



ORIGINAL ARTICLE: HEPATOLOGY

Acute Alcohol Tissue Damage: Protective Properties of Betaine

Lucy Petagine¹, Hannah Everitt¹, Victor R. Preedy², Roy A. Sherwood³, Vinood B. Patel^{1,2}

¹Centre for Nutraceuticals, School of Life Sciences, University of Westminster, London, UK; ²Nutrition and Dietetics, King's College London, London, UK; ³Department of Clinical Biochemistry, King's College Hospital, London, UK

Abstract

Teenage binge drinking is a major health issue; however, there is a paucity of data on liver injury. Herein, we investigated how acute ethanol affects juvenile hepatic cells through changes in oxidative stress, apoptosis, and liver function, as well as the ability of betaine, which can replenish the antioxidant glutathione and mitigate oxidative injury. Juvenile male Wistar rats were given either water or betaine (2% w/v) for 6 days and treated with either saline 0.15 mol/L NaCl or ethanol (75 mmol/kg bodyweight). After 24 h, liver enzymes, oxidative damage, apoptosis, and parameters of antioxidant enzyme activity were examined. Acute ethanol increased hepatic enzymes (99%, $P < 0.05$). Total protein and albumin levels were reduced by 14 and 18% ($P < 0.001$), respectively, which was prevented by betaine treatment. Cytosolic cytochrome c increased by 59% ($P < 0.05$), corresponding to a decrease in mitochondrial cytochrome c content, which was ameliorated with betaine. Cytosolic glutathione peroxidase was reduced with alcohol ($P < 0.05$) and was prevented with betaine. Subtle changes were observed in catalase, superoxide dismutase, glutathione reductase, and complex I activity after ethanol treatment. In summary, whilst juvenile animals appear to have higher basal levels of antioxidant enzymes, betaine conferred some protection against alcohol-induced oxidative stress.

Keywords: adolescents; antioxidants; binge drinking; liver; oxidative stress

Received: 2 February 2021; *Accepted after revision:* 3 March 2021; *Published:* 19 March 2021

Author for correspondence: Dr. Vinood Patel, School of Life Sciences, University of Westminster, 115 New Cavendish Street, London W1W 6UW, UK. Email: v.b.patel@westminster.ac.uk

How to cite: Petagine L, et al. Acute Alcohol Tissue Damage: Protective Properties of Betaine. *J Ren Hepat Disord.* 2021;5(1): 19–29.

Doi: <https://doi.org/10.15586/jrenhep.v5i1.96>

Copyright: Petagine L, et al.

License: This open access article is licensed under Creative Commons Attribution 4.0 International (CC BY 4.0). <http://creativecommons.org/>

Introduction

Worldwide, alcoholic liver disease (ALD) is a major cause of chronic liver disease, which develops due to excessive, prolonged consumption of alcohol. This results in the formation of fatty liver, which can develop to steatohepatitis, cirrhosis/fibrosis, and lastly hepatocellular cancer (1).

ALD is traditionally associated with the adult population; however, recent statistical reports have identified an alarming increase in the percentage of teenage binge drinking. The proportion of adolescents who binge drink remains high with a shift toward young people drinking more frequently

with the intention of becoming intoxicated (2). Adolescents are now exposing their livers to the damaging effects of ethanol more frequently and more seriously than at any time in the past. The United Kingdom, in particular, is consistently categorized as a high prevalence country for underage drinking (3), with a third of British teenagers reporting intoxication by the age of 13 (4). The National Survey on Drug Use and Health has estimated that in the United States 22% of adolescents used alcohol in 2016 (5), whereas the European School Survey Project on Alcohol and Other Drugs has revealed that at least half of the students in three-quarters of the countries surveyed had drunk an alcoholic beverage

at age 13 or younger (3, 4). Whilst the clinical consequences of ethanol intake have been studied in adults, it remains unclear as to whether adolescents are more susceptible to ethanol-mediated liver damage, or if they can withstand the adverse effects better than adults. Animal studies with binge ethanol treatment have shown to decrease adenosine triphosphate (ATP) production as well as increase reactive oxygen species (ROS) and mitochondrial dysfunction, thus altering the electron transport chain components, resulting in mitochondrial failure (6–8).

Antioxidant enzymes are capable of catalyzing the decomposition of ROS (9). The three main antioxidant enzymes are superoxide dismutase (SOD), which can protect against toxic effects of superoxide radicals (10); glutathione peroxidase (GPx), which protects against oxidative injury (11); and catalase, which promotes the breakdown of hydrogen peroxide H₂O₂ (11).

