

Original article

# The effects of biotin supplementation on serum and liver tissue biotinidase enzyme activity and alopecia in rats which were administrated to valproic acid

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## Abstract

Valproic acid (VPA) is a widely used and well-tolerable antiepileptic drug in epileptic patients. However, VPA has many side effects dose-dependent or non-dose-dependent. It is reported that VPA treatment may lead to biotin deficiency and low serum and liver tissue biotinidase enzyme activity (BEA). Major clinical manifestations in biotin deficiency are seborrheic dermatitis, dry skin, fine and brittle hair, and alopecia. We aimed to investigate the effects of biotin supplementation on serum and liver tissue BEA and alopecia during VPA therapy. Rats were randomly divided into 4 groups, each consisted of 15 rats (VPA-B1, VPA-B2, VPA, and control). Except the control group, all groups were administrated VPA dose of 600 mg/kg/d per oral (PO) for 60 days with 12 h intervals two divided doses. VPA-B1 was administrated biotin dose of 6 mg/kg/d and VPA-B2 was administrated biotin dose of 0.6 mg/kg/d. In the third week of the study, we determined alopecia in the study groups. Alopecia was seen in the subjects of 13.3% of VPA-B1 ( $n = 2$ ), 13.3% of VPA-B2 ( $n = 2$ ), and 40% of VPA ( $n = 6$ ). But statistical significant effect on alopecia by biotin supplementation was not able to be determined between the study groups. In the control group, alopecia was not observed. The ratios of alopecia in the study groups were statistically higher than the control group ( $p = 0.028$ ). Itchiness was more obvious in the study groups compared with the control group. Serum biotin levels of the biotin supplemented groups (VPA-B1 and VPA-B2) were higher than the other groups (VPA and control group). Serum biotin levels of the VPA group were lower than the control group. There were significant decreases in the levels of serum and liver tissue BEA of the study groups compared with the control group. In conclusion we showed that VPA usage reduced the serum and liver tissue BEA and impaired the biotin utilization by affecting the liver. Partial biotinidase deficiency may lead to alopecia. It might be prevented by biotin supplementation in the patients receiving VPA therapy. We considered that further studies are necessary to find out the effective and safe biotin dose.

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**Keywords:** Valproic acid; Biotinidase activity; Biotin supplementation; Alopecia

## 1. Introduction

Valproic acid (VPA) is a widely used and well-tolerable antiepileptic drug in epileptic patients. However, VPA has many side effects dose-dependent or non-dose-dependent. Although dose-dependent side effects of VPA such as tremor, skin eruption and alopecia

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do not necessitate the cessation of the treatment, reducing of the VPA dose may be necessary [1,2]. Biotinidase enzyme found in high concentrations in serum and liver tissue, and provides biotin transport to histones and proteins by the transferase activity [3,4]. In addition to, the enzyme provides biotin to apocarboxylases ensuring biotin releasing by the hydrolase activity, and contributes to formation of apocarboxylases (pyruvate carboxylase, propionyl-CoA carboxylase, acetyl-CoA carboxylase, and  $\beta$ -methyl crotonyl-CoA carboxylase) functioning in the amino acid, fatty acid and glucose metabolism [5]. Although biotin, a member of the B vitamin complex, may be synthesized by bacteria in intestinal flora, organisms ensure their essential biotin requirements from the biotin found in foods [6]. It is reported that antiepileptic drugs may lead to biotin deficiency [7,8]. There are also some studies reporting that VPA treatment may lead to low serum and liver tissue biotinidase enzyme activity (BEA) [8,9,10]. A major clinical manifestation in biotin deficiency is alopecia [7].

In the literature, there is no study investigating effects of biotin supplementation on serum and liver tissue BEA and alopecia during VPA treatment. We aimed to investigate the effects of biotin supplementation on serum and liver tissue BEA and alopecia during VPA therapy.

## 2. Materials and methods

This study was carried out in the Gülhane Military Medical Academy (GMMA) Research and Development Central Office Laboratory, and the GMMA Biochemistry and Clinical Biochemistry Laboratory between February 2006 and July 2006.

