Original Article

TIPS Trends in Pharmaceutical Sciences 2017: 3(2): 63-70.

Valproic Acid-Induced Hepatotoxicity and the Protective Role of Thiol Reductants

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Abstract

Valproic acid (VPA) is a widely administered drug against epilepsy and several other neurological disorders. On the other hand, liver injury is a deleterious side effect associated with VPA. Oxidative stress seems to play a critical role in VPA-induced hepatotoxicity. The current investigation was designed to evaluate if N-acetylcysteine (NAC) and dithiothreitol (DTT) as thiol reducing agents have any protective effects against VPA-induced liver injury. Isolated rat hepatocytes (*in vitro*) were exposed to increasing concentrations of VPA (25, 50, 100, 150, and 250 µM) and markers of cytotoxicity were evaluated. Furthermore, animals received VPA (250 and 500 mg/kg, i.p for 15 consecutive days) (*in vivo*) and markers of liver injury were monitored. It was found that 250 µM of VPA caused marked cytotoxicity toward isolated hepatocytes as judged by trypan blue exclusion test. Moreover, markers of oxidative stress including glutathione depletion and lipid peroxidation were detected in VPA-treated hepatocytes. On the other hand, VPA caused a significant increase in plasma markers of hepatotoxicity in drug-treated group. Liver histopathological changes and markers of oxidative stress were also detected in VPA-treated animals. It was found that administration of NAC (1 mM), and DTT (1 mM) significantly alleviated VPA-induced cytotoxicity (*in vitro*). NAC (250 and 500 mg/kg) and DTT (15 and 30 mg/kg) also significantly mitigated VPA hepatotoxicity (*in vivo*). The data obtained from the current investigation indicate potential therapeutic properties of thiol reductants against VPA-induced liver injury.

Keywords: Glutathione, Hepatoprotective, Hepatotoxicity, Oxidative stress, Sodium valproate.

1. Introduction

Valproic acid (2-propyl-pentanoic acid, VPA) is clinically administered against epilepsy and several other psychiatric disorders (1). On the other hand, several drug reactions have been reported in relation to VPA therapy (1-3). Hepatotoxicity, hyperammonemic encephalopathy, hypersensitivity reactions, neurological toxicity, and renal injury have been reported after VPA administration (1, 4, 5). Hepatotoxicity is a deleterious and life-threatening side effect of VPA (2, 3, 6). Hence, finding hepatoprotective agents against VPA-induced liver injury could have clinical value.

VPA hepatotoxicity is believed to be mediated through hepatocytes mitochondrial dysfunction (6, 7). Several investigations also men-

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Recieved: 01/05/2017; Accepted: 25/05/2017

tioned the important role of oxidative stress and its consequences in the VPA hepatotoxicity (8-11). Reactive oxygen species (ROS) formation, lipid peroxidation, defect in cellular antioxidant enzymes, and glutathione depletion is documented in different experimental models of VPA hepatotoxicity (6-10, 12). Hence, the thiol reducing agents (NAC and DTT) which preserve the glutathione molecule in its reduced form (GSH), might provide protection against VPA-induced hepatotoxicity.

The current study was designed to evaluate the effect of VPA on isolated hepatocytes (*in vitro*) and animal liver (*in vivo*). NAC and DTT were administered to investigate if these thiol reducing agents have any protective properties against VPA hepatotoxicity.

2. Materials and methods

2.1. Chemicals

Trichloroacetic acid (TCA), Sodium valproate, Glutathione (GSH), Malondialdehyde (MDA), Bovine serum albumin (BSA), and Thiobarbituric acid (TBA) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Kits for evaluating biomarkers of liver injury were obtained from Pars Azmun® (Tehran, Iran). 1-Bromoheptane, Trypan blue, Ethylenediaminetetraacetic acid (EDTA), 5,5-bis-dithio-nitro benzoic acid (DTNB), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), phosphoric acid, n-Butanol, and 2 Amino 2-hydroxymethyl-propane-1, 3-diol (Tris), were obtained from Merck (Darmstadt, Germany). All salts used for making buffer solutions were of analytical grade and obtained from Merck (Darmstadt, Germany).

