Damage to Liver Tissue Caused by Valproic Acid Used for Treating Epilepsy: Protective Effects of Vitamin B_6

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Abstract

Objective: We intended to determine the vitamin B_6 (Vit B_6) protection on valproic acid (VPA)-induced liver injury.

Methods: Male Sprague-Dawley rats were used. The control group: Vit B_6 (50 mg/kg/day) given rats; VPA (500 mg/kg/day) given rats; VPA and Vit B_6 given rats at the same dose and time for 7 days.

Results: Liver glutathione and total antioxidant capacity were decreased while, lipid peroxidation, advanced oxidized protein products, tumor necrosis factor-alpha, interleukin-6, nitric oxide, total oxidant status, oxidative stress index, reactive oxygen species levels and myeloperoxidase, antioxidant enzyme activities, glucose-6-phosphate dehydrogenase, and adenosine deaminase activities were increased in the VPA group. Vit B_6 eased these parameters in the VPA group. In the histological determinations, nuclei including dense chromatin material, hyperemia, sinusoidal dilation, collagen accumulation in connective tissue, and large and dense granules in the cytoplasm were increased in the VPA group according to the control groups, microscopically.

Conclusion: As a result, Vit B_6 supplementation reversed biochemical results in VPA-induced liver damage by regulating the antioxidant status.

Keywords: Valproic acid, vitamin B_6, hepatotoxicity, oxidative stress, antioxidant effect

INTRODUCTION

2-propyl valeric acid or generally known as valproic acid (VPA) is an effective anticonvulsant and preferred all around the worldwide for treatments of epilepsies for both childhood and adults.[1,2] Although it has been reported for this disease, its side effects have reached a dangerous level. Day by day, there is an increasing number of studies that indicate its unwanted effects on many organs and tissues in both human and experimental rat studies, listed as liver, lung, heart, etc.[3-5]

Albeit with the existence of an unpredictable mechanism for liver toxicity, there is a common opinion for reactive oxygen species (ROS) production because of either VPA-sourced or its metabolites-sourced.[6] This production can be associated with many metabolic pathways. One of these pathways is the interfering effect of VPA on the beta oxidation of fatty acids.[7] Likewise, the electron transferring alterations in the mitochondrial electron transport system (ETC), disrupted ATP generation, and asymptomatic hyperammonemia are the reasons that were related to the hepatotoxic effects of VPA.[6-8] The clinical symptoms for VPA hepatotoxicity during its usage in humans can be numbered as vomiting, jaundice, fatigue, etc.[9] To decrease these effects and find a solution for its serious side effects, researchers have been trying to use different plant extracts or vitamin sourced substances.[10,11]

Vitamin B_6 (Vit B_6) is an important water-soluble vitamin. Its derivatives can be produced at its 4th position, which are named as pyridoxal (with an aldehyde group), pyridoxamine (with an aminomethyl group) and pyridoxine (with an hydroxymethyl group).[12] Their phosphate forms (at 5th position) are also necessary. Vit B_6 is an indispensable cofactor for approximately over a hundred enzymatic reactions in metabolism and the most known reactions are known as transaminationand alpha decarboxylation.[13] Since it isnot produced in humans, it is necessary for being consumed with milk, meat, and various fruits and vegetables.[12] The antioxidant and radical scavenging activities of Vit B_6 have been well proven by Turkyilmaz et al.[14]

Based on this information, we planned to investigate the unique antioxidant capacity of Vit B_6 on VPA-induced hepatotoxicity by evaluating both histochemically and biochemically.

METHODS

Chemicals

All chemicals used were of analytical grade. They were purchased from Sigma-Aldrich and Merck.

Animals and Ethics Statement

In this study, male Sprague-Dawley rats (4-months aged) were preferred, and the ethic permission was taken from the Local Ethics Committee of Animal Research of İstanbul University (number: 2015/09, date: 05.02.2015). All animals were fed with a standard pellet and free access water ad libitum.

Experimental Design

Rats were divided into four groups.

Group 1: Control animals.

Group 2: Animals given Vit B_6 at a dose of 50 mg/kg for 7 days.

Group 3: Animals treated VPA at a dose of 500 mg/kg for 7 days.

Group 4: Animals received Vit B_6 and VPA at the same dose and time for 7 days.