### 2.1. Establishment of study design

Permission for the study was taken from the Ethics Committee of our institute and the study conformed to the ARVO Resolution on the Use of Animals in Research. Sixty male rats (Wistar Albino) of 6-week-old, weighing average  $110 \pm 30$  g were included in the study. Rats were kept in metal cages at a temperature of 21–22 °C with 45–55% humidity, illuminated with an artificial lightning system for 12 h to simulate day-time. All rats were fed ad libitum on a 24% protein rodent chow during the study. Rats were randomly divided into 4 groups, each consisted of 15 rats (VPA-B1, VPA-B2, VPA, and control). Except the control group, all groups were administrated VPA dose of 600 mg/kg/d (DEPAKIN® 200 mg/ml solution, Sanofi Doğu) per oral (PO) for 60 days with 12 h intervals two divided doses via gavaj prepared specifically by bending its tip of metal tube and coating with silicon. VPA-B1 was administrated biotin of 6 mg/kg/d (Biotin

V-CAPS® 1000 mcg capsule, Solgar) and VPA-B2 was administrated biotin of 0.6 mg/kg/d by the same route. Distilled water was administrated via gavaj PO 12 h intervals for 60 days. Demographic features of the study and the control groups were showed in Table 1.

Before the sacrifice, number of the rats which have apparent alopecia was determined for each group. After the application was completed into 60-day all rats were sacrificed within 2–5 h following the last dose administrated. Sacrifice was carried out appropriate to the provisions of the ethic board after general anesthesia ensured by ketamin. The chest walls of the rats were incised and the hearts were reached by releasing peripheral tissues. Blood samples were taken from the left ventricles of the hearts by apical cut. After that, livers were preserved in 154 mmol/L NaCl by means of ice-cooling.

### 2.2. Laboratory methods

Blood samples were centrifuged 10 min at 2500 rpm, serum was obtained and stored at  $-80$  °C until the time of study of the samples. The liver tissues were cut into pieces finely, and homogenized at  $-4$  °C with 154 mmol/L NaCl in a homogenizer and the homogenates which were tissue fractions of 0.1 were prepared and preserved in ice. Classical kinetic UV method was used to determine serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Serum albumin was measured by the bromocresol green calorimetric method, Olympus AU 2700 Autoanalyser® (Olympus Optical Co. Ltd., Shizuoka-ken, Japan). AST and ALT levels were expressed as IU/L, and albumin as g/dl. For measuring serum VPA level Fluorescence Polarization method in the Roche Cobas Integra 400® Analyser was used and serum VPA was expressed as  $\mu$ g/ml.

Serum biotin level was measured by Immune Diagnostic Biotin ELISA Kit®. Serums, standards and controls were incubated with streptavidin-avidin conjugate for 15 min at 25 °C. After that 100  $\mu$ l of incubation mixture was put into wells coated with biotin-albumin conjugate. After the second incubation performed at 18–25 °C, enzyme-substrate TMB solution was added to wells, and the procedure was stopped after the adding of acidic solution. By BIO-TEK Synergy<sup>HT</sup>, USA absorbances of the samples were measured at 450 nm and expressed as nmol/L.

Both liver and serum BEA were determined by colorimetric measurements of *p*-aminobenzoate released from *N*-biotinyl-*p*-aminobenzoate in accordance with colorimetric method of Wolf et al. [9,10]. After preincubation of 15 min at 37 °C, the enzyme assay was initiated by addition of 0.1 ml serum and liver tissue homogenate, to 1.9 ml of mixture consisting of 200  $\mu$ mol potassium phosphate buffer, pH 6.0, 20  $\mu$ mol

Table 1  
Demographic features of the study and the control groups

Groups	<i>n</i>	Valproic acid dose (mg/kg/d)	Biotin dose (mg/kg/d)	Duration of administration (day)
VPA-B1	15	600	6	60
VPA-B2	15	600	0.6	60
VPA	15	600	–	60
Control	15	Distilled water	–	60

EDTA, 0.5 mg serum albumin and 0.3  $\mu$ mol *N*-biotinyl-*p*-aminobenzoate reaching the final volume to 2 ml (0.3 ml of diluted serum was used in experiments to find out the activity as a function of amount of enzyme). The mixture was incubated for 30 min at 37 °C and the reaction was ceased by the addition of 0.2 ml of 30% trichloroacetic acid. After that the mixture was centrifuged at 2000 rpm for 15 min and 1.5 ml supernatant was added to 0.5 ml water. At room temperature, and with 3 min intervals, 0.2 ml of 0.1% sodium nitrite (made fresh daily), 0.2 ml of 0.5% ammonium sulfamate and 0.2 ml of 0.1% *N*-1-naphthyl ethylenediamine hydrochloride were added and allowed to incubate for 10 min before measuring absorbance at 546 nm. Serum and liver BEA was expressed as IU/L and mU/100 mg protein, respectively.

