Arch Biol Sci. 2018;70(3):531-541 https://doi.org/10.2298/ABS180320014R

The effects of whey and pumpkin seed oil on blood biochemical parameters of liver function and lipid profile in rats chronically drinking low concentrations of ethanol

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Received: March 20, 2018; **Revised**: March 30, 2018; **Accepted**: March 30, 2018; **Published online**: April 17, 2018

Abstract: We studied the effects of whey and pumpkin seed oil supplementation on the biochemical parameters in blood serum of male rats after chronic *ad libitum* alcohol consumption. The levels of AST, ALT, total bilirubin, ALP, LDH, triglycerides, total cholesterol, HDL, LDL, VLDL, triglyceride/HDL ratio, total cholesterol/HDL ratio (cholesterol ratio) and LDL/HDL ratio (index of atherosclerosis) were determined in rats after six weeks of treatment with: (i) ethanol (12% ethanol, *ad libitum*), (ii) whey (2 g/kg per day), (iii) pumpkin seed oil (2 mL/kg per day), (iv) both ethanol and whey, and (v) both ethanol and pumpkin seed oil. The results showed no changes in the levels of AST, ALT, total bilirubin, ALP, total cholesterol, HDL and VLDL in alcoholic rats when compared to the controls (fed with a standard laboratory diet *ad libitum*) and rats supplemented with whey and pumpkin seed oil. Our results suggest that alcohol consumption in small doses for 6 weeks changes lipid metabolism and significantly elevates the LDL/HDL ratio (index of atherosclerosis) but does not induce extensive liver damage. Ethanol consumption in our experimental conditions lowered the triglyceride level as well as the triglyceride/HDL ratio, suggesting lipid redistribution and the induction of some cardio-protective effect. However, ethanol induced a higher index of atherosclerosis. Pumpkin seed oil showed some protective potential in alcoholic rats by lowering the total cholesterol/HDL ratio, but it elevated the LDH. Whey consumption prevented elevation of the atherosclerosis index, pointing to its protective role, probably through the redistribution of lipids. However, whey in combination with ethanol elevated LDH.

Key words: whey protein; pumpkin seed oil; ethanol; liver biomarkers; rat

Abbreviations: Alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), body weight (b.w.), cholesteryl ester transfer protein (CETP), high density lipoprotein cholesterol (HDL), high density lipoprotein particles (HDL-P), lecithin-cholesterol acyltransferase (LCAT), lactate dehydrogenase (LDH), low density lipoprotein cholesterol (LDL), low density lipoprotein particles (LDL-P), polyunsaturated fatty acids (PUFA), reactive oxygen species (ROS), total cholesterol (TC), triglycerides (TG), very low density lipoprotein cholesterol (VLDL).

How to cite this article: Radić I, Nestorović V, Mijović M, Tatalović N, Joksimović B, Lukić V, Mitić M, Adžić M, Blagojević D, Veličković S, Bulajić S, Đerković B, Mirić M, Janićijević-Hudomal S. The effects of whey and pumpkin seed oil on blood biochemical parameters of liver function and lipid profile in rats chronically drinking low concentrations of ethanol. Arch Biol Sci. 2017;70(3):531-41.

INTRODUCTION

Contemporary lifestyle imposes sedentary behavior and increased consumption of alcohol. Ethanol is contained in popular drinks and is the most abused substance that causes liver injury [1]. Ethanol induces hepatotoxicity directly or by its metabolites that involve ROS, acetaldehydes and the release of endotoxin from the gastrointestinal tract, inducing the immune response and the release of various cytokines and proinflammatory mediators from infiltrating leukocytes [2-6]. Ethanol can also induce hepatocyte hypoxia and oxidative stress induced by increased oxygen consumption during its metabolism [7]. Persistent oxidative stress causes fatty liver, which leads to inflammation, fibrosis and cirrhosis [8].

The effect of ethanol on the lipid status is known and laboratory analyses usually detect the increase of triglycerides in the blood. However, research has indicated that changes in lipids depend on the amount of consumed alcohol as well as on the duration of consumption. Alcohol acts on different metabolic pathways. Studies on the effect of alcohol on lipid metabolism have revealed alterations in gene expression that lead to the reduction of apoprotein B and stimulation of very low density lipoprotein (VLDL) cholesterol secretion. Acute alcohol intake can reduce the activity of lipoprotein lipase, or lipolysis of circulating chylomicrons and VLDL. In contrast, chronic ethanol consumption at low concentrations increases the activity of lipoprotein lipase, thus preventing hypertriglyceridemia, although low doses of ethanol are insufficient to affect the levels of "bad cholesterol", chylomicrons, low density lipoprotein (LDL) cholesterol and VLDL [9]. Usually, when the amount of alcohol intake is low, but if it is consumed daily, it takes time before analyses of blood biochemical parameters show any signs of liver toxicity. Therefore, in our experiment we exposed rats to daily intake of a 12% ethanol solution *ad libitum* with no access to water, in order to explore how lower doses of alcohol affect lipid metabolism and liver function. Moreover, it is important to obtain a time threshold for the potential treatment of chronic alcoholics.

It has been suggested that ingestion of different supplements can prevent liver injury induced by chronic alcohol intake. One of them is whey [10], which has been suggested as a general liver protective mix. Whey is a popular dietetic product for reducing body weight and increasing muscle mass during body building exercises. It is derived from milk and has been studied as a functional food due to its biological constituents, such as lactoferrin, β-lactoglobulin, α-lactalbumin, glycomacropeptide, immunoglobulins, lactose, vitamins and minerals. Whey has the ability to act as an antihypertensive, antitumor, hypolipidemic, antiviral, antibacterial, and it has a general healing factor; its antioxidant properties have been described in recent years [11,12].

Pumpkin seed (*Cucurbita pepo* L.) oil, as a nutritional antioxidant, is a rich source of unsaturated fatty acids, antioxidants and fibers, and its antiatherogenic and hepatoprotective potential has been documented [13]. The high content of unsaturated fatty acids (especially linoleic and oleic acids), carotenoids, γ-aminobutyric acids, sterols, proteins, polysaccharides, polyphenols, phytosterols, vitamins A and E among other substances, qualifies pumpkin seed oil seed as a healthy supplement to human nutrition [14]. Previous studies have shown that both whey and pumpkin seed oil possess hepatoprotective effects [15,16]. Therefore, in the present study we investigated the effects of whey and pumpkin seed oil on serum biochemical parameters that describe body fat and liver functionality during chronic low-dose alcohol consumption: AST, ALT, total bilirubin, ALP, LDH, TG, TC, HDL, LDL, VLDL, and cardiovascular risk factors: the TG/HDL, TC/HDL (cholesterol ratio) and LDL/HDL (index of atherosclerosis) ratios.

MATERIALS AND METHODS

Ethics Statement

All animals were treated according to directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. This study was approved by the Ministry of Agriculture and Environmental Protection, the Veterinary Administration of the Republic of Serbia, Decision no.: 323-07-10690/2015-05, and by the ethical committee of development, regulatory affairs and quality division, Galenika a.d., Belgrade, Decision no. 4121/2015.

Animals

The study was conducted on mature male Wistar rats aged from 9 to 11 weeks and weighing 200-220 g. The animals were housed in standard cages with 3 animals per cage, at a constant room temperature (22±1°C) and circadian rhythm of 12 h day/night; they were fed *ad libitum* with standard food for laboratory animals (produced in the Veterinary Institute, Zemun).

Experimental design

The study included 36 male Wistar rats that were divided into 6 groups: (i) control – C (standard *ad libitum* access to food and tap water); (ii) ethanol – E (provided with 12% ethanol *ad libitum* for 42 days without any access to water); (iii) whey – W (supplemented with 2 g/kg per day during 42 days, standard *ad libitum* access to food and tap water); (iv) pumpkin seed oil – P (supplemented with 2 mL/kg per day for 42 days, standard *ad libitum* access to food and tap water); (v) ethanol and whey E+W (provided with 12% ethanol *ad libitum* for 42 days, without access to water, supplemented with 2 g/kg per day of whey for 42 days, standard *ad libitum* access to food); (vi) ethanol and pumpkin seed oil – E+P (provided with 12% ethanol *ad libitum* for 42 days, without access to water, 2 mL/kg per day of pumpkin seed oil for 42 days, standard *ad libitum* access to food).

The supplements, whey product and commercial oil derived from pumpkin seeds, were administered with a rat oral gavage tube daily, always at 9 a.m., for 6 weeks. Supplementation was based on the average daily intake of whey and pumpkin seed oil in a diet extrapolated to the daily intake of dry food from humans to rats through the "metabolic mass". The cow whey powder contained carbohydrates (61 g/100 g), proteins (11 g/100 g) and lipids (2 g/100 g). Whey powder was dissolved in 0.5 g/mL of distilled water. The total administered dose was 2 g/kg body weight (b.w.)/day for each animal (in 1 mL of the solution). Commercial oil derived from pumpkin seeds contained saturated fatty acids (19.4 g/100 mL), monounsaturated fatty acids (26.2 g/100 mL) and PUFA (46.3 g/100 mL).

After 6 weeks of treatment, all animals were killed by intraperitoneal administration of ketamine (Ketamidor 10% 10 mL RighterFaraAG) at a single dose of 0.224 mg/kg b.w., leading to immediate death. Blood samples for biochemical analysis were collected from the heart.

Measurement of water and food consumption and changes in body weight

Every third day the volume of consumed water and the mass of consumed food were measured as the difference between delivered and remaining water and food in the cages. The changes in body weight were measured for each animal using a beam scale every week during the experiment.

Estimation of biochemical parameters

The serum was separated in the blood samples by centrifugation (3000 x g for 5 min). The concentration of ethanol in the blood was measured by gas chromatography with the headspace technique GC-FID [17]. Analysis of the biochemical parameters (AST, ALT, total bilirubin, ALP, LDH, triglycerides, total cholesterol, LDL, HDL) were performed on an automated analyzer (COBAS INTEGRA 400 plus, Roche Diagnostic GmbH, Mannheim, Germany), using commercial laboratory tests according to the provided instructions. The concentration of VLDL was calculated by subtracting the concentrations of HDL and LDL from total cholesterol concentration. Three cardiovascular risk factors were calculated: TG/HDL, TC/HDL (cholesterol ratio) and LDL/HDL (index of atherosclerosis) ratios.

Statistical analysis

Statistical analyses were performed according to the protocols described by Hinkle et al. [18]. Values for liquid and food consumption and b.w. are expressed as means. All other values are expressed as the mean±SEM. The concentrations of ethanol in the blood were compared using one-way ANOVA. Changes in b.w. were tested by three-way ANOVA on logarithmically transformed data (factors were time, i.e. weeks of consumption of ethanol, and supplements) and post hoc compared by Tukey's HSD test. The concentrations of blood biochemical parameters

were compared by two-way ANOVA (factors: ethanol consumption and supplementation) and post hoc compared by Tukey's HSD test. The level of statistical significance for all tests was $p<0.05$.

RESULTS

Liquid consumption

The consumption of liquid was almost uniform (90.52 mL/kg b.w./day on average) in all groups of animals except in the group treated with pumpkin seed oil (P) where the water consumption was higher than in all other groups (106.75 mL/kg b.w./day) (Fig. 1A).

Concentration of ethanol in the blood

Chronic daily consumption of 12% ethanol *ad libitum* resulted in the presence of ethanol in the rats' blood. There was no difference in the ethanol content in the blood of rats that were provided ethanol daily with either pumpkin seed oil (E+P) (0.372±0.085 g/L) or whey (E+W) (0.331±0.060 g/L), and was comparable to the ethanol (E) group (0.254±0.070g/L) (Fig. 1B).

Food consumption

The consumption of food was higher in the non-ethanol groups (C, W, P; 95.47 g/kg b.w./day on average) compared to the ethanol groups (E, E+W and E+P; 67.82 g/kg b.w./day on average) (Fig. 1C).

Weight change of animals during the experiment

The highest increase in b.w. was observed in the group of animals treated with pumpkin seed oil (amounting to 103.77% of the initial b.w.), followed by groups treated with ethanol (E) (89.73% of the initial b.w.), ethanol and whey (E+W) (80.82% of the initial b.w.) and ethanol and pumpkin seed oil (E+P) (76.16% of the initial b.w.) (Fig. 1D).

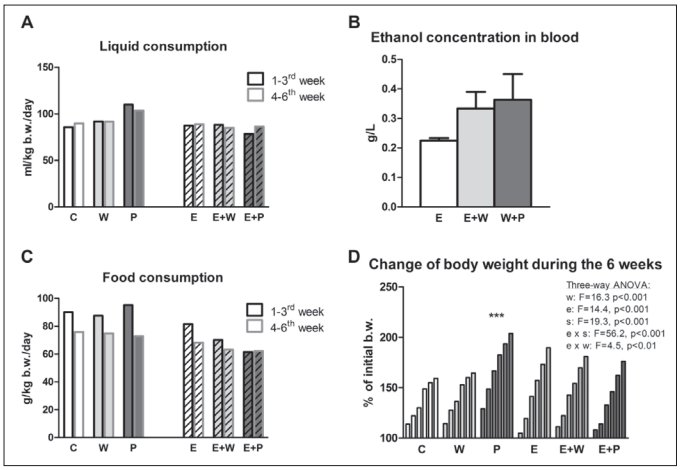

Fig. 1. Liquid and food consumption, concentration of ethanol in the blood and change in body weight during the treatment with whey, pumpkin seed oil and ethanol. **A** – Liquid consumption, expressed as the average consumption per animal during the first three weeks and during the second three weeks of the experiment. **B** – Concentration of ethanol in the blood at the end of the experiment. One-way ANOVA showed no significant differences between the groups. **C** – Food consumption, expressed as the average consumption per animal during the first three weeks and during the second three weeks of the experiment. **D** – Change in b.w. during 6 weeks of the experiment, expressed as the average b.w. for each week relative to the initial b.w. Differences between the groups were tested by three-way ANOVA (factors: time i.e. weeks (w), consumption of ethanol (e) and consumption of supplements (s); $p<0.05$ was the lower limit, and post hoc tested by Tukey's HSD test. Three-way ANOVA showed a significant effect of all three factors: w – there was an increase in the average b.w. in every week; e – groups treated with ethanol exhibited a smaller increase in b.w. when compared to the groups that were not treated with the ethanol; s – groups treated with pumpkin seed oil displayed a greater increase in b.w. as compared to groups not treated with pumpkin seed oil; interaction of w and e – the non-ethanol groups exhibited a greater increase during the early phase of the experiment while the ethanolic groups exhibited a greater increase of b.w. during the later phase of the experiment; interaction of e and s – treatments with whey and pumpkin seed oil had the opposite effects on the increase in b.w. in ethanolic and non-ethanol groups, namely groups W and P displayed a greater increase in b.w. as compared to C, while groups E+W and E+P exhibited a smaller increase in b.w. as compared to E. Tukey's HSD test which showed that the group treated with pumpkin seed oil only (P) displayed the greater increase in b.w. when compared to all of the other groups. C – control group; W – group treated with whey; P – group treated with pumpkin seed oil; E – group treated with ethanol; E+W – group treated with ethanol and whey; E+P – group treated with ethanol and pumpkin seed oil. *** $p<0.001$.

The effect of whey and pumpkin seed oil on blood biochemical parameters of alcoholized rats

Six weeks of treatment of rats with ethanol, whey and pumpkin seed oil did not lead to significant changes in AST, ALT, total bilirubin, ALP, total cholesterol, and VLDL levels between the groups (Figs. 2 and

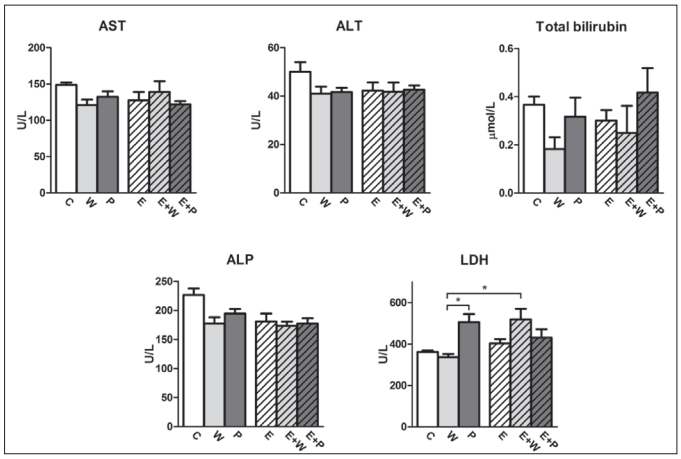

Fig. 2. Blood indicators of hepatic function: AST, ALT, total bilirubin, ALP and LDH after the six-week treatment with whey, pumpkin seed oil and ethanol. Differences between the groups were tested by Two-way ANOVA (factors: consumption of ethanol – e and consumption of supplements – s). Only significant ANOVA values are presented ($p<0.05$ was the lower limit). When ANOVA showed significant differences, Tukey's HSD post-hoc test was performed to show particular differences. Consumption of supplements and ethanol showed no significant effect on AST, ALT, total bilirubin and ALP levels. Two-way ANOVA showed that consumption of supplements did not have the same effect on the LDH level with and without ethanol (significant interaction of e and s, $p<0.01$). Tukey's HSD post-hoc test showed a significant difference between the groups W and P ($p<0.05$) and groups W and E+W ($p<0.05$). C – control group; W – group treated with whey; P – group treated with pumpkin seed oil; E – group treated with ethanol; E+W – group treated with ethanol and whey; E+P – group treated with ethanol and pumpkin seed oil. * $p<0.05$.

3). Two-way ANOVA showed that consumption of supplements did not have the same effect on the LDH level with and without ethanol (significant interaction of the two factors, $F=6.77$, $p<0.01$) (Fig. 2). Tukey's HSD post-hoc test showed that there was a statistically significant increase ($p<0.05$) in LDH levels in the group supplemented with pumpkin seed oil (P) when compared to the group of animals supplemented with whey (W). LDH was also higher in the group supplemented with pumpkin seed oil as compared to the control group, but at the margin of statistical significance. A statistically significant increase ($p<0.05$) was recorded in the group of animals that both consumed ethanol and were supplemented with whey (E+W) as compared to the group of animals that consumed only ethanol (E).

The concentrations of triglycerides were lower in alcoholic rats (two-way ANOVA, ethanol consumption effect, $F=6.92$, $p<0.05$; Tukey's HSD post-hoc test showed no significant differences) irrespective of the supplementation (Fig. 3). On the other hand, consumption of alcohol led to a general increase in LDL (two-way ANOVA, ethanol consumption effect, $F= 4.39$, $p<0.05$; Tukey's HSD post-hoc test showed no significant differences). Two-way ANOVA also showed that consumption of supplements did not have the same effect on the concentration of HDL with and without ethanol consumption (significant interaction of the two factors, $F=4.06$, $p<0.05$; Tukey's HSD post-hoc test showed no significant differences).

The TG/HDL ratio was lower in the alcoholized rats (two-way ANOVA, ethanol consumption effect, $F=5.01$, $p<0.05$; Tukey's HSD post-hoc test showed no significant differences) irrespective of the supplementation (Fig. 4). Two-way ANOVA also showed that consumption of supplements did not have the same effect on the TC/HDL ratio with and without ethanol (significant interaction of the two factors, $F=4.00$, $p<0.01$). Tukey's HSD post-hoc test showed that there was a significant decrease ($p<0.05$) in the TC/HDL ratio in the group treated with ethanol and pumpkin seed oil (E+P) when compared to the group treated with ethanol only (E). The LDL/HDL ratio was higher in the alcoholized rats (two-way ANOVA, ethanol consumption effect, $F=5.60$, $p<0.05$), irrespective of the supplementation. Two-way ANOVA also showed that consumption of supplements did not have the same effect on the LDL/HDL ratio in rats with and without ethanol (significant interaction of the two factors, $F=6.40$, $p<0.01$). Tukey's HSD post-hoc test showed that there was a significant increase ($p<0.05$) in the LDL/HDL ratio in the groups treated with ethanol (E) and ethanol and pumpkin seed oil (E+P) as compared to the control (C).

DISCUSSION

There are no randomized clinical studies that can provide an answer as to whether the supplementation of whey and pumpkin seed oil affects serum biomarkers of liver function, but in clinical practice it is often suggested that whey and herbal supplements can induce changes of the biochemical parameters that point to their effects on the liver in different diseases

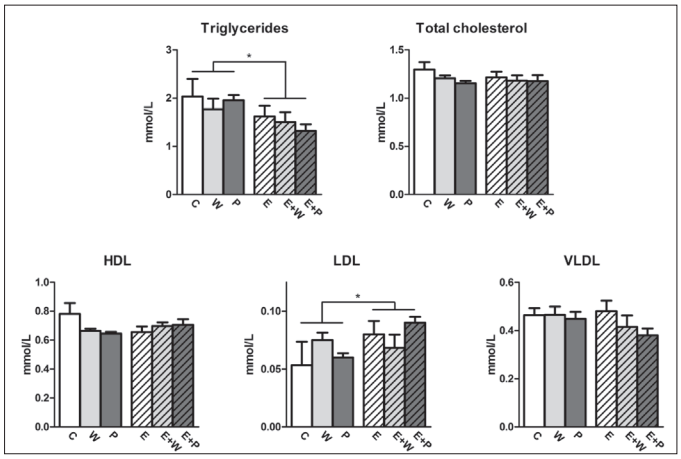

Fig. 3. Lipid profile determined after the six-week treatment with whey, pumpkin seed oil and ethanol. The levels of: triglycerides, total cholesterol, HDL, LDL and VLDL are shown. Differences between the groups were tested by two-way ANOVA (factors: consumption of ethanol – e and consumption of supplements – s). Only significant ANOVA values are presented ($p<0.05$ was the lower limit). When ANOVA showed significant differences, Tukey's HSD post-hoc test was performed to show specific differences. Consumption of supplements showed no significant effect on the level of any of these lipids/lipoproteins, while consumption of ethanol had a significant effect on the level of triglycerides and LDL. Ethanol in general decreased the level of TGs ($p<0.05$) and increased the level of LDL ($p<0.05$). Two-way ANOVA also shows that consumption of supplements did not have the same effect on the HDL level with and without ethanol (significant interaction of e and s, $p<0.05$). C – control group; W – group treated with whey; P – group treated with pumpkin seed oil; E – group treated with ethanol; E+W – group treated with ethanol and whey; E+P – group treated with ethanol and pumpkin seed oil. * $p<0.05$.

[10]. Here we studied the effects of whey and pumpkin seed oil supplementation on rats that chronically consumed low ethanol doses *ad libitum* daily for 6 weeks. Our study showed that daily ingestion of the studied supplements did not change the level of consumption of ethanol (estimated by the total liquid intake) or its concentration in the blood. Ethanol consumption decreased food intake and led to elevated body weight. Pumpkin seed oil treatment itself also elevated body weight, suggesting that it changed the metabolic profile of the rats.

Blood aminotransferase concentrations represent an important marker of chronic exposure to ethanol. Studies in the human population have shown the increase in plasma aminotransferases and body weight after chronic alcohol intake [19-21]. Liangpunsakul et al. [21] pointed to the insignificance of tests that determine the levels of aminotransferases if the intake of alcohol is less than or about two drinks per day. The consumption of alcohol higher than the indicated amount leads to an increase in aminotransferase activities. Our results confirm this. Estimation of ethanol intake showed that the daily alcohol intake is comparatively low and that AST and ALT did not exhibit significant changes after 6 weeks of consumption. On the other hand, there are data that show that acute exposure to alcohol, irrespective of the dose, does not lead to significant changes in the activity of aminotransferases in rats [22]. However, chronic exposure to alcohol increases the level of ALT in plasma, without changing the level of AST [23]. Previous research has shown that 35% alcohol treatment led to an increase in aminotransferases [24,25]. However, consumption of 12% alcohol *ad libitum* (0.254 g/L) during six weeks in our study did not cause a significant increase in these biochemical parameters, suggesting either that the total consumed amount of alcohol or its concentration were low. Supplementation with whey and pumpkin seed oil did not change the levels of AST and ALT in either the alcoholized or control rats.

Furthermore, the total bilirubin levels were not changed, suggesting that liver damage did not progress. Bilirubin metabolism depends on the activity of microsomal UDP-glucuronyl transferase, which is important for its conjugation, as well as the availability of UDP-glucuronic acid and its precursor, UDP-glucose [26]. Studies have shown that chronic consumption of ethanol leads to an increase in UDP-glucuronyl transferase activity [27,28]. The formation of the glucuronide conjugate depends on the continuous production of UDP-glucuronic acid and can be inhibited by increasing the ratio of $NADH/NAD^+$ during the oxidation of ethanol [29]. In our study, total bilirubin was not elevated after chronic ethanol consumption and the reduction in UDP-glucuronic acid production during ethanol oxidation can explain this finding.

The alkaline phosphatase level did not change in the different groups of animals. The effects of chronic ethanol consumption on serum and hepatic ALP levels have been the subject of controversy [30]. Although most studies showed increased levels of ALP [31,32], some researchers did not detect an increase

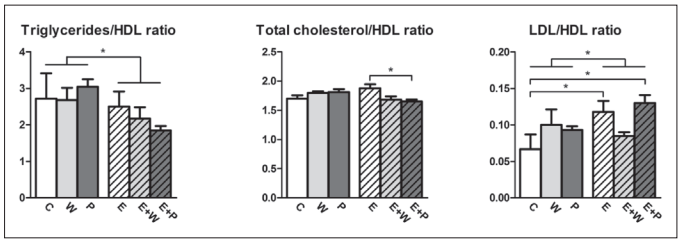

Fig. 4. Cardiovascular risk factors: TG/HDL ratio, TC/HDL ratio (cholesterol ratio) and LDL/HDL ratio (index of atherosclerosis) after the six-week treatment with whey, pumpkin seed oil and ethanol. Differences between the groups were tested by two-way ANOVA (factors: consumption of ethanol – e and consumption of supplements – s). Only significant ANOVA values are presented ($p<0.05$ was the lower limit). When ANOVA showed significant differences, Tukey's HSD post-hoc test was performed to show particular differences. Consumption of supplements showed no significant effect on any of these three ratios, while consumption of ethanol had a significant effect on the TG/HDL and LDL/HDL ratios. Ethanol decreased the TG/HDL ratio ($p<0.05$) and increased the LDL/HDL ratio ($p<0.05$). Two-way ANOVA also showed that consumption of supplements did not have the same effect on the TC/HDL and LDL/HDL ratios with and without ethanol (significant interaction of e and s, $p<0.01$ for both TC/HDL and LDL/LDH ratios). Tukey's HSD post-hoc test showed a significant difference between the groups E and E+P ($p<0.05$) for TC/HDL ratio and significant difference between groups C and E and groups C and E+P ($p<0.05$ both) for LDL/HDL ratio. C – control group; W – group treated with whey; P – group treated with pumpkin seed oil; E – group treated with ethanol; E+W – group treated with ethanol and whey; E+P – group treated with ethanol and pumpkin seed oil. * $p<0.05$.

in ALP concentration [33]. Our results also suggest that the alcohol consumption in our study was insufficient to change ALP and hence liver metabolism to a significant extent.

Pumpkin seed oil intake elevated the concentration of LDH and suppressed the increase induced by ethanol consumption. On the other hand, whey supplementation along with alcohol consumption elevated LDH levels. This points to the different potential of these supplements. Elevation of LDH caused by pumpkin seed oil suggest caution in its recommendation, but further parallel consumption of ethanol and supplementation with pumpkin seed oil had no effect on the already established LDH level. On the other hand, supplementation with whey had no effect on the LDH level, but parallel consumption of alcohol and whey supplementation maintained a healthy liver, estimated by the LDH level.

Supplementation of ethanol consumption with whey and the intake of pumpkin seed oil alone led to a significant increase in LDH. Some authors have found that LDH-1, LDH-2 and LDH-3 activities were increased in rats treated with a high percentage of starch, and it was suggested that the disruption of the nutritional status can lead to an increase in this enzyme [34]. LDH is an enzyme with a role in the conversion of lactate to pyruvate. This reaction requires NADH. The mechanism by which whey in combination with ethanol and pumpkin seed oil alone increases the activity of this enzyme could be through a disturbance of the NAD/NADH ratio during their metabolism, and a consequent increase in the activity of this enzyme. It was found that whey protein supplements that precede exercise can lead to an elevation of LDH [35].

Total cholesterol levels were not different among the groups. Preliminary studies [36] in human population have shown that alcohol does not lead to an increase in total cholesterol, which was also confirmed in our study. Whey and pumpkin seed oil supplementation had no effects on the cholesterol levels. However, ethanol consumption decreased triglyceride and the TG/HDL ratio, sensitive markers of the lipid profile used for detecting coronary disease in humans. There is a growing number of studies pointing to the fact that drinking small amounts of alcohol is associated with a fall in triglycerides in the blood [37]. These paradoