage (De Groot et al., 1999).

In the present study, the granule cells appeared small in size, deeply stained cytoplasm and became clumped together in groups and separated by wide spaces. These findings are in agreement with the results of Abolfazl et al., 2014 who found that the mature cerebellar granule neurons are more sensitive to potassium dichromate toxicity than immature neurons, the toxicity of potassium dichromate in these cells is concentration dependent but not time dependent, exposure to toxic concentration of Cr (VI) produced ROS in both mature and immature neurons.

In addition, lipid peroxidation was observed in neurons that exposed to toxic concentration of Cr (VI) (Patlolla et al., 2009).

The metals deactivate acetyl cholinesterase (AChE) enzyme that is important for cholinergic neurotransmission and neurobehavioral, thus inhibiting acetylcholine from binding to acetyl cholinesterase and degradation (Guilhermino et al., 1998). There are some studies reporting interactions between the Ach system and a variety of heavy metals. The wide spaces between the cells could be due to degeneration of these cells, leaving empty spaces.

In the present study, Immunohistochemical stains for caspase-3 in chromium treated guinea pigs revealed strong cytoplasmic immunoreactivity in the purkinje cells and granule cells and some cells in the molecular layer. Some studies reported that soluble hexavalent chromium is metabolized inside the cells by reductive agents such as glutathione and ascorbic acid, and genetic lesions are produced, Cr damage produce physical barriers to DNA replication/transcription and promote a terminal cell apoptosis or growth arrest (Quinteros et al., 2008).

In this study, prominent increase in astrocytes was observed after chromium exposure. This could reflect a compensatory mechanism in neurodegeneration. Some studies have found an increased GFAP content in different regions of the brain tissue mainly in the cerebellar cortex after metal exposure. Gliosis that develops in metal exposure could be caused by free radicals generation (Gonzalez et al., 2007).

The antioxidant in diet has an important role in inhibition of metal induced toxicity. Selenium is an essential element in humans and has antioxidant action (Agar et al., 2013). In the present study, co-administration of selenium with chromium decreased the degenerative changes and restored the cerebellum integrity. There was a nearly normal appearance of the molecular, granular and Purkinje cell layers.

The protective effects of selenium against neurotoxicity induced by metals are well reported, it is known that selenium binds to the metal by a simple quenching reaction (Farina et al., 2003).

Selenium supplementation could reduce lipid peroxidation level in rats during Parkinson's and after cerebral ischemia, and could prevent hydroxyl radical formation (Lix et al., 2001).

Selenium may be a therapeutic regenerative material that scavenges ROS formed on Cr (VI) exposure (Mohamed et al., 2016).

The most important roles of selenium are related to its function on many antioxidant enzymes active sites, like glutathione peroxidase (Flora et al., 2002).

Selenium could reduce the destructive oxidative stress caused by toxicants (Parveen et al., 2014). It was reported that selenium could prevent aflatoxicosis induced apoptosis by inhibiting mitochondrial pathway (Yu et al., 2015). Selenium administration could improve the activities of glutathione peroxidase, enhance mechanisms of selenium-dependent and selenium –independent ROS scavenging and restore their antioxidative capacity (Kassem and Jakob, 2006).

Selenium supplementation restored cholinesterase activity in chromium treated rats. The selenoenzymes like thioredoxin reductase have role in increasing the ability of selenium to protect against cancer (Irons et al., 2006). Selenium plays an important role in preventing thyroid toxicity and has a protective effect against heart, liver and kidney damage induced by mercury chloride (El-Shenawy and Hassan, 2008).

Conclusion

It is concluded that chromium has deleterious morphological, histological and immunohistochemical effects on the cerebellum. Such hazardous effects could be ameliorated with concomitant administration of selenium.

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