Edaravone Ameliorates Valproate-Induced Gingival Toxicity by Reducing Oxidative-Stress, Inflammation and Tissue Damage

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ABSTRACT
Valproic acid (2-n-propylpentanoic acid, VPA), the most widely used antiepileptic drug, has potential adverse effects and it can disrupt the oxidant and antioxidant balance. Edaravone (3-methyl-1-phenyl-2-pyrazoline-5-one, EDA) is a potent free radical scavenger. In this study, the effect of EDA on gingiva in VPA induced toxicity was investigated. Female Sprague Dawley rats were randomly divided into four groups: control group, EDA (30 mg/kg/day) given group, VPA (0.5 g/kg/day) given group, and VPA+EDA (in same dose and time) given group. EDA and VPA were given intraperitoneally for seven days. Total protein, lipid peroxidation (LPO), sialic acid (SA) and reduced glutathione (GSH) levels and catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD), myeloperoxidase (MPO), alkaline phosphatase (ALP), acid phosphatase (ACP), sodium potassium ATPase (Na’/K’-ATPase) and tissue factor (TF) activities were determined in gingiva homogenates. The VPA-induced increases were statistically significant for MPO (p<0.01), ACP (p<0.01), Na’/K’-ATPase (p<0.05) and TF (p<0.01) activities, but not for LPO level and ALP activities. EDA treatment markedly blunted all such elevated anomalies. Conclusively, VPA induced oxidative and inflammatory gingival tissue damage, reactions that were appreciably reversed by concurrent administration of EDA.

Keywords: Gingiva, valproic acid, edaravone, oxidative stress, inflammation

INTRODUCTION
Valproic acid (2-n-propylpentanoic acid, VPA) is the most widely used antiepileptic drug in the treatment of certain types of seizures (1). Although VPA has good efficacy and it is often well tolerated, it has several side effects on the neurological, gastrointestinal, hematological and reproductive systems. The most common symptoms of VPA toxicity include anorexia, nausea, vomiting and also hyperammonemic encephalopathy, pancreatitis, elevated liver enzymes, leukopenia, thrombocytopenia, coagulation disorders and development of coma (2). The toxicity of VPA has been investigated in various studies, but the knowledge about the incidence and occurrence of these effects is still unclear (3-7). Besides the damage caused to other tissues, taking VPA can cause primarily gingival overgrowth and other dental problems (8).

Epilepsy is a chronic neurologic disorder and affects 1-3 % of the population. Most epilepsy patients receive long-term
anticonvulsant therapy and may require medications for other transient conditions on body. Both epilepsy and its medical management may affect oral health. Therefore, it is important to consider the adverse effects of anticonvulsant usage on tissues (9). Evidence suggests that the incidence of dental disease is increased in epileptic patients, thus they have tendency of dental disease and increased oral health needs. The positive correlation between severity of seizure and periodontal disease was shown in a study with refractory epilepsy patients (10).

Edaravone (3-methyl-1-phenyl-2-pyrazoline-5-one, EDA) is a potent free radical scavenger, and has been widely used to treat acute ischemic stroke. In experimental studies EDA has been suggested to show protective effects on inflammation and tissue damage by eliminating the deleterious effects of oxidants in various tissues (11-13). It acts as an antioxidant by quenching hydroxyl radicals and by preventing lipid peroxidation caused by hydroxyl radicals. In this study, the effect of EDA in VPA induced toxicity was investigated on gingiva by some biochemical parameters.

