Original Article

The Possible Protective Role of Selenium Against Long-Term Phenytoin Administration Effects on Cerebellar Cortex of Adult Male Albino Rats: Histological and Immunohistochemical Study

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ABSTRACT

Background: Phenytoin is a commonly prescribed antiepileptic drug. Chronic phenytoin treatment has been documented to be associated with cerebellar degeneration. Selenium is one of the main antioxidant coenzymes of glutathione peroxidase and it is a key component of seleno-proteins that play important roles in brain development and metabolism.

Aim: This work aimed to study the possible protective role of selenium against long-term phenytoin administration effects on cerebellar cortex of the adult male albino rats.

Material and Methods: Forty-five adult male albino rats were used in this study, aged from 6-8 months, weighting 180 -200 gm. Rats were equally divided into three groups: Group I: rats were further equally subdivided into: group IA: kept as negative control, group IB: each rat received 1ml of distilled water /day orally for 45 days and group IC: each rat received selenium at a dose of 0.5 mg/kg BW(body weight)/day orally for 45 days. Group II: each rat received phenytoin at a dose of 20mg/ Kg BW /day orally for 45 days. Group III: each rat received phenytoin as group II in addition to selenium at a dose of 0.5 mg/kg BW/day orally for 45 days.

Results: The present work revealed that long term phenytoin administration induced histological changes of rat's cerebellar cortex in the form of decreased thickness, irregular deeply stained Purkinje cells and increased perineural space around Purkinje cells and granule cells. Selenium administration with phenytoin nearly preserved the histological structure of the cerebellar cortex.

Conclusion: Long term phenytoin administration led to histoarchitectural changes of rat's cerebellar cortex. However, Selenium administration with phenytoin greatly protected the cerebellar cortex from such changes.

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Key Words: Cerebellar cortex, phenytoin, selenium.

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INTRODUCTION

Epilepsy is a disorder that affect up to1% of humans[1], the main target in its treatment is to attain a strict control over seizures to improve the quality of life[2].

Phenytoin is a commonly prescribed antiepileptic drug since 1938, in generalized seizures, partial seizures and in status epilepticus. It has a narrow therapeutic index that needs clinician's caution to avoid the high possibility of toxicity[2,3]. It acts by blockage of neuronal sodium channels which leads to increase the membrane threshold for depolarization and subsequently decreasing the neuronal susceptibility to epileptogenic stimuli[4]. Patients with phenytoin intoxication may experience nystagmus, ataxia, dysarthria and cognitive difficulties, chronic phenytoin treatment has been documented to be associated with cerebellar degeneration[5].

Selenium is a trace element which has many functions, it is an essential micronutrient for the brain function, and it is a key component of seleno-proteins that play important roles in brain development and metabolism[6]. Selenium is one of the main antioxidant coenzymes of glutathione peroxidase[7].

Previous studies proved the protective effect of selenium on many tissues against toxic compounds and oxidative stress[8-10]. Selenium depletion was an important triggering factor of intractable seizures and subsequent neuronal damage amongst patients with epilepsy[11].

There was a controversy whether cerebellar dysfunction and atrophy that occurs with phenytoin therapy is due to the drug itself or a consequence of the underlying epilepsy[12]. Selenium showed neuroprotective effects against many central neurotoxic drugs[13]. Therefore, the present work aimed to study the possible protective role of selenium against long-term phenytoin administration effects on rat's cerebellar cortex.

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MATERIALS AND METHODS

Chemicals

- Phenytoin was purchased in the form of capsules (Pfizer Company, Egypt). Each capsule contained 100mg of phenytoin that was dissolved in 10 of distilled water.
- Selenium was purchased in the form of 5gm powder (Sigma Aldrich, Egypt). The calculated dose for each rat was dissolved in 1ml of distilled water.

Animals

Forty-five adult male albino rats were used in this study, aged from 6-8 months, weighting 180 -200 gm, they were obtained and locally bred at the animal house of the medical research center of Faculty of Medicine, Ain-Shams University. Rats were housed in medium sized metal cages in a room temperature with regular dark/light cycles with good ventilation. Free diet and water access were allowed, and all rats were kept under the same circumstances throughout the experiment. the experiment followed the guidelines of Ain Shams University Ethics Committee.

Experimental Protocol

Rats were Equally Divided into Three Groups

Group I (Control): included fifteen rats that were further equally subdivided into: group IA (negative control): included five rats that were kept as negative control, group IB (vehicle control): contained five rats, each rat received 1ml of distilled water /day orally by gastric tube for 45 days and group IC (Selenium control): contained five rats that received selenium at a dose of 0.5 mg/kg BW(body weight)/day orally by gastric tube for 45 days.

Group II (Phenytoin): included fifteen rats, each rat received phenytoin at a dose of 20mg/ Kg BW/day orally by gastric tube for 45 days[14].