### 2.3. Statistical analysis

Kolmogorov–Smirnov Goodness of fit test was used to control whether the distribution of parameters is normal or not. Homogeneity of variance of the groups was tested with Levene's test. For all groups the parameters had normal distribution. Thus, the groups were compared with the one-way ANOVA with Tukey HSD test and chi-square test as appropriate. All *p* values reported were for a two-sided test; *p* < 0.05 were considered statistically significant. Correlation between the groups was compared with Pearson's correlation test. Data were analyzed by using the package program of SPSS for Windows 10.0.

### 3. Results

No subject was lost during the study. Demographic features of the study and the control groups were shown in Table 1. In the third week of the study, we determined alopecia in the study groups. It was more obvious in the VPA group (Fig. 1).

Alopecia was seen in the subjects of 13.3% of VPA-B1 (*n* = 2), 13.3% of VPA-B2 (*n* = 2), and 40% of VPA (*n* = 6). In the control group, alopecia was not observed (Fig. 2). There is a statistically significant differences between the groups with respect to alopecia (*p* = 0.028). The statistical significance is due to the dif-



Fig. 1. In the study groups, alopecia was seen in the third week of the study.

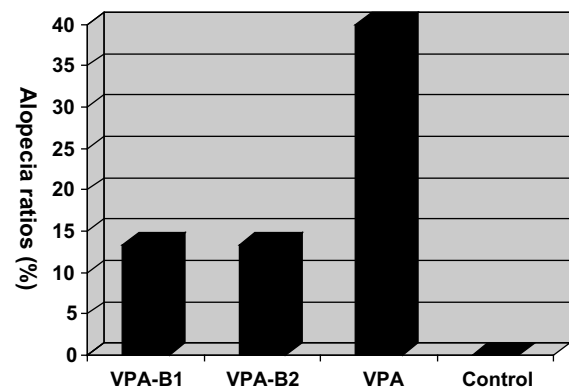


Fig. 2. The ratios of alopecia in the study groups were statistically higher than the control group (*p* = 0.028).

ference between VPA and control groups. There is no statistically significant difference between the ratios of alopecia in the study groups (*p* = 0.128). Itchiness was more obvious in the study groups compared with the control group.

The levels of liver tissue and serum BEA, and the serum biotin levels of the study and control groups are demonstrated in Table 2. Significant differences between the study and control groups for serum biotin levels were determined (*p* < 0.05). Serum biotin levels of the biotin supplemented groups (VPA-B1 and VPA-B2) were higher than the other groups (VPA and control group) (*p* < 0.05). Serum biotin levels of the VPA group

Table 2  
The levels of liver tissue and serum BEA, and the serum biotin levels of the study and control groups

Groups	Serum biotin (nmol/L)	Serum BEA (IU/L)	Liver BEA (mU/100 mg protein)
VPA-B1	79 ± 8.78	12.60 ± 0.96	8.37 ± 0.79
VPA-B2	42.86 ± 10.0	12.96 ± 1.53	9.17 ± 0.68
VPA	16.06 ± 2.43	12.85 ± 1.36	9.20 ± 0.48
Control	21.66 ± 4.11	15.40 ± 0.85	11.49 ± 0.96
<i>p</i> values	<i>p</i> < 0.05*	<i>p</i> < 0.05*	<i>p</i> < 0.05*

Table 3  
Sub-group comparisons for serum biotin, serum and liver tissue BEA

Groups	Serum Biotin (nmol/L)	Serum BEA (IU/L)	Liver tissue BEA (mU/100 mg protein)
VPA-B1/Control	$p < 0.05^*$	$p < 0.05^*$	$p < 0.05^*$
VPA-B2/Control	$p < 0.05^*$	$p < 0.05^*$	$p < 0.05^*$
VPA/Control	$p = 0.145$	$p < 0.05^*$	$p < 0.05^*$
VPA-B1/VPA-B2	$p < 0.05^*$	$p = 0.85$	$p = 0.11$
VPA-B1/VPA	$p < 0.05^*$	$p = 0.94$	$p = 0.09$
VPA-B2/VPA	$p < 0.05^*$	$p = 0.99$	$p = 0.99$

