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The Effects of Lipoic Acid on Markers of Oxidative Stress and Inflammation in the Lungs of Valproic Acid-Treated Rats

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Abstract: In this experimental study, the effect of lipoic acid (LA) on lung damage caused by valproic acid (VPA) was investigated. The antioxidant, oxidative stress, and inflammation indicators such as glutathione (GSH), lipid peroxidation (LPO), catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), nitric oxide (NO), sialic acid (SA), myeloperoxidase (MPO), and tissue factor (TF) were examined. Sprague Dawley rats were used, and they were randomly divided into four groups as follows: Control group, LA group received 50 mg LA/kg/day for 15 days, VPA group received 500 mg VPA/kg/day for 15 days, and VPA+LA group received the same doses of VPA and LA for 15 days. On day 16, lung tissues were taken. VPA caused the decreases in GSH, SA and SOD values and the increases LPO, NO, and TF values. LA reversed the changes in GSH, SOD, and TF values. GST and CAT activities did not change significantly by the effect of VPA or LA. On the other hand, the inhibitory effect of VPA on MPO, which is an inflammatory marker, and the pro-oxidant effects of LA causing the increases in both LPO and MPO values were observed in lung tissue. These regulations may help LA to overcome oxidative stress caused by VPA in the lung. Further studies are needed to confirm the mechanism underlying VPA-induced MPO inhibition in the lung.

Keywords: Lung, Lipoic acid, Valproic acid, Oxidative stress, Antioxidant.

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1. INTRODUCTION

Valproic acid (VPA) is a widely used antiepileptic drug for the treatment of various seizure and bipolar disorders. Although generally well tolerated, VPA induces oxidative stress, resulting in an inability to balance the generation of reactive oxygen species (ROS) with the body's capacity to detoxify these harmful compounds. As a result, cellular components such as lipids, proteins, and DNA can be damaged, contributing to deterioration of tissue and organ function (1). Oztay et al. (2), suggested that VPA-induced oxidative stress may cause structural distortion and fibrotic changes in the rat lung. Lungs are susceptible to many environmental pollutants, toxicants, oxidants, and many infections that can cause oxidative damage. Several studies have demonstrated the role of ROS produced by lung epithelium and inflammatory cells in the pathogenesis of lung diseases, including acute respiratory syndrome, acute lung injury, chronic

obstructive pulmonary disease, pulmonary fibrosis, asthma, and lung cancer (3).

α-lipoic acid (1,2-dithiolane-3-pentanoic acid, LA), a natural organo-sulfur compound, contains a disulfide bond as a part of a dithiolane ring with a five–carbon tail. LA can be found in plant and animal food sources such as tomatoes, spinach, broccoli, kidney, liver, and heart. It is also endogenously produced by the liver. It exists in cells as dihydrolipoic acid (DHLA), LA's reduced form. It is essential for mitochondrial aerobic metabolism. The fact that LA is soluble in both fat and water makes it special compared to other antioxidants. This means that it can act in the plasma membrane as well as in the cytoplasm (4). It is also the redox regulator of several proteins including, thioredoxin, myoglobin, the transcription factor nuclear factor kappa B (NF-κB), and prolactin, and helps recycle cellular antioxidants involving vitamin C, vitamin E, and glutathione (5,6). High electron density due to the special position of two sulfur atoms in the 1,2-dithiolane ring gives LA a high tendency to reduce other redox-sensitive molecules depending on environmental conditions (6). The antioxidant properties of both LA and DHLA consist of quenching ROS, regeneration of antioxidants, and chelation of redox metals. In recent decades, through its pharmacological effects, such as anti-cancer, anti-oxidant, anti-inflammatory, and anti-viral effects, numerous studies have reported the effects of LA in improving many diseases (4).

This study was designed to investigate the potential protective effect of LA on the lungs of VPA-treated rats by measuring important markers of tissue oxidative stress and inflammation.

2. EXPERIMENTAL SECTION

2.1. Chemicals

VPA was obtained from Merck (Darmstadt, Germany) and LA from Sigma (USA). All other chemicals used in the experiments were of analytical purity and were purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (St. Louis, MO, USA), and Fluka (Buchs, Switzerland).