Group III (Phenytoin+ Selenium): contained fifteen rats, each rat received phenytoin as group II in addition to selenium at a dose of 0.5 mg/kg BW/day[15] orally by gastric tube for 45 days.

Processing of Samples

Paraffin Blocks Preparation and Staining Methods

Cerebellar specimens (Right hemispheres) were fixed in 10% buffered formalin, processed and embedded in paraffin blocks, sectioned (parasagittal) at 5µm, cut and stained by Hematoxylin and Eosin (Hx. & E.)[16].

For immunohistochemical studies for Glial fibrillary acidic protein (GFAP) for glial cells. deparaffinized paraffin sections 5 µm thickness were hydrated and peroxidase activity was blocked with 10% hydrogen peroxide. The sections were placed in 0.01 mol/l citrate buffer for 10 min. slides then were incubated with the primary antibody (1: 500 monoclonal mouse anti-GFAP (Dako Carpenteria, Ca, USA) at 4 C. for 20hrs. Then incubated with biotinylated secondary antibodies (ABC kit, 1: 200), then with avidin-biotin complex. Finally, sections were developed with 0.05% diaminobenzidine and counterstained with Mayer's hematoxylin[17].

For immunohistochemical study for caspase-3 as an indicator of apoptosis, sections were washed in phosphate-buffered saline for 5 mins and then incubated with antibody to cleaved Caspase-3 at a dilution of 1:200 (Invitrogen, Sweden AB Stockholm Sweden) overnight at 4°C. Then they were washed and incubated with (1:500) secondary anti mouse antibody (Invitrogen, Molecular Probes, Eugene, Oregon, USA) for 1h in room temperature. Slides were then incubated in 3,3-diaminobenzidene for 10 min and counterstained by Mayer's hematoxylin[18].

Epon Blocks Preparation for Semithin Sections and Staining Method

Cerebellar specimens (Right hemispheres) were fixed immediately in 2.5 % glutaraldehyde in phosphate buffer for 3 hours. Fixed tissue samples were washed with phosphate buffer and post fixed in 1 % osmium tetra oxide. After dehydration in ascending grades of alcohol and embedding in epon, semithin sections one um thick were cut using L.K.B. ultra-microtome, picked up on a gelatinized glass slides and stained with toluidine blue[19].

Stained paraffin and semithin sections were examined and photographed using light microscope (Olympus 268M microscope) equipped with an automatic photomicrographic camera system.

Image Analysis

Morphometric analysis was carried out on routine Hx. & E. stained slides using image analyzer Leica Q win V.3 program installed on a computer in the Histology Department, Faculty of Medicine, Ain Shams University. The computer was connected to a Leica DM2500 microscope (Wetzlar, Germany). Six randomly chosen fields in six sections obtained from six different animals from the same group were used for measuring the number of Purkinje cells/field and the thickness of the cerebellar cortex. Pixels were calibrated for actual measurements in micrometer. The magnification used was 400X with an objective lens of 40X for counting Purkinje cells number and 100X with an objective lens of 10X for the thickness of the cerebellar cortex.

Statistical Analysis

Data analysis was performed using PSPP freeware with one-way ANOVA and Bonferroni Post Hoc test to detect the significance between every two groups. Results were considered highly significant when $P\ value \leq 0.001$, significant when $P\ value \leq 0.05$ and nonsignificant when $P\ value > 0.05$.

RESULTS

Histological Results

Groups I (Control)

Light microscopic examination of Hx. & E. stained sections of cerebellar cortex of the control subgroups IA, IB and IC showed almost the same regular structure of the cerebellar cortex, it was formed of three layers, outer molecular layer, middle Purkinje cell layer and inner granular layer. The molecular layer was formed of fibers and cells (outer stellate and inner basket cells). Purkinje cells were large pyriform cells arranged in one row and having vesicular nuclei with prominent nucleoli. The granular layer was formed of small deeply stained cells and in-between the cells there were noncellular areas representing the cerebellar glomerulus (Figures 1,2). In semithin sections stained with toluidine blue, Purkinje cell appeared to have large infolded vesicular nucleus with prominent nucleolus. The granule cells were small cells with granular chromatin (Figure 3). Immunohistochemically stained sections for GFAP showed positive immune reaction of scattered small astrocytes with thin processes (Figure 4). While, caspase-3 immunohistochemically stained sections revealed negative cytoplasmic immunoreaction of Purkinje cells and granule cells (Figure 5).

Group II (Phenytoin)

Light microscopic examination of Hx. & E. stained sections of cerebellar cortex of group II showed, apparent decrease of cerebellar cortex thickness. Irregular deeply stained Purkinje cells with dense nuclei were noticed. An apparent increase in perineural spaces around Purkinje cells and granule cells were also noticed (Figures 6,7). In semithin sections stained with toluidine blue, Purkinje cells appeared irregular with dark poorly defined nuclei. The granular layer showed many vacuolated cells with loss of the granular chromatin (Figures 8,9). Immunohistochemically stained sections for GFAP showed intensely stained astrocytes with an apparent increase in cell body size and length of their processes (Figure 10). Whereas, caspase-3 immunohistochemically stained sections revealed intense cytoplasmic immunoreaction of Purkinje cells and granule cells (Figure 11).