2.2. Animals

Male BALB/c mice (20-25 g) were obtained from the Laboratory Animal Breeding Center, Shiraz University of Medical Sciences, Shiraz, Iran. Animals were housed in an environmental temperature of 23±1 ºC with a 40% of relative humidity. Animals had free access to water and a normal chow diet during experiments. All procedures were in accordance with the guidance for care and use of experimental animals and were approved by a local ethic committee at Shiraz University of Medical Sciences, Shiraz, Iran (95-01-36-12163).

2.3. In vitro experimental setup

Rat hepatocytes were freshly isolated according to a previously described method using collagenase enzyme (13). The cells (1×10^6 cell/mL) were suspended in Krebs Henseleit buffer containing 12.5 mM HEPES and incubated under a stream of 95% O_2 and 5% CO_2 in continuously rotating round-bottomed flasks at 37 ºC water bath (14).

2.4. Cell viability

Hepatocyte viability was assessed by plasma membrane disruption as determined by trypan blue uptake test (15, 16). Cell viability was determined immediately after isolation and at scheduled time intervals following hepatocytes incubation. Approximately 85–90% of hepatocytes were viable at the time of isolation (14).

2.5. In vivo experimental setup

Animals were randomly divided into three groups (n=8) as follows:

1) Control (Vehicle-treated group),
2) VPA (250 mg/kg/day, i.p);
3) VPA (500 mg/kg/day, i.p).

It has been previously reported that a dose of 500 mg/kg/day of valproic acid for 5 consecutive days caused marked liver and renal injury (7). At the end of experiments (day 6^{th}), animals were anesthetized with ketamine/xylazine (100 mg/kg/10 mg/kg, i.p) and their blood and liver were collected (17, 18).

2.6. Plasma biochemistry and liver histopathology

A Mindray BS-200® auto analyzer (Mindray chemistry analyzers for low-volume laboratories, Guangzhou, China), and standard kits were employed to assess serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and bilirubin (19-22). For histopathological assessments, samples of liver tissue were fixed in a buffered formalin solution (0.4% sodium phosphate monobasic, NaH_2PO_4, 0.64% sodium phosphate dibasic, Na_2HPO_4, and 10% formaldehyde in dis-

tilled water; pH=7.4)(22, 23). Finally, paraffin-embedded sections of liver tissue were prepared and stained with hematoxylin and eosin (H&E) before light microscope viewing (21, 24). Liver slides were analyzed by a pathologist in a blind fashion.

2.7. Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) were assessed as an index of lipid peroxidation. Briefly, 500 µl of liver tissue homogenate (10% w/v in KCl, 1.15%) was added to a reaction mixture consisted of 1 mL of thiobarbituric acid (0.375%, w/v) and 3 mL of phosphoric acid (1% w/v, pH=2) (21-23, 25). Samples were mixed well and heated in boiling water (100 °C, 45 minutes). Finally, 2 mL of n-butanol was added and vigorously mixed. Samples were centrifuged (10,000 g, 5 minutes) and the absorbance of developed color in the n-butanol phase was measured at 532 nm using an Ultrospec 2000®UV spectrophotometer (26, 27).

2.8. Hepatocytes glutathione content

Hepatic glutathione (GSH) content was assessed by the method described by Sedlak et al.(25, 28). Briefly, tissue samples (200 mg) were homogenized (Heidolph, Germany), in 8 mL of ice-cooled EDTA solution (20 mM, 4 ºC). Then, 5 mL of the prepared homogenate was mixed with 4 mL of distilled water (4 ºC) and 1 mL of trichloroacetic acid (50% w/v, 4 ºC) (20). Samples were mixed and centrifuged (15,000 g, 4 °C, 20 minutes) (29). Then, 2 mL of the supernatant was mixed with 4 mL of Tris buffer (pH=8.9), and 0.1 mL of the Ellman's reagent (DTNB, 10 mM in methanol). The absorbance of the developed yellow color was measured at 412 nm using an Ultrospec 2000®UV spectrophotometer (29, 30).

