# The effects of thiamine and thiamine pyrophosphate on alcohol-induced hepatic damage biomarkers in rats

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**Abstract.** – OBJECTIVE: This study examined the effects of thiamine and thiamine pyrophosphate on oxidative damage developing in association with hepatic injury caused by alcohol toxicity in rats and on hepatic injury markers.
MATERIALS AND METHODS: Four groups of rats were used; control, a group receiving thiamine+ethanol, a group receiving thiamine pyrophosphate+ethanol and a healthy group. The experimental protocol was repeated over 30 days. Malondialdehyde, glutathione and DNA damage product levels in liver tissue were measured at the end of the study. Alanine amino transferase and aspartate amino transferase, markers of liver damage, levels were determined. The results were then compared among the groups.
RESULTS: A statistically significant difference between antioxidant markers and markers of liver damage was determined between the group given thiamine pyrophosphate ethanol and the group given ethanol alone (*p* < 0.01) No statistically significant difference was observed between the group given thiamine and ethanol and the group given ethanol alone (*p* > 0.01).
CONCLUSIONS: Our results suggest that thiamine pyrophosphate may have a protective effect against liver damage caused by alcohol toxicity.

*Key Words:*
  Ethanol, Rat, Oxidants, Thiamine.

## Introduction

Alcohol consumption is rising across the world. Liver damage concludes with a high rate of death in chronic alcohol consumers[1]. Alcohol has been shown to lead to oxidative stress in living tissues[2]. A large part of the alcohol ingested is metabolized by alcohol dehydrogenase into ac-

etaldehyde in the liver[3]. Acetaldehyde is a highly reactive and toxic metabolite that leads to GSH loss by compromising L-cysteine in the glutathione (GSH) molecule in the liver. Acetaldehyde is oxidized by oxidase in the presence of iron, leading to the formation of free radicals. Additionally, it leads to lipid peroxidation and also to liver damage by reacting with malondialdehyde (MDA)[4,5]. The liver is, therefore, thought to be the organ most affected by alcohol. Alcohol is also exposed to oxidization by the microsomal ethanol oxidizing system (MEOS), resulting in the formation of hydrogen peroxide. If hydrogen peroxide is not sufficiently neutralized with GSH, it leads to lipid peroxidation[6,7]. This information from the literature shows that alcohol causes oxidative damage in the liver. Alcohol has been reported to induce experimental liver damage[8]. Alanine amino transferase (ALT) and aspartate amino transferase (AST) screening has been shown to be beneficial in alcohol-related liver damage[9,10]. The AST/ALT ratio rises in alcoholic liver damage. If this level (AST/ALT) exceeds 1.5, damage is thought to be alcohol dependent, but not if the level is less than 1[11]. The information obtained from the literature shows that alcohol causes oxidative damage in the liver and that determination of the markers AST and ALT and their ratios is important in the detection of that damage. In addition, it indicates that antioxidant therapy can be useful in preventing oxidative liver damage caused by alcohol.

The thiamine pyrophosphate (TPP) tested in this study is an active metabolite of thiamine. It emerges with the phosphatization of thiamine with thiamine pyrophosphokinase in the liver. Thiamine uses the pentose phosphate pathway to increase antioxidant formation and NADPH lev-

els[12]. Studies in recent years have shown that TPP also exhibits antioxidant activity[13,14]. However, thiamine has been tested and found to have no effect on ethanol-related oxidative liver damage[15]. Alcohol and various drugs have been reported to prevent the conversion of thiamine into TPP in the body. Alcohol has been reported to reduce thiamine pyrophosphokinase enzyme expression[16,17]. This information from the literature explains why thiamine is ineffective in alcohol-related oxidative liver damage. This, therefore, suggests that TPP, an active metabolite of thiamine, may be beneficial in oxidative liver damage caused by alcohol. Our scan of the literature revealed no data concerning the effect of TPP on oxidative liver damage induced with alcohol (ethanol). The purpose of this study was, therefore, to investigate the effect of TPP on oxidative liver damage induced with ethanol in rats and to assess this in comparison with thiamine.