2.2. Laboratory Animals and Experimental Design

All the experiments in this study were approved by Marmara University Experimental Animals Ethics Committee (Decision No: 34.2015.mar).

Thirty-two Sprague Dawley rats (six-month-old, female) were used in the study. The animals were housed in the standard cage with optimal temperature (20°C±2) and light/dark (12 h light/12 h dark) conditions. All rats were fed orally with standard rat chow and fresh tap water.

The animals were divided into four groups: Control group (n=7), LA given group (50 mg/kg/day, by intraperitoneal, n=8), VPA given group (500 mg/kg/day, by intraperitoneal, n=7), and VPA+LA given group (in same doses and same way, n=10). LA was administered 1 h prior to VPA administration for 15 days. On day 16, the rats were sacrificed, and lungs were taken. Lung homogenates (10% w/v) were prepared in physiological saline (NaCl, 0.9%).

2.3. Biochemical Analysis

Lung homogenates were analyzed for glutathione (GSH), lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), nitric oxide (NO), sialic acid (SA), myeloperoxidase (MPO), and tissue factor (TF).

2.3.1. Estimation of GSH (μg GSH per g tissue)

GSH levels were determined by the method using metaphosphoric acid and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (7). The extinction coefficient of 1.36×10^4 $M^{-1}cm^{-1}$ was used for the calculation. The absorbance was measured spectrophotometrically at 412 nm.

2.3.2. Estimation of LPO (nmol MDA per g tissue)

Malondialdehyde (MDA) is an end product of the peroxidation of lipids. LPO levels were determined using thiobarbituric acid (TBA) assay (8). The pink colour obtained at the end of the reaction was measured with a spectrophotometer at 532 nm.

2.3.3. Estimation of SOD (U SOD per g tissue)

SOD activities were measured as the ability to increase the rate of photooxidation of riboflavin-sensitized o-dianisidine (9). The absorbance was measured spectrophotometrically at 460 nm.

2.3.4. Estimation of CAT (kU CAT per g tissue)

CAT activities were determined based on the reduction of hydrogen peroxide (H_2O_2) to water (H_2O) (10). The decrease in absorbance was measured spectrophotometrically at 240 nm.

2.3.5. Estimation of GST (U GST per g tissue)

GST activities were assayed by measuring the absorbance at 340 nm of the product obtained by conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) (11).

2.3.6. Estimation of NO (nmol NO per g tissue)

In order to measure NO levels, nitrate was converted to nitrite using vanadium (III) chloride. The complex diazonium compound was obtained by reacting nitrite with sulfanilamide in an acidic medium. This was then coupled with N-(1-naphthyl) ethylenediam-ine dihydrochloride, and the coloured complex formed was measured spectrophotom-etrically at 540 nm (12).

2.3.7. Estimation of SA (mg SA per g tissue)

Sodium periodate was used to oxidize SA in concentrated phosphoric acid. Next, TBA was combined with the product of periodate oxidation. A pink chromophore was obtained, which was then extracted into cyclohexanone. The absorbance was measured spectrophotometrically at 549 nm (13).

2.3.8. Estimation of MPO (U MPO per g tissue)

MPO activity was measured by the method using phenol, 4-amino antipyrine (4-AAP), and H_2O_2. The absorbance was measured spectrophotometrically at 510 nm (14).

2.3.9. Estimation of TF (Second)
TF activities were determined by mixing lung homogenate with plasma, then adding calcium chloride and measuring the time for fibrin formation (15). There is an inverse relationship between the clotting time and the activity of the TF.

2.4. Statistics
Statistical analysis (GraphPad Prism 9.0, California, USA) was performed using analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Data are presented as mean ± standard deviation (SD). P-value below 0.05 is considered significant.

