**Acute alcohol tissue damage: Protective properties of betaine**

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# Abstract

Alcohol 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, 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 hours, liver enzymes, oxidative damage, apoptosis and antioxidant enzyme activity parameters 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 was conferred some protection against alcohol-induced oxidative stress.

**Key words: adolescents; antioxidants; binge drinking; liver; oxidative stress**

# 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 towards 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 UK, 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 US 22% of adolescents used alcohol in 2016 (5), whereas the European School Survey Project on Alcohol and Other Drugs survey has revealed that at least half of 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 whether adolescents are more susceptible to ethanol-mediated liver damage, or if their livers can withstand the effects better than adults. Animal studies with binge ethanol treatment have shown to decrease 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 able to catalyze 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 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 GLC guidelines at the Biological Services Unit at Kings College London. This study was been approved by the local Ethics Committee and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Animals were age and weight matched and divided into 4 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; (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 one 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 hours 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). 5 µL of cytosolic or mitochondrial protein 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 minutes.

## Malondialdehyde Levels

Malondialdehyde (MDA) levels were detected using a colorimetric thiobarburitic acid reactive substances assay adapted from Bar-Or *et al* (27). 500 µg of protein was mixed with 400 µL of 20 mM phosphate buffer (pH 7.4) and 500 µL reaction buffer (5 mg/mL thiobarburitic acid, 25 mM NaOH, 50% glacial acetic acid). Samples were tightly sealed and boiled for 1 hour, rested on ice for 10 minutes 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 mitochondria 40 µg) 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 (1:10000). 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. 10 μL samples 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 hour 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 will be prepared with 40 mM NADH. This method will determine 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 increases in absorbance caused by the reduction of DTNB at 412 nm. The Catalase Assay Kit (Abcam, UK) was used to measure catalase activity. Unconverted H2O2 reacts with OxiRedTM 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, in the betaine plus ethanol group a 5% increase, 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 32% (*p*<0.001) following betaine and ethanol. 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).

## 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 1, B-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 1).

## 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 (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 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 treatment (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).

# Discussion

To date, there have been few studies assessing the effects of both ethanol and betaine on oxidative damage and apoptosis in the teenage population. Binge ethanol treatment has been shown to decrease ATP and increase ROS (6). Recent *in vivo* studies using chronic ethanol models have examined the protective effects of betaine (14–18) and in acute ethanol models, dietary betaine has been shown to ameliorate injury (19). Therefore, this study aimed to investigate how ethanol affects juvenile hepatic cells through changes in oxidative damage, apoptosis and liver function and whether supplementation of betaine can provide protection or minimize the effects of oxidative liver injury.

## Liver Function

The administration of ethanol caused a rise in the levels of hepatic enzymes, indicating structural membrane damage to the hepatocytes. However, betaine was also unable to prevent the rise in ALT levels following ethanol exposure (Table 2). Although, it is perhaps the length of betaine treatment or concomitant betaine treatment that is essential for complete protection. This has been demonstrated previously in experiments where chronic betaine (1% for 2 weeks) ameliorated acute ethanol or lipopolysaccharide challenge in mice (19,29) and reduced chronic effects of ethanol in rats (30) and guinea pigs (31). Ethanol also led to a slight decrease in protein synthesis as measured by albumin. This is thought to be due to perturbed elongation factors reducing protein translation (32). These findings also show protein and albumin levels were normalized in the ethanol-treated animals pre-treated with betaine (Table 2), supporting previous research and demonstrating the beneficial effect of betaine (33).

## Apoptosis

Cytochrome c levels were significantly raised in the cytoplasm and the levels were consequently decreased in the mitochondria. As alcohol metabolism results in oxidative damage to the mitochondria as well as the production of ROS, destabilization of the cardiolipin anchor may have occurred, thus, releasing cytochrome c into the cytosol. Betaine pre-treatment followed by ethanol maintained the levels of mitochondrial cytochrome c and reduced the release of cytochrome c into the cytosol (Figure 1). Previous studies *in vitro* and *ex vivo* studieshave shown protection against apoptosis and research has shown that betaine prevented adenosine-induced apoptosis by preventing intracellular S-Adenosylmethionine (SAM) levels from increasing in rat hepatocytes (21). SAM supplementation has also shown to protect against apoptosis in rat hepatocytes after 24-hour incubation with ethanol (34). This suggests induction of the methionine-homocysteine recycling mechanism reduces cytochrome c release, which may in part be mediated by oxidative stress. However, this did not translate into an increase in caspase-3 activity, which is an ATP-dependent activation step (35). Since ATP levels drop following ethanol metabolism (36–38) or can be depleted following 24 hours starvation (39) in conjunction with oxidative damage (40,41), this may explain the lack of caspase activity.

## Oxidative Damage

Studies have shown that the metabolism of ethanol reduces the level of GSH (42–44), which ultimately increases susceptibility to oxidative damage, mitochondrial dysfunction and impairment of ATP synthesis (45). However, surprisingly, cytosolic and mitochondrial GSH levels were generally maintained or slightly increased (Figure 2), and MDA levels were generally correspondingly decreased (Figure 2). These findings, therefore, suggest some compensatory mechanisms involved preventing oxidative damage in response to acute ethanol administration (43,46). This may be due to an increased synthesis of GSH in the cytoplasm along with its subsequent import to the mitochondria or increased synthesis of antioxidant enzymes available to remove ROS. This shows the liver is capable of responding to an acute ethanol dose and that the increased GSH production is protecting against basal oxidative damage.