MATERIAL and METHODS
All experimental protocols were approved by the Marmara University Animal Care and Use Committee (134.2013.mar). VPA (Merck, Germany) and EDA (Fluka, Switzerland) were dissolved in saline (0.9% NaCl) separately. Thirty seven female Sprague Dawley rats were randomly divided into four groups as follows; Control group (n=8), EDA (30 mg/kg/day) given group (n=8), VPA (0.5g/kg/day) given group (n=10), VPA + EDA (in the same dose) given group (n=11). VPA was given to the animals one hour later than the EDA administration everyday. EDA and VPA were administered to the experimental group intraperitoneally for 7 days. On the 8th day of the experiment, animals were fasted overnight and then sacrificed under anesthesia. Gingiva samples were homogenized in saline (0.9% NaCl) in a bowel filled ice cubes for 15 second at high speed by using by Ika-Werkrw-14 homogenizer to obtain 10% (w/v) homogenates. Oxidant and antioxidant parameters; lipid peroxidation (LPO) and reduced glutathione (GSH) levels, superoxide dismutase (SOD), glutathione-S-transferase (GST), catalase (CAT), glutathione peroxidase (GPx) and myeloperoxidase (MPO) activities, inflammation, bone resorption and thrombosis parameters; sialic acid (SA) levels, Na-K ATPase, alkalen phosphatase (ALP)-acide phosphatase (ACP), tissue factor (TF) activities were determined in gingiva homogenates.

**Determination of total protein**
The method of Lowry (14) was used to determined total protein levels of gingiva samples. Briefly, alkali proteins are reacted with copper ions and then reduced by Folin reative. The absorbance of the product was evaluated at 500 nm spectrophotometrically and calculated to express the results of the parameters per protein.

**Determination of LPO**
Malondialdehyde (MDA), results from LPO, was determined as thiobarbituric acid reactive substances by the method of Yagi (15). The extinction coefficient of 1.56 x 10^5 M^{-1} cm^{-1} was used and LPO was expressed in terms of MDA equivalents as nmol MDA/mg protein.

**Determination of GSH**
The method of Beutler (16) was used to determine GSH levels of gingiva samples. Metaphosphoric acid was used for protein precipitation and 5,5′-Dithiobis-2-nitrobenzoic was used for color development. For calculation, the extinction coefficient of 1.36 x 10^4 M^{-1} cm^{-1} was used and results were expressed as mg GSH/g protein.

**Determination of SOD activity**
The method based on the ability of SOD to increase the effect of riboflavin-sensitized photo-oxidation of o-dianisidine was used to determine SOD activities in gingiva samples. The absorbance of the product was measured in 460 nm spectrophotometrically. The net absorbance was calculated by measuring absorbances at 0 and 8th minutes of illumination. The results were expressed as U/mg protein (17).

**Determination of GST activity**
GST is an antioxidant enzyme that catalyzes the conjugation of GSH. The absorbance of the mixture is measured in 340 nm spectrophotometrically and GST activities were calculated by using the extinction coefficient of 9.6 mM^{-1} cm^{-1}. Results were expressed as U/g protein (18).

**Determination of CAT activity**
The method of Aebi (19) was used to determine CAT activities of gingiva samples. The method is based on converting hydrogen peroxide (H_2O_2) to water by the effect of CAT. The absorbance was measured in 240 nm spectrophotometrically and results were expressed as U/mg protein.

**Determination of GPx activity**
The method of Paglia and Valentine (20) was used to determine GPx activities of gingiva samples. Glutathione reductase reduced glutathione disulfide to GSH. During the
oxidation of NADPH to NADP, the decreasing absorption was measured in 366 nm spectrophotometrically. For calculation, the extinction coefficient of 6.22 mM⁻¹cm⁻¹ was used and results were expressed as U/g protein.

**Determination of SA**

The method of Warren (21) was used to determine SA levels of gingiva samples. The hydrolysate was prepared by incubation of the homogenates with 0.1N H₂SO₄ at 80°C for 1 hour. In concentrated phosphoric acid, SA is oxidized with sodium periodate and the product of periodate oxidation is coupled with thiobarbituric acid. The formed chromophore is extracted into cyclohexanone and the absorbances of gingiva samples were evaluated in 549 nm spectrophotometrically. The results were expressed as mg SA/g protein.

