Does Dihydromyricetin Impact on Alcohol Metabolism

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Summary

Dihydromyricetin (DHM) is a natural flavonoid showing several health promoting effects such as protective activity during severe alcohol intoxication. The mechanism underlying the effects of DHM on alcohol metabolism is virtually unknown. The present paper is focused on clarifying the role of DHM in the liver alcohol elimination at its molecular level. First, impact of DHM on alcohol dehydrogenase (ADH) activity *in vitro* and the enzyme induction *in vivo* was examined. Neither the ADH activity nor the enzyme expression were influenced by DHM. Next, the effect of DHM during alcohol intoxication were studied on primary hepatocytes isolated from EtOH-premedicated and untreated rats. The viability of cells exposed to alcohol, estimated based on the released enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), was slightly affected by DHM. Although the expected hepatoprotective effect of DHM was not fully achieved, DHM (in a concentration manner) proved to reduce the level of ROS/RNS in hepatocytes. However, no change in the rate of alcohol metabolism *in vivo* was found when rats were administered with a single or repeated dose of ethanol supplemented with DHM. In conclusion, the proposed positive effect of DHM during alcohol intoxication has not been proven. Moreover, there is no effect of DHM on the alcohol metabolism. The "hoped-for" DHM hepatoprotective activity can be attributed to the reduction of ROS/RNS levels in cells.

Key words

Alcohol dehydrogenase • Hepatocytes • Flavonoid

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Introduction

Dihydromyricetin (DHM), also known as ampelopsin, is a natural flavonoid compound, which is found in large quantities in Asian plant species. One of them – *Hovenia dulcis* – is known for centuries in traditional Chinese medicine as a cure for alcohol poisoning and hangover (Liang *et al.* 2014).

Dihydromyricetin, like other flavonoid compounds, is known for several effects on the human body. DHM has an anti-inflammatory, cardioprotective or dermaprotective effect. By affecting cholesterol metabolism, DHM inhibits the onset of atherosclerosis. Positive effects on insulin resistance are related to its ability to lower glucose levels in the blood. Thus, dihydromyricetin suppresses diabetic cardiomyopathy. And most importantly, DHM has been shown to provide significant hepatoprotective effects. Its positive influence on alcohol-induced and other liver diseases was suggested in the study of acute liver failure (Liu *et al.* 2017).

The impact of DHM on ethanol consumption is explained in terms of the competitive inhibition of the benzodiazepine sites on GABA receptors. It was published that ethanol intoxication of the experimental rats was reduced by administration of DHM, and the symptoms of alcohol withdrawal syndrome were mitigated (Shen *et al.* 2012). The hepatoprotective effect of DHM is likely connected with its ability to protect liver cells against reactive oxygen species (ROS) and inflammatory reactions in the organism. Studies on human umbilical vein endothelial cells and on human liver cancer cell line (HepG2) proved that DHM affects

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the regulatory mechanisms in the liver cells which can cause the decrease of ROS level (Hou *et al.* 2015, Silva *et al.* 2020).

The data show that DHM, when used as a dietary supplement, helps to reduce oxidation damage of hepatocytes. The mechanism of hepatoprotective effects against alcohol is not yet completely understood. The studies were mostly carried out with an inaccurate experimental model such as HepG2 cells which does not have functional enzymes for oxidative metabolism of EtOH (Silva *et al.* 2020).

Another study using hepatoblastoma cells, as a model, shows that supplementation with DHM significantly increases the activity and expression of alcohol dehydrogenase and aldehyde dehydrogenase (Silva *et al.* 2020). Interestingly, the expression of CYP2E1, the enzyme, which is also involved in the metabolism of ethanol, especially during chronic ethanol consumption, was significantly reduced by DHM in the mice model (Silva *et al.* 2020, Zakhari 2006).

Even though it appears that DHM health promoting activity is probably due to a reduction of oxidative stress, the role of DHM in ethanol metabolism is still unknown. Although several hypotheses have been suggested, the exact mechanism has not been found yet.

The aim of this study is to determine the impact of DHM on ADH, CYP2E1 and ethanol metabolism *in vivo*, under conditions closely mimicking human alcohol consumption, and to reveal a possible hepatoprotective effect of this flavonoid.