Group III (Phenytoin+ Selenium)

Light microscopic examination of Hx. & E. stained sections of cerebellar cortex of group III showed apparent average thickness of cerebellar cortex (Figure 12), Purkinje cells were mostly pyriform in shape and having vesicular nuclei with prominent nucleoli and minimal perineural spaces, the granular layer showed deeply stained cells with minimal perineural spaces (Figure 13). In semithin sections stained with toluidine blue, Purkinje cells showed large infolded vesicular nuclei with prominent nucleoli and the granule cells were having granular chromatin (Figure 14). Immunohistochemically stained sections for GFAP showed positive immune reaction of scattered small astrocytes with thin processes (Figure 15). While, caspase-3 immunohistochemically stained sections revealed negative cytoplasmic immunoreaction for caspase-3 of Purkinje cells and granule cells (Figure 16).

Morphometrical Results

Using morphometric studies for counting the mean number of Purkinje cells/field and measuring the mean thickness of the cerebellar cortex in µm of the three groups. Statistical analysis revealed highly significant reduction of the mean number of Purkinje cells and the mean thickness of the cerebellar cortex of group II (Phenytoin) as compared to group I (Control group) with a *P-value* <0.001. Similarly, a highly significant reduction between group II and group III (Phenytoin+ Selenium) has been found for both measures, with a *P-value* < 0.001. On the other hand, non-significant statistical reduction between group I and group III for both measures were found with a *P-value* > 0.05 (Tables 1 and 2). The comparisons between the morphometric results were further illustrated in column (Charts 1 and 2).

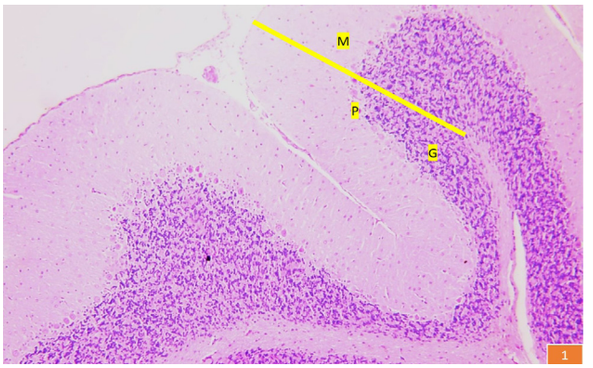


Fig. 1: A photomicrograph of a section of the cerebellum of the group I showing, the thickness of the cerebellar cortex (line) with its forming three layers the molecular layer (M), Purkinje cell layer (P) and the granular layer (G). (Hx. & E. X100)

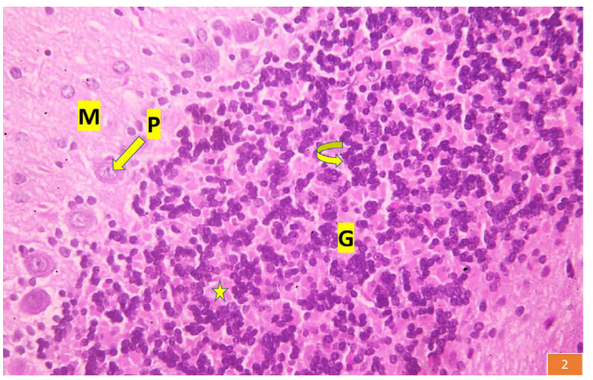


Fig. 2: A photomicrograph of a section of the cerebellar cortex of the group I showing, the molecular layer (M), Purkinje cell layer (P) formed of large pyriform cells arranged in one row with vesicular nuclei (long arrow). The granular layer (G) formed of small deeply stained granule cells (curved arrow) separated by noncellular areas representing the cerebellar glomerulus (star). (Hx. & E. X400)

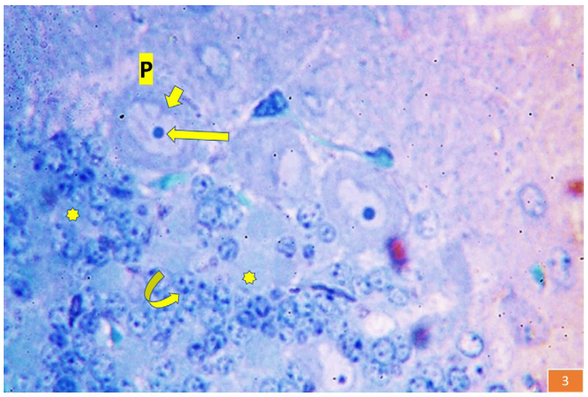


Fig. 3: A photomicrograph of a semithin section of cerebellar cortex of group I showing, Purkinje cells (P) having large infolded vesicular nuclei (short arrow) with prominent nucleoli (long arrow). Granule cells appear small and having granular chromatin (curved arrow). Notice the cerebellar glomeruli (stars) between the granule cells. (Toluidine blue X 1000)