**Determination of MPO activity**

The method of Wei and Frenke (22) was used to determine MPO activities of gingiva samples. The method contains a solution of homogenate, phenol, H₂O₂ and 4-Aminoantipyrine. The change in absorbance of reaction mixture per minute at 460 nm was recorded. The results were expressed as U/g protein.

**Determination of ALP and ACP activity**

The method of Walter (23) was used to determine ALP and ACP activities of gingiva samples. Depending on the pH of the medium, ACP and ALP hydrolyze p-nitrophenyl phosphate to p-nitrophenol. The absorbances were measured in 405 nm spectrophotometrically and ALP and ACP activities were expressed as U/g protein.

**Determination of Na⁺/K⁺-ATPase activity**

The method of Riddertap (24) was used to determine Na⁺/K⁺-ATPase activities of gingiva samples. The reaction solution contained 5 mmol/L KCl, 150 mmol/L NaCl, 2.5 mmol/L MgCl₂ and 0.1mmol/L EDTA in 600 ml of 20 mmol/L imidazole buffer. 0.5 ml of ghost suspension was added and the mixture was placed for incubation in 37° for 10 min. Adding 0.1 ml of 2.5 mmol/L disodium ATP was started the reaction. After 1 h, the tubes were cooled and 1ml of 15% trichloroacetic acid was added to stop the reaction. Na⁺/K⁺ ATPase activities were expressed as mmol Pi/ mg protein per hour.

**Determination of TF activity**

The method of Quick (25) was used to determine TF activities of gingiva samples. Pooled plasma collected from healthy subjects was used. The reagents were brought to 37°C which was the reaction temperature. 0.1 ml tissue homogenate with 0.1 ml of 0.02 M CaCl₂ was mixed and by adding of 0.1 ml of plasma the clotting reaction being started. Since the clotting time is inversely proportional to the TF activity, the lengthening of the clotting time is a manifestation of decreased TF activity.

**STATISTICS**

All statistical analyses were performed using SPSS 15.0 (Statistical Package for the Social Sciences) Software program. Results were expressed as mean ± standart error of the mean (SEM). Groups of data were compared using ANOVA followed by Tukey’s multiple comparison tests. p <0.05 values were regarded as significant.

**RESULTS**

**Oxidant and antioxidant parameters**

The increase in LPO levels of the VPA group was not statistically significant (Fig.1A). There was a tendency towards decreased antioxidant status in VPA group as evidenced by insignificant decreases in GSH levels, SOD, CAT, GPx activities compared to controls (Fig.1B,1C,1E,1F). GST activities decreased significantly (p<0.01) in VPA group compared with the control group (Fig.1D). EDA was also increased GSH levels, SOD, GST, CAT, GPx activities and decreased LPO levels unsignificantly (Fig. 1A, 1B,1C,1D,1E, 1F).

**Inflammation, bone resorption and thrombosis parameters**

The VPA-induced increases were statistically significant for MPO (p<0.01) (Fig.2A), ACP (p<0.01) (Fig.2B), Na⁺/K⁺-ATPase (p<0.05) (Fig.2C) and TF (p<0.01) (Fig.2D) activities, but not for SA levels (Fig.2E) and ALP activities (Fig.2F) compared to control group. EDA treatment markedly blunted all such elevated anomalies. TF activity was expressed as seconds. Shorthened clot formation time shows increased TF activity. EDA treatment caused to significant decreases in MPO (p<0.01) (Fig.2A), ACP (p<0.01) (Fig.2B), Na⁺/K⁺-ATPase (p<0.05) (Fig.2C) and TF activities (p<0.01) (Fig.2D) in VPA group. EDA was also decreased SA and ALP levels unsignificantly (Fig.2E and Fig.2F).
Figure 1. Oxidant and antioxidant parameters in four groups. Values are given as mean±standard error; C: Control group; EDA: Edaravone given group; VPA: Valproic acid given group; VPA+EDA: Valproic acid and edaravone given group; P:protein; LPO: lipid peroxidation; GSH: glutathione; SOD: superoxide dismutase; GST: glutathione-S-transferase; CAT: catalase; GPx: glutathione peroxidase.