The administration of Vit B_6 was performed by orally (gavage technique), and VPA was intraperitoneally. The doses of Vit B_6 and VPA were referenced according to the study of Tunali.[15] Before the termination of the experiments, the rats were fasted for one night. On 8th day, they were sacrificed under anesthesia. Liver tissue samples were taken for both histologic and biochemical analyses.

Histological Assay

The Bouin's solution was used for fixation of liver tissues. Fixed liver tissues were dehydrated in an ethanol series. It was cleared in xylene and embedded in paraffin. The tissues were cut as 5 µm-thick sections. The sections were stuck on a microscope slide. It was stained with Masson's trichrome for histological determination. The Olympus CX-45 microscope was used for histological analysis (X40 objective and X10 ocular system). The histological score, which has a grade from 0 to 3 as negative (0), weak (1), moderate (2), and strong (3) was used for explanation the results.

MAIN POINTS

- Valproic acid (VPA) is an effective anti-epileptic drug used for the treatments of various seizures and migraine.
- Nevertheless, VPA has many side effects on many organs by triggering oxidative stress.
- Liver is an important organ and contains many macromolecule pathways, but this organ is open to different side effects associated with free radical and oxidative conditions.
- Vitamin B_6 (Vit B_6) is a vital water-soluble vitamin and an important free radical scavenger. This feature leads to this vitamin to be used in preventing toxicities sourced by free radicals.
- The obtained biochemical and histological results from this study support the potential antioxidant feature of Vit B_6 on liver injury induced by VPA.

Biochemical Experiments

Liver tissues were homogenized in cold saline (0.9% NaCl) for preparing 10% (w/v) homogenates. They were centrifuged, and the supernatants were collected. They had been kept at -80 ºC until the biochemical experiments were performed. The reduced glutathione (GSH), lipid peroxidation (LPO), and advanced oxidized protein products (AOPP) levels were performed according to the methods of Beutler[16], Ledwozyw et al.[17] and Witko-Sarsat et al.[18], respectively. Tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels were determined as using ELISA kits as the manufacturers' procedure. Myeloperoxidase (MPO) activity and nitric oxide (NO) levels were determined according to the methods as follows Wei and Frenkel[19] and Miranda et al.[20]. Total antioxidant capacity (TAC), total oxidant status (TOS) and oxidative stress index (OSI), and reactive oxygen species (ROS) levels were performed according to Erel[21] and Erel[22], Zhang et al.[23] methods, respectively. Catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) activities were determined as based on the methods of Aebi[24], Mylroie et al.[25], Wendel[26], and Beutler[27], respectively. Glucose-6-phosphate dehydrogenase (G6PD) and adenosine deaminase (ADA) activities were performed according to the methods of Beutler[28] and Karker[29], respectively.

Statistical Analysis

The statistical analysis of the biochemical results was determined via GraphPad Prism 6.0 (GraphPad Software, San Diego, California, USA). The data were expressed as mean ± standard deviation. The results were assessed with an unpaired t-test and analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. $P<0.05$ was considered significance. The biochemical results were also evaluated using Origin for performing principal component analysis (PCA).

RESULTS

Histological Results

The histological results are presented in Figure 1. The nuclei including dense chromatin material, hyperemia, sinusoidal dilation, collagen accumulation in connective tissue, and large and dense granules in the cytoplasm were increased in the VPA group according to the control group given physiological saline and control group given Vit B_6 in the histological determinations. There was not any alteration in VPA+Vit B_6 group according to the VPA group, microscopically (Figure 1).

Biochemical Results

Figure 2 represents liver GSH, LPO, AOPP, TNF-α and IL-6 levels, MPO activity and NO levels.

After both VPA and VPA+Vit B_6 administration, the alterations in GSH levels were observed as insignificant. LPO and AOPP levels were significantly increased in the VPA group compared to the control group ($^{**}p<0.01$). Vit B_6 reversed LPO and AOPP levels significantly in the VPA group ($^{\#}p<0.01$) (Figure 2).