## Materials and Methods

### Animals

Twenty four male albino Wistar rats weighing 200-210 g were used. These were obtained from the Atatürk University Medical Experimental Practice and Research Center, Turkey, and kept in groups at normal room temperature (22°C). All studies were performed in accordance with the ethical guidelines set out by the Local Ethical Committee and fully compatible with the "NIH Guide for the Care and Use of Laboratory Animals".

### Chemical Substances

Of the chemical substances used for the experiments, thiopental sodium was provided by IE Ulagay, Turkey. Thiamine and TPP were obtained from Biopharma, Russia.

### Experimental Groups

The experimental groups consisted of four groups of six rats each, all selected at random. These consisted of a healthy group (HG), a control group receiving ethanol (ETC), a group receiving thiamine+ethanol (TAE) and a group receiving thiamine pyrophosphate+ethanol (TPPE).

### Experimental Protocol

The ETC (n-6) group was administered 5 mg/kg ethanol orally, the TAE (n-6) group was administered 25 mg/kg thiamine intraperitoneally (ip) + 5 mg/kg ethanol orally and the TPPE (n-6) was given 25 mg/kg TPP (ip) + 5 mg/kg ethanol orally. The HG (n-6) group was given distilled water as solvent by the same route. Thiamine and TPP were injected 5 min before administration of ethanol. This procedure was repeated over 1 month. At the end of this period blood samples were collected from all animals for ALT and AST measurement. Animals were subsequently sacrificed under high-dose anesthesia. The livers were removed and oxidant and antioxidant measurements were performed. Biochemical results from the TAE and TPPE groups were analyzed by comparison with those from the HG and ETC groups.

## Biochemical Analyses

### MDA, GSH and DNA Damage Measurement

#### Biochemical Analysis of Liver Tissue

Homogenates were prepared from liver tissue in order to measure hepatic enzyme activities. tGSH and MDA levels in supernatants obtained from these homogenates were determined using appropriate methods based on those described in the literature.

#### Specimen Preparation

At this stage, 0.2 g was weighed from each liver extracted. Livers were homogenized in 0.5% HDTMAB (0.5% hexadecylthrimethylammonium bromide) containing 1.15% potassium chloride solution for MDA assay and pH = 7.5 phosphate buffer for other measurements, all made up to 2 ml in an iced environment. They were subsequently centrifuged at 10,000 rpm for 15 min at +4°C. The supernatant part was used as a specimen for analysis. For all the measurements the tissue-protein estimation was performed according to Bradford's method[18].

#### Malondialdehyde (MDA) Assay

Based on spectrophotometric measurement at an emission wavelength of 532 nm of the absorbance of the pink complex formed at high temperature (95°C) by thiobarbituric acid (TBA) and MDA[19].

#### Total Glutathione (GSH) Assay

Based on the method described by Sedlak et al[20] DTNB [5,5'-Dithiobis (2-nitrobenzoic acid)] is a disulfide chromogen easily reduced by

sulfhydryl group compounds. The resulting yellow color is measured spectrophometrically at 412 nm.