The use of antioxidants serves as a prospective therapeutic approach for the treatment of ALD. Glutathione (GSH) is an intracellular antioxidant, which can neutralize basal levels of ROS (12). Other nutrients such as betaine play a role in the cysteine production pathway (13), ultimately forming GSH. Recent chronic experimental studies have examined the protective effects of betaine against alcohol-induced liver damage (14–18). In animal studies, dietary betaine has been shown to ameliorate the adverse effects of acute (19) and chronic ethanol dosing on liver steatosis and oxidative stress (20–23).

However, there are few studies exploring the effect of ethanol intoxication in the teenage population. Therefore, we aimed to investigate how ethanol affects hepatic cells through changes in oxidative damage, apoptosis, and liver function in juvenile rats. The effects of betaine supplementation on ethanol-induced injury in very young animal models is also limited. The use of betaine will determine if any such damage (and by extension, protection) is mediated through oxidative damage and the antioxidant capacity of the liver.

Materials and Methods

Animals

Juvenile male Wistar rats (50–55 g) were obtained from Charles Rivers (Bicester, UK) and housed according to good laboratory practice guidelines at the Biological Services Unit at Kings College, London. Animals were age and weight matched and divided into four groups: (1) Control, i.p. treated 0.15 mol/L NaCl; (2) Ethanol, i.p. treated ethanol (75 mmol/kg bodyweight); (3) Betaine 2% (w/v) in drinking water and i.p. treated 0.15 mol/L NaCl; and (4) Betaine (2% w/v) in drinking water and i.p. treated ethanol (75 mmol/kg bodyweight). Groups 1 and 2 were given water for 1 week and groups 3 and 4 received free access to food and freshly prepared betaine (2% w/v) in the drinking water. On the 7th

day, animals were i.p. treated with either saline or ethanol. Following treatment, food was removed, and the rats were sacrificed 24 h later. Hepatic cytosol and mitochondria were prepared as previously described (24). Blood serum was collected and stored for subsequent liver function test analysis. Liver function tests were measured by standard laboratory diagnostic procedures as previously described (25).

Glutathione levels

GSH levels were determined using an assay adapted from Tietze (26) based on the conversion of 5'5'-dithio-bis-2-(nitrobenzoic acid) (DNTB) to 5-thio-2-nitrobenzoic acid (TNB) by glutathione reductase (GR). Cytosolic or mitochondrial protein (5 µL) was added to the reaction buffer (100 mM sodium phosphate, 1 mM EDTA, 0.5 mM DTNB, 0.175 mM NADPH, 1.7U/mL GR) and the absorbance measured at 412 nm for 25 min.

Malondialdehyde levels

Malondialdehyde (MDA) levels were detected using a colorimetric thiobarbituric acid reactive substances assay adapted from Bar-Or et al (27). Protein (500 µg) was mixed with 400 µL of 20 mM phosphate buffer (pH 7.4) and 500 µL reaction buffer (5 mg/mL thiobarbituric acid, 25 mM NaOH, 50% glacial acetic acid). Samples were tightly sealed and boiled for 1 h, rested on ice for 10 min, and the absorbance measured at 532 nM.

Cytochrome c levels

The expression of Cytochrome c protein was quantified by an immunoblotting technique. Protein (60 µg cytosol or 40 µg mitochondria) was loaded onto gels and following transfer, the membranes were incubated with mouse anti-cytochrome c (1:1000) overnight, followed by the secondary antibody rabbit anti-mouse (1:10,000). Signals were detected using Pierce ECL reagent (ThermoFisher, UK). The resultant images were analyzed using the Biorad GS-800 Calibrated Densitometer.

Caspase-3 activity

Caspase-3 activity was evaluated by measuring the fluorescence of N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin (Ac-DEVD-AFC) after cleavage by caspase-3 to 7-amino-4-trifluoromethyl-coumarin (AFC) following incubation with 25 µg of protein. Samples (10 µL) were incubated in darkness with 100 µL of the substrate solution (10 µg/mL Ac-DEVD-AFC, 100 mM HEPES, 10 mM DTT) for 1 h at 37°C. The fluorescence was measured using an excitation wavelength of 380 nm and emission wavelength of 520 nm (28).

Complex I activity

The Complex I Enzyme Activity Microplate Assay Kit (Abcam, UK) was used to determine the activity of mitochondrial OXPHOS Complex I in the electron transport chain. Assay solution was prepared with 40 mM NADH. This method determined whether treatment of cells causes damage at complex I. Absorbance was read at OD 450 nm.