Liver TNF-α and IL-6 levels, MPO activity and NO levels mentioned in Figure 2 were significantly increased in VPA group compared to the control group ($^{*}p<0.05$, $^{**}p<0.01$, $^{\#\#\#}p<0.001$, respectively). The

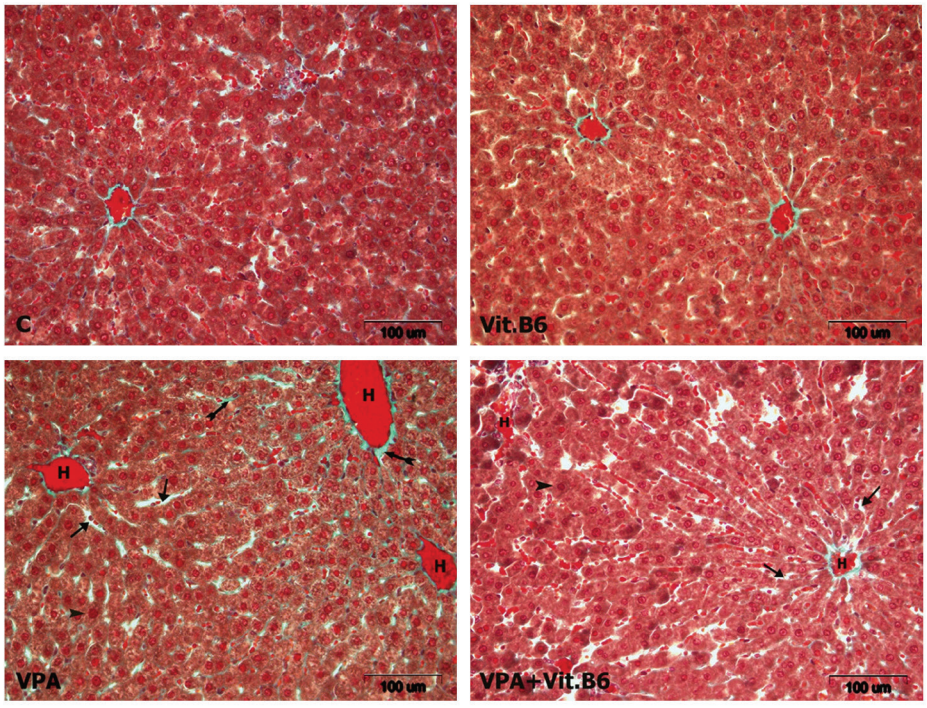

Figure 1. Histological appearance of the liver tissues of all groups.
The control group: C, control group given vitamin B_6 (50 mg/kg): Vit B_6, experimental group given VPA (100 mg/kg): VPA, the experimental group given vitamin B_6: VPA+Vit B_6 (doses were given at the same and concentration). Nuclei including dense chromatin material; ➤, hyperemia; H, sinusoidal dilation; ➔, collagen accumulation in the connective tissue; ➡ can be seen in VPA and VPA+Vit B_6 groups. Masson's trichrome. X40 objective and X10 ocular system
VPA: Valproic acid, Vit B_6: Vitamin B_6