### DNA Damage Product Assay

50-200 mg was homogenized with 1 mL homogenization buffer (30 mM Tris pH 8, 10 mM EDTA, 10 mM/L 2-merkapto ethanol, 0.5% (v/v) Triton X-I00) ice or at +4°C with a mechanical homogenator. The supernatant resulting after centrifugation for 10 min at 1000 g was discarded. The pellet obtained was again suspended with 1 mL extraction buffer (0.1 M Tris pH 8, 0.1 M NaCI, 20 mM EDTA) and homogenized by being vortexed for 30 s. It was subsequently centrifuged at 1000 g for 2 min. The pellet obtained was further suspended with extraction buffer. Good mixing was ensured by vortexing the suspension. Subsequently, 400 μL phenol was added to the mixture and strongly vortexed for 1 min. It was then kept at room temperature for 10 men to separate out the phases. The top phase was removed and placed into a clean tube; 400 μL chloroform-isopropanol was then added to the part in the tube (24:1) and centrifuged at 10,000 g for 10 min. The topmost phase was placed into another clean tube. To the mixture obtained from the final centrifugation was added 40 μL 3 M sodium acetate (pH=5) and 800 μL ice-cold ethanol and mixed by being slowly revolved. This was than centrifuged at 10,000 g for 15 min and the uppermost part was completely removed. To the part beneath was added 1 mL 70% ethanol[21]. Finally, 0.5 mL 60% formic acid was added to 1 mL of mixture. The tubes were sealed and stored for 60 min at 150°C. After waiting for the tubes to cool, in order to remove the formic acid in the cooling tubes they were kept at room temperature and approximately 1 mL of mixture was stored at -20°C until the day of the study[22].

8-OH/Gua and deoxyguanine (dG) levels were measured in HPLC with HPLC-UV and HPLC-ECD electrochemical detectors at various wavelengths with previously described systems. Before HPLC analysis, hydrolyzed DNA specimens were again dissolved with eluent. The final volume was 1 mL. Subsequently, 20 μL of final hydrolysate HPLC-ECD was injected. (HP, HP 1049A ECD detector, Agilent 1100 modular systems HP 1049A ECD detector, Germany) A reverse phase C18 (RP-C18) analytic column (250 mm × 4.6 mm × 4.0 um, Phenomenex, CA, USA). The mobile phase forms from 0.05 M potassium phosphate [pH 5.5] buffer containing acetonitrile (97: 3, v/v) with a flow rate of 1 mL per minute. dG concentration absorbance at 245 nm was measured and 8-OHdG observed with electrochemical reading (600 mV). dG and 8-OH/Gua levels were determined using Sigma brand dG and 8-OH/Gua standards. 8-OH Gua molecules/$10^5$ Gua molecules was given as a marker of DNA damage[21,23].

### Liver Function Tests Analysis

Venous blood samples were collected into tubes without anticoagulant. Serum was separated by centrifugation after clotting and stored at -80°C until assay. Serum AST and ALT activities were measured spectrophotometrically as liver function tests, and LDH activity as a marker of tissue injury, using a Cobas 8000 (Roche) autoanalyzer with commercially available kits (Roche Diagnostics, GmBH, Mannheim, Germany).

### Aspartate aminotransferase (AST) Assays

The International Federation of Clinical Chemistry (IFCC), pyridoxal-5' phosphate method was used. The 3,4 AST inside the specimen catalyzes the transfer of one amino group between L-aspartate and 2-oxoglutarate for oxaloacetate and L-glutamine to form. Oxaloacetate then enters into reaction with NADH in the presence of malate dehydrogenase (MDH) for $NAD^+$ to form. Pyridoxal phosphate serves as a co-enzyme in the amino transfer reaction.

L-Aspartate + 2-oxoglutarate $\rightarrow$ (AST) oxaloacetate + L-glutamate.

Oxaloacetate + NADH + H+ $\rightarrow$ (LDH) L-malate + $NAD^+$.

The speed of oxidation of NADH is directly proportional to catalytic AST activity.

### Alanine aminotransferase (ALT) Assay

The International Federation of Clinical Chemistry (IFCC) pyridoxal-5' phosphate method was used. 3,4 ALT catalyzes the reaction between L-alanine and 2-oxoglutarate. The pyruvate that forms is reduced by NADH in a reaction catalyzed by the lactate dehydrogenase formed from L-lactate and $NAD^+$. Pyridoxal phosphate serves as a co-enzyme in the amino transfer reaction. It permits full enzyme activation.

L-alanine + 2-oxoglutarat $\rightarrow$ (ALT) pyruvate + L-glutamate

Pyruvate + NADH + $H^+$ $\rightarrow$ (LDH) L-lactate + NAD + NADH oxidation rate is directly proportional to ALT activity.