Antioxidant enzyme activity

The Glutathione Peroxidase Cellular Activity Assay Kit (Sigma, UK) was used as an indirect determination method based on the oxidation of GSH to GSSG. The decrease in NADPH absorbance was measured at 340 nm, indicating GPx activity. Glutathione Reductase Assay Kit (Sigma, UK) was used to measure the activity of GR, analyzed by spectrophotometric measurement. The activity was measured by any increase in absorbance caused by the reduction of DTNB at 412 nm. The Catalase Assay Kit (Abcam, UK) was used to measure catalase activity. Unconverted H_2O_2 reacts with OxiRed™ probe, which was analyzed by spectrophotometric measurement at 570 nm. The SOD Assay Kit-WST (Sigma, UK) was used to measure the activity of SOD via the utilization of Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. SOD activity was measured spectrophotometrically at 440 nm.

Statistical analysis

Results were analyzed using a one-way ANOVA. Data are presented as mean + SEM (n = 3–8) and P < 0.05 was considered statistically significant.

Results

Food, water, and body parameters

For the period of betaine treatment, food, water consumption, and body weights were recorded (Table 1). Betaine had no effect on food or water intake in the 6 days preceding the ethanol treatment, and body weights were also unchanged on the final day. On the other hand, liver weights were increased by 13 and 17% (P < 0.05), respectively, in the ethanol and betaine-/ethanol-treated groups, when compared to their corresponding controls. The ratio of liver weight to body weight was used to account for any differences in individual rat weights and liver sizes. A slight increase was observed in the liver weight/body weight ratio in ethanol-treated animals when compared to their corresponding control. In the ethanol group, a slight increase of 6% was observed, while there was a 5% increase in the betaine plus ethanol group, with the latter significantly increasing by 12% when compared to control animals (P < 0.01) (Table 1).

Liver function

Following ethanol treatment, ALT levels increased by 99% (P < 0.05) and AST levels showed an increase of 10% (Table 2). Although betaine alone showed no effect, betaine followed by ethanol led to an increase of 160% (P < 0.001) in ALT levels and 82% (P < 0.05) in AST levels. Following ethanol treatment, the AST/ALT value decreased by 41% (P < 0.001), and decreased by 32% (P < 0.001) following betaine and ethanol treatment. Total protein and albumin levels were also reduced following ethanol treatment by 14% (P < 0.001) and 18% (P < 0.001), respectively. However, this decrease was completely prevented in the betaine and ethanol group when compared to both control and betaine-alone treatment groups. There were no changes in circulating globulin levels in all treatment groups (Table 2).

Table 1: The effect of betaine on food and water intake, body weights, and liver weights.

	Control	Ethanol	Betaine	Betaine + Ethanol
Food intake (g/ rat/ day)	12.2 ± 0.4	11.8 ± 0.6	11.0 ± 0.4	10.7 ± 0.4
Water intake preinjections (mL/rat/day)	21.7 ± 1.1	21.4 ± 1.2	24.1 ± 1.6	21.9 ± 1.6
Water intake (post-injections (mL/rat/day)	20.5 ± 1.4	20.0 ± 1.6	16.5 ± 1.9*	17.5 ± 1.3*
Final body weight (g)	72.8 ± 1.6	77.3 ± 2.4	69.8 ± 1.6	75.6 ± 2.1
Liver weight (g)	2.67 ± 0.0	3.01 ± 0.1	2.69 ± 0.1	3.15 ± 0.2*
Liver weight (g)/body weight (kg)	36.6 ± 0.3	39.0 ± 0.8	38.4 ± 1.0	41.5 ± 1.0**

During the pretreatment stage, animals were housed in groups in cages and both food and water intake was monitored daily. During the treatment, animals were caged singly, deprived of food but allowed free access to water (either control or supplemented with 2% betaine). The body weights were recorded on the morning of sacrifice and the liver weights were recorded as soon as they were excised. *P < 0.05, **P < 0.01 compared to overall control.

Table 2: The effect of ethanol and betaine on liver function.