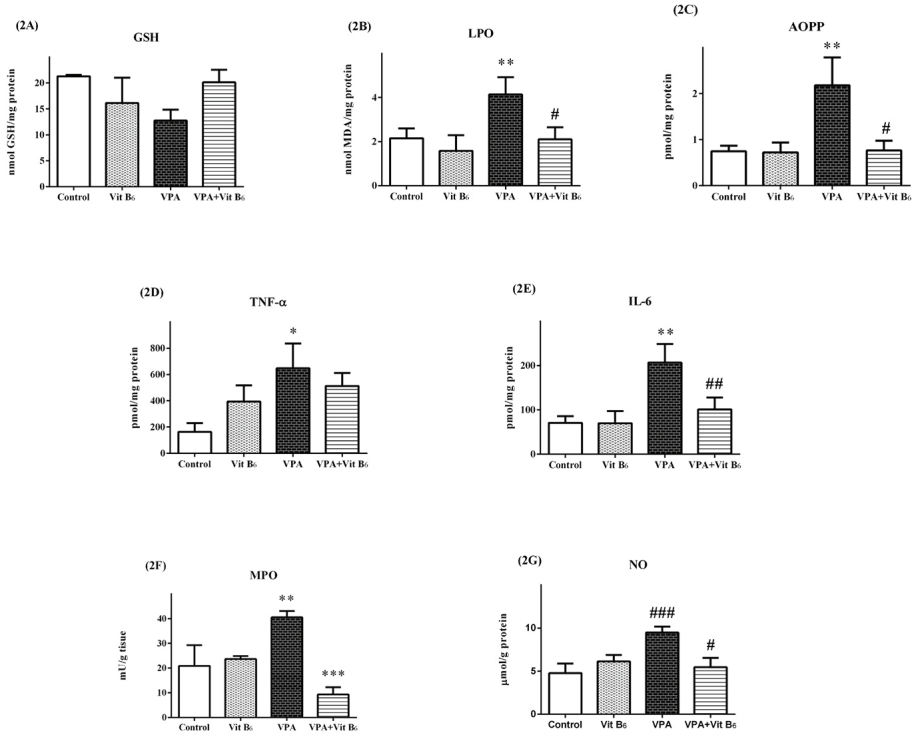

Figure 2. The liver (A) reduced glutathione, (B) lipid peroxidation and (C) advanced oxidized protein products, (D) tumor necrosis factor-α, (E) interleukin-6 levels, (F) myeloperoxidase activity, and (G) nitric oxide levels of all groups. Each column represents mean±standard deviation. The control group: Intact group, Vit B_6 group: animals received 50 mg/kg Vit B_6 per day, VPA group: 500 mg/kg administered animals, VPA+Vit B_6 group: animals received the same doses at the same time. $^{**}p<0.01$ versus control group, $^{\#}p<0.01$ versus VPA group, $^{*}p<0.05$ versus control group, $^{\#\#}p<0.05$ versus VPA group, $^{***}p<0.001$ versus VPA group, $^{\#\#\#}p<0.001$ versus control group
VPA: Valproic acid, Vit B_6: Vitamin B_6

administration of Vit B_6 decreased IL-6, MPO, and NO significantly in the VPA group ($^{\#\#}p<0.05$, $^{***}p<0.001$, $^{\#}p<0.01$, respectively) (Figure 2).

Liver TAC, TOS, OSI and ROS levels, CAT, SOD, GPx and GR activities are presented in Figure 3. Vit B_6 caused a decrease in TAC levels and an elevation in OSI of control group ($^{**}p<0.01$, $^{*}p<0.05$). VPA administration decreased TAC levels ($^{***}p<0.001$) and increased TOS, OSI, and ROS levels significantly as compared the control group ($^{**}p<0.01$, $^{\#\#}p<0.0001$, respectively). In VPA+Vit B_6 group, all the levels given in this Figure 4 were altered significantly compared to the VPA group ($^{****}p<0.0001$, $^{\#}p<0.01$) (Figure 3).

VPA administration increased CAT, SOD, GPx, and GR activities significantly as compared the control group ($^{**}p<0.01$, $^{*}p<0.05$, $^{***}p<0.001$). The administration of Vit B_6 to the VPA group significantly reduced CAT, SOD, GPx, and GR activities ($^{\#}p<0.01$, $^{\#\#\#}p<0.001$) (Figure 3).

Liver G6PD and ADA activities are given in Figure 4. Vit B_6 significantly reduced G6PD in the control group ($^{*}p<0.05$). GP6D activity was significantly increased after VPA administration in the control group ($^{**}p<0.01$). The administration of Vit B_6 decreased G6PD and ADA activities in the VPA group significantly, respectively ($^{****}p<0.0001$, $^{\#}p<0.05$) (Figure 4).

PCA was used to determine the correlation between all biochemical parameters and the results are shown in Figure 4. According to the PCA, the first two components were determined around 83.41% (as total result). PC1 and PC2 values were calculated as 74.60% and 8.81%, respectively. At the first part, MPO, SOD, ADA, GPx, G6PD, GR, OSI, CAT, NO, AOPP, LPO, ROS, IL6, TOS, TNF-alpha data were observed to be clustered together. These parameters were negatively correlated with PON, GSH, and LPO (Figure 4D).

DISCUSSION

VPA and its metabolites can cause mitochondrial oxidative phosphorylation inhibition, impairments of the electron transport chain, and hence disruption of ATP generation.[6] The interruption of energy functioning also affects the pyruvate uptake and transportation in the mitochondrial inner membrane.[30] Systemic insulin resistance alteration and obesity-affected inflammation conditions are the well-documented reasons for VPA hepatotoxicity.[6,30] All the defined reasons are strictly associated with the production of ROS on VPA-induced hepatotoxicity.