3. RESULTS AND DISCUSSION

VPA is generally well tolerated but has been shown both clinically and experimentally to cause several adverse effects, including lung injury. Symptoms of VPA-induced pleural effusion or interstitial lung disease have been reported to resolve within days of discontinuing the drug (16,17). Oztay et al. proposed that VPA administration at a dose of 500 mg kg^{-1} day^{-1} caused pulmonary toxicity via changes in both biochemical and inflammatory markers (2). Therefore, we used a VPA dose of 500 mg kg^{-1} day^{-1} in the present study. VPA primarily damages tissue by inducing oxidative stress, which is then followed by inflammation and apoptosis (18). ROS in the lung are generated from both exogenous and endogenous sources, such as environmental gases and the mitochondrial electron transport system (19). VPA induces elevation of mitochondrial ROS (20). Excessive and uncontrolled oxidative stress leads to cell death, but at lower and less harmful levels it can act as a signal to redox signaling that helps restore cellular balance (21).

The present study showed that the administration of VPA was associated with a decrease in the levels of GSH ($p<0.05$) and SA ($p<0.0001$), the activities of SOD ($p<0.01$) and MPO ($p<0.0001$), and an increase in the levels of MDA, is an end product of the LPO, ($p<0.0001$), NO ($p<0.0001$), and activity of TF ($p<0.0001$) compared to the control group (Figures 1 and 2).

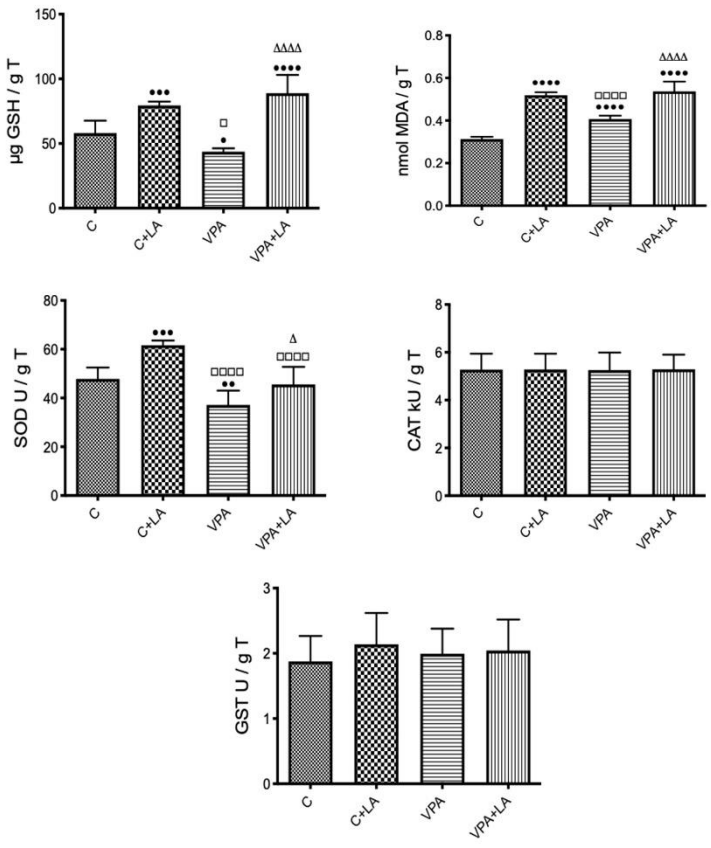

Figure 1: Lung GSH and LPO levels and SOD, CAT, GST activities of the groups. C, control group; C+LA, lipoic acid given control group; VPA, valproic acid given group; VPA+LA, lipoic acid given valproic acid group, T, tissue; GSH, glutathione; MDA, malondialdehyde; LPO, lipid peroxidation; GST, glutathione-S-transferase; SOD, superoxide dismutase; CAT, catalase. Values are represented as mean±standard deviation.
•p < 0.05, ••p < 0.01, •••p < 0.001, ••••p < 0.0001 vs control group; □p < 0.05, □□□□p < 0.0001 vs C+LA group, △p < 0.05, △△△△p < 0.0001 vs VPA group.