Methods – Materials

Materials

Rabbit monoclonal antibody to alcohol dehydrogenase and rabbit monoclonal antibody to CYP2E1 were obtained from Abcam (UK), dihydromyricetin from APIChem (China), reagents for Western Blot from Bio-Rad (USA), ^{L}ALT (GPT) kit, ^{L}AST (GOT) kit, ^{D}Biocal standard from Biovendor (Czech Republic), NAD^+ from Cambrian Chemicals (Canada) and FBS from Gibco® by Life TechnologiesTM (USA). Collagen and NADH were provided by ROCHE s.r.o., Diagnostic Division (Germany), heparin and collagenase by Zentiva Group, a.s. (Netherlands) and all other used reagents and chemicals of reagent grade or better were purchased from Sigma-Aldrich (Czech Republic).

Animals

Male Wistar rats (300-400 g body weight) (AnLab, Czech Republic) were used for all experiments in this study. Animals were allowed tap water and standard granulated diet *ad libitum* and were maintained under the standard conditions – light (12/12-h dark/light), temperature (20±2 °C) and relative humidity (50±100 %). The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (419/2019, Ministry of Agriculture, Czech Republic) which is in compliance with the Declaration of Helsinki.

Preparation of primary hepatocyte cell culture

Primary hepatocytes were isolated from rats premedicated by gastric gavage with 40 % EtOH diluted in PBS (3 g/kg body weight) for four consecutive days and 20 % EtOH was available as an only resource of water for two days before isolation. Rats from a control group were premedicated by gastric gavage with PBS.

The standard two-phase collagenase perfusion (Moldeus *et al.* 1978) with minor modifications (Farghali *et al.* 1986) was used for hepatocytes isolation. Bürker chamber was used for the counting hepatocytes and the cell viability was determined *via* Trypan blue exclusion method (Strober 2001).

Primary hepatocytes were cultivate using William's medium containing 5 % FBS, 2 mM L-glutamine, 6 μg/ml insulin, 100 μg/ml streptomycin, and 100 IU/ml penicillin. Hepatocytes were diluted to the final concentration of 6.7×10^5 viable cells/ml medium and were cultivated in collagen coated 6 or 24 wells with a density of 10^4 cells/cm^2. Cultivation of cells took place at standard conditions (37 °C, 95 % air, and 5 % CO_2) for three hours in the incubator SANYO MCO-20 AIC. After that medium was replaced for a fresh one and non-adherent hepatocytes were removed. The cultivation of cells continued for another 21 h under the standard conditions as described above. Then, the medium was replaced with medium lacking FBS with tested compounds added. The first group contained LPS in final concentration 15 μg/ml and the second ethanol in final concentrations 100-800 mM with or without dihydromyricetin in final concentrations 1-1000 μM. Because of alcohol evaporation EtOH was added after 7 h. Cultivation continued again for approx. 15 h under the standard conditions.

Then hepatocytes were washed two times with ice-cold PBS and harvested by scraping. Cells were spin down at 5000 RPM for 5 min at 4 °C (Spectrafuge

C1301B). The pellets were immediately frozen in liquid nitrogen and stored at -80 °C. Protein concentration of samples was determinate using Lowry method (Lowry *et al.* 1951) with minor modifications (Hartree 1972). The medium (collected last day) was used for determination of ALT and AST.

Cell viability assays

Cytotoxicity of tested compounds was determined using MTT test (Mosmann 1983) as well as based on the concentration of released enzymes, ALT and AST, in the medium. For the determination of concentration of ALT and AST commercial tests (^{L}ALT (GPT) kit, ^{L}AST (GOT) kit) were used. Enzyme concentrations were determined in medium collected from 6-WP last day of primary hepatocyte cultivation. Groups of hepatocytes cultivated in medium containing EtOH and with or without DHM were used. MTT test and enzyme assays were carried out in triplicates.

Determination of ROS in primary hepatocytes

Hepatocytes cultivated on 24-WP were washed with cold PBS and incubated with 20 µM DCFDA for 60 min under 37 °C in the dark (Chen *et al.* 2010, LeBel *et al.* 1992). The fluorescence intensity was measured at 485/535 nm, Ex/Em (Infinite® 200 PRO, Tecan Trading AG, Switzerland). The results were standardized to the number of cells seeded and related to the control group.