After being taken to the organism, Vit B_6 serves at many reactions such as transamination, decarboxylation, etc. as a cofactor. Vit B_6 can form a Schiff base with the amino groups of lysine, which can exist at the active sites of enzymes and then helps electron transfers by stabilizing the reaction intermediates.[31] Its antioxidant capacity on different metabolic disorders has been proved by many researchers.[15]

GSH, a unique tripeptide for the antioxidant system, is capable of detoxifying many toxicants with its thiol (-SH) group. Increased free radical levels have been related to lower GSH levels in different VPA-induced hepatotoxicity models studied by Sokmen et al.[10] Besides, Kiang et al.[32] reported that different VPA metabolites had dramatically depleted GSH levels in rat hepatocytes. In our rat-modelled study, we got diminished GSH levels in the livers of the VPA-treated group compared to the control group. To maintain GSH levels at a constant ratio may help better antioxidant system functioning either regulating GSH-dependent enzymes or total antioxidant status. For this purpose, we administered Vit B_6 to the VPA group. Vit B_6 has been defined as serving like a cofactor for the transsulfuration pathway for the transformation of homocysteine to cysteine, which is necessary for the formation of GSH.[33] As considering this approach, we may say that

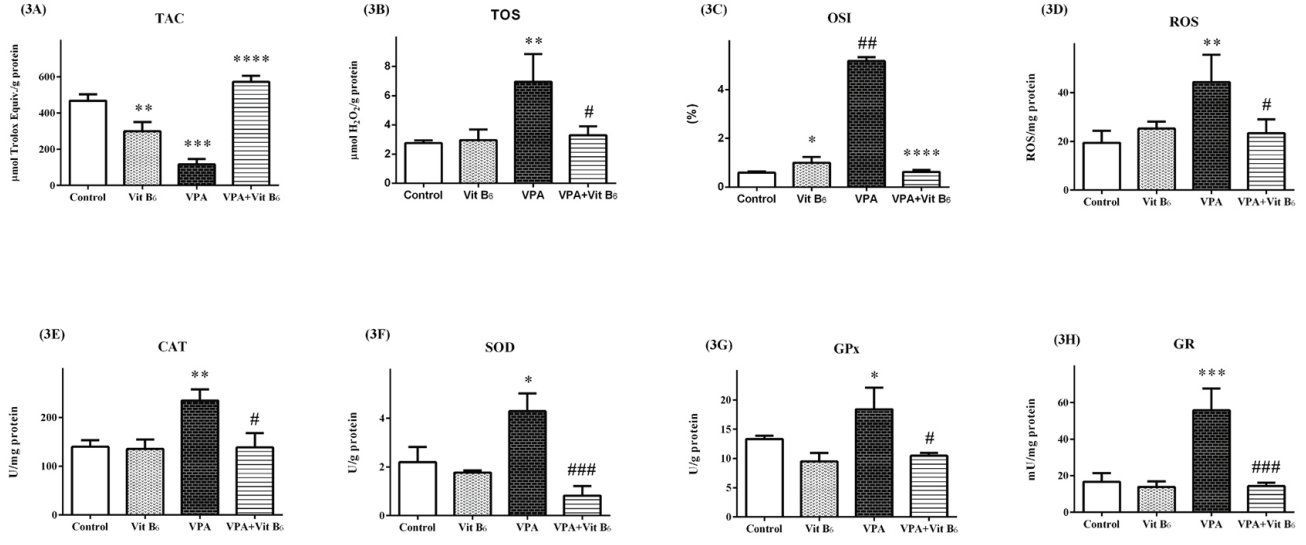

Figure 3. The liver (A) total antioxidant capacity, (B) total oxidant status, (C) oxidative stress index and (D) reactive oxygen species levels, (E) catalase, (F) superoxide dismutase, (G) glutathione peroxidase and (H) glutathione reductase activities of all groups.
Each column represents mean±standard deviation. The control group: Intact group, Vit B_6 group: animals received 50 mg/kg Vit B_6 per day, VPA group: 500 mg/kg administered animals, VPA+Vit B_6 group: animals received the same doses at the same time.$^{**}p<0.01$ versus control group, $^{***}p<0.001$ versus control group, $^{****}p<0.0001$ versus VPA group, $^{\#}p<0.01$ versus VPA group, $^{*}p<0.05$ versus control group, $^{\#\#}p<0.0001$ versus control group, $^{\#\#\#}p<0.001$ versus VPA group
VPA: Valproic acid, Vit B_6: Vitamin B_6