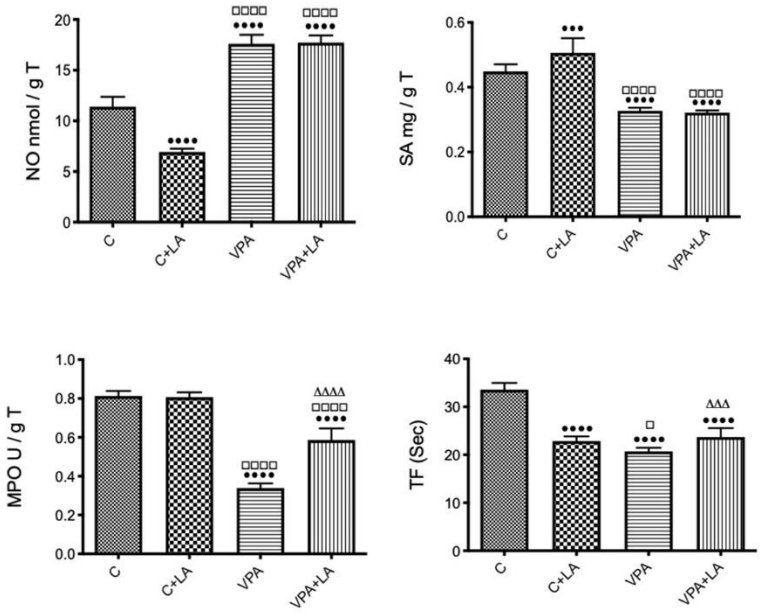

Figure 2: Lung NO, SA levels and MPO, TF activities of the groups
C, control group; C+LA, lipoic acid given control group; VPA, valproic acid given group; VPA+LA, lipoic acid given valproic acid group, T, tissue; NO, nitric oxide; SA, sialic acid; MPO, myeloperoxidase; TF, tissue factor; Sec, second. Values are represented as mean±standard deviation.
•••p < 0.001, ••••p < 0.0001 vs control group; □p < 0.05, □□□□p < 0.0001 vs C+LA group,
△p < 0.05, △△△p < 0.001, △△△△p < 0.0001 vs VPA group.

The two primary antioxidants in the anti-oxidative system are GSH and SOD. These antioxidants reduce the damaging effects of harmful free radicals. LPO is one of the biomarkers of oxidative stress status and indicates oxidative lipid degradation (22). Their levels are closely linked to how cells respond to oxidative stress. Impairment of the lung antioxidant system is linked to a reduction in GSH levels, SOD activities, and an elevation in LPO levels (23). This is supported by our findings. Besides SOD, CAT, and GST are the key antioxidant enzymes that eliminate free radicals from cells. GST is responsible for catalyzing the reaction between GSH and a variety of reactive electrophilic compounds to protect against oxidative stress. CAT prevents the conversion of H_2O_2 to hydroxyl radicals and reduces oxidative stress by catalyzing the conversion of H_2O_2 to water and oxygen. It has been reported that the activities of CAT, SOD, and GST were decreased in the lungs of VPA-treated rats compared to the control group (2). In contrast to these findings, in the present study, VPA did not alter CAT and GST activities.

NO reacts with ROS products to produce peroxynitrite and can alter protein and lipid structure. NO has important neurotransmitter and regulatory roles when released at low levels. However, when combined with superoxide to form harmful peroxynitrite, high levels of NO (produced by the increased activity of inducible NO synthase (iNOS) during inflammation) have a negative effect (24). VPA administration has been reported to increase NO levels in brain tissue (25) and bovine aortic endothelial cells and mouse serum (26) in various VPA toxicity models. In our study, VPA also caused a significant increase in NO values in lung tissue compared to the control group. SA is a major component of the secreted mucins of the airways and is highly expressed along the epithelial border lining the respiratory tract (27). For lung physiology and respiratory balance, this surface anionic shield may provide a repulsive structure, a hydration barrier and a protective barrier (28). SA has also been suggested to have antioxidant activity against hydroxyl radicals in mucin in the respiratory and gastrointestinal mucus layers (29). Higher levels of SA have also been linked with inflammation (30). So, there are conflicting studies on the effect of VPA on SA levels. While VPA administration increased SA levels in the small intestine (31), they did not change in the gastric tissue (29). In the present study, the damaged respiratory mucosa and the decrease in the activity of defense mechanisms may have caused to a reduction in SA levels.