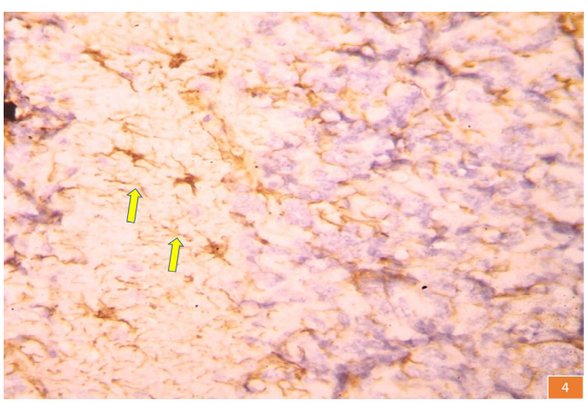


Fig. 4: A photomicrograph of a section of cerebellar cortex of group I showing, positive immunoreaction for GFAP of scattered small astrocytes with thin processes (arrows). (GFAP X400)

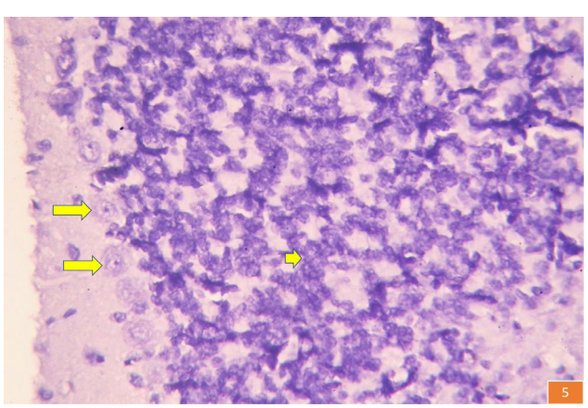


Fig. 5: A photomicrograph of a section of cerebellar cortex of group I showing, negative immunoreaction for caspase-3 of Purkinje cells (long arrows) and granule cells (short arrow). (Caspase-3, X400)

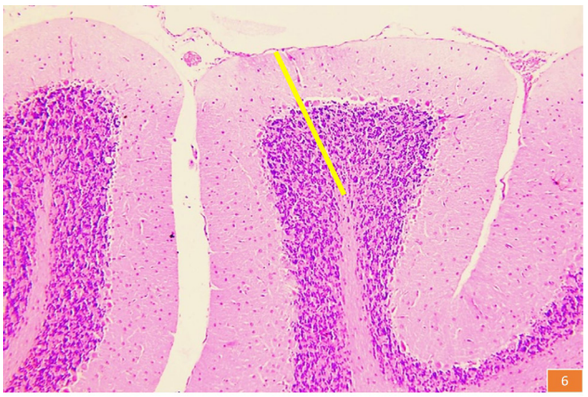


Fig. 6: A photomicrograph of a section of the cerebellum of group II showing, apparent decrease of cerebellar cortex thickness (line). (Hx. & E. X100)

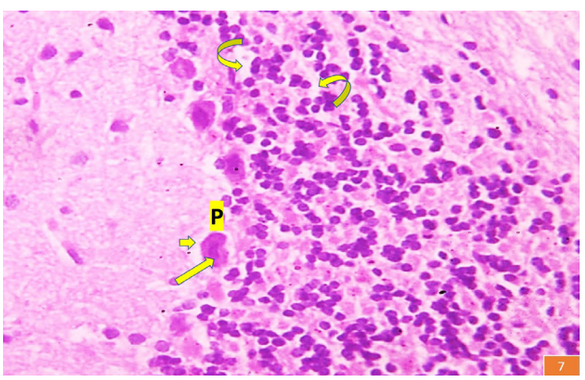


Fig. 7: A photomicrograph of a section of the cerebellar cortex of group II showing, irregular deeply stained Purkinje cell (P) with dense nucleus (long arrow). Notice, an apparent increase in perineural spaces around Purkinje cells (short arrow) and granule cells (curved arrows). (Hx. & E. X400)

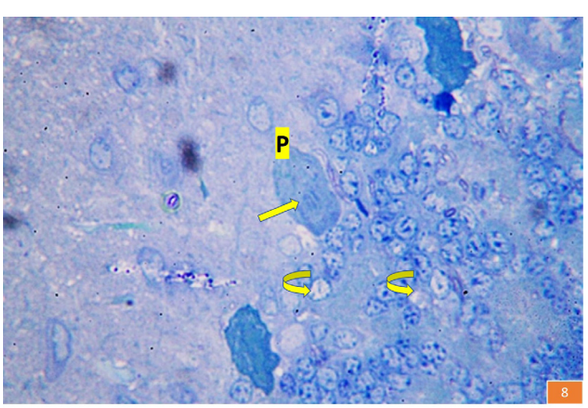


Fig. 8: A photomicrograph of a semithin section of the cerebellar cortex of group II showing, irregular Purkinje cells (P) with dark poorly defined nuclei (long arrow) and vacuolated granule cells (curved arrows). (Toluidine blue X 1000)