Activity of alcohol dehydrogenase

Alcohol dehydrogenase catalyzes the oxidation of ethanol to acetaldehyde in the presence of cofactor NAD^+, which is reduced to NADH. To determine activity of alcohol dehydrogenase in samples of hepatocytes the increase of absorbance at 340 nm every two minutes for 60 min under 37 °C and constant shaking was measured (Program medium shaking, Multiskan GO). In brief, the reaction mixture contained 82 µl of 0.1 M phosphate buffer pH 7.4; 8 µl of 10 mM NAD^+; 50 µl of samples (1 mg/ml) in 150 µl final volume and was started by addition of 10 µl of EtOH. Enzyme activity was calculated based on a calibration curve.

Determination of CYP2E1 activity

Activity of CYP2E1 was determined based on 6-hydroxylation of chlorzoxazone, a specific substrate of CYP2E1. To follow the formation of 6-hydroxychlorzoxane the HPLC (Agilent Technologies 1200, USA) equipped with C18 column (Macherey-Nagel, Nucleosil 100-5 column C18) was used (Peter *et al.* 1990).

Western blot analysis

Discontinuous SDS-PAGE analysis using Mini-PROTEAN® Tetra Cel (Bio-Rad, USA) was performed (Laemmli 1970). Samples of hepatocytes or cytosolic samples from liver (40 µg) prepared in reducing sample buffer were separated in a 10 % polyacrylamide gel at 200 V. The transfer of separated proteins onto PVDF membranes was carried out at 0.8 mA/cm^2 for 10 min and to 2 mA/cm^2 for 45 min. Membranes were incubated with primary rabbit antibody against alcohol dehydrogenase and against CYP2E1, respectively and diluted 1:1000, 1:5000 respectively and then with secondary goat anti-rabbit IgG-alkaline phosphatase conjugate diluted 1:1428. For visualization of a specific protein band the membrane was incubated in developing solution (SigmaFast tablet dissolved in 10 ml of distilled water) for 5 min.

Alcohol metabolism in vivo

Rats were separated into two groups (n=4) and premedicated by gastric gavage with 40 % EtOH or with combination of 40 % EtOH and DHM (10 mg/kg body weight). Animals were fasted for approx. 16 h before premedication. Blood of all animals was collected from eye in 5, 15, 30 and 60 min from the gastric gavage. The concentrations of ethanol and acetaldehyde in full blood samples were measured immediately using headspace-gas chromatography (in triplicates).

After 24 h, half of rats of both groups (n=2) were repeatedly premedicated by gastric gavage with 40 % EtOH again. Blood sample collection and measuring of concentrations of ethanol and acetaldehyde proceeded similarly to the procedure described above. All samples were measured at Institute of Forensic Medicine and Toxicology, Branch of Toxicology, Ke Karlovu 2, 128 08 Prague 2, Czech Republic.

Preparation of liver microsomal and cytosolic fractions

Standard differential centrifugation procedure was used for isolation of cytosolic fractions (Hodek *et al.* 2011, Krizkova *et al.* 2008). The whole procedure was carried out in a cold room at 4 °C. All materials and buffers were cooled down before using.

In brief, liver tissues were washed and homogenized in 0.1 M Tris-HCl buffer pH 7.4 with 50 µM tocopherol (from methanol stock) in a Potter-Elvehjem homogenizer and centrifuged at 600×g for

10 min (383K, HERMLE). Pellets were rehomogenized under the same conditions. Subsequently, supernatants from both centrifugations were pooled and centrifuged at 15000×g for 20 min (Allegra X-30R, Beckman Coulter). Then supernatants were ultracentrifuged at 123000×g for 90 min (Optima XPN-90, Beckman Coulter). Collected microsomal and cytosolic fractions were immediately frozen and stored at -80 °C.

Statistics

Statistical analyses were performed using Student's *t*-test and approximated with Welch´s *t*-test. Means ± standard deviations of parallel experiments are shown and $P<0.05$ were considered as significant.