**p<0.01 significantly different from group C
Figure 2. Inflammation, bone resorption and thrombosis parameters in four groups.

Values are given as mean±standard error; C: Control group; EDA: Edaravone given group; VPA: Valproic acid given group; VPA+EDA: Valproic acid and edaravone given group; P: protein; MPO: myeloperoxidase; ACP: acid phosphatase; Na+/K+ ATPase: sodium-potassium ATPase; TF: tissue factor (the lengthening of the clotting time is a manifestation of decreased TF activity); SA: sialic acid and ALP: alkalen phosphatase.

*p<0.05, **p<0.01 significantly different from group C; °p<0.05, °°p<0.01 significantly different from group EDA;  +p<0.05, ++p<0.01 significantly different from group VPA
DISCUSSION

VPA is still the broadest spectrum used in antiepileptic drugs for all types of seizures in children and adults, although serious complications may occur in some patients due to toxic side effects of VPA. Gingival enlargement is highly seen in patients with gingivitis and periodontal diseases and is a common adverse effect of drugs such as calcium channel blockers, anti-convulsants, and immunosuppressants. Sodium valproate induced gingival enlargement in a patient with pre-existing chronic periodontitis has been reported (26). Twenty two months old child suffering from gingival enlargement following intake of sodium valproate has been reported (27). The association between chronic carbamazepine, valproic acid and phenytoin medication on the periodontal condition in epileptic children and adolescents was studied and it was reported that the gingival and sulcus bleeding indices were higher in epileptics than in controls, gingival enlargement was also found in 30% of the epileptic patients (28). In the study of Gurbuz and Tan, epileptic children have been shown to possess mental and motor deficits and also these patients were at risk for oral health due to side effects of anticonvulsant treatment. The dental conditions of epilepsy patients were significantly worse than non-epileptic age-matched groups. Furthermore, poorly controlled epilepsy patients had worse oral health compared to patients who had better controlled epilepsy (29).

Disrupted balance between oxidants and antioxidants cause oxidative stress, which results in the activation of antioxidant system to neutralize the toxicity in the cell. But excessive production of reactive oxygen species (ROS) leads to depletion of antioxidant enzymes, such as SOD, GST, CAT and GPx. These enzymes are part of protection system of the organism against harmful effects of ROS by detoxify peroxides. Antiepileptic drugs increase ROS by several mechanism and cause some damages in certain tissues (2,30,31). In our previous study, significantly decreased GST activities and also, although the results were insignificant, decreased SOD, CAT and GPx activities were found in the intestinal tissues of VPA group compared to controls (12). The results of this study were consisted with the previous studies related with the VPA toxicity (32-35). GSH is an effective nonenzymatic antioxidant factor in defence system. Although the result was insignificant, decreased GSH level was found in VPA treated group compared to controls. Reduced capacity of defence system may be a result of decreased levels of antioxidants in gingival tissue. Elevation in LPO levels was frequently seen in VPA treatment (7,30,35,36), which is an evidence of disruption of oxidant-antioxidant balance and reduced antioxidant defence by peroxides. In the present study, although insignificantly, increased LPO levels were found in VPA group compared to controls. Edaravone was an effective agent in eliminating the deleterious effects of VPA by increasing antioxidants. Unsignificantly decreased LPO levels and increased GSH levels and SOD, GST, CAT, GPx activities were found in edaravone given VPA groups compared to VPA group. Unsignificant LPO and GSH results may be related with the dose of VPA for the induction of damage in gingival tissue.