## Statistical Analysis

All data were subjected to one-way analysis of variance using Statistical Package for Social Sciences 18.0 (Armonk, NY, USA) software. Differences among groups were obtained using the least significant difference option, and significance was declared at $p \leq 0.01$. The results are expressed as mean ± SEM.

## Results

MDA levels in the ETC group were 14 ± 1.2 μmol/g protein, and 11.3 ± 1.7 ($p > 0.01$), 5 ± 0.7 ($p < 0.01$) and 3 ± 0.5 ($p < 0.01$) μmol/g protein, respectively, in the TAE, TPPE and HG groups (Figure 1). GSH levels in the ETC, TAE, TPPE and HG groups were 1.4 ± 0.3, 1.7 ± 0.8 ($p > 0.01$), 5.6 ± 0.9 ($p < 0.01$) and 7.8 ± 0.9 ($p < 0.01$) nmol/g protein, respectively (Figure 2). Levels of 8OH Gua, a DNA damage marker, were 2.2 ± 0.1, 1.8 ± 0.4 ($p > 0.01$), 0.8 ± 0.06 ($p < 0.01$) and 0.6 ± 0.04 ($p < 0.01$) pmol/L, respectively (Figure 3).

Data for groups' AST and ALT levels and AST/ALT ratios are shown in Table I.

Mean AST level in the ETC group was 284.6 ± 2.5 U/L, compared to 243.1 ± 16.7 U/L ($p > 0.01$), 98 ± 11.2 U/L ($p < 0.01$) and 36.8 ± 3.5
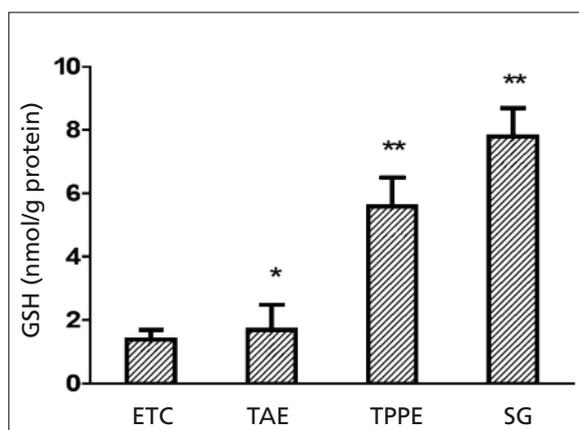


**Figure 2.** Comparison of total glutathione (GSH) levels of groups. *$p > 0.01$, groups' data were compared versus ETC groups; **$p < 0.01$, groups' data were compared versus ETC groups. *Notes:* Differences among groups were obtained using ANOVA post hoc the least significant difference option. Each group contained six animals. GSH levels defined in nmol/g protein. Bars are means ± standard error mean. ETC, control group receiving ethanol; TAE, group receiving thiamine+ethanol; TPPE, group receiving thiamine pyrophosphate+ethanol; HG, healthy group.

U/L ($p < 0.01$), respectively in the TAE, TPPE and HG groups. Mean ALT levels were 105 ± 0.9 U/L, 91.3 ± 5.1 U/L ($p > 0.01$ 39.6 ± 10.9 U/L ($p < 0.01$) and 33.6 ± 6 ($p < 0.01$), respectively.
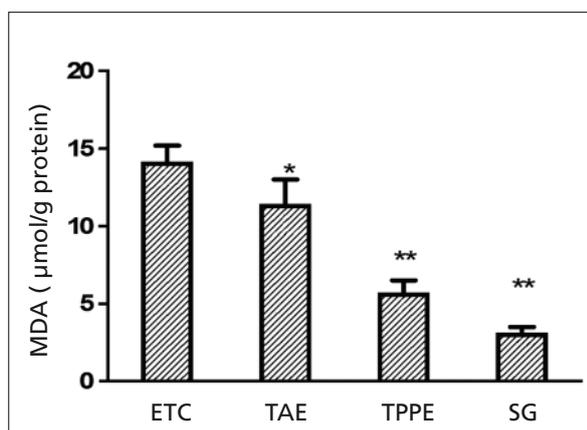