Results and Discussion

To study the effect of DHM on ethanol metabolism introductory *ex vivo* experiments using primary hepatocytes isolated from EtOH-premedicated and untreated rats were performed. Cells were exposed to ethanol and DHM. Markers of liver damage (ALT and AST) were used for testing the cell viability along with MTT test and the determination of ROS. Next, an impact of DHM on ADH and CYP2E1 was examined on the level of protein expression, protection and modulation of the enzyme activity. Finally, *in vivo* experiments with rats simultaneously treated with EtOH and DHM were performed. Ethanol metabolism was monitored based on the concentration of ethanol and acetaldehyde in full blood.

Effect of DHM on viability of primary hepatocytes

The cell viability and degree of damage of primary hepatocytes were determinate using MTT test and based on the amount of enzymes – ALT and AST, known markers of liver damage. Released ALT and AST were measured using their enzyme activity (Fig. 1).

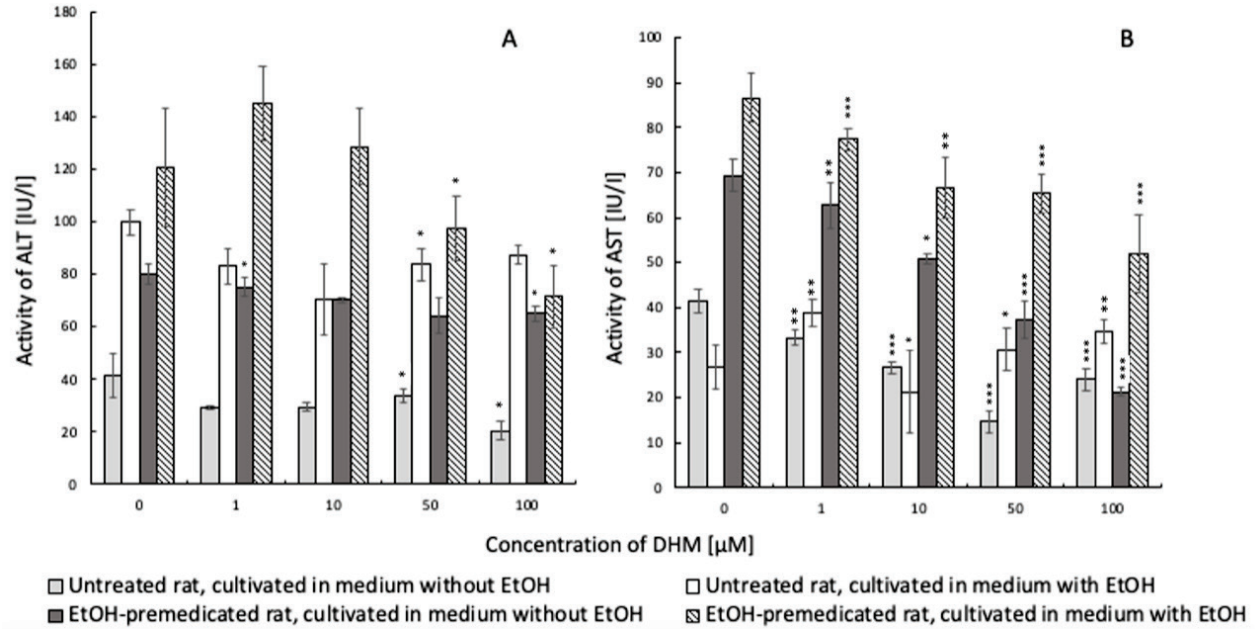

Fig. 1. Effect of DHM on activity of released ALT/AST. Concentrations of ALT (**A**) and AST (**B**) were measured in medium of hepatocytes isolated from EtOH-premedicated and untreated rats with or without EtOH. Primary hepatocytes were incubated in medium with 1-100 μM DHM in the presence or absence of 300 mM EtOH. Medium was collected from 6-WP. All samples were measured in triplicates. Data are means ± standard deviations of parallel experiments; $P<0.05$ were considered as significant (* $P<0.05$, ** $P<0.01$ and *** $P<0.001$).