MPO plays an important role in inflammation and tissue damage (32). One of the major ROS, superoxide anion, is transformed into H_2O_2. Activated MPO converts this to the potent oxidant hypochlorite. As a marker of neutrophil recruitment and lung tissue damage, MPO activity is measured in the lung. MPO activity has been found to be significantly increased in the pancreatic, lung, and liver tissue of VPA-treated rats (2,33,34). However, in contrast to these findings, there have also been studies showing that VPA, as a histone deacetylase inhibitor (HDACI), attenuated lung injury by inhibiting inflammatory cytokine production and NF-κB activation (35). In a model of rat intestinal ischemia-reperfusion study, VPA treatment caused a decrease in MPO activity,

and it has been suggested that VPA decreases neutrophil migration into the lungs (36). The reduction in MPO activity in the present study may be due to the anti-inflammatory effect of VPA through its HDACI action. HDACIs have been shown in the literature to have an anti-inflammatory effect (37, 38). TF is a transmembrane glycoprotein and acts as a cellular initiator of coagulation. The lung is one of the tissues that contain high levels of TF, along with the brain, uterus, heart, and placenta. Alveolar macrophages and airway epithelial cells express TF in the lung. The abnormal expression of TF is associated with thrombotic complications in a variety of diseases, including atherosclerosis, cancer, and inflammation. Alveolar TF production suggests that alveolar epithelium expresses TF when exposed to inflammatory cytokines (39–41). Additionally, it has been suggested that elevated levels of ROS may be an inducer of TF gene expression. The procoagulant activity of TF is strictly regulated to maintain hemostasis and avoid thrombosis (42). VPA is a cause of hematological problems, and coagulation abnormalities are frequently observed during VPA treatment (43). It has been shown that VPA increased TF activity in gingival tissue (44). In parallel with the findings of previous studies, this present study suggests that increased TF activity in the lung in response to inflammation and/or oxidative stress may provide extra hemostatic protection against tissue damage.

LA is a powerful reductant and has the ability to scavenge free radicals. Administration of LA has been shown in several studies to reduce oxidative stress and restore depleted levels of other antioxidants *in vivo*. On the other hand, data show that LA may have pro-oxidant effects (6). The oxidative status of cells and physiological parameters may determine the ability of LA and/or DHLA to act as pro- or antioxidants. According to its chemical structure, LA functions as an oxidative molecule without generating endogenous ROS because of its oxidized dithiolane ring. DHLA may also produce thiol and disulfide radical anion and function as a pro-oxidant. *In vitro* and *in vivo* studies suggest that DHLA causes the production of ROS through the Fenton reaction by reducing ferric iron and the stimulation of the Ca^{2+}-induced mitochondrial permeability transition (MPT) by the production of ROS, which depletes mitochondrial antioxidant capacity (45).

In the current study, when the VPA group was treated with LA, the changes in GSH levels ($p<0.0001$) and SOD ($p<0.05$), MPO ($p<0.0001$), TF ($p<0.001$) activities were reversed by LA compared to VPA, whereas LPO levels were increased more ($p<0.0001$) (Figure 1 and 2). In addition, LA in combination with VPA had no effect on SOD, CAT activities, or NO, SA levels ($p>0.05$) (Figures 1 and 2). In the C+LA group, the NO levels were significantly reduced ($p<0.001$) and SA levels and TF activity were significantly increased ($p<0.001$) compared to the control group (Figure 2).