Treatment with betaine alone led to increased cytosolic GSH levels (Figure 2), however, this was slightly reduced with ethanol. This may provide some explanation to the lower cytosolic MDA levels in the betaine and ethanol treated group indicating that betaine is conferring some protection, perhaps via utilization of cytosolic GSH. In the mitochondria, a similar pattern occurred, whereby in the ethanol only treated group GSH levels were slightly increased and MDA levels lowered following betaine and ethanol treatment. These results indicate some compensation to oxidative stress and betaine providing some protection against ethanol-mediated injury. Overall, the ameliorative effect of betaine on hepatic GSH and MDA levels has been shown in a chronic model of ethanol intake using guinea pigs (20) and in an acute ethanol model using mice (19). These studies attributed the protective effects of betaine on the maintenance of cellular GSH levels. Currently, little research relates to acute ethanol administration in juvenile rats. These results do however support previous studies whereby betaine protects against the effects of ethanol on GSH levels, the mechanism causing the increase in GSH rather than a decrease remains to be elicited.

Ethanol consumption affects mitochondrial morphology, which alters the capacity of oxidative phosphorylation (18,47). Studies have shown that chronic treatment of ethanol induces loss of oxidation phosphorylation proteins which can be prevented by betaine supplementation (15). However, here we found complex I activity increased after acute ethanol treatment. When ethanol and betaine treatment was combined, an increase in complex I activity was also observed. Further studies are required to explore this finding.

## Antioxidant Enzymes

It has previously been reported that chronic ethanol treatment led to decreases in both cytosolic and mitochondrial GPx (48). In this study, acute ethanol exposure led to a significant decrease in GPx activity in the cytoplasm as well as betaine alone and betaine ethanol groups, leading to a decrease in activity in the cytoplasm. This lower GPx indicates less clearance of H2O2 taking place. GPx activity remained unchanged in the mitochondria as well as catalase activity which remained unchanged across all treatment groups. Treatment with ethanol alone and ethanol and betaine led to decreased SOD activity which is consistent with previous studies. The reduction in SOD activity may be due to ROS-induced enzyme degradation as well as a reduced synthesis of enzymes (49). However, as SOD activity was increased in the cytoplasm with betaine treatment alone, it may be possible betaine provides some protection to oxidative damage via an increase in antioxidant enzymes.This demonstrates that the antioxidant enzymes are generally maintained after acute ethanol treatment in juvenile rats. Age-related decreases in antioxidant enzymes are exacerbated by ethanol which causes aged animals to become more vulnerable to oxidative damage (50–52). This may be explained by a higher antioxidant capacity in adolescents; therefore, less damage occurs to the liver.

# Conclusion

These findings indicate juvenile rat livers react differently to acute ethanol than livers from aged animals by increasing GSH levels in response to ethanol. The mechanism behind this increase remains unclear and warrants further investigation. However, this could be partly due to higher basal levels and capacity of antioxidant enzymes in adolescents, causing less damage to the liver. This study showed betaine pre-treatment was able to ameliorate or prevent some oxidative changes. Thus, betaine may serve as a potential effective therapeutic in the treatment of liver disease as well as other diseases associated with oxidative damage.

# Acknowledgments

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## Conflict of Interest

None

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

During the pre-treatment stage, animals were housed in group cages and both food and water intake 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 the morning of sacrifice and the liver weights recorded as soon as they were excised. \**p*<0.05, \*\**p*<0.01 compared to overall control.

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| --- | --- | --- | --- | --- |
|  | 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 pre-injections (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\*\* |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | ALT  (U/L) | AST  (U/L) | AST/ALT | Total Protein  (g/L) | Albumin  (g/L) | Globulin  (g/L) |
| Treatment |  |  |  |  |  |  |
| 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 |

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

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 hours later the animals were sacrificed. Serum was analysed 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.

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**Figure 1. The effect of betaine on ethanol-induced changes in A) cytosolic and mitochondrial cytochrome c detection, B) cytosolic cytochrome c levels, C) mitochondrial cytochrome c levels and D) caspase-3 activity.** Cytochrome c levels were detected by Western blotting. Lanes 1-3 – control; Lanes 4-7 – ethanol; Lanes 8-10 – betaine; Lanes 11-13 – betaine and ethanol. Caspase-3 activity in 25 µg of cytosolic protein was determined based on the cleavage of Ac-DEVD-AFC (3 mg/mL) to AFC. Values are expressed as mean + SEM (n = 3-4). \* *p*<0.05 compared to control.

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**Figure 2. The effect of betaine on ethanol-induced changes in A) cytosolic GSH levels, B) mitochondrial GSH levels, C) cytosolic MDA formation** **and D) mitochondrial MDA formation**. The ‘betaine’ 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 hours later the animals were sacrificed. Liver mitochondrial and cytosolic fractions were prepared as described in the methods. Values are expressed as mean + SEM (n = 3-4). \* *p*<0.05 compared to control.

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Description automatically generatedFigure 3. The effect of betaine on ethanol-induced changes in A) cytosolic GPx activity and B) mitochondrial GPx activity, C) cytosolic GSH reductase activity, D) mitochondrial GSH reductase activity.** Following treatment liver mitochondrial and cytosolic fractions were prepared as previously described and the antioxidant enzyme activity was determined as described in the methods. Values are expressed as mean + SEM (n = 3-4). \* *p*<0.05 compared to control.

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**Figure 4. The effect of betaine on ethanol-induced changes in A) cytosolic SOD activity, B) mitochondrial SOD, C) catalase activity and D) complex I activity.** Following treatment liver mitochondrial and cytosolic fractions were prepared as previously described and the antioxidant enzyme activity was determined as described in the methods. Values are expressed as mean + SEM (n = 3-4).