Data in Figure 1 show differences in the cell viability among primary hepatocytes isolated from EtOH-premedicated and untreated rats cultivated in medium with or without EtOH. In general, hepatocytes isolated from EtOH-premedicated rats had lower viability compared to hepatocytes isolated from control groups (data not shown). This observation is in agreement with results of determination of ALT and AST (Fig. 1). Activities of these two enzymes were higher in EtOH-premedicated groups. As data show the presence of DHM in a concentration-dependent manner slightly reduced the level of released enzymes. This hepatoprotective effect of DHM has been also reported by others (Jiang *et al.* 2015, Murakami *et al.* 2004).

Effect of DHM on alcohol dehydrogenase and CYP2E1 activity

The activity of recombinant equine alcohol dehydrogenase was determined at increasing concentration of DHM. As shown in Figure 2A DHM in concentrations 10 and 100 μM did not affect the activity of ADH. Some extent of inhibition is apparent at the highest DHM concentrations (1000 μM).

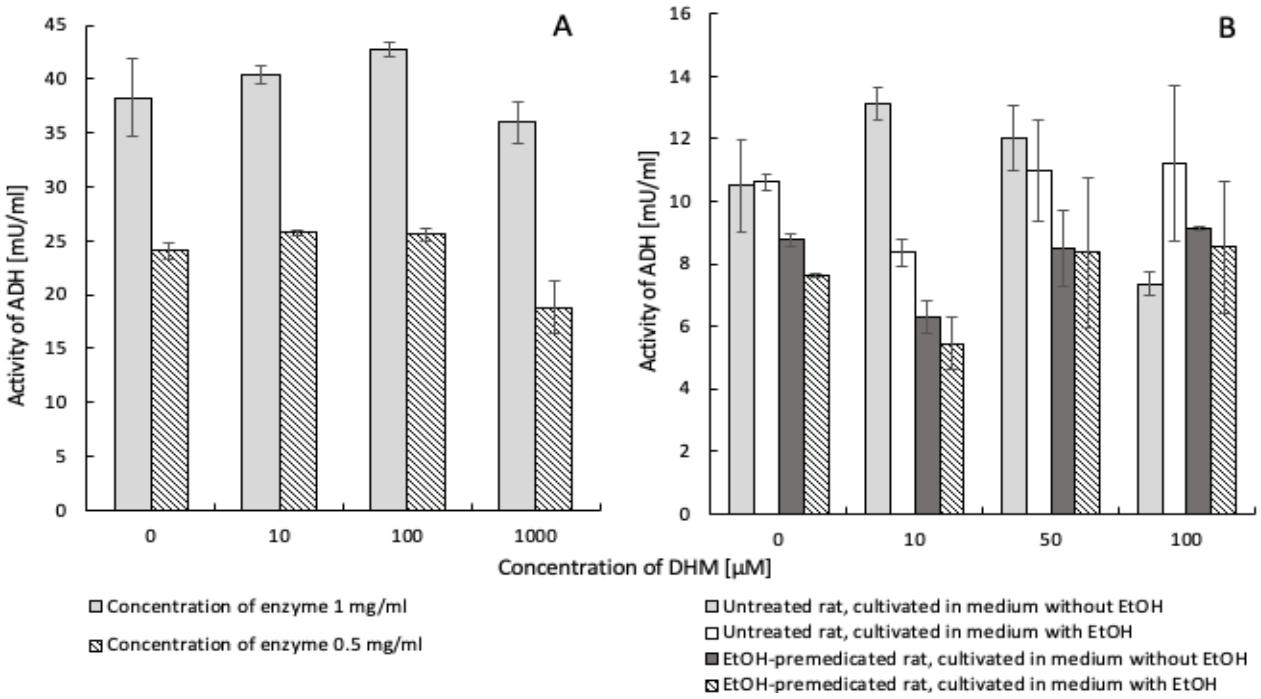

Fig. 2. Effect of DHM on activity of ADH. The assay was carried out with recombinant equine alcohol dehydrogenase (**A**) and alcohol dehydrogenase in primary hepatocytes isolated from EtOH-premedicated and untreated rats (**B**). The amount of reduced NADH cofactor was determined spectrophotometrically at 340 nm for 60 min (A). Same as described in previous figure (B). The reaction was monitored as an increase of absorbance in time. Enzyme activity was determined based on the calibration curve. All samples were measured in triplicates. Data are means ± standard deviations of parallel experiments; $P<0.05$ were considered as significant (* $P<0.05$, ** $P<0.01$ and *** $P<0.001$).