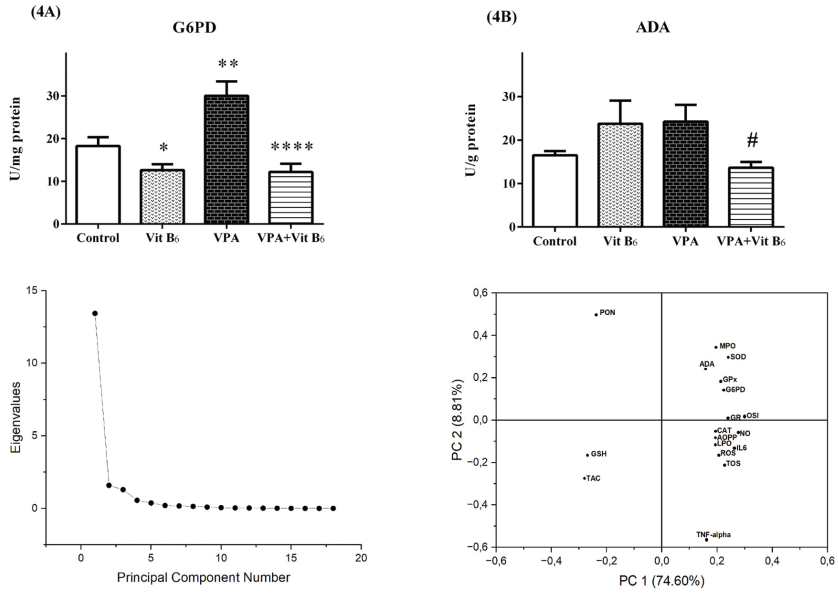

Figure 4. The liver (A) glucose-6-phosphate dehydrogenase and (B) adenosine deaminase activities and principal component analysis (PCA) (C, D) results of all groups.

Each column represents mean±standard deviation. The control group: Intact group, Vit B_6 group: animals received 50 mg/kg Vit B_6 per day, VPA group: 500 mg/kg administered animals, VPA+Vit B_6 group: animals received the same doses at the same time. $^{*}p<0.05$ versus control group, $^{**}p<0.01$ versus control group, $^{****}p<0.0001$ versus VPA group, $^{\#}p<0.05$ versus VPA group. PCA (C, D) results for all biochemical parameters. (C) Plot presentation, (D) the presentation of PCA total result as 83.41% with PC1 and PC2

VPA: Valproic acid, Vit B_6: Vitamin B_6

administration of Vit B_6 elevated GSH levels probably supporting this mechanism and helping scavenge free radicals in the VPA group.

A sign for increased free radical level is LPO. Its elevated level is a major indicator for destructing cell membranes, which could begin with VPA administration. Different concentrations of VPA affect LPO levels in both liver and kidney, by represented by Tong et al.[34] and Chaudhary et al.[35], respectively. In addition, the elevation of LPO may be associated with AOPP levels because proteins can be affected at the same conditions as much as the lipid structure of membranes in cell media with the existence of VPA. Tunali et al.[11] showed that VPA elevated AOPP levels of VPA-induced brain injury by proving the increased ROS levels after VPA administration. Our results are in accordance with this approach, and we got elevated levels of LPO and AOPP in the livers of the VPA-administered group. The administration of Vit B_6 decreased these levels in the VPA group. This diminishing effect of Vit B_6 can be related to the radical scavenging effect of VPA, whose protective effect has also been also indicated in sepsis -induced lung and liver damage by Giustina et al.[36]

Immune system -mediated drug hypersensitivity is another unwanted consequence for the patients who use VPA.[37] Affected energy metabolism is evidence for cytokine-related liver toxicities.[38] VPA increases TNF-α and interleukin gene expression levels in liver tissue.[39] Unfortunately, increasing TNF-α levels lessen ATP levels in cell media and enhances the strong harmful effects of ROS.[37] Likewise, MPO, as forming hypochlorite by using chloride and hydrogen peroxide, which are initiators for the formation of hydroxyl radicals and singlet oxygen and NO, as being a diffusible gas, are important factors for cells.[40,41] Exemplarily, elevation of NO levels is related to increased union with ROS molecules and hence, covalently binding of proteins in mitochondrial respiration complex IV occurs.[42] In our study, TNF-α, IL-6, NO levels and MPO activities were found dramatically increased in VPA group. Our results are parallel with different VPA studies.[39,43,44] However, a diminishment in plasma Vit B_6 levels has been associated with altered inflammation conditions.[45] Taking into consideration of the protective effect of Vit B_6 on both ROS scavenging and stimulation of inflammation, we can assume that Vit B_6 is effective in decreasing TNF-α, IL-6, MPO and NO in liver toxicities.

