Protective role of Edaravone against valproic acid induced changes in skin

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Valproic acid (VPA) is an antiepileptic drug known to have some adverse effects on gastrointestinal, neurologic, hematologic and reproductive systems. In this study, we investigated the effect of edaravone (3-methyl-1-phenyl-2-pyrazoline-5-one), a potent free radical scavenger, on skin oxidative and inflammatory changes caused by VPA. Female rats were randomly divided into four groups: control, edaravone (30 mg/kg/day, i.p.), VPA (0.5 g/kg/day, i.p.), and VPA+edaravone (in same dose). On the 8th day of experiment, all the animals were fasted overnight and then sacrificed under anesthesia. Malondialdehyde, glutathione, total sialic acid and total protein levels, superoxide dismutase, glutathione-S-transferase, catalase, glutathione peroxidase, Na+/K+ATPase, myeloperoxidase and tissue factor activities were determined in homogenized skin samples. VPA administration significantly altered the skin oxidant and antioxidant balance which may cause skin cell damage. Edaravone, as an antioxidant, demonstrated its radical scavenger effect especially on malondialdehyde level and glutathione-S-transferase activity. Edaravone, by preventing inflammatory and oxidative reactions, partly protected the skin against the changes observed after the VPA administration.

Keywords: Adverse drug reaction, Antiepileptic drug, Oxidative damage, Scavenger, Side effects, Tissue Factor Activity

Valproic acid (2-propylvaleric acid, 2-propylpentanoic acid or n-dipropyl acetic acid, VPA) is a well-known drug for treatment of convulsive seizures related to epilepsy, as a mood stabilizer and in the treatment of schizophrenia1. The use of VPA can be limited by either loss or lack of efficacy, or by adverse drug reactions. The adverse drug reactions associated with the VPA usage include drowsiness, dizziness, headache, diarrhea, constipation, heartburn, changes in appetite, weight changes, back pain, agitation, mood swings, abnormal thinking, memory loss, body tremor, loss of coordination, uncontrollable movements of the eyes, blurred or double vision, ringing in the ears, stuffy or runny nose, sore throat and hair loss2. VPA is also associated with the adverse drug reactions including purple spots on the skin, cases of stomatitis, cutaneous leukoclastic vasculitis, and psoriasiform eruption3,4. As the skin is constantly under attack by reactive oxygen species (ROS) from both endogenous and exogenous sources5, VPA can disrupt the oxidant and antioxidant balance of the skin. Studies on the oxidative role of VPA, both in human and animal models are not uncommon6,9. In these reports, the generation of oxidative damage is associated with VPA, as a consequence of VPA biotransformation6, alterations in glutathione homeostasis6,8, and/or depletion of co-factors required for antioxidant defense.

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is drug that acts as an antioxidative radical scavenger, is used in the treatment of cerebrovascular diseases9. In addition, edaravone has been reported to have protective effects on inflammation-induced pain and cisplatin-induced nephrotoxicity in rats9,10. However, effects of edaravone on skin antioxidant metabolism after the VPA treatment has not been investigated until now. Therefore in this study, we evaluated the possible antioxidative effect of edaravone on skin following the VPA administration.

Material and Methods

All experimental protocols were approved by the Marmara University Animal Care and Use Committee (134.3013 mar). Thirty-seven Sprague Dawley female rats were randomly divided into four groups as follows: Control; edaravone (30 mg/kg/day); VPA (0.5 g/kg/day); and VPA + edaravone (same dose). Edaravone, VPA and VPA+edaravone were given

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intra-peritoneally (i.p.) for 7 days. VPA was given to the animals one hour after the edaravone administration every day. On the 8th day of the experiment, animals were fasted overnight and then sacrificed under anesthesia. The skin samples were taken from the back (dorsal side) of each rat after removing the local fur. After the epidermis was removed, the skin samples were homogenized in 0.9% NaCl solution. Homogenates [10% (w/v)] were prepared. Total protein, lipid peroxidation (LPO), glutathione (GSH), sialic acid (SA) levels and glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), myeloperoxidase (MPO), sodium potassium ATPase (Na+/K+ ATPase) and tissue factor (TF) activities were determined in skin homogenates.

**Determination of total protein**

Total protein levels of tissues were determined by the method described by Lowry et al.\textsuperscript{11}. Briefly, in alkali medium proteins are reacted with copper ions and reduced by Folin reactive. The absorbance of the blue colored product was evaluated at 500 nm. Bovine serum albumin was used as a standard and total protein levels of skin tissues were used to express the results of the parameters per protein.