The effect of DHM on activity of ADH was determined also in primary hepatocytes isolated from EtOH-premedicated and untreated rats (Fig. 2B). Preliminary experiments with primary hepatocytes show that concentration 1000 μM of DHM is lethal for cells, thus, such high concentration of DHM was not used for further experiments. The ADH activity was monitored depending on the concentrations of DHM or EtOH added to the cultivation medium. Figure 2 shows that DHM has no significant influence on the activity of ADH. Hence, the published data showing DHM as an agent which can increase the metabolic rate of alcohol degradation in the body (Shen *et al.* 2012), cannot be attributed to the ADH activity stimulation by this flavonoid.

Moreover, the effect of DHM on the metabolic activity of CYP2E1, the second most important enzyme involved in the metabolism of ethanol, was examined. Up to 100 μM concentration DHM did not affect CYP2E1 mediated 6-hydroxylation of chlorzoxazone.

Effect of DHM on expression of alcohol dehydrogenase and cytochrome P450 2E1

Alternatively, the effect of DHM on alcohol metabolism might be associated with the induction of ADH and/or CYP2E1 in liver. The expression of ADH and CYP2E1 were examined at its protein level by Western blot technique. Data show that DHM has no effect on the expression of ADH and CYP2E1 at *in vivo* experiments (data not shown).

Determination of the level of ROS/RNS in primary hepatocytes

To test the hypothesis that DHM impacts the alcohol metabolism *via* its hepatoprotective properties, the antioxidant effect of DHM on primary rat hepatocytes was examined using a fluorescent probe, 2′,7′-dichloro-fluorescin diacetate (DCFDA). This probe is the most commonly used for determination of total level of ROS/RNS in the cells (LeBel *et al.* 1992).