SA is a member of neuraminic acid derivatives and plays a crucial role in metabolic events. Increased levels of SA is known as an inflammation marker due to increased levels of sialylated acute-phase glycoprotein (37). MPO is an enzyme found in polymorphonuclear leucocytes and also used as a marker in inflammation. Anticonvulsant drugs may directly influence gingival connective tissues by stimulating an increase in number of gingival fibroblasts and production of connective tissue matrix (38). The inflammation causing the changes in the gingival tissues influence the interaction between drug and fibroblastic activity. Epithelial width, odema, infiltrates of lymphocytes and plasma cells were reported (38). In the present study, increased SA level in VPA group may be a protection way of gingival tissue and significantly increased MPO activity is an evidence of damaged gingival tissue by VPA caused inflammation. Oxidative damage, in turn, leads to the generation of oxidized molecules, which are bioactive and induce inflammation. There is a relationship between oxidative damage and inflammation in several stages during the pathogenesis of many diseases. The antioxidant effect of EDA and following decreasing of inflammation may cause the decrease in SA levels and MPO activities in VPA group.

Epilepsy patients are at risk of fracture due to enzyme-inducing antiepileptic drugs. These drugs alter the metabolism and cause osteopenia and osteomalacia by leading clearance of vitamin D (29). Anticonvulsant drugs cause changes in bone metabolism and calcium levels and these drugs may lead to a decrease in bone mass (39). In previous studies increased ALP activities were reported in serum and different tissues (40,41). Consistent with these results, insignificantly increased ALP activity was found in VPA group compared to controls. ALP, which is an important enzyme for calcification and altered activities can be useful to detect inflammation. Edaravone administration reduced ALP activity in VPA group in this study. As VPA has been shown to alter bone and calcium metabolism, the patients using VPA may have special dental needs during treatment.
ACP is an enzyme which is widely distributed in tissues. In many diseases, especially prostate cancer, diseases of bone, diseases of blood increased levels of ACP is found to be elevated (42). In the present study, significantly increased ACP activity was found in VPA group compared to controls. Applying of edaravone was effective in decreasing gingival ACP activity in the VPA group. 

Na+/K+-ATPase is a membrane bound enzyme and has been identified in various human tissues (43). The Na+/K+-ATPase, also known as the Na+/K+ pump, converts the free energy of ATP into transmembrane ion gradients. Also, it is a signal transducer that modulates cell energy-transducing ion pump on the plasma membrane protein. It does not only maintain the membrane potential of excitable neuron and different cells but also is involved in reabsorption of Na⁺ in the kidney (44) and in salivary glands (45). The interaction between the inducing drug and the fibroblastic activity is influenced by the inflammatory changes within the gingival tissues. Increasing gingival fibroblasts DNA synthesis may involve stimulation of Na+/K+ -ATPase activity including phosphorylation of Na⁺ channels. This may be the reason of the significant increase of Na⁺/K⁺-ATPase activity found in VPA group compared to controls in the present study. Na⁺/K⁺-ATPase activity was decreased in edaravone applied VPA group compared to VPA. EDA reversed this increase.

TF is a cell membrane component. It is a cellular initiator of coagulation and many tissues have TF activity (46-50). VPA cause hematological problems and coagulation defects are commonly seen in VPA treatment. VPA administration significantly increased TF activity compared to controls and decreased TF activity in gingival tissue was found in edaravone given VPA group compared to VPA group. Edaravone may be useful in reducing trombosis and coagulation problems.

**CONCLUSION**

To our knowledge, there are few reports published on the effects of antiepileptic drugs on oral tissues. Dentists should carry out dental treatments to epileptic patients under antiepileptic drug treatment. Increased oxidant and inflammation marker levels and decreased antioxidant enzyme activities suggest the damage in gingiva caused by VPA treatment. EDA was effective in these parameters and it may be useful to prevent side effects of VPA in gingival tissues. EDA can be added to the new pharmaceutical formulations and may be used to alleviate stomatitis, gingivitis and buccal inflammations in dental clinics. However, further investigations in details are necessary.

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**Edaravone, valproik asit nedeniyle oluşan dişeti toksisitesini, oksidatif stresi, inflamasyonu ve doku hasarını azaltarak düzeltebilmektedir**

**ÖZ**


**Anahtar kelimeler:** Dişeti, valproik asit, edaravon, oksidatif stres, inflamasyon

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