**Determination of lipid peroxidation**

Malondialdehyde (MDA), one of the products of LPO in tissues, was determined as thiobarbituric acid reactive substances according to the method described by Yagi.\textsuperscript{12} LPO was expressed in terms of MDA equivalents using the extinction coefficient of 1.56 × 10^5 M$^{-1}$ cm$^{-1}$. Results of skin tissues are expressed as nmol MDA/mg protein.

**Determination of glutathione**

GSH levels were determined in skin tissues according to the method of Beutler\textsuperscript{13} using metaphosphoric acid for protein precipitation and 5,5-dithiobis-2-nitro benzoic acid for color development. The extinction coefficient of 1.36 × 10^4 M$^{-1}$ cm$^{-1}$ was used for calculation of GSH levels of skin tissues and results were expressed as mg GSH per gram of protein.

**Determination of sialic acid**

SA levels of skin tissues were determined by the thiobarbituric acid method of Warren.\textsuperscript{14} Homogenates were incubated with 0.1 N H$_2$SO$_4$ at 80°C for 1 h and hydrolysate was used for analysis. SA was oxidized with sodium periodate in concentrated phosphoric acid and the product of periodate oxidation was coupled with thiobarbituric acid, and the resulting chromophore was extracted into cyclohexanone. The absorbances of samples were measured at wavelength of 549 nm and the results were expressed as mg SA/g of protein.

**Determination of glutathione peroxidase activity**

GPx activity of skin tissues were determined by the method of Paglia and Valentine\textsuperscript{15}. In this method, the conversion of H$_2$O$_2$ to water is catalyzed by GPx. The generated glutathione disulfide is reduced to GSH with consumption of NADPH by glutathione reductase. The decreased absorbance during the oxidation of NADPH to NADP was measured by spectrophotometer at 366 nm. The extinction coefficient of 6.22 mM$^{-1}$ cm$^{-1}$ was used for calculation and results were expressed as U/g protein.

**Determination of glutathione S transferase activity**

GST catalyzes the conjugation of GSH\textsuperscript{16}. The absorbance of mixture at 25°C was measured by spectrophotometer at 340 nm. GST activities of skin tissues were calculated by using the extinction coefficient of 9.6 mM$^{-1}$ cm$^{-1}$ and results were expressed as U/g protein per min.

**Determination of superoxide dismutase activity**

SOD activities were determined by the method\textsuperscript{17} based on the ability of SOD to increase the effect of riboflavin-sensitized photooxidation of o-dianisidine. The activity of superoxide is generated by illuminating the reaction mixture which contains o-dianisidine dihydrochloride and riboflavin by light of a fluorescent lamp. The oxidation of o-dianisidine, as sensitized by riboflavin, is enhanced by SOD and the increase is linearly dependent on SOD concentration. The absorbance of the coloured product is quantified by spectrophotometer at 460 nm. Absorbances at 0 and 8 min of illumination were measured and the net absorbance was calculated. Bovine SOD (Sigma Chemical Co, S-2515-3000 U) was prepared as reference and the results were expressed in U/mg protein.

**Determination of catalase activity**

CAT activities of skin tissues were determined by the method of Aebi\textsuperscript{18} which is based on the conversion of hydrogenperoxide (H$_2$O$_2$) to water by the effect of CAT enzyme. This conversion was observed as a decrease in absorbance measured at 240 nm and CAT activity of samples were expressed as U/mg protein.
Determination of myeloperoxidase activity
MPO is a natural constituent of primary granules of neutrophils. Enzymatic activity of MPO was determined by the method of Wei and Frenke, which contains a solution of tissue homogenate, phenol, H2O2 and 4-AAP as colour generating substance. One unit of enzyme activity was defined as the amount of the MPO present per gram of protein which caused a change in absorbance per minute at 460 nm and 37°C. The results were expressed as U/g protein.

Determination of Na+/K+-ATPase activity
Na⁺/K⁺-ATPase activity was measured by the method of Riddersnap & Bontinck. The reaction medium for total standard reaction mixtures contained 5 mM KCl, 150 mM NaCl, 2.5 mM MgCl₂ and 0.1 mM EDTA in 600 mL of 20 mM imidazole buffer. The ghost suspension was added in a volume of 0.5 mL and the mixture was placed in a water bath at 37°C for 10 min. The reaction was started with the addition of 2.5 mM of disodium ATP in a volume of 0.1 mL. At the end of 1 h, the tubes were placed in an ice bath. To stop the reaction, 1 mL of 15% trichloroacetic acid was added. Pi released by the reaction was measured. Na⁺/K⁺-ATPase activities were expressed as µmol of Pi per milligram of protein per hour.