The lessened GSH levels, increased LPO and AOPP levels, as well as altered inflammatory conditions by VPA, it is inevitable to make a relation with ROS levels. VPA-induced elevation of TOS-ROS levels and diminishment of TAC levels are good evidence for an altered antioxidant system. When we evaluated the activities of CAT, SOD, GPx, and GR, we realized that there had been an increase in these activities in the liver samples of the VPA group. Jafarian et al.[8] reported at their study that VPA-induced ROS generation on isolated mitochondria of the liver would be associated with its harmful effect on mitochondrial ETC system complexes. Pourahmad et al.[46] also revealed that VPA has helped ROS distribution by destabilizing the lysosomal membrane structure, and as H_2O_2 can easily pass, it can form hydroxyl radical at liver tissues. Although we got diminished levels of GSH, we can make a relationship between the elevated activities of GSH-dependent enzymes such as GPx, GR, and increased free radical production. The administration of Vit B_6 may have helped ease all these antioxidant parameters by reducing ROS levels with its antioxidant activity.

G6PD is an important enzyme for the pentose phosphate pathway (PPP), which is a vital source for NADPH production. VPA was reported that it has decreased mitochondrial bioenergetics in yeast. This effect has also been explained with many reasons as the inhibitory function of VPA on some TCA cycle enzymes, an increasing effect on glycolysis. Besides, Salsaa et al.[47] emphasized that 6-phosphogluconic acid levels, related to PPP, were increased by the presence of VPA

because of elevated need for NADPH because of oxidative stress. They also indicated that the elevation of NADPH in cell media would help protect GSH levels for scavenging ROS. Additionally, inflammatory factors have been associated with increased G6PD activities and decreased cAMP levels.[48] Therefore, our results, which we obtained as elevated G6PD activities in the VPA-treated liver may be relate to this approach. Vit B_6 reversed this effect on the VPA group as using its protection against ROS.

ADA is a key enzyme for purine metabolism. It catalyzes the deamination of adenosine. Its excess activity was accompanied by ammonia production. Elevated ammonia levels can be dangerous for causing impairment of energy metabolism and other important macromolecule transformation in the liver. In addition, VPA treatment has been associated to cause asymptomatic hyperammonemia in patients.[7] As parallel to these approaches, our results indicated elevated liver ADA activity in the VPA group. The administration of Vit B_6 decreased this activity by its protective effect.

VPA is a hepatotoxic drug that can be used as a medicine in various diseases such as epilepsy and migraine. However, it shows toxicity in the liver in relation to liver diseases such as steatosis and liver failure. Additionally, in a study, it was shown that administration of VPA to mice exacerbates existing liver damage.[49] Histological changes in liver damage caused by anti-tuberculosis drugs have been shown to be partially improved by the administration of Vit B_6 and it was stated that it is a little toxicity in mice.[50] However, the effects of Vit B_6 against the damage caused by VPA in the liver are unknown. Our results have shown that the damage caused by VPA is not enough to reverse morphological changes by the administration of Vit B_6.

Study Limitations

The study limitation of this study is to fully lighten the beneficial effects of Vit B_6 on liver biochemical and histological parameters, thus further liver disease or toxicity models must be developed and protection of Vit B_6 must be examined on these models.

CONCLUSION

VPA is a widely used anti-antiepileptic drug. Although it has beneficial effects, there are many affected systems and organs due to their serious side effects. The liver is the most affected organ against to toxicity and free radical species exposure. To protect this tissue is a vital target for all research. For this purpose, Vit B_6 was chosen as the protector due to its well-known antioxidant and protective effects. The obtained biochemical and histological results from this study support the protection of Vit B_6 on liver tissue, which has been exposure to VPA.

Ethics

Ethics Committee Approval: The ethic permission was taken from the Local Ethics Committee of Animal Research of İstanbul University (number: 2015/09, date: 05.02.2015).

Informed Consent: Animal experiment.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: İ.B.T., Ş.B., R.Y., Concept: İ.B.T., Ş.B., R.Y., A.K.K., Design: İ.B.T., Ş.B., R.Y., A.K.K., Data Collection or Processing: R.Y., Ş.B., Analysis or Interpretation: R.Y., Ş.B., İ.B.T., Literature Search: R.Y., Ş.B., İ.B.T., Writing: R.Y., Ş.B., İ.B.T., A.K.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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