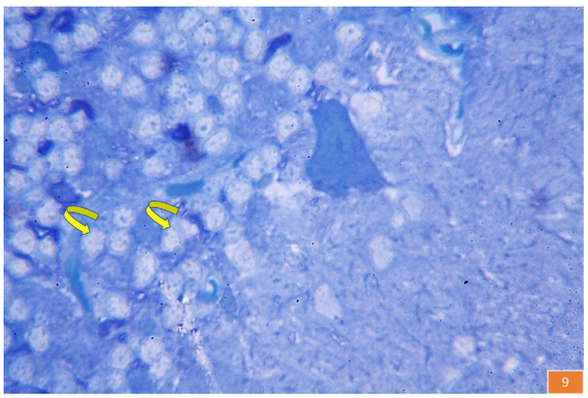


Fig. 9: A photomicrograph of a semithin section of the cerebellar cortex of group II showing, the granule cells with loss of their granular chromatin (curved arrows). (Toluidine blue X 1000)

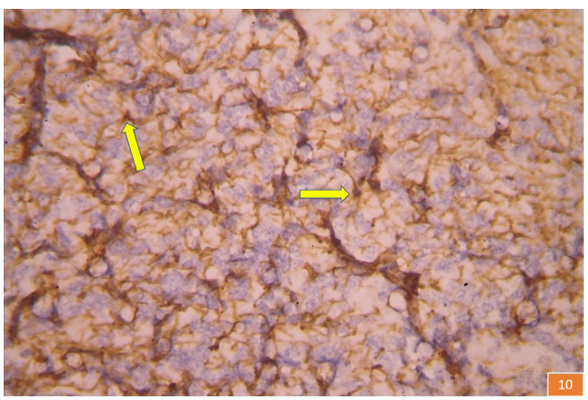


Fig. 10: A photomicrograph of a section of the cerebellar cortex of group II showing, intense positive immunoreaction for GFAP of astrocytes with an apparent increase in cell body size and length of their processes (arrows). (GFAP X400)

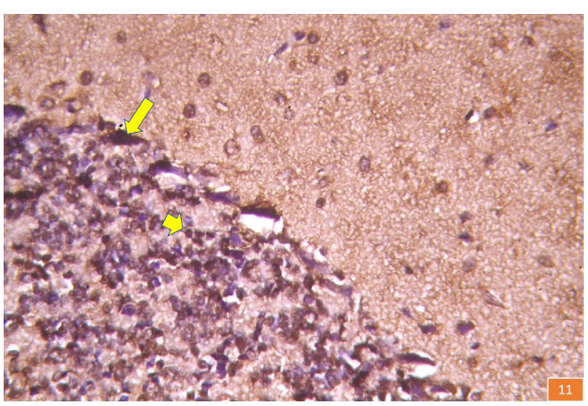


Fig. 11: A photomicrograph of a section of the cerebellar cortex of group II showing, intense cytoplasmic immunoreaction for caspase-3 of Purkinje cells (long arrow) and granule cells (short arrow). (Caspase-3, X400)

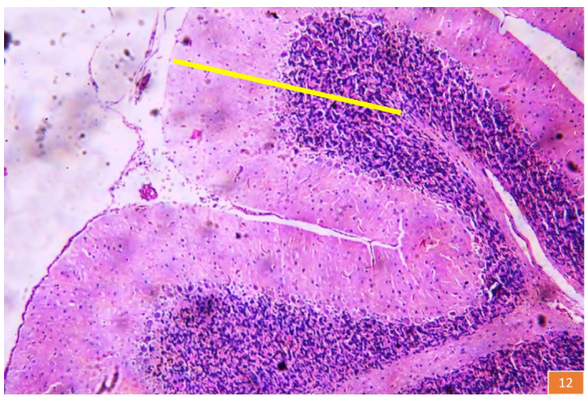


Fig. 12: A photomicrograph of a section of the cerebellum of group III showing, apparent average thickness of the cerebellar cortex (line). (Hx. & E. X400)