Determination of tissue factor activity
TF activities of skin tissues were determined by Quick’s one stage method. Pooled plasma collected from healthy subjects was used and all reagents were brought to 37°C, which is the reaction temperature, before admixture. TF activity of skin tissues were performed by mixing 0.1 mL homogenate with 0.1 mL of plasma, then the clotting reaction being started in addition of 0.1 mL of 0.02 M CaCl₂. The clotting time is inversely proportional to the TF activity, and for that reason the increase of the clotting time is a manifestation of decreased TF activity. TF results were expressed as seconds.

Statistical analysis
Statistical analyses were carried out using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). All data were expressed as mean ± SD (standard deviation). Groups of data were compared using ANOVA followed by Tukey’s multiple comparison tests. Values of P < 0.05 were regarded as significant.

Table 1—Skin MDA level and SOD and CAT activities

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<th>C+E</th>
<th>VPA</th>
<th>VPA+E</th>
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<td></td>
<td>(n=6)</td>
<td>(n=6)</td>
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<tr>
<td>MDA (nmol/Mg p)</td>
<td>Mean/SD</td>
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<tr>
<td>1.89/0.36</td>
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<td>SOD (U/mg p)</td>
<td>4.12/0.06</td>
<td>3.32/0.37</td>
<td>3.40/0.37</td>
<td>3.57/0.72</td>
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<tr>
<td>CAT (U/mg p)</td>
<td>36.00/6.07</td>
<td>37.33/7.95</td>
<td>35.49/4.14</td>
<td>36.00/8.72</td>
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Table 2—Skin TP, SA levels and TF, Na⁺/K⁺-ATPase, MPO activities

<table>
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<th>VPA</th>
<th>VPA+E</th>
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<td></td>
<td>(n=6)</td>
<td>(n=6)</td>
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<tr>
<td>TP (µg/g wet tissue)</td>
<td>Mean/SD</td>
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<td>2.08/0.34</td>
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<td>SA (mg/g)</td>
<td>Mean/SD</td>
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<td>13.60/3.85</td>
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<td>39.32/4.75</td>
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<tr>
<td>TF (sec)</td>
<td>Mean/SD</td>
<td>Mean/SD</td>
<td>Mean/SD</td>
<td>Mean/SD</td>
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<tr>
<td>122.8/33.55</td>
<td>101.5/24.19</td>
<td>70.83/21.94</td>
<td>126.7/21.07b</td>
<td></td>
</tr>
<tr>
<td>Na⁺/K⁺-ATPase (µmol Pi/mg p h)</td>
<td>Mean/SD</td>
<td>Mean/SD</td>
<td>Mean/SD</td>
<td>Mean/SD</td>
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<tr>
<td>0.28/0.05</td>
<td>0.26/0.07</td>
<td>0.15/0.03c</td>
<td>0.21/0.06</td>
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<tr>
<td>MPO (U/g p)</td>
<td>Mean/SD</td>
<td>Mean/SD</td>
<td>Mean/SD</td>
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<td>3.20/0.64</td>
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<td>5.92/1.64c</td>
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Results
Administration of VPA to the rats did not significantly change the skin total protein. MDA level increased, though insignificantly, in the VPA administered group compared to the control group. Similarly, VPA did not significantly change GSH levels and GPx, SOD and CAT activities when compared with the control group (Table 1 and Fig. 1). SA levels, TF and MPO activities significantly increased with the administration of VPA (Table 2). VPA also decreased the Na⁺/K⁺-ATPase activity (Table 2).

Edaravone administration to VPA group, significantly decreased MDA level and TF activities (Table 1). It also significantly decreased MPO level (Table 1) in VPA administered group. GST activity significantly increased with the edavarone administration to the VPA administered group (Table 1).
and Fig. 1). Edaravone administration to the control group also significantly increased GSH and GPx level (Fig. 1). Edaravone also increased GPx level in VPA administered group but this increase was not significant.

**Discussion**

The antiepileptic drug, valproic acid (VPA) treatment may cause some skin diseases such as stomatitis, cutaneous leukoclastic vasculitis, and psoriasiform eruption. Here, we investigated the possible effects of VPA and/or edaravone on skin, in terms of oxidative stress and tissue damage. As the rates of skin rash with VPA are very low\textsuperscript{22} as compared to another antiepileptic drug lamotrigine\textsuperscript{23}, the mechanism of skin rashes under the VPA therapy still unknown. There are reports on the antiepileptic drug, sodium valproate increasing the lipid peroxidation in patients receiving it\textsuperscript{24,25}. Chang & Abbott\textsuperscript{25} also showed that oxidative stress has a potential role on sodium valproate-induced hepatotoxicity. On the other hand, Verrotti et al.\textsuperscript{27} found that sodium valproate therapy does not cause oxidative stress in epileptic children who remained non-obese during the treatment.