2.9. Statistical analysis

Data are given as the Mean±SEM. Data comparison was performed by the one-way analysis of variance (ANOVA) with Tukey's multiple comparison test as a post hoc. Differences were considered statistically significant when $P<0.05$.

3. Results and Discussion

VPA was cytotoxic toward isolated rat hepatocytes (Table 1). Increasing concentrations of VPA caused cytotoxicity time-dependently (Table 1). It was found that addition of NAC (1 mM) and/or DTT (1 mM) diminished toxicity induced by VPA (Table 2). The cellular GSH content was significantly lower in VPA-treated hepatocytes (Table 3). Treating rat hepatocytes with NAC (1 mM) or DTT (1 mM) significantly mitigated VPA-induced GSH depletion (Table 3). When hepatocytes were incubated with VPA (250 µM), a significant amount of lipid peroxidation was detectable (Table 4). It was found that NAC (1 mM) or DTT (1 mM) prevented VPA-induced lipid peroxidation in isolated rat hepatocytes (Table 4).

VPA hepatotoxicity was biochemically evident in animals by increased serum level of liver injury biomarkers (Figure 1). It was found that serum ALT, AST, and LDH were significantly higher in VPA (250 and 500 mg/kg)-treated animals (Figure 1). Markers of oxidative stress were

Table 1. Valproic acid cytotoxicity in isolated rat hepatocytes (Trypan blue exclusion test).

VPA (µM)	Time (min)			
	30	60	120	180
0	21±1	27±4	26±1	28±1
25	44±2*	61±3*	68±4*	83±7*
50	44±1*	61±3*	75±6*	85±5*
100	48±4*	65±5*	77±7*	86±4*
150	57±6*	67±7*	76±9*	93±6*
250	61±2*	75±5*	90±4*	97±3*

Data are presented as Mean±SEM for three individual experiments. VPA: Valproic acid. *Indicates significantly different as compared with control (VPA 0 µM) ($P<0.05$).

Table 2. Effect of thiol reductants on valproic acid cytotoxicity toward isolated rat hepatocytes (Trypan blue exclusion test).

Treatment	Time (min)			
	30	60	120	180
Control	20±1	22±2	26±1	28±4
VPA 250 µM	59±4*	74±6*	86±5*	100±8*
NAC 1 mM	18±2[a]	20±3[a]	25±4[a]	27±3[a]
DTT 1 mM	19±2[a]	21±3[a]	26±3[a]	28±5[a]
VPA 250 µM + NAC 1 mM	37±2[a]	43±3[a]	50±3[a]	59±6[a]
VPA 250 µM + DTT 1 mM	41±5[a]	43±2[a]	48±4[a]	58±6[a]

Data are given as Mean±SEM for three individual experiments. VPA: Valproic acid; DTT: Dithiothreitol; NAC: N-acetyl cysteine. *Indicates significantly different as compared with control (P<0.05). [a]Indicates significantly different as compared with VPA-treated group (P<0.05).

also increased in VPA-treated mice (Figure 2). It was found that liver lipid peroxidation was significantly increased and hepatic glutathione content was decreased in VPA-treated animals (Figure 2). Histopathological presentations of VPA-induced hepatotoxicity include inflammation, sinusoidal dilation and pyknosis (Figure 3 and Table 5).