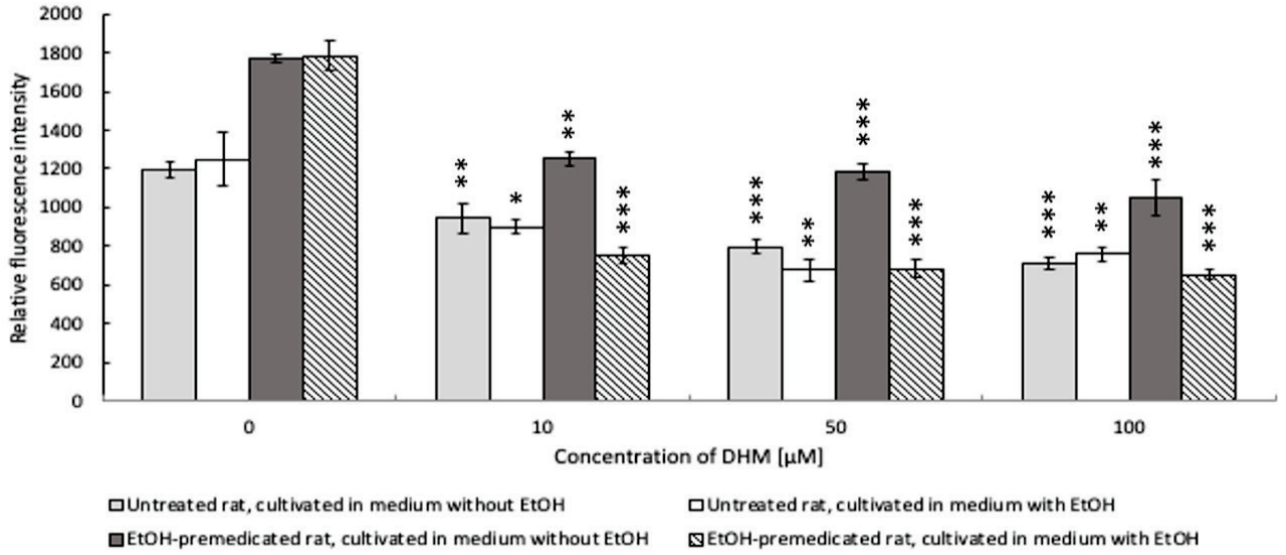

Fig. 3. Effect of DHM on level of ROS/RNS in primary hepatocytes. Cells were isolated from EtOH-premedicated and untreated rats. ROS/RNS was measured in 24-WP after 60 min incubation with DCFDA at 37 °C in the dark. The fluorescence intensity was measured at 485/535 nm, Ex/Em (Infinite® 200 PRO, Tecan Trading). Six wells were measured for each group. Data are means ± standard deviations of parallel experiments; P<0.05 were considered as significant (* P<0.05, ** P<0.01 and *** P<0.001).

Results shown in Figure 3 confirmed presumption that DHM is able to decrease the level of ROS/RNS in primary hepatocytes (Silva *et al.* 2020). Hepatocytes isolated from EtOH-premedicated rats had significantly higher level of relative intensity of fluorescence compared to cells isolated from untreated rats. This observation accounts for a cell damage induced by EtOH-premedication. Hepatocytes isolated from EtOH-premedicated rats incubated with 300 mM EtOH did not show a proportional dependence of ROS/RNS levels with the increase of DHM concentration. The most marked decrease of ROS/RNS levels was caused by 10-50 μM DHM. The adaptation of cells to ethanol or ability of EtOH to scavenge radicals, resulting in reduction of the amount of ROS/RNS in cells may explain this observation. Moreover, it was published that DHM regulates level of ROS *via* liver catalase (Silva *et al.* 2020). Another study also confirmed ability of DHM to lower the level of ROS and to induce apoptosis in HepG2 (Liu *et al.* 2014).

Effect of DHM on metabolism of ethanol in vivo

The proposed impact of DHM on the acceleration of ethanol metabolism was finally tested in rats. Rats were divided into two groups. First group received 40 % ethanol by gastric gavage while the second group was premedicated with a 40 % EtOH containing DHM (10 mg/kg body weight). Within 60 min from the animal treatment the blood was collected from the eye of the rats. The amount of EtOH and acetaldehyde in the blood was determined. Data show that DHM does not significantly affect the EtOH metabolism rate (Fig. 4A, C). Next, the effect of DHM on repeated application of EtOH was examined after 24 h. Both groups were administered with 40 % EtOH and the amounts of EtOH and acetaldehyde were determined in collected blood. Amount of EtOH was markedly higher in the blood of rats being premedicated the first day with DHM compared to rats that were administered with EtOH, only (Fig. 4B, D). Our results of a single EtOH administration experiment did not confirm the data presented by Shen *et al.* (2012).

As it is clear from Figure 4, DHM does not decrease concentration of either EtOH or acetaldehyde in the blood. A sole decrease of EtOH concentration (time 30 min) is not statistically significant (group of DHM-premedicated rats). The reason for these diverse results between our and Shen's studies might be caused by different method of administration of EtOH and DHM. In our experiment the gastric gavage of EtOH (which simulates better the real human intake) was used while Shen *et al.* (2012) administered EtOH intraperitoneally. The recent findings indicate the use of intraperitoneal administration for the purpose of studying the metabolism of EtOH as inappropriate (Silva *et al.* 2020). Thus our data show that DHM do not speed up the alcohol metabolism but even slows down this process when alcohol was repeatedly administered.