In the present study, as a marker of lipid peroxidation, MDA level was determined. The VPA administration did not significantly change the skin MDA and as an antioxidant edaravone administration to VPA group significantly decreased the MDA level. Neither VPA, nor VPA + edaravone did not significantly change SOD, GPx and CAT activities and GSH level in the skin. The skin GST activity significantly decreased in VPA group compared to the control and the edaravone administered control group. Edaravone significantly increased skin GST activity in VPA administered group. In the glutathione metabolism, the net effect of edaravone was found on skin GST activity after the VPA administration. GST catalyze the conjugation of GSH, via a sulfhydryl group, to electrophilic centers on a wide variety of substrates in order to make the compounds more soluble\textsuperscript{28}. It has been reported that GST null genotype increases the susceptibility of skin cancer and also shows lower enzymatic activity than GST positive genotype\textsuperscript{29}. These studies suggest that lower GST activity possibly induces cell damage that causes skin cancer. Similarly, VPA induced skin damages observed here might be attributed to the decreased GST activity after the VPA administration. As edaravone markedly increased the GST activity after the VPA administration, the usage of edaravone may prevent the skin disorders observed after the VPA treatment.

TF, also known as thromboplastin or Factor III, is a cell membrane component. As TF is a labile protein, its activity can be changed by the disturbances in membrane composition, heating and pH changes\textsuperscript{30}. In the present study, the VPA administration significantly increased skin TF activity when compared to the control group. Increase in skin TF activity may be relevant to the skin damage seen following VPA administration. Asero et al.\textsuperscript{30} also observed an increased TF expression in the skin of the patients with urticaria. They revealed that if autoantibodies, complement, and mast cell-derived factors such as histamine, tryptase, and/or different cytokines are responsible for activation of endothelial cells causing TF expression, then the observed activation of the extrinsic coagulation pathway would be a secondary consequence of urticaria and they recommended using anticoagulants in patients with urticaria. Cadroy et al.\textsuperscript{31} demonstrated the activation of coagulation by free radicals and also the fact that polymorphonuclear leukocytes modulates the production of TF by monocytes via the release of free radicals. In the present study, the skin TF activity was used as an indicator of tissue damage, which increased due to the VPA administration, whereas edaravone administration to the VPA group
significantly decreased the TF activity. The increased TF activity following the VPA administration can be attributed to the skin cell damage, and the normalized TF activity with edaravone administration to the free radical scavenger effect of the edaravone.

Sialic acid (SA) is the generic term of a family, which is the acetylated derivative of neuraminic acid. These are suggested to be major participants in many biological functions. Increased SA concentrations have been reported during inflammatory processes, probably resulting from the increased levels of richly sialylated acute-phase glycoprotein. It was suggested that elevated SA is a way for the organism to protect itself. This study showed the increased skin SA level in VPA group. Edaravone administration did not change the skin SA level in the VPA group.

The accumulation of MPO, an enzyme found at high content in polymorphonuclear leukocytes, has been used as a biomarker to assess the inflammatory response to a number of well characterized skin irritants and tumor promoters. In the present study, with the similar purpose, skin irritation that may occur due to the VPA administration was determined using MPO activity. There are some conflicting results about this enzyme and VPA treatment in the literature. MPO accumulation could be an additional and useful toxicological tool for determining the dermal irritancy of chemicals. Suda et al. reported that VPA treatment reduced the MPO infiltration in ischemia reperfusion injury in rat brain. Our study showed increased skin MPO activity due to the VPA administration. However, edaravone significantly decreased MPO activity in the VPA administrated group. The increased MPO activity may be the reason for the skin damages observed in the VPA treated group. Edaravone, as an antioxidant agent, prevented VPA-induced MPO increases, indicating that the antioxidant treatment may help people under VPA treatment.

In the present study, Na⁺/K⁺-ATPase activity was also determined for testing the possible effect of VPA and/or antioxidant edaravone on Na⁺/K⁺-ATPase activity that may have role in the skin energy metabolism. Zugno et al. showed that the administration of valproate reversed the increased Na⁺/K⁺-ATPase activity in rat hippocampus induced by amphetamine. However, in the present study, VPA decreased Na⁺/K⁺-ATPase activity and edaravone administration did not change the activity of this enzyme statistically. Decreased Na⁺/K⁺-ATPase activity in skin could be due to the skin damages caused by the VPA treatment.

**Conclusion**

Increased skin SA, TF and MPO activities and decreased GST and Na⁺/K⁺-ATPase after valproic acid (VPA) administration are symptoms of the damaged skin cells subsequent to the VPA treatment. Edaravone by preventing the inflammatory and oxidative responses, partially protected the skin tissue against the changes observed after the VPA administration.

**References**