The antioxidant effects of LA may act either directly by restoring endogenous antioxidants such as GSH or indirectly by balancing ROS. It has been suggested that increasing the availability of cysteine is the mechanism by which LA can increase GSH levels (46). Similarly, Cadirci et al. (22) reported that decreased SOD activity and GSH amounts in septic lung tissue were increased by the administration of LA to rats. The both studies (46, 22) support our findings that GSH and SOD values were increased in LA-treated rats. Lung LPO levels were found to be significantly increased in LA+VPA treated rats in our study, which appears to contradict previous findings from a systematic review and meta-analysis suggesting that LA consumption significantly reduces MDA levels (47). It is possible that an induction of SOD activity is involved in the mechanism of LPO induction by LA. The elevated SOD activity and unaltered catalase activity in LA-exposed rats would be expected to result in the deposition of H_2O_2 and hence elevated LPO in the presence of ferrous ions as a consequence of the Fenton reaction. When exogenous LA enters the cell, it is reduced to DHLA with the use of NADPH by the cytosolic enzymes glutathione reductase and thioredoxin reductase. Although DHLA has been demonstrated to have both pro-oxidant and antioxidant effects, both LA and DHLA have been classified as strong antioxidants based on their unique characteristics. Ferric and ferrous iron are both chelated by DHLA *in vitro*, preventing oxidative damage to iron. However, LA also removes iron from ferritin and has the ability to convert Fe^{3+} to Fe^{2+}, increasing the risk of oxidative damage. This is because iron is a redox-active metal. Through Fenton chemistry, it can significantly increase oxidative stress by producing hydroxyl radicals. (4,6,45). Briefly, LA may have caused lipid peroxidation by acting as a pro-oxidant and accelerating the formation of iron-dependent hydroxyl radicals in the lungs. Turkyilmaz et al. (25), showed that administration of LA in the VPA group increased CAT activity, decreased GST activity, and the change in SOD activity was insignificant in brain tissue. Differently from Turkyilmaz et al. (25), in the current study, the CAT and GST activities were not modified by LA in the C+LA and VPA+LA groups compared to the respective groups. These results suggested that H_2O_2 degradation processes were unchanged and may have increased the accumulation of superoxide anion and the formation of hydroxyl radicals. In the literature, it has been shown that LA induces apoptosis by increasing mitochondrial superoxide anion production in cancer cells (48). However, more research is needed to explain the mechanism behind these changes.

Preclinical studies have shown that LA can reduce inflammation by inhibiting the iNOS/NO pathway (4). In another study, Oktay & Caliskan (49) found that LA reduced NO levels but had no effect on SA levels compared to the methotrexate group during methotrexate-induced oxidative stress in rat hearts. In the present study, LA reduced NO levels and increased SA levels in LA given control group. This may be due to the antioxidant effect of LA. However, it was not sufficient to reverse the NO and SA levels in the VPA+LA group compared to VPA.

LA (25 mg/kg/day for 21 days) increased colon MPO activity in the mouse model of acute ulcerative colitis

(50), and LA at higher doses (100 mg/kg/day for 45 days) may have a pro-oxidant effect on cardiac tissue in a rat-diabetic model (51). In line with these observations, 15 days of LA (50 mg/kg/day) at proposed doses may be pro-inflammatory based on our available data. Future studies are recommended to clarify the effects of LA in a dose- and time-dependent and oxidative stress-type-dependent manner.

It is known that LA affects iron metabolism. Although findings from clinical studies are conflicting, LA has been shown to reduce serum iron levels. It has been suggested that this may be because DHLA chelates iron, preventing its use (52). Based on this, LA, which increases TF activity in the C+LA group, may be beneficial to keep normal blood clotting to prevent further bleeding when serum iron is low**.** Iron deficiency causes iron deficiency anemia, which may be associated with decreased thrombogenesis, aberrant platelet function, and elevated inflammatory levels, all of which raise the risk of bleeding (53). Moreover, LA inhibits platelet activation, and co-administration with vitamin E prolongs the clotting time (54). Accordingly, in the current study, LA decreased lung TF activity in VPA group, which may prevent lung tissues from the risk of thrombosis and coagulation problems.

4. CONCLUSION

In conclusion, the present data do not allow the conclusion that LA could prevent VPA-induced lung injury; the data are consistent with LA may contribute to the reduction of TF activity and increase in GSH levels, SOD, and MPO activities in VPA treatment. However, more studies are required to determine the mechanism underlying VPA-induced MPO inhibition in the lung. Also despite its dual modulation of oxidative stress and inflammation, LA may have beneficial effects on lung health.

5. CONFLICT OF INTEREST

There are no conflicts to declare.

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