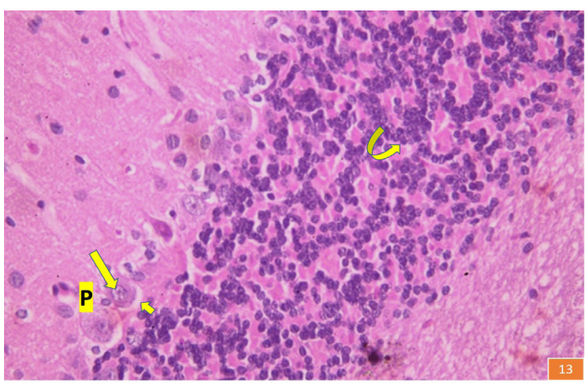


Fig. 13: A photomicrograph of a section of the cerebellar cortex of the group III showing, Purkinje cell (P) with vesicular nuclei (arrow). Notice, minimal perineural spaces around Purkinje cells (short arrow) and granule cells (curved arrow). (Hx. & E. X400)

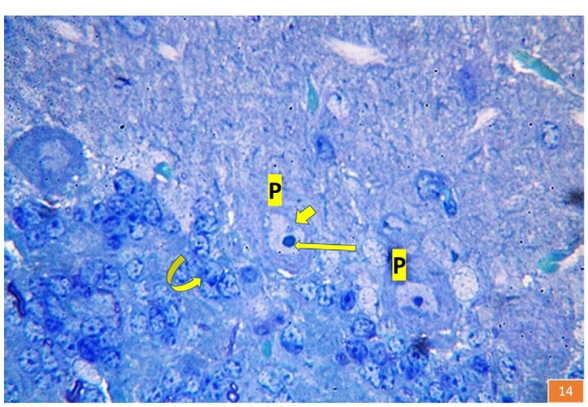


Fig. 14: A photomicrograph of a semithin section of the cerebellar cortex of group III showing, Purkinje cells (P) having large infolded vesicular nuclei (short arrow) with prominent nucleoli (long arrow) and granule cells having granular chromatin (curved arrow). (Toluidine blue X 1000)

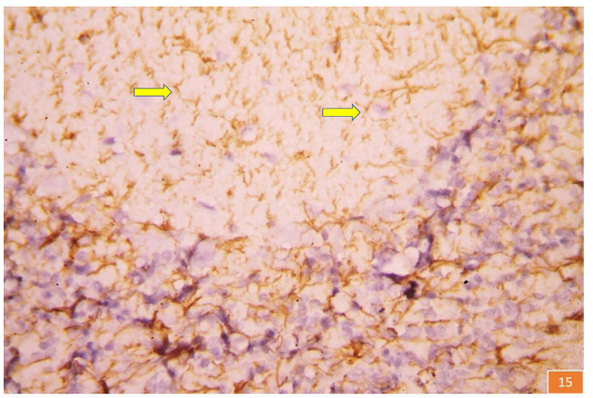


Fig. 15: A photomicrograph of a section of the cerebellar cortex of group III showing, positive immunoreaction for GFAP of scattered small astrocytes with thin processes (arrows). (GFAP X400)

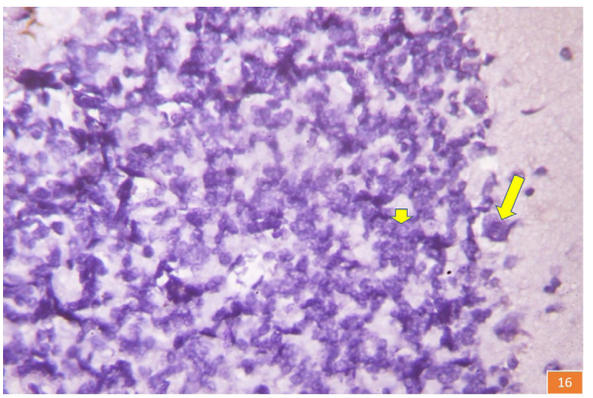


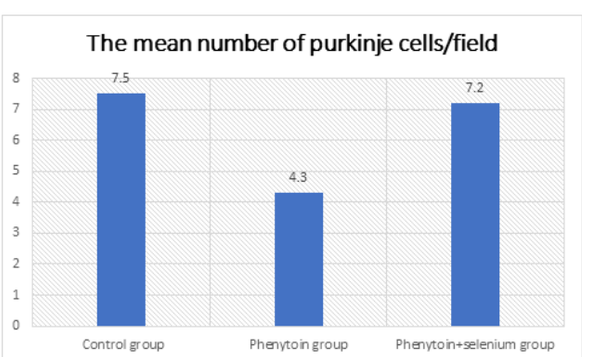
Fig. 16: A photomicrograph of a section of the cerebellar cortex of group III showing, negative immunoreaction for caspase-3 of Purkinje cells (long arrow) and granule cells (short arrow). (Caspase-3, X400)

Table 1: Comparing the mean number of Purkinje cells/field between the three groups showing, P-value either; non-significant (*) or highly significant (**)