NAC and DTT administration mitigated plasma biomarkers of liver injury in VPA-treated animals (Figure 1). Moreover, thiol reductants alleviated oxidative stress (Figure 2) as well as VPA-induced liver histopathological changes (Figure 3 and

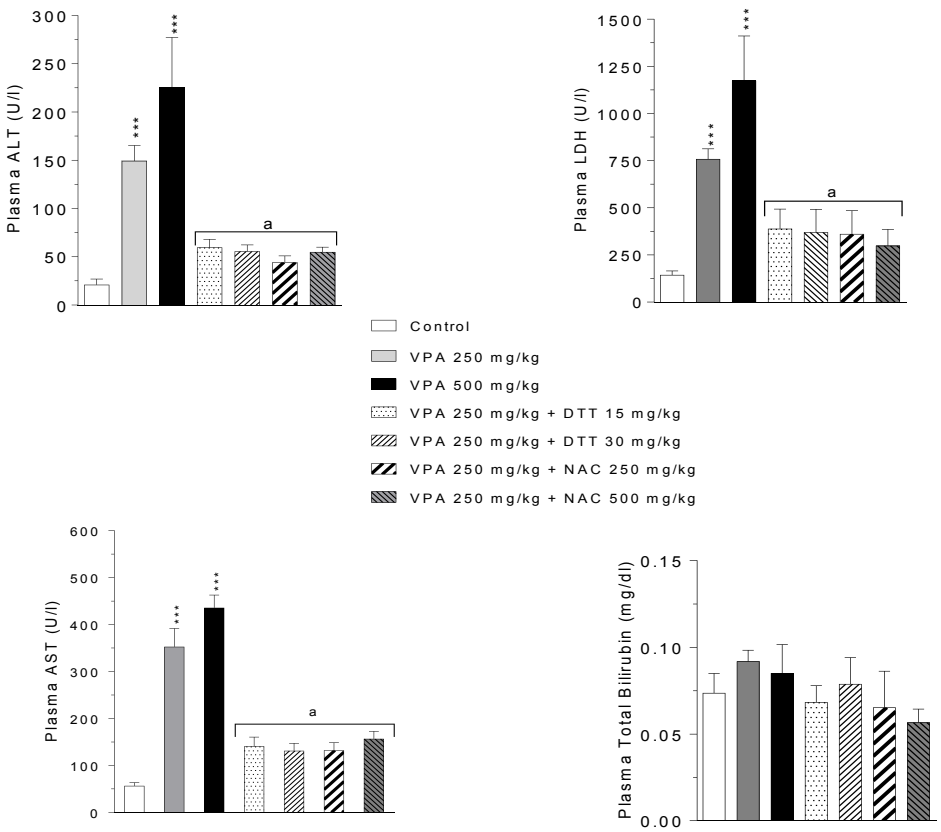

Figure 1. Plasma biochemistry in VPA-treated animals (*in vivo*). VPA: Valproic acid; DTT: Dithiothreitol; NAC: N-acetyl cysteine. Data are presented as Mean±SEM (n=8). ***Indicates significantly different as compared with control (P<0.001). [a]Indicates significantly different as compared with VPA (250 mg/kg)-treated group (P<0.001).

Table 3. Effect of valproic acid on hepatocytes GSH content (µmol/10^6 cells).

Time (min)	Treatments			
	Control	VPA 250 µM	VPA 250 µM + NAC 1 mM	VPA 250 µM + DTT 1 mM
30	33±3	19±1*	33±1[a]	32±2[a]
60	30±2	14±1*	29±1[a]	27±1[a]
120	27±1	10±1*	24±1[a]	25±1[a]

Data are presented as Mean±SEM for three individual experiments. VPA: Valproic acid; DTT: Dithiothreitol; NAC: N-acetyl cysteine. *Indicates significantly different as compared with control (P<0.05). [a]Indicates significantly different as compared with VPA-treated group (P<0.05).

Table 5).

4. Discussion

VPA effectively controls epilepsy and several other psychiatric disorders. On the other hand, there is evidence of hepatic injury in patients treated with VPA (4). The present study aimed to investigate the role of thiol reducing agents against VPA-induced hepatotoxicity. VPA caused toxicity in both isolated hepatocytes (*In vitro*) and drug-treated animals (*In vivo*) as judged by a significant increase in cytotoxicity, increased biomarkers of oxidative stress, elevated plasma markers of liver injury, and tissue histopathological changes. The abovementioned markers of toxicity caused by VPA were alleviated by administration of thiol-reducing agents.