were lower than the control group but, it was not statistically significant ( $p = 0.145$ ). There was no significant difference between the study groups for serum and liver tissue BEA ( $p > 0.05$ ). However, there were significant decreases in the levels of serum and liver tissue BEA of the study groups compared with the control group ( $p < 0.05$ ). The levels of serum AST, ALT, albumin, and VPA of the study and the control groups are demonstrated in Table 3. There were statistically significant differences among the groups for serum AST and ALT levels ( $p < 0.05$ ). But, no significant difference was determined among the groups for serum albumin levels ( $p = 0.06$ ). Serum AST and ALT levels of the study groups were higher than the control group ( $p < 0.05$ ). The levels of VPA in the study groups were determined as similar ( $p = 0.112$ ).

#### 4. Discussion

VPA has many side effects dose-dependent such as hair loss, skin eruption, tremor, and non-dose-dependent such as metabolic disorders, liver toxicity, teratogenicity and hemostasis disturbances. The PO minimum effective and LD<sub>50</sub> doses of VPA in rats were suggested as 200 mg/kg, and 1530–1788 mg/kg, respectively [11,12]. In some studies carried out with rats, VPA was used as PO 200–400 mg/kg/d, divided in two doses for 90 days or 200–400–600 mg/kg/d, divided in two doses for 60 days [9,13,14]. In our study, except the control group, all groups were administrated VPA of 600 mg/kg/d, divided in two doses for 60 days that was shown in Table 4.

Table 4  
The levels of serum AST, ALT, albumin, and VPA of the study and the control groups

Groups	Serum AST (IU/L)	Serum ALT (IU/L)	Serum albumin (g/dL)	Serum VPA (μg/ml)
VPA-B1	132.73 ± 23.08	58.53 ± 13.15	3.10 ± 0.49	30.30 ± 4.29
VPA-B2	132.13 ± 29.47	56.33 ± 7.43	3.20 ± 0.57	32.72 ± 3.28
VPA	135.06 ± 26.97	52.06 ± 10.91	3.14 ± 0.29	28.98 ± 4.48
Control	90.13 ± 9.67	39.06 ± 5.04	3.45 ± 0.22	–
<i>p</i> values	$p < 0.05^*$	$p < 0.05^*$	0.06	0.112

Table 5  
Sub-group comparisons for serum AST, ALT, and albumin levels

Groups	Serum AST (IU/L)	Serum ALT (IU/L)	Serum albumin (g/dL)
VPA-B1/Control	$p < 0.05^*$	$p < 0.05^*$	$p = 0.13$
VPA-B2/Control	$p < 0.05^*$	$p < 0.05^*$	$p = 0.40$
VPA/Control	$p < 0.05^*$	$p < 0.05^*$	$p = 0.24$
VPA-B1/VPA-B2	$p = 0.99$	$p = 0.99$	$p = 0.92$
VPA-B1/VPA	$p = 0.99$	$p = 0.26$	$p = 0.29$
VPA-B2/VPA	$p = 0.98$	$p = 0.26$	$p = 0.09$

Biotin is an essential vitamin ensured from the foods for all mammals [6]. Daily recommendations for biotin are as follow: for infants 50 mcg/d, for children less than 4 years-old 150 mcg/d and for adults 300 mcg/d. Approximately the daily biotin dose of 0.06 mg/kg/d for rats is equilibrium the biotin dose of 300–400 mcg/d for a 70-kg person [15]. Daily biotin requirement is 10-fold higher for the treatment of biotin-responsive disorders, such as biotinidase deficiency and multiple carboxylase deficiency [16,17]. In our study, rats were administrated 6 mg/kg/d or 0.6 mg/kg/d biotin for 60 days which was shown in Table 5.

Alopecia is a frequent and a temporary side effect seen in children and adults during VPA therapy. It was reported that alopecia, skin eruption, and itch seen during the VPA usage in therapeutic doses were 38%, 11%, and 14%, respectively [1]. Korkmazer et al. reported the ratio of alopecia as 27% in rats which were administrated 600 mg/kg/d VPA for 60 days [9]. In another study carried out in epileptic children, hair loss was found as 12% in children receiving 41 mg/kg/d VPA and 24% receiving 55 mg/kg/d VPA [8]. In our study, alopecia and itch began after 2 weeks from the beginning of VPA therapy, and appeared in the third week. Alopecia was observed in Group-1, Group-2, and Group-3 as 13.3% ( $n = 2$ ), 13.3% ( $n = 2$ ), and 40% ( $n = 6$ ), respectively.