Treatment	ALT (U/L)	AST (U/L)	AST/ALT	Total Protein (g/L)	Albumin (g/L)	Globulin (g/L)
Control	59.7 ± 5.6	382 ± 43	6.4 ± 0.5	54.0 ± 1.3	36.3 ± 0.8	17.7 ± 0.6
Ethanol	119 ± 15*	423 ± 34	3.8 ± 0.2***	46.5 ± 1.4**	29.6 ± 0.9***	16.9 ± 0.7
Betaine	51.8 ± 2.3	313 ± 26	6.0 ± 0.3	52.3 ± 0.9	35.0 ± 0.7	17.3 ± 0.3
Betaine/Ethanol	135 ± 4.2***	569 ± 37*	4.1 ± 0.2***	52.3 ± 1.8	33.7 ± 0.9	18.6 ± 0.9

The betaine-supplemented experimental animals were given drinking water containing 2% betaine (w/v) for 6 days. Saline (0.15 mol/L NaCl) or ethanol (75 mmol/ kg bodyweight) was administered i.p. on day 7, and 24 h later the animals were sacrificed. Serum was analyzed by standard biochemistry tests. ALT = alanine aminotransferase; AST = aspartate aminotransferase. Values are mean ± SEM (n = 5–8). *P < 0.05; **P < 0.01; ***P < 0.001 compared to relevant control values.

Apoptosis

Ethanol exposure led to a significant increase (59%; P < 0.05) in cytosolic cytochrome c levels in comparison to controls. However, cytochrome c release was prevented by betaine pre-treatment followed by ethanol (6% decrease) (Figure 1B–C). In the mitochondria, a contrasting pattern occurred following ethanol treatment, whereby cytochrome c levels were lower (18%), confirming the release into the cytosol. Similarly, there was no change in the betaine-alone or betaine-ethanol exposed groups (Figure 1).

Caspase-3 is one of the final effector caspases in the apoptosis pathway (along with caspase-6 and caspase-7). Therefore, caspase-3 levels were assayed to determine if the cytochrome c release into the cytosol resulted in apoptosis and if betaine could prevent this release. However, ethanol treatment alone led to a 19% decrease in caspase-3 activity. No changes were observed in either betaine alone or betaine followed by ethanol groups (Figure 1D).

Oxidative damage

GSH levels were generally increased in ethanol and betaine supplementation. Ethanol treatment and betaine supplementation alone led to a 23 and 31% increase in cytosolic GSH levels, respectively (Figure 2A). A similar pattern occurred with mitochondrial GSH levels where ethanol caused an increase of 22% in mitochondrial GSH levels, and betaine followed by ethanol resulted in a 24% increase (Figure 2B). To assess whether betaine supplementation provided oxidative protective properties to the liver, MDA levels were assessed as a parameter for lipid peroxidation. Surprisingly, ethanol treatment led to a 50% reduction in cytosolic MDA levels (P < 0.05). Betaine alone had little effect (13% decrease), whereas betaine followed by ethanol caused a 22% reduction (Figure 2C). In the mitochondria, ethanol caused a 47% increase in MDA formation (Figure 2D). Betaine

alone as well as the betaine and ethanol group also showed no changes.

Antioxidant enzyme activity

Ethanol exposure alone led to a 31% decrease (P < 0.05) in cytosolic GPx activity when compared to the control (Figure 3A). Both the betaine only and ethanol- and betaine-treated groups led to a 10% and an 8% decrease, respectively, when compared to the corresponding controls (Figure 3B). In the mitochondria, no changes were seen in the ethanol and betaine-only groups. However, in the ethanol- and betaine-treated group, a 16% increase was observed (Figure 3B). GR catalyzes the reduction of GSSG to reduced GSH. In the cytoplasm, no changes were observed in GR activity in any treatment group (Figure 3C). On the other hand, in the mitochondria, GR was increased by 175 and 575% following treatment with ethanol and betaine supplementation, respectively (Figure 3D). In the ethanol- and betaine-treated group, a 15% reduction was observed when compared to the betaine only group (Figure 3D).

In the cytoplasm, ethanol treatment alone led to a 14% decrease in SOD activity, whereas in contrast, betaine treatment alone led to a 34% increase (Figure 4A). Ethanol and betaine treatment led to a 53% reduction in SOD activity in the cytoplasm when compared to the betaine control (Figure 4A). In the mitochondria, no changes to SOD activity were observed across all treatment groups (Figure 4B). The activity of catalase was also measured to assess its function. No changes in catalase activity were observed in either the ethanol alone, betaine alone, or a combination of ethanol and betaine treatments (Figure 4C).

Ethanol treatment alone led to a 50% increase in complex I activity and betaine supplementation alone led to a 13% decrease in complex I activity, respectively (Figure 4D). Betaine treatment followed by ethanol led to a 43% increase in complex I activity when compared to the betaine only group (Figure 4D).