**Figure 1.** Comparison of groups' in terms of Malondialdehyde (MDA). *$p > 0.01$, groups' data were compared versus ETC groups; **$p < 0.01$, groups' data were compared versus ETC groups. *Notes:* Differences among groups were obtained using ANOVA post hoc the least significant difference option. Each group contained six animals. MDA levels defined in μmol/g protein. Bars are means ± standard error mean. ETC, control group receiving ethanol; TAE, group receiving thiamine+ethanol; TPPE, group receiving thiamine pyrophosphate+ethanol; HG, healthy group.



**Figure 3.** Comparison of DNA damage products levels of groups. *$p > 0.01$, groups' data were compared versus ETC groups; **$p < 0.01$, groups' data were compared versus ETC groups. *Notes*: Differences among groups were obtained using ANOVA post hoc the least significant difference option. Each group contained six animals. DNA damage products levels defined in pmol/L. Bars are means ± standard error mean. ETC, control group receiving ethanol; TAE, group receiving thiamine+ethanol; TPPE, group receiving thiamine pyrophosphate+ethanol; HG, healthy group.
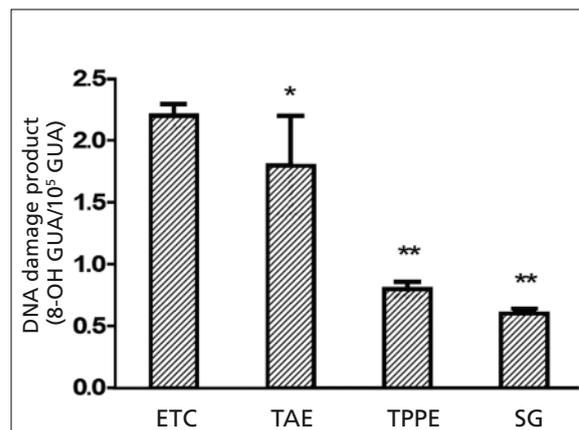
**Table I.** Comparison of the groups' rat liver damage marker levels.

| Groups | ETC | | | TAE | | | TPPE | | | SG | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Animal no | AST | ALT | AST/ALT ratio | AST | ALT | AST/ALT ratio | AST | ALT | AST/ALT ratio | AST | ALT | AST/ALT ratio |
| 1 | 275 | 102 | 2.7 | 195 | 95 | 2.05 | 116 | 52 | 2.23 | 21 | 40 | 0.53 |
| 2 | 291 | 103 | 2.83 | 250 | 112 | 2.23 | 134 | 31 | 4.32 | 47 | 28 | 1.68 |
| 3 | 288 | 108 | 2.67 | 280 | 90 | 3.11 | 70 | 55 | 1.27 | 35 | 39 | 0.9 |
| 4 | 279 | 106 | 2.63 | 295 | 87 | 3.4 | 65 | 36 | 1.8 | 38 | 31 | 1.23 |
| 5 | 286 | 104 | 2.75 | 241 | 91 | 2.65 | 90 | 34 | 2.65 | 41 | 26 | 1.58 |
| 6 | 289 | 107 | 2.7 | 198 | 73 | 2.71 | 113 | 30 | 3.77 | 39 | 38 | 1.03 |

*Notes:* AST and ALT levels defined in U/L. ETC, control group receiving ethanol; TAE, group receiving thiamine+ethanol; TPPE, group receiving thiamine pyrophosphate+ethanol; HG, healthy group.