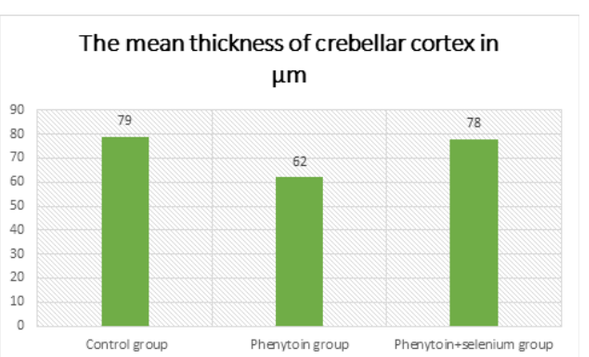
		Group I (Control)	Group II (Phenytoin)	Group III (Phenytoin+selenium)
	Number of Purkinje cells / field (mean ± standard deviation)	7.5± 0.2	4.3 ± 0.5	7.2 ± 0.1
T test	Between Group I&II		P= 0.0002**	
	Between Group II&III		P = 0.0001**	
	Between Group I&III		P= 0.2*	

Table 2: Comparing the mean thickness of cerebellar cortex in µm between the three groups showing, P-value either; non-significant (*) or highly significant (**)

		Group I (Control)	Group II (Phenytoin)	Group III (Phenytoin+selenium)
	Thickness of cerebellar cortex in µm (mean ± standard deviation)	79± 0.7	62.6 ± 3.2	78.1 ± 1.6
T test	Between Group I&II		P= 0.0001**	
	Between Group II&III		P = 0.0007**	
	Between Group I&III		P= 0.1*	



Column chart 1: Demonstrating the morphometric comparison as regards; the mean number of Purkinje cells/field



Column chart 2: Demonstrating the morphometric comparison as regards; the mean thickness of cerebellar cortex in µm

DISCUSSION

Cerebellar cortex plays a critical role in motor-coordination, long term memory storage and in consolidation of memory[20,21]. Long term phenytoin therapy has been proven to affect the process of learning and memory consolidation, motor-coordination and motor activity leading to ataxia, nystagmus and slurred speech[22-24]. Phenytoin as a widely used anticonvulsant interacts with many drugs and foods which may be significant for researches to reduce its toxicity[25].

The results of the present work revealed that prolonged use of phenytoin greatly affected rat's cerebellar cortex, Hx. and E. stained sections showed that Purkinje cells were irregular and deeply stained with increased perineural spaces around Purkinje and granule cells. In semithin sections stained with toluidine blue, Purkinje cells appeared irregular with dark poorly defined nuclei and the granular layer showed many vacuolated cells with loss of granular chromatin. Moreover, the morphometric results revealed highly significant statistically reduction in the mean number of Purkinje cells and in the mean thickness of cerebellar cortex as compared to the control group. It has been reported that long-term phenytoin administration increases lipofuscin storage and alter the metabolism of biogenic amines of rat cerebellar Purkinje cells, these findings were considered indicators of cerebellar degenerative changes[26]. Autopsy analysis of different brain regions of adult patients with chronic epileptic syndromes on phenytoin therapy, revealed high levels of phenytoin in their cerebellum with the highest concentration in the Purkinje cell layer[27].

In addition, immunohistochemical staining for caspase-3 as an indicator of cellular apoptosis, revealed intense cytoplasmic immunoreaction of Purkinje cells and granule cells. Phenytoin markedly raised the brain lipid peroxidase and acetylcholine esterase activity with subsequent oxidative damage to the neuronal membranes and disturbance of the neuronal functions that led to marked degeneration of different brain regions[28]. Oxidative stress ultimately leads to cellular death by apoptosis through specific signaling pathways with sequential activation of cysteine proteases known as caspases, caspase-3 activation often considered the point of no return during the process of apoptosis[29-31]. It has been reported that phenytoin induced apoptosis of the cerebellar granule cells at high doses[32]. Immunohistochemical staining for GFAP showed intensely stained astrocytes with increased cell body size and length of their processes. GFAP is thought to have a role in modulating astrocytes motility and shape by providing structural stability to astrocytic processes[33]. Increase astrocytic GFAP was considered a hallmark of reactive astrocytes to oxidative damage [reactive gliosis] that has been reported in many neurodegenerative conditions[34].

On the other hand, Hx. and E. stained sections of group III that received selenium with phenytoin showed nearly a preserved cerebellar cortical structure, Purkinje cells were mostly pyriform in shape and having vesicular nuclei with prominent nucleoli. Minimal perineural spaces were detected around Purkinje cells and granule cells. In semithin sections stained with toluidine blue, Purkinje cells appeared having large infolded vesicular nuclei with prominent nucleoli and the granule cells showed preserved granular chromatin. The morphometric results revealed a non-significant difference between group III and the control group and a highly significant difference between group III and group II regarding the mean number of Purkinje cells and the mean thickness of cerebellar cortex. It has been reported that selenium protected the brain tissue from reactive oxygen species that induce cellular damage and attenuated the toxic effects of many compounds on rat cerebellum such as lead, fluoride and lithium[35-38]. The antioxidant effect of selenium on brain cells was explained to be due to its role in elevating glutathione and reducing lipid peroxidation through expression of seleno-proteins which were involved in regulation of redox status and thus protected the cells from death[39]. Therefore, selenium plays a critical role in maintaining the proper function of the nervous system under physiological conditions and in oxidative stress[38].