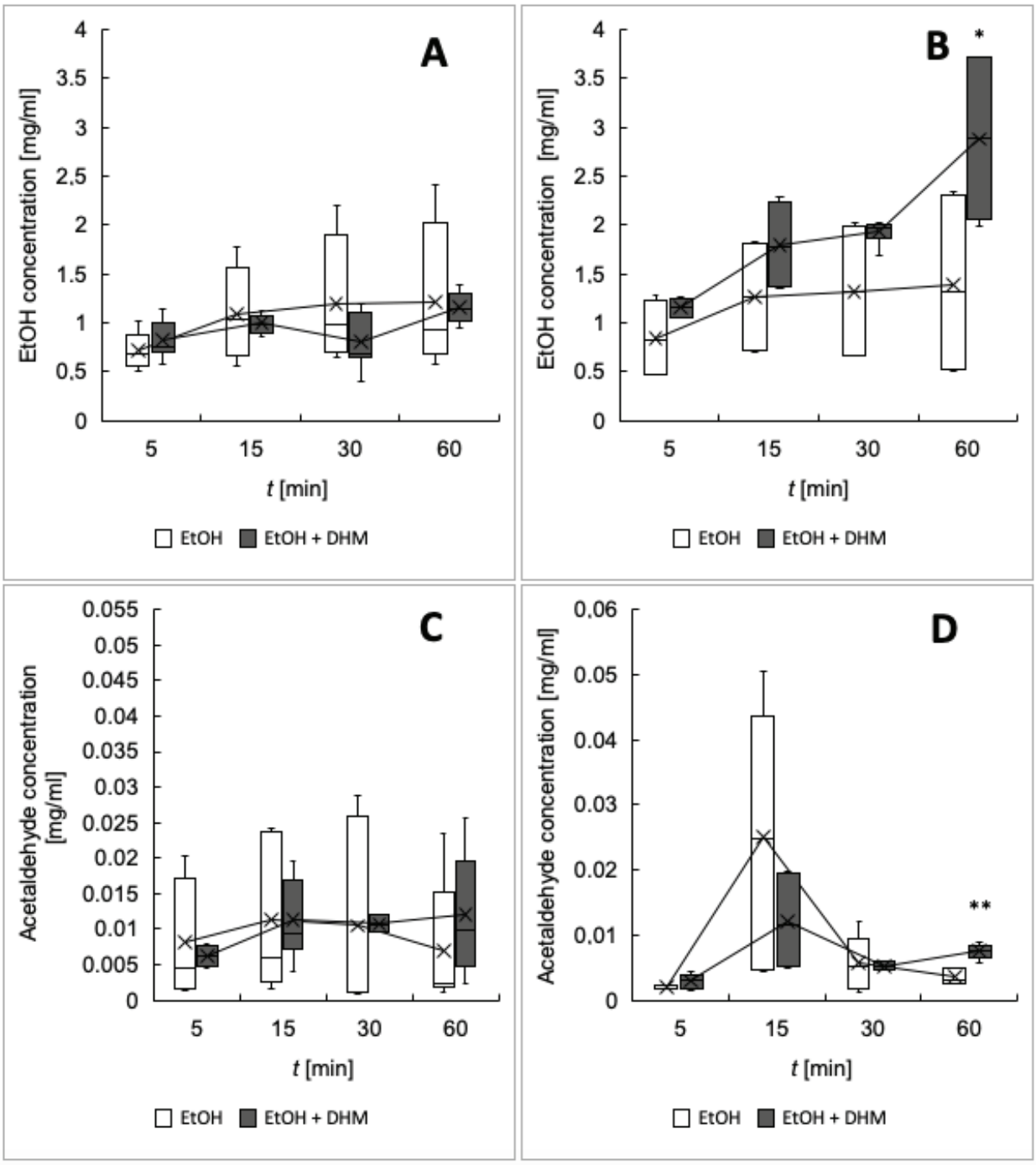

Fig. 4. Effect of DHM on ethanol metabolism. After the first gastric gavage of ethanol/DHM concentrations of ethanol (**A**) and acetaldehyde (**C**) was determined in blood samples from rats (n=4). Concentrations of ethanol (**B**) and acetaldehyde (**D**) in blood samples from rats (n=2) was also determined after the second premedication with EtOH only. Blood samples were collected and immediately measured (in triplicates) by headspace-gas chromatography. Data are means ± standard deviations of parallel experiments; P<0.05 were considered as significant (* P<0.05, ** P<0.01 and *** P<0.001).

Conclusions

Regardless our concentrated effort we were not able to reveal the basic of DHM effect on ethanol metabolism if there is any. Our data show that suggested hepatoprotective effect of DHM during alcohol intoxication cannot be attributed to the modulation of ADH or CYP2E1 activities at any level. Thus, the exact mechanism underlying the hepatoprotective properties of DHM is still not fully understood. However, we cannot exclude that DHM-mediated reduction of oxidative stress is partially involved.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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