## Discussion

This study investigated the effects of thiamine and TPP on oxidative damage induced with ethanol in the rat liver. The experimental results showed a significant increase in MDA and a decrease in GSH levels in the livers of rats receiving ethanol compared to the healthy group. These results indicate that ethanol induces oxidative stress in liver tissue. Several authors have shown experimentally that ethanol give rises to oxidative stress[8]. Under physiological conditions, the oxidant/antioxidant balance is maintained with antioxidant superiority. Impairment of that balance leads to tissue damage, known as oxidative stress. In other words, the presence or absence of tissue damage is assessed with the oxidant/antioxidant balance. The oxidant/antioxidant balance changes in favor of oxidants in various models established in living tissues, and a rise in oxidant and decrease in antioxidant levels is observed[24]. As described, MDA levels in the liver tissue of rats receiving ethanol was statistically significantly higher compared to those in the HG and TPPE groups. MDA is a product of lipid peroxidation. Lipid peroxidation is a pathological event initiated by toxic oxygen radicals and that compromises cell structure and functions[25]. Alcohol has been reported to lead to lipid peroxidation in liver tissue[26]. Studies have shown that MDA levels rise in association with ethanol in liver tissue[27]. Seo et al[28] reported that ethanol raises MDA levels in rat liver tissue.

GSH levels were also statistically significantly lower in rat liver tissue administered ethanol compared to those in the groups receiving TPP and the healthy group. GSH is a tripeptide synthesized in the liver with no requirement for genetic information. An endogenous antioxidant, GSH reacts with free radicals to protect the cells against oxidative damage. GSH protects against oxidation, binding-SH groups in protein in the reduced state. The liver is the tissue in which GSH is present in the highest level[29]. Studies have shown that a single 0.5 g/kg of alcohol causes a significant decrease in the amount of GSH in the rat liver[30]. Mladenovi et al[31] reported that levels of GSH decreased in rat liver tissue in which ethanol was applied. These findings from the literature are compatible with our own results.

Alcohol is metabolized in liver tissue with the enzyme P450 2E1. As a consequence of this metabolism, levels of superoxide ($O_2^-$) and hydroxyl ($OH^-$) radicals increase. Hydroxyl radicals cause DNA oxidative damage by interacting with DNA. This leads to irreversible cell damage[32]. Hydroxyl radicals lead to the oxidation of DNA by extracting hydrogen from nucleic acids or entering into reactions with double bonds[33]. 8-OHGua is regarded as the most important marker reflecting DNA oxidation[34]. In our study, too, levels of 8-OH/Gua, a product of DNA damage, in the ETC group given ethanol were significantly higher than those in the TPP and healthy rat groups; however, thiamine failed to significantly prevent a rise in MDA and 8-OH/Gua levels with ethanol. Recent studies have reported that 8-OH/Gua levels induced liver tissue with methotrexate rose significantly in comparison to a healthy control group; one study also reported that TPP significantly prevented 8-OH/Gua levels rising in oxidative liver damage induced with methotrexate, while thiamine failed to prevent this[35].

ALT and AST levels were significantly elevated in blood specimens in the ETC group rats administered ethanol compared to the TPP and healthy groups. AST and ALT are used to determined liver damage and alcoholic liver disease[36]. These aminotransferases are a sensitive marker of liver cell damage[37]. ALT is more specific than AST in liver diseases, although AST rises more in alcoholic liver disease. This is attributed to alcohol increasing mitochondrial AST activity. An AST/ALT ratio above 2 is regarded as alcoholic liver disease[38]. The AST/ALT ratio in the ETC group blood specimens in our study being above 2 shows that this is compatible with the information from the literature. Studies have shown that ALT and AST values rise in alcoholic liver toxicity[28,39]. AST/ALT activities and ratios in the TPP and healthy group blood specimens being close to one another indicates that TPP is effective. Previous studies have also reported that TPP significantly prevented an oxidative liver damage-related rise in ALT and AST[35].

## Conclusions

Ethanol at a dose of 5 mg/kg led to oxidative stress in the rat liver and to compromised hepatic functions. The thiamine used in the experiment failed to prevent ethanol-related oxidative stress and a rise in AST and ALT in the rat liver. However, TPP significantly prevented oxidative stress induced with ethanol and a rise in AST and ALT. These results show that TPP can be used in the treatment of alcohol-related liver toxicity.

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**Conflict of Interest**

The Authors declare that there are no conflicts of interest.

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