In addition, immunohistochemical staining for caspase-3 of the same group revealed negative cytoplasmic immunoreaction for caspase-3 of Purkinje cells and granule cells which was nearly similar to those of the control group. Selenium has a suppressive effect against DNA damage and Poly ADP-ribose polymerase (PARP) cleavage, PARP is a family of proteins involved in DNA repair and programmed cell death, so selenium is considered a protector against apoptotic cell death[40]. While, immunohistochemical staining for GFAP showed positive immune reaction of scattered small astrocytes with thin processes. Seleno-proteins experimentally enhanced astrocytic survival and increased the resistance of these cells to ischemia and oxidative stress[41].

Reviewing the literature, there was not enough data for selenium and phenytoin interaction. However, it has been proven that old antiepileptic drugs like phenytoin induced overproduction of reactive oxygen species and depleted vital nutrients, such as selenium, calcium, copper, and zinc, these nutrients were found to be critical in restoring the antioxidant redox systems in the brain[42,43]. Additionally, the present study revealed the protective capability of selenium on the structure of rat's cerebellar cortex when administrated with phenytoin.

CONCLUSION

Long term phenytoin administration led to histoarchitectural changes of rat's cerebellar cortex. However, Selenium administration with phenytoin greatly protected the cerebellar cortex from such changes.

CONFLICTS OF INTEREST

There are no conflicts of interest

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الملخص العربى

الدور الوقائي المحتمل للسيلينيوم ضد تأثيرات الاستخدام طويل الأجل للفينيتوين على قشرة المخيخ لذكور الجرذان البيضاء البالغة: دراسة هستولوجية وهستوكيميائية مناعية

ايناس أنور بخيت

قسم التشريح كلية الطب جامعة عين شمس

المقدمة:الفينيتوين هو دواء يوصف كمضاد للصرع. تم الربط بين التناول المزمن للفينيتوين وتدهور المخيخ. السيلينيوم هو أحد الإنزيمات الرئيسية المضادة للأكسدة المساعدة للجلوتاثيون وهو ايضا عنصر رئيسي في بروتينات السيلينوم التي تلعب دورا هاما في نمو المخ والتمثيل الغذائي.

الهدف: هدف هذا العمل إلى دراسة الدور الوقائي المحتمل للسيلينيوم ضد تأثيرات الاستخدام طويل الأجل للفينيتوين على قشرة المخيخ للجرذان البيضاء البالغة.

المواد والطرق المستخدمة: تم استخدام خمسة وأربعين جرذا من الذكور البالغين في هذه الدراسة ، الذين تتراوح أعمارهم بين ٦-٨ أشهر ، وتتراوح اوزانهم من ١٨٠ -٢٠٠ جم. تم تقسيم الجرذان بالتساوي إلى ثلاث مجموعات:

المجموعة الأولى: تم تقسيمها إلى ثلاث مجموعات فرعية متساوية:

المجموعة IA: تم الاحتفاظ بها كمجموعه ضابطه سلبية .

المجموعة IB: تلقى كل جرذ ١ مل من الماء المقطر يوميا عن طريق الفم لمدة ٤٥ يومًا.

المجموعة IC: تلقى كل جرذ السيلينيوم بجرعة ٠,٥ ملجم / كجم من وزن الجسم يوميا عن طريق الفم لمدة ٤٥ يوما.

المجموعة الثانية: تلقى كل جرذ الفينيتوين بجرعة ٢٠ ملجم / كجم من وزن الجسم يوميا عن طريق الفم لمدة ٤٥ يوما.

المجموعة الثالثة: تلقى كل جرذ الفينيتوين كما فى المجموعة الثانية بالإضافة إلى السيلينيوم بجرعة ٠,٥ ملجم / كجم من وزن الجسم يوميا عن طريق الفم لمدة ٤٥ يوما.

النتائج: كشف العمل الحالي أن تناول الفينيتوين على المدى الطويل تسبب في تغييرات نسيجية لقشرة المخيخ فى الجرذان في شكل انخفاض سماكه القشرة، و عدم انتظام خلايا بيركنجي و زيادة الفراغات حول خلايا بيركنجي والخلايا الحبيبية, وقد ادى استخدام السيلينيوم مع الفينيتوين الى الحفاظ على التركيب النسيجي للقشرة المخية الى حد كبير.

الخلاصة: اوضحت هذه الدراسة ان تناول الفينيتوين على المدى الطويل ادى إلى تغييرات فى قشرة المخيخ فى الجرذان. وقد ادى استخدام السيلينيوم مع الفينيتوين الى الحماية من هذه التغييرات الى حد كبير.