Urinary biotin expression begins to reduce within 2–3 weeks in insufficient biotin intake. Insufficient biotin intake with diet also decreases the serum biotin level [18,19]. In biotin deficiency, intestinal system, skin, hair, central and peripheric nervous systems are affected. There is not a precise explanation why these systems are involved in biotin deficiency. It is reported that decreased activity of acetyl-CoA carboxylase enzyme and associated alterations in fatty acid synthesis may play a role in affecting these systems in biotin deficiency [15,20].

BEA is found in higher concentrations in the liver and serum compared to the brain [21]. It was reported that liver tissue and serum BEA diminished as 50% and 30% in rats undergone partial hepatectomy, respectively [22]. This result suggests that serum BEA may originate from liver. In addition to, it was reported that serum BEA was lower levels in the patients with chronic hepatitis than that of healthy individuals [23].

VPA treatment decreases the serum and liver tissue BEA by influencing liver functions. Schulpis et al. reported that serum BEA were statistically reduced in children with epilepsy receiving VPA of 35 mg/kg/d [8]. In another study carried out with rats it was shown that VPA usage lowered the liver tissue and serum BEA [9]. Therefore decline of serum biotin levels may be resulted from affecting of liver created by VPA usage with an indirect mechanism. It is reported that VPA may affect the functions of some carboxylase enzymes associated with biotin cycle. It may explain how VPA decrease the serum BEA levels [8].

In our study, when compared with the control group there were statistically significant decreases in the study groups for serum and liver tissue BEA. There were no significant differences between VPA and biotin supplemented VPA groups for serum and liver tissue BEA. Alopecia was observed in the subjects of 40% ( $n = 6$ ) of VPA group. But in the groups of VPA-B1 and VPA-B2, receiving biotin dose of 6 mg/kg/d or 0.6 mg/kg/d, respectively, ratio of alopecia was 13.3% ( $n = 2$ ). The ratios of alopecia in biotin supplemented VPA groups were lower than VPA group but it was not statistically significant. This situation may be due to small sample size because in VPA group alopecia was three times more than biotin supplemented groups. There was also no statistically significant difference between VPA-B1 and VPA-B2 groups for alopecia and serum and liver tissue BEA. This situation may be related to preserving of apocarboxylase enzyme pool. It is suggested that apocarboxylase enzyme pool is preserved in biotin deficiency. Rodriguez-Melendez et al. reported that apocarboxylase enzyme pool reached to the adequate levels within 24 h after the biotin supplementation in biotin deficient rats [24]. They also showed that biotin supplementation increased apocarboxylase enzyme expression via a regulation at the posttranscriptional level. These effects of biotin at molecular level may be an explanation for the same alopecia ratios seen in VPA-B1 and VPA-B2 groups in our study. It was reported that rats consuming biotin-free diets had reduced levels ranged from 50% to 60% of biotinylated forms of carboxylases after 21-day [15]. In our study we observed that hair loss and itch began after the second week in rats administered VPA. At the same time, these findings became more apparent after the third week.

Lewis et al. reported that apocarboxylase enzyme levels decreased in rats which supplemented with excessive biotin of 100 mg/kg/d [15]. It is not known that why large doses of biotin reduce the enzyme levels. Furthermore, it is reported that large doses of biotin can cause undesired side effects.

During VPA treatment, decreased levels of trace elements may be related with hair loss. It is suggested that lowness of selenium can cause hair loss and hair fragility [25]. At the same time there is a weak relation-

ship between serum VPA and zinc levels and zinc supplementation may be useful in reducing hair loss [26,27].

In the patients receiving VPA therapy, dose-dependent increase in the levels of liver transaminases may be seen [1]. Likewise there are many studies showing increased levels of liver transaminases in rats administered VPA [8,9]. In addition to, lowness of serum albumin and BEA levels is determined in rats with liver disease [28]. In our study, we determined statistically significant increases in the levels of serum AST and ALT in study groups. Serum albumin levels of the rats in the study groups were lower than the control group but not statistically significant.

In conclusion we showed that VPA usage reduced the serum and liver tissue BEA and partially impaired the biotin utilization by affecting the liver. Partial biotinidase deficiency may lead to alopecia. It might be prevented by biotin supplementation in the patients receiving VPA therapy. We considered that further studies are necessary to find out the effective and safe biotin dose.

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