Previous investigations proposed oxidative stress as a mechanism for VPA-induced liver injury (8, 10, 11, 31). In the current study, we found that VPA caused a significant amount of lipid peroxidation as an index of oxidative biomembranes

Table 4. Lipid peroxidation in valproic acid-treated hepatocytes (nmol of TBARS/10^6 cells).

Time (min)	Treatments			
	Control	VPA 250 µM	VPA 250 µM + NAC 1 mM	VPA 250 µM + DTT 1 mM
30	1.45±0.4	3.65±0.12*	3.25±0.3[a]	2.7±0.15[a]
60	2.2±0.5	4.6±0.31*	4.45±0.02[a]	3.5±0.6[a]
120	2.8±0.2	5.6±0.24*	5.45±0.38	5.0±0.28

Data are expressed as Mean±SEM for three independent experiments. VPA: Valproic acid; DTT: Dithiothreitol; NAC: N-acetyl cysteine. *Indicates significantly different as compared with control (P<0.05). [a]Indicates significantly different as compared with VPA-treated group (P<0.05).

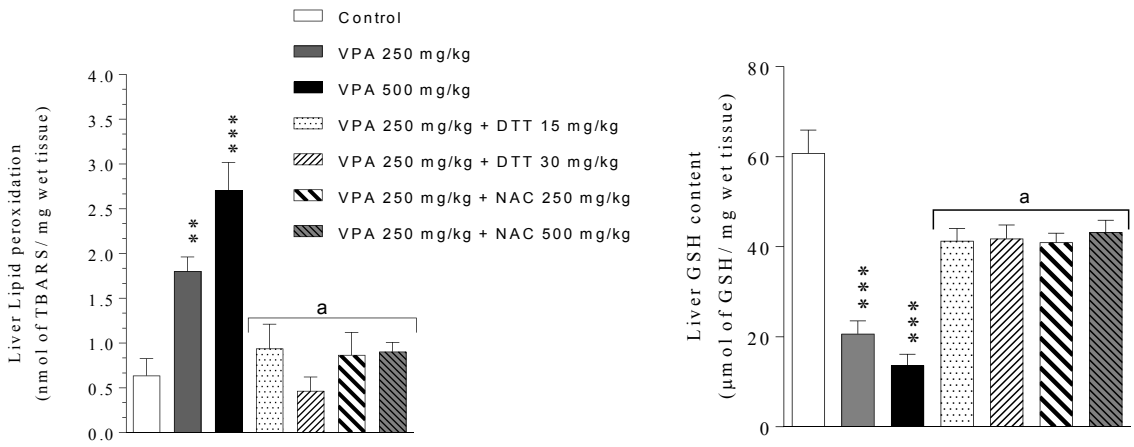

Figure 2. Liver tissue glutathione content and lipid peroxidation in VPA-treated animals. Data are presented as Mean±SEM (n=8). VPA: Valproic acid; DTT: Dithiothreitol; NAC: N-acetyl cysteine. ***Indicates significantly different as compared with control (P<0.001). [a]Indicates significantly different as compared with VPA (250 mg/kg)-treated group (P<0.001).

Table 5. Liver histopathological changes in valproic acid-treated animals.

	Nuclear Fragmentation	Cytoplasm Degeneration	Inflammation	Congestion	Total grade
Control	-	-	-	-	0
VPA 250 mg/kg	+++	+++	+	+	8
VPA 500 mg/kg	+++	+++	++	++	10
VPA +NAC 250 mg/kg	+++	+++	-	+	7
VPA + NAC 500 mg/kg	++	+++	-	-	5
VPA + DTT 15 mg/kg	++	++	+	+	6
VPA +DTT 30 mg/kg	++	++	-	-	4

VPA: Valproic acid; NAC: N-acetyl cysteine; DTT: Dithiothreitol.

