## EXPERIMENCAL RESEARCH

# Influence of Vitamin E on Electron Microscopic Findings Caused by Pharmacologically Induced Epilepsy

## Deneysel Farmakolojik Epilepside E Vitamininin Elektron Mikroskopik Bulgular Üzerine Etkisi

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**Abstract**: Our previous study showed that epileptic seizures induced by DL-methionine DL-sulfoximine administration to rats could be prevented and T1 relaxation time shortened as a sign of avoiding the brain edema fluid by prior administration of an antioxidant vitamin E. This present study also consisted of three randomized groups: The first was the control group, the second the epilepsy group, the third the prophylaxis group. In each group seven male swiss albino rats of the same age and similar weight were used. In all of the animals in the second-epilepsy group, epileptic generalized seizures were induced pharmacologically by DL-methionine DL-sulfoximine i.v.. In the third prophylaxis group, alpha-tocopherol ( vitamin E ) was intravenously given fifteen minutes before injecting the same epileptic agent of the same dose. None of the animals in this third group developed any convulsions. Thirty minutes after the application of the epileptic agent, all rats in the last two groups were decapitated. The cerebral hemispheric tissue removed from the same site was histologically investigated in all animals of each group by an electron microscope. The clinical as well as neuropathological outcome confirmed that the DL-methionine DL-sulfoximine-induced epileptic activity could be arrested and epileptic cell and tissue damage could be prevented by prophylactic administration of vitamin E.

**Key Words**: Epilepsy, glutamate, epileptic cell damage, electron microscope, vitamin E

**Özet:** Bir önceki çalışmamızda sıçanlara DL-metiyonin DL-sulfoksimin verilmesiyle meydana gelen epilepsinin önceden antioksidan vitamin E verilmesiyle önlenebileceği ve bein ödeminin önlendiğinin T1 gevşeme zamanının kısalmasıyla gösterildiğini bildirmiştik. Bu çalışmamızda da üç grup vardır: Kontrol, epilepsi ve profilaksi grupları. Epilepsi grubundaki deneklere i.v. DL metiyonin DL sulfoksimin verilerek yaygın epileptik nöbetler oluşturuldu. Profilaksi grubundaki deneklere epileptik madde verilmeden onbeş dakika önce intra venöz olarak alfa tokoferol uygulandı. Üçüncü gruptaki deneklerin hiç birinde nöbet görülmedi. Epileptik madde uygulanmasından otuz dakika sonra son iki gruptaki sıçanlar dekapite edildi. Aynı bölgeden alınan hemisferik beyin dokusu elektron mikroskopla histolojik olarak incelendi. Klinik ve nöropatolojik sonuçlar DL metiyonin DL sukfoksiminle meydana gelen epilepsinin durdurulabileceğini ve epilepsiye bağlı doku ve hücre hasarının vitamin E verilmesiyle engellenebileceğini göstermiştir.

**Anahtar Sözcükler:** Elektron mikroskobu, epilepsi, epileptik hücre hasarı, glutamat, vitamin E

## INTRODUCTION

Vitamin E (alpha-tocopherol) seems to be the first line of defense against peroxidation of cellular and subcellular membrane phospholipids such as those of mitochondria, endoplasmic reticulum and plasma membranes (17). This antioxidant activity of vitamin E is thought to be caused by three main moleculer mechanisms; first, reduction of lipid oxidants; second, stabilization of the moleculer components of the membrane lipid bilayer through formation of complexes between tocopherol and the polyunsaturated fatty acids in the membrane; and third, quenching of nonlipid radicals such as singlet oxygen. Thus the biochemical action of vitamin E appears to be prevention of peroxidative damage to cellular and subcellular elements, which thereby preserves the organelles necessary to cope with disease, physical or chemical environmental insults and other stresses including the effect or cause of epilepsy (17,26,29 ).

In our preceding study we have also discussed in detail on a preventing effect of alpha-tocopherol on the experimentally induced clinical epilepsy and its influences on the cerebral vascular permeability with its subsequent effect on water and protein concentration -the brain edema fluid or exudate- in brain tissue (10).

In this present study we aimed to investigate in greater detail using the electron microscopy the influence of vitamin E on the extent and type of structural damage that occur in the rat cerebral hemispheric tissue after DL-methionin DL-sulfoximine- induced epilepsy.

## MATERIALS AND METHODS

Twenty-one adult male Swiss albino rats of the same age weighing 200-250 g were used. They were randomly divided into three groups (The first group is the control group, the second is the epilepsy group, the third group is the prophylaxis group). Each group consisted of seven rats. Animals were anesthetized with diethyl ether. Catheters filled with 100 IU heparin in isotonic saline (0.9 % w/vol NaCl) were inserted into a femoral vein and artery. Each animal in the control group also received the same amount of isotonic saline (0.9 % wt/vol NaCl). The hindquarters of the animals were immobilized in a loosefitting plaster cast and the rats allowed to recover. The body temperature was monitored with a rectal temperature probe, and external heat lamps were utilized to maintain body temperature at 35-37° C.

When the animal was entirely un conscious 30 minutes after ether anestesia, the rats in the control group were decapitated. Brains were quickly removed from the calvaria and freed from the cerebrospinal fluid (CSF).

Tissue samples were taken from the same cerebral frontal cortical-area in each animals, and prefixed in 2.5 % glutaraldehyde in phosphate buffer. They were postfixed in 1 % buffered osmium tetraoxide ($OsO_4$), en bloc-stained with uranyl acetate and Reynold's lead dye, dehydrated and embedded in epon 812. For histopathological examination a JEOL 1200 electron microscope was used. Animals in the epilepsy group were given a single rapid intravenous injection of DL-methionine DL-sulfoximine [ DL - [3 - Amino - 3 carboxypropyl ] methyl sulfoximine , crystalline [ 1987 - 67- 8 ] $C_5H_{12}N_2O_3S$ FW 180.2, Sigma, product number M 9503 ] 200 mg/kg dissolved in water given 30 minutes after ether anestesia to induce epileptic seizure ( 10 ) . In all rats, a single rapid intravenous administration of DL-methionine DL-sulfoximine resulted in immediate generalized tonic-clonic convulsions with a mean duration of 120±20 s. No significant difference in seizure pattern and duration was observed. At the end of the experiments, i.e. 30 minutes after DL-methionine DL-sulfoximine, all of the seven rats included in this epilepsy group were decapitated and prepared in the same manner used in the first group to examine in an electron microscope.

In the third prophylaxis group all rats were given a single intravenous administration of vitamin E [a - Tocopherol nicotinate (pts) (Vitamin E nicotinate) [ 51898 - 34 - 1 ] $C_{35}H_{53}NO_3$ FW 535.8 Sigma, Product number T 5134] 30 mg/kg at normal speed. During and after the injection of vitamin E no significant reaction was observed. Fifteen minutes later a single rapid i.v. injection of DL-methionine DL-sulfoximine 200 mg/kg dissolved in water, was given. However, none of the animals developed any kind of epileptic seizure, in contrast to the second group. The consciousness level of the animals was not in any way affected. Thirty minutes after the injection of the epileptic agent, the animals were decapitated and their brain tissues were prepared and dealt with in the same fashion utilized in the first and second group of this study to undertake the electron microscopic examination.

## RESULTS

The cerebral hemispheres of animals in each group looked macroscopically normal. Animals in the control group showed by electron microscopy a closely woven structural relation formed by outer membrane of endothelial cells, their tight junctions, vascular basal membrane, outer membrane of the astrocytes, dense contact between them and finally good connections of astrocytes with neuron cells; all of these which are known to establish the blood-brain barrier, showed by electron microscopy no difference from what is generally considered normal (Figure 1).
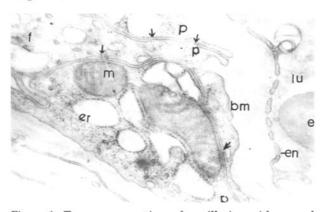


Figure 1: Transverse section of capilleries with normal blood - brain barrier in the control group: erytrocyte (et), endothelial cells (en) are seen in the capillary lumen ( lu ). Around the basal membrane ( bm ) processes of astrocytes ( p ) and dense contact between them (↓) are clearly seen. Mitochondria ( m ), endoplasmic reticulum ( er ), fibrils ( f ) and other organels in the astrocytic cytoplasm (X30000).

Electron microscopic examination of the animals in the second group showed that epilepsy induced by DL-methionine DL-sulfoximine administration to rats produced narrowing and fragmentation of the basal membrane; the endothelial cells were swollen. Perivascular tissue edema was present. These pathologic changes were not only in the basal membrane but also in the neuropil. Depending on the density of edema the relation between astrocytes and vasculature were demolished (Figure 2). In some vessels endothelial cells appeared in ovoid and lengthened-shape together with narrowed basal membrane. Separation and loss of the tight junctions could be seen. Within the vessels stasis existed (Figure 3). As a sign of reduced functional activity there were swelling of

endoplasmic reticulum including loss of the ribosomes and homogenity of the mitochondria; in places increases in neuropathologic alterations were encountered such as distropic microvacuoles and pinocytic vesicles, massive swelling of endothelial cells loosing contact to each other due to large increase in extracellular space caused by edema. In
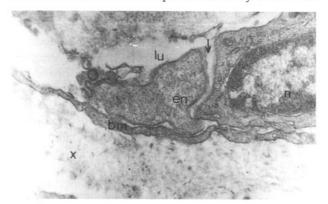


Figure 2: Microphotograph through part of cerebrovascular structures in the epileptic brain tissue: Basal membranes ( bm ) are electron - dense and narrowed. Their endothelial cells ( en ) are swollen and spaces between them widened (↓). Some intravascular stasis and perivascular edema exist ( X ); white appearances present edema. Connection between astrocytes and vessels are disturbed. Vascular lumen (lu), nucleus of endothelial cell (n) (X33000).
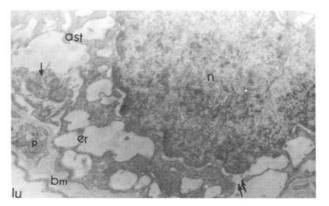


Figure 3: Microphotograph of cerebrovascular structures in the epileptic brain tissue: Basal membrane (thin and white) ( bm ) next to astrocyte ( ast ) astrocytic processus ( p ) appears to be narrowed and fragmentated due to destructive epileptic changes. Endothelial cells are disoriented and separated in favor of extracellular space. Uneven nucleus ( n ); endoplasmic reticulum ( er ) habouring organels brokendown inside (↓) are widened and fragmentated (↓↓), ( lu ) vascular lumen (X 35000).

the subendothelial parts plasma proteins, fibrin and diapedesis in some places were seen . In the vicinity of these vascular changes the vessels were surrounded by swollen astrocytic processes and cytoplasma. The outer membrane of astrocytes was generally disturbed. Swollen mitochondria with attenuated matrix and loss of their crests, but increased number of the lysosomes were encountered. This neuropathological alterations with dense edema, plasma proteins and fibrin caused an increased volume of the extracellular compartment between astrocytes and neurons.

Animals in the prophylaxis group showed generally thickened widened basal membrane without any fragmentation (Figure 4). The outer membrane of the endothelial cells could be clearly discerned. Tight junctions were so strongly narrowed that their compact contact areas came into view like a line. There was very few perivascular infiltration (Figure 5). The outer membrane of the astrocytes could be sharply seen. There was no any astrocytic edema; astrocytes seemed to be closely connected. As a sign of having some activation the cytoplasmic organelles showed no abnormality.

## DISCUSSION

The investigations established over the past two decades indicate that the amino acid glutamate functions as a fast and most important excitatory neurotransmitter in the central nervous system (CNS) of the mammalian (4,8). This action of glutamate within the CNS is · mediated by four pharmacologically distinct receptor subtypes;
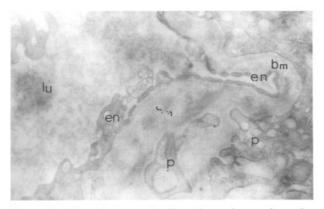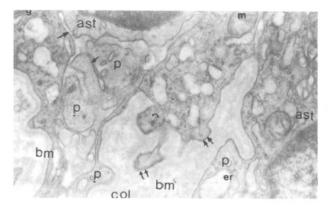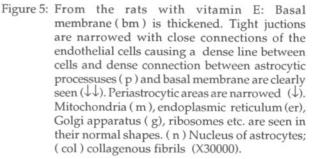


Figure 4: Microphotograph of basal membrane from the vitamin E - injected rats: basal membranes ( bm) are seen thickened with very few perivascular infiltration. Minimal destructive changes in endothelial cells ( en ) and astrocytic processus ( p ). Capillary lumen ( lu ) (X30000).



Figure 5: From the rats with vitamin E: Basal membrane ( bm ) is thickened. Tight juctions are narrowed with close connections of the endothelial cells causing a dense line between cells and dense connection between astrocytic processuses ( p ) and basal membrane are clearly seen ( $\downarrow\downarrow$ ). Periastrocytic areas are narrowed ( $\downarrow$ ). Mitochondria ( m ), endoplasmic reticulum (er), Golgi apparatus ( g), ribosomes etc. are seen in their normal shapes. ( n ) Nucleus of astrocytes; ( col ) collagenous fibrils (X30000).

kainate, N-methyl-D-aspartate (NMDA), and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors; these three receptors are linked to membrane ion channels and the fourth receptor subtype, the metabotropic (or $GLU_G$) stimulates phosphatidylinositol turnover (5). Recent experiments have provided definitive evidence supporting a hypothesis that glutamate is a powerful neurotoxin, capable of killing neurons when its extracellular accumalation is sufficiently high (6,7). This neurotoxic effect is thought to be mediated predominantly via the excessive activation of NMDA receptor causing, the lethal influx of calcium through the NMDA channels (2,13,16). Therefore the concept that either blockade of of synaptic transmission or the specific antagonism of excitatory glutamate receptors attenuates the transmembrane ionic fluxes and that the drugs which decrease the accumulation of glutamate or block its postsynaptic effects may be a rational therapy offers a therapeutic approach to neurological disorders such as stroke, cerebral palsy, epilepsy, aging and Alzheimer's disease, Huntington's disease, and other chronic degenerative disorders (5,20,21).

In our present study as did in the previous study DL-methionine DL-sulfoximine proved again to be an epileptic seizure-inducing agent which inhibits the glutamine synthetase. This is a key enzyme for

handling ammonia and glutamate in the brain. Glutamine is formed from these substances, induced by glutamine synthetase to remove ammonia from the brain tissue. If this enzyme does not act, the excitatory neurotransmitter glutamate accumulates around in large amounts in the microenvironment of functional neurons and may also act as a potent excitotoxin leading to seizures and to gray matter edema in the acute phase or/and to neuronal death and glial scar in the chronic phase (1,4,5,6,7,12,20,21,). A neurological syndrome has been observed in rats with severe and prolonged experimental vitamin E deficiency, comprising muscle weakness, tremor, ataxia and hyperaesthesia in association with the development of axonal dystrophy and a disturbance of axonal transport (23).

Kovalenko et al. (11) showed in an uncontrolled, nonblinded clinical trial therapeutic efficacy of add-on therapy of vitamin E in a therapy-resistant epileptic population. Ogunmekan and Hwang (19) also suggested in a double-blind placebo controlled add-on trial a significant improvement in seizure control in therapy-resistant epileptic children when the active group was compared to the placebo group. In other clinical studies in children confirmed reduced plasma levels of vitamin E without age-related increase in seizure patients; however brain mechanisms underlying epilepsy are not completely understood as yet (15,18,19).

As shown in our previous as well as present study, in some experimental models, vitamin E reduces or prevents seizure activity if it is given prior to administration of epileptic agents; the mechanism of changes induced by vitamin E addition is not exactly known. That is mostly attributed to its important protective role on membrane integrity which has been suggested to be mediated by free radical scavenging and a stabilizing structural role of vitamine E (4,9,10,24,27,28). Although a contrary report is to also be encountered in the literature (22).

First of all, the results of this second part of our study are in agreement with those of the first part which has dealt with the protective effect of prior vitamin E administration on occurrence of both clinical seizures and intensity of brain edema fluid in experimental epileptic lesions induced by intravenous injection of DL-methionine DL-sulfoximine (10). Our present observations on the histopathologie changes can answer the question raised in the first part of the study positively, as to whether vitamin E prevents the arterial wall from

histopathological changes such as endothelial degeneration and edema, constriction internal lamina and ultimately brain edema and vascular degeneration of media.

Our study has been aimed to draw attention to the relation between astrocytes and vessels since metabolic exchanges between blood and neuron cells do not occur directly but via astrocytes which have been suggested also to play an important role in antioxidative processes in the brain (14). Extracellular diffusion is selectively hindered by the blood-brain barrier and the metabolites circulate intracellular between endothelium, astrocyte and neuron.

The present results obviously indicate that the prophylactic administration of alpha-tocopherol to rats prevents both induction of epilepsy following the administration of a glutamine synthetase inhibitor, DL-methionine DL-sulfoximine, and occurence of seizure related histopathologic perturbations similar to ischemic brain tissue. These two responses must be considered in view of the highly significant correlation between epileptic activity and brain edema. One can speculate that either brain edema can trigger epileptic activity or that epileptic activity can lead to or enhance brain edema or that both phenomena are caused by and are quantitatively dependent on a common tissue disorder (12). In our observations obtained by administration of a glutamine synthetase inhibitor which should cause an excessive extracellular accumulation of an excitotoxin, glutamate, in large amounts we suggest that the last hypothesis should be considered (3,20,21,25). In our present study it can be assumed that vitamin E have in some way prevented the glutamate from acting as potent excitatory neurotransmitter as described in detail above. However we can not certainly exclude the possibility that this epileptic agent was not allowed to pass through the capillary basal membrane, to the blood-brain barrier to inhibit the glutamine synthetase in the extracellar spaces of neuropil since as also shown in our specimens the tocopherol stabilizes the state of the membrane. Therefore we can not conclude at which level the vitamin E affects the process. Further studies on this epilepsy model may be relevant for an understanding of the mechanism by which the epilepsy occurs or is inhibited. In order to shed light on this matter we are also investigating the correlation between the plasma concentration of the vitamin E and the tissue concentration of glutamate yet. In the light of the results of our both studies on this subject we should emphasize at least the

importance of the other attempts to find out the understanding of the relationship between vitamin E and glutamate receptor channel ionic selectivity.

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