Original Article

Light and Electron Microscopic Study on the Effect of Valproic Acid on Cerebellar Cortex of Adult Male Albino Rats and The Possible Protective Effect of L-Carnitine

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ABSTRACT

Introduction: Valproic Acid (VPA) is one of the most widely prescribed antiepileptic drugs and is regarded as a first choice for most forms of seizures. Although valproic acid has a wide therapeutic window, yet it is associated with many adverse effects. L-carnitine is a naturally occurring compound widely distributed in all animal cells. It has neurotropic, neuroprotective and antioxidant properties.

Aim of the Work: The aim of the present study was to evaluate the possible neurotoxic effect of valproic acid (Depakene) on the cerebellar cortex of adult male albino rats when used alone and when given concomitantly with L-carnitine.

Material and Methods: In this study twenty four adult male albino rats were used and divided into four groups (six rats each): Group I was the control group, group II (L-carnitine group): Each animal received 100mg/kg L-carnitine, group III (Valproic acid treated group): Each animal received 50 mg/kg valproic acid and group IV received valproic acid concomitantly with L-Carnitine. Both drugs were given orally once daily for three months.

Results: Light microscopic examination of cerebellar cortex of valproic acid treated animals revealed its prominent neurotoxic effect on Purkinje cells and granule nerve cells in association with vacuolation in the molecular layer. Ultrastructural study of the cerebellum of the same group showed dilated Golgi complex and accumulation of secondary lysosomes in association with nuclear shrinkage and irregularity within Purkinje cell perikarya. Many myelinated nerve fibers and nerve cell processes in the molecular and granular layer belonging to the affected nerve cells displayed similar degenerative changes. On the other hand group IV revealed resolving of most of these alterations. However, few Purkinje and granule nerve cells were seen affected in between the normal ones.

Conclusion: It could be concluded according to this research that valproic acid has a prominent neurotoxic effect on the cerebellar cortex of the adult male albino rats that can be reduced by concomitant administration of L- carnitine.

Key Words: Valproic acid, l-carnitine, cerebellar cortex.

INTRODUCTION

Valproic acid (VPA) (Depakene) is a branched–chain carboxylic acid similar in structure to endogenous fatty acids. It was approved by Food and Drug Administration (FDA) for treatment of epilepsy either as monotherapy or in combination with other antiepileptic drugs. Recently, it was added as one of the mood stabilizing agents in patients with affective and anxiety disorder and as a prophylactic treatment of migraine. It is absorbed rapidly from the gastro-intestinal tract to be metabolized in the liver by conjugation and oxidation in the mitochondria. It exerts its pharmacological effects through interaction with sodium channels on the cell membrane inhibiting repetitive firing of neurons and reduces the release and effects of excitation amino acid in some specific brain regions thought to be involved in the control of seizure generation and propagation. Although VPA was thought to have minimal neurological adverse effects, yet its long term administration was associated with many side effects primarily gastrointestinal in nature, weight gain and transient hair loss. The most commonly observed neurological adverse effects were sedation, tremors, ataxia and impairment of cognitive function. Undesired toxic symptoms from the CNS especially from the cerebellum and extra pyramidal system defined as valproate encephalopathy were also recorded. Clinical and experimental studies have shown that some anatomic structures of the central nervous system especially cerebellum and hippocampus are particularly sensitive to long term effects of various antiepileptic drugs including valproic acid. These drugs could cause functional disorders and organic lesions in nerve tissue even at their therapeutic serum concentration.
distributed in virtually all cells of higher animals. It is synthesized in liver, kidney and brain to be stored in skeletal muscles, heart, brain and sperms. L-carnitine possess unique neuroprotective, neuromodulatory, and neurotrophic properties which play an important role in countering various disease processes. It plays an important role in energy translocation by transporting activated fatty acids (acyl-CoA) into the mitochondrial matrix for metabolism and transporting intermediate compounds out of the mitochondrial matrix preventing their accumulation. It also has antioxidant effect and plays an important role in the translocation of acetyl moieties from mitochondria to the cytoplasm for acetylcholine synthesis in neurons.

Acquired L-carnitine deficiency was commonly recorded in VPA treated persons. This finding was also elucidated by Hiraoka who reported that prolonged treatment with VPA enhances renal loss of carnitine esters and lowers serum carnitine level. In addition it was reported that L-carnitine treatment produced improvement of some symptoms in patients with degenerative cerebellar ataxia.

Based on these facts, the current study was conducted to evaluate the neurologic effects of VPA on cerebellar cortex of adult male albino rats as well as the protective effect of L-carnitine.

MATERIAL AND METHODS

Chemicals: Valproic acid (Depakene) was supplied as a syrup form by Sanaofi Synthelabo Company, while L-Carnitine 30% (Carnitol) was supplied as a syrup by Goba, NAPI Company.

This study was carried out on twenty four adult male albino rats weighing 180-250 gm each. They were kept in clean properly ventilated cages and fed a commercial laboratory diet. Both drugs were given orally by gastric tube once daily for three months. The animals were divided into four groups (six rats each):

Group I (Control group): Each animal received no medication.

Group II (L-carnitine group): Each animal received L-Carnitine at a dose of 100 mg/kg body weight.

Group III (Valproic acid treated group): Each animal received orally 50 mg/kg body weight which is the maximum allowable human therapeutic dose.

Group IV: Each animal received valproic acid at a dose of 50mg/kg body weight in combination with L-Carnitine at a dose of 100 mg/kg body weight.

Cerebellar samples were taken after 24 hour from the last dose. At the appropriate time, animals were anaesthetized with 4 % halothane and perfused with 0.1 M sodium phosphate buffer (pH 7.4) containing 2.5 glutraldehyde solution. After perfusion, Specimens of cerebellum were taken from the lateral lobes. The cerebellum was dissected out as follows; small slit was made between the pinnae to be extended forwards to the snout (nose) and backwards between the levels of the fore-limbs. Muscles were cut away, while bones of the skull were chipped by gentle dissection. Specimens of cerebellum were taken from the lateral lobes and processed for light and electron microscopic study.

For electron microscopy, very small pieces were fixed in 2.5% of 0.1 M phosphate buffered glutraldehyde solution (pH 7.4) at 4°C for 2 hours. The specimens were then washed 3 times (5 mins each) with phosphate buffered osmium tetroxide at room temperature for 30 minutes. Later on, the specimens were washed in buffer, dehydrated in a graded series of alcohol and embedded in epon. Ultrathin sections (80-90 nm) were cut with ultramicrotome, stained with uranyl acetate and lead citrate and examined by a JEOL electron microscope at 80 kV in Faculty of Science, Ain-Shams University.

RESULTS

Light microscopic results:

Light microscopic examination of the cerebellar cortex of the control group revealed its well known architecture. The cortex of the cerebellum which constituted its grey matter was formed of three layers; outer molecular layer, middle Purkinje cell layer and inner granular cell layer (Fig. 1). The outer molecular layer consisted of horizontally directed nerve fibers with relatively few scattered nuclei of basket cells. The Purkinje cell layer showed large flask shaped cells arranged typically in single row at the junction of the molecular layer with the granular layer. These cells displayed a characteristic centrally located rounded open face nucleus surrounded by prominent nucleolus surrounded by cytoplasmic Nissl's granules. The next granular cell layer contained numerous compactly disposed granule cell bodies with darkly stained nuclei surrounded by very little cytoplasm. Scattered golgi type II nerve cells with typical open face nuclei were also observed in this layer near the Purkinje cells. Small clear lightly stained areas called cerebellar islands (glomeruli) were also seen in between the granule nerve cells, they are areas of synaptic connections between the ascending fibers and the dendrites of granule cells (Fig. 2).

Group II (animals treated with L-carnitine only) showed histological picture more or less similar to control group.
Light microscopic examination of the cerebellar cortex of valproic acid treated rats (Group III) revealed widespread neuronal affection specifically of the Purkinje cell layer which was reflected on the other two layers. The Purkinje cells revealed disturbed normal linear organization with marked disarrangement, where some cells were displaced upwards in the molecular layer (Fig. 3) while others were displaced downwards in the granular cell layer (Fig. 4). They appeared, irregular, distorted, shrunk with pericellular unstained haloes (Figs. 3, 4, 5, 6). The cytoplasm of these cells showed eosinophilic homogenization with irregular darkly stained nuclei (Fig. 5). Some nuclei also showed peripheralization and pyknosis (Fig. 6). In parallel with morphological alterations seen in Purkinje layer, the molecular layer displayed prominent spongiosis in the form of multiple vacuolated areas (Figs. 3, 5). Granular cell layer showed pyknosis of their nuclei with pericellular unstained haloes (Figs. 4, 5). In between the affected cells, the cerebellar islands (Glomeruli) showed increased eosinophilia (Fig. 4).

Animals treated with valproic acid and L-carnitine simultaneously (Group IV) showed mild neurotoxic effect of valproic acid as only few Purkinje cells were affected in between the normal ones. The affected cells showed cytoplasmic eosinophilic homogenization in association with focal vacuolated areas in the molecular layer. The granular cell layer exhibited apparently normal cerebellar islands in the vicinity of normal granule nerve cells (Fig. 7).

Electron microscopic results:

Electron microscopic examination of cerebellar cortex of control group revealed the well known picture of perikaryon of Purkinje cells and their surrounding neuropil. It was rich in rough endoplasmic reticulum organized into aggregates of parallel cisternae around regularly contoured, spherical nucleus with dispersed chromatin, golgi complexes formed of parallel arrays of smooth cisternae were seen around the nucleus together with many scattered mitochondria (Fig. 8). Concerning the molecular layer, many unmyelinated nerve fibers were seen either in their longitudinal sections revealing regularly arranged parallel microtubules (Fig. 9) or in their cross section containing many mitochondria and microtubules (Fig. 10). In between these nerve fibers, few scattered myelinated nerve fibers were also seen. They revealed compact lamellar structure of myelin around smooth contoured axons. The axoplasm of these nerve fibers contained regularly arranged microtubules and mitochondria (Fig. 10). Ultrastructural observations of granular layer of this group showed closely packed spherical nuclei of granule nerve cells with their characteristic condensed chromatin surrounded by very little cytoplasm (Fig. 11). The cerebellar islands were also commonly detected in the vicinity of the granule nerve cells containing many myelinated and unmyelinated nerve fibers (Fig. 12).

Group II (L-carnitine treated animals) showed ultrastructural features more or less similar to control group.

Ultrastructural examination of the cerebellum of valproic acid treated group (Group III) showed shrunk electron dense perikarya of Purkinje cells (Fig. 13). The nuclei of these cells displayed condensation of their chromatin and irregularity of their outlines (Figs.13,14). The cytoplasm of these affected cells revealed dilated golgi apparatus (Fig. 14), secondary lysosomes and lipofuschin pigments (Fig. 15). The surrounding neuropil revealed spongiotic changes. Concurrently with morphological changes encountered in the perikaryon of Purkinje cells, their belonging dendrites in the molecular layer in their transverse section showed marked swelling with rarefied dendroplasm, focally disrupted plasma lemma and destroyed mitochondria with rarefied matrix (Fig. 16). Others were seen shrunken, contracted, and darkly stained (Fig. 17). The granular cell layer showed irregular hyperchromatic and pyknotic nuclei. The neuropil of the cerebellar islands showed amalgamation and loss of most of the structural details and exhibiting spongiosis (Figs.18,19). They have swollen mitochondria with partial or total destroyed cristae and rarefied matrix (Fig. 19). The myelinated axons seen in this region revealed severe degenerative changes in the form of disruption, splitting and loss of the compact lamellar structure of myelin layers. Axons of these degenerated myelin sheath revealed disorganization of their microtubules and many swollen irregular mitochondria with destroyed cristae and rarefied matrix (Figs. 19,20).

Concerning group IV (animals that received Valproic acid and L-carnitine simultaneously), their ultrathin sections showed apparent decrease in the cerebellar damaging effect of valproic acid as most of the Purkinje nerve cells appeared nearly similar to the control group and few of them displayed rarefied cytoplasm (Fig. 21). The molecular layer showed that few dendrites were affected in between the normal ones and the regular compact lamellar structure of the myelin sheath was preserved around the apparently normal axons (Fig. 22).

![Fig. 1: A photomicrograph of cerebellar cortex of control group showing Purkinje cell layer(P), molecular cell layer (M) and granular cell layer (G).](image-url)
Fig. 2: A photomicrograph of cerebellar cortex of control group showing nuclei of basket cells (►) in the molecular layer (M), Purkinje cell (—►) with open face nucleus and prominent Nissl's granules, granular cell layer with open face nucleus of Golgi type II cells (wavy arrow), granular cells with darkly stained nuclei (curved arrow) and cerebellar islands (C). H&E. X 1000.

Fig. 3: A photomicrograph of a section of the cerebellar cortex of valproic acid treated group showing upward displacement of Purkinje cells (—►) into the molecular layer, distortion in their shape, eosinophilic homogenization of their cytoplasm and pericellular unstained halo (curved arrow). Notice spongiosis of the molecular layer (►). H&E. X 1000.

Fig. 4: A photomicrograph of a section of the cerebellar cortex of valproic acid treated group showing downward displacement of distorted Purkinje cells (—►) into the granular layer. The granular cell layer showed pyknotic nuclei (curved arrow). Notice increased eosinophilia of some cerebellar islands (Glomeruli) (*). H&E. X 1000.

Fig. 5: A photomicrograph of a section of the cerebellar cortex of valproic acid treated group showing distorted and darkly stained Purkinje cells (—►) with pericellular unstained halos, spongiosis of molecular layer (►) and pyknotic nuclei of granular cells (curved arrow). H&E. X 1000.

Fig. 6: A photomicrograph of a section of the cerebellar cortex of valproic acid treated group showing shrunken small Purkinje cell with pyknosis and peripheralization of the nucleus (►). H&E. X 1000.

Fig. 7: A photomicrograph of a section of the cerebellar cortex of group IV showing distortion of only one Purkinje cell (—►) in between more or less normal others. H&E. X 200.
Fig. 8: An electron micrograph of cerebellar cortex of control group showing perikaryon of Purkinje cell with part of its nucleus (N) and cytoplasm containing rER (R), Golgi apparatus (G) and mitochondria (M).

X 12000.

Fig. 9: An electron micrograph of longitudinal section in unmyelinated axon of control group showing regular arrangement of the microtubules (mt).

X 6000.

Fig. 10: An electron micrograph in the molecular layer of cerebellar cortex of control group showing transverse section of myelinated nerve fibers containing mitochondria (M) and regularly arranged microtubules (mt). Notice the nearby T.S of densities (——).

X 15000.

Fig. 11: An electron micrograph in the granular layer of cerebellar cortex of control group showing normal structure of the granular cells (*) with part of cerebellar islands (C).

X 4000.

Fig. 12: An electron micrograph of cerebellar cortex of control group showing cerebellar islands containing myelinated nerve fiber (*). Notice: transverse section in unmyelinated nerve fibers containing mitochondria (M).

X 15000.

Fig. 13: An electron micrograph of cerebellar cortex of valproic acid treated group showing contracted darkly stained Purkinje cell having small irregular nucleus (N). Notice vacuolization of the surrounding neuropil (-).

X 3000.
Fig. 14: High power magnification of the previous field showing irregular outline of the nucleus (N) and dilated Golgi apparatus (G). X 15000.

Fig. 15: An electron micrograph of cerebellar cortex of valproic acid treated group showing cytoplasm of Purkinje cell containing secondary lysosomes (S) and lipofuscin pigments (L). X 15000.

Fig. 16: An electron micrograph of cerebellar cortex of valproic acid treated group showing transverse section in swollen dendrites with rarefied dendroplasm, focally disrupted plasma membrane (→) and swollen mitochondria with destroyed cristae (M). X 25000.

Fig. 17: An electron micrograph in the molecular layer of cerebellar cortex of valproic acid treated group showing shrunken and contracted nerve cell and its axon (→). X 6000.

Fig. 18: An electron micrograph in the granular layer of cerebellar cortex of valproic acid treated rats showing granule nerve cells with chromatin condensation (N1) and chromatin margination (N2). Notice vacuolation of the surrounding neuropil (*). X 5000.

Fig. 19: An electron micrograph in the cerebellar island of the granular layer of cerebellar cortex of valproic acid treated rat showing amalgamation of multiple nerve cell processes which display many swollen mitochondria with destroyed cristae (M). Notice intramyelinic oedematous clefts and splitting of myelin of a myelinated nerve fiber (→) together with spongiosis of the surrounding neuropil (*). X 12000.
Fig. 20: An electron micrograph in the granular layer of cerebellar cortex of valproic acid treated rat showing myelinated nerve fiber with disruption of myelin sheath (—) and splitting of its lamellae (►). The axoplasm contains swollen irregular mitochondria with destroyed cristae (M). X 12000.

DISCUSSION

The present work revealed that valproic acid induced morphological changes in the cerebellar cortex of adult male albino rats, mostly on the Purkinje and granule nerve cells which were in turn reflected on the morphological structure of all layers of the cerebellar cortex.

In the present work the appearance of distorted shrunken electron dense Purkinje cells was similar to the previous findings of earlier study that reported that long-term VPA administration causes considerable damage to the system associated with structural and functional biosynthesis of cell proteins manifested in markedly increased electron density in cytoplasm of Purkinje cell19. Some investigators believed that presence of dark neurons situated in various regions of grey matter of the CNS is usually due to ischemia that occurs as a result of substantial abnormalities in the capillary wall of the cerebellar cortex. Subsequently, there were disorders in the transportation of sodium VPA and/or its toxic metabolites through structural elements of the blood-brain barrier to neurons and vice versa25-27. Others suggested that the appearance of dark neurons might reflect a certain phase of apoptosis as they displayed markedly condensed cytoplasm and nucleoplasm21-29.

The major ultrastructural changes were mostly seen within the perikarya of Purkinje cells. These changes were in the form of dilated channel of Golgi together with accumulation of secondary lysosomes and lipofuscin pigments. All these cytoplasmic changes were accompanied with profound abnormalities in the nuclei where they appeared irregular and hyperchromatic. Similar changes were previously observed in other studies and were attributed to inhibition of oxidative phosphorylation processes in Purkinje cell mitochondria30. This was also supported by biochemical studies which have been performed in vitro on isolated mitochondria of the brain, the liver and the kidneys in the course of acute VPA administration31-33. Many investigators mentioned also that direct toxic effect of the drug or its metabolites on neuronal cells induced profound disorder of intracellular biochemical events, such as inhibition of oxidative phosphorylation, abnormal production of proteins and dysfunction of the detoxication and secretory processes as well as the disorders of morphological cell integrity34-36.

Valproic acid treated rats revealed will evidenced unstained haloes around Purkinje cell perikarya and vacuolation in the nearby molecular and granular layer. This was mostly attributed to shrinkage of purkinje cells and withdrawal of their protoplasmic processes secondary to disintegration of the cytoskeletal elements of these cells34,35.

The processes of Purkinje cells showed interesting morphological changes where they appeared shrunken...
and ischemic with electron dense cytoplasm. This was consistent with similar changes in their corresponding perikarya. Similarly Sobaniec- Lotowska revealed the same findings and attributed them to severe damage to cytoskeletal elements within the perikaryon and protoplasmic processes of Purkinje cells.

In the present work, electron microscopic examination revealed markedly affected mitochondria of Purkinje cells as well as their processes. These findings were in accordance with results of Sobaniec-Lotowska who attributed them to profound disorder of inter cellular biochemical events such as inhibition of oxidative phosphorylation due to direct toxic effect of the drug or its metabolites.

Disruption of the cell membrane of the Purkinje cell perikarya and their dendrites was attributed by Mitchell et al., to dissociation of the cytoskeletal elements which could result in detachment of the cell membrane, rendering it susceptible to stretch and rupture. They added that defective mitochondrial function secondary to inhibition of oxidative phosphorylation results in decreased phospholipid synthesis which affects all cell membranes.

Myelinated axons of cerebellar cortex of valproic acid treated rats revealed considerable degenerative structural changes within their axoplasm as well as splitting and disruption. This was similar to that recorded by Lotowska et al. It was reported that, the myelinated nerve fibers seen in the molecular layer of cerebellar cortex were thought to belong to the parallel fibers of granule nerve cells or to the recurrent collaterals of Purkinje cell axons. While those seen in the granular layer were mostly believed to belong to the axons of Purkinje cells. So injury of Purkinje and granular cells made them incapable of maintaining their distal processes. Others also recorded that axon degeneration in the cerebellum is a component of a dying – back process of neuronal injury.

Sobaniec- Lotowska & Sobaniec believed that the loss of myelin or its disruption (demyelination) was attributed to changes in myelin basic protein secondary to a toxic insult which can occur following a direct perturbation to the myelinating cell or its myelin sheath or as a response to axonal degeneration. They also added that dysmyelination including folding and splitting at various levels of the myelin lamellae was attributed to increased water content in degenerating nerve causing intramyelinic edema with separation of myelin lamellae.

In the present work, the concomitant administration of L-carnitine and valproic acid revealed decreased neurotoxic effect of valproic acid on the cerebellar cortex of adult male albino rats. It has been shown that the most nerve cells appeared remarkably healthy and more or less similar to that of control group while few focally scattered others exhibited mild degenerative changes. These results were in agreement with those recorded by Sarhan et al. and Raskind & El-Charr. They reported that the efficiency of the treatment of L-carnitine seems to be through the prevention of accumulation of lethal concentrations of ammonia induced by valproic acid in the brain by easily crossing the blood-brain barrier. Valproic acid was also recorded to lower blood levels of carmitine and cause carmitine deficiency by interfering with its renal reabsorption and increasing its excretion in urine. In addition, it was previously reported by Di Marzio et al. and Milosevi et al., that L-carnitine has anti-apoptotic effect which enhances cell survival through retarding DNA fragmentation and nuclear condensation as well as increasing the activity of BcI-2 gene which act as anti apoptotic gene.

From this current work, it could be concluded that valproic acid has a toxic effect on the cerebellar cortex of adult male albino rats and the concomitant administration of L-carnitine together with valproic acid is highly effective in retarding the cerebellar neurotoxic insults caused by valproic acid.

REFERENCES


دراسة بالمجهز الضوئي والإلكتروني على تأثير حمض الفالبرويك على قشرة مخيخ ذكر الفأر الأبيض البالغ والتأثير الوقائي المحتمل لـ كارتينين

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ملخص البحث

يعتبر عقار حمض الفالبرويك من أكثر الأدوية استخداماً لتشويه الضرر والصداع التصاعدي والاضطراب الوجداني. إلا أنه قد وجد أن له العديد من الأعراض الجانبية على كثير من أعضاء الجسم. ويعتبر ل- كارتينين مادة طبيعية مركبة وموزعة بكثرة في كل خلايا الجسم وهي تعزز الأعصاب وتحميها ضد الأكسدة. لذلك يهدف هذا البحث إلى دراسة تأثير عقار حمض الفالبرويك على قشرة مخيخ الفأر الأبيض البالغ عندما يستخدم بمفرده وعندما يعطى مصاحباً لـ كارتينين. وقد أجريت الدراسة على أربع وعشرين فأرا، قسمت إلى أربع مجموعات (ستاند فوران في كل مجموعة): مجموعة الأولى تمثل المجموعة الضيقة، المجموعة الثانية أُعطيت ل- كارتينين 100 مجم / كجم، أما المجموعة الثالثة فقد أُعطيت عقار حمض الفالبرويك 50 مجم/كجم و أُعطيت المجموعة الرابعة عقار حمض الفالبرويك و ل- كارتينين نفس الجرعات السابقة. كل العقاران أعطاهما يومياً لمدة ثلاث شهور عن طريق الفم. وقد أظهر الفحص بالميكروسكوب الضوئي للمجموعة الثالثة التأثير السبيسي لحمض الفالبرويك على الخلايا العصبية لقشرة المخيخ خصوصاً خلايا بيركنجي والخلايا الحبيبية بالإضافة إلى وجود فجوات في الطبقة الجزئية. أما التركيب الدقيق لخلايا بيركنجي فقد أظهر اتساع في جهاز جويج مع زيادة في النفوذات التائية مع عدم تأسخ. وتشير نتائج تلك التجارب إلى أن posses إستراتيجية في العديد من الجوانب وبيئات المعطيات الخاصة بهذه الخلايا و موجودة في طبقة الخلايا الحبيبية و الطبقة الجزئية. وعلى الآخر فالحيوانات التي أعطت ل- كارتينين وحمض الفالبرويك معًا فقد فضت فيما التغيرات الهيستولوجية السابقة وتأثرت عضيات الخلايا بدرجة أقل. من هذه الدراسة نستنتج أن حمض الفالبرويك له تأثير سبيسي على قشرة مخيخ ذكر الفأر الأبيض البالغ والذي يمكن تقليله باستخدام ل- كارتينين مصاحباً له.