injury (Table 4 and Figure 2). Moreover, hepatocytes glutathione content were decreased (Table 3 and Figure 4), which might be a consequence of VPA-induced oxidative stress in hepatocytes. It was found that administration of the thiol-reducing agents effectively mitigated VPA-induced oxidative stress. Thiol reductants might provide protection against VPA by counteracting oxidative stress in hepatocytes. As mentioned, glutathione depletion and oxidation have been documented in VPA hepatotoxicity (31). This mention the important role of glutathione in preventing VPA hepatotoxicity. Hence, thiol reducing agents preserve glutathione in its reduced form and could provide protection against VPA hepatotoxicity (Table 3 and Figure 2).

Mitochondrial dysfunction could play a pivotal role in the mechanism of drug-induced liver injury (32, 33). It has been found that mitochondria are a major target for VPA to induce cytotoxicity (31, 34). On the other hand, glutathione reductants such as NAC could effectively protect mitochondria against xenobiotics (35, 36). Although it was not investigated in the current study, a part of protective properties of thiol reducing agents might be attributed to their effect on mitochondria.

Collectively, the data obtained from current investigation indicate the potential protective properties of thiol reducing against VPA-induced oxidative stress and liver injury. The hepatoprotective properties of these agents deserve further

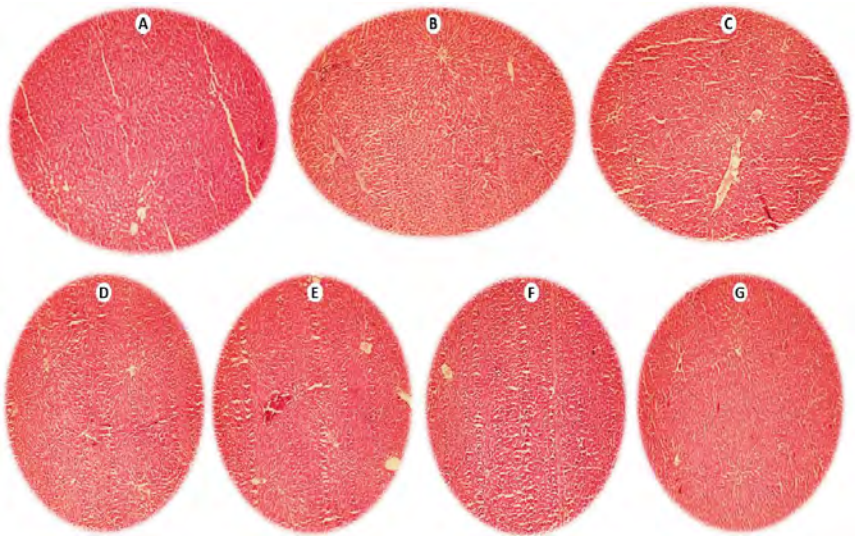

Figure 3. Liver histopathological changes in valproic acid-treated mice. Control liver showed no significant histopathological changes (A). Liver histopathological changes were revealed as inflammation, sinusoidal dilation and pyknosis in valproic acid treated animals (250 mg/kg and 500 mg/kg, B and C respectively). D: VPA 250 mg/kg +NAC 250 mg/kg; E: VPA 250 mg/kg +NAC 500 mg/kg; F: VPA 250 mg/kg +DTT 15 mg/kg; G: VPA 250 mg/kg +DTT 30 mg/kg. Refer to Table 5 for the grade of histopathological changes in each group. VPA: Valproic acid; DTT: Dithiothreitol; NAC: N-acetyl cysteine.

investigation in other models of drug-induced liver injury. Moreover, evaluating the effect of thiol reductants on VPA-induced mitochondrial dysfunction could be the subject of future investigations.

Acknowledgements

The instrumental facility providing of the Pharmaceutical Sciences Research Center of Shiraz University of Medical Sciences is gratefully acknowledged. This investigation was financially supported by the Vice Chancellor of Research Affairs of Shiraz University of Medical Sciences Grant number: (95-01-36-12163).

Conflict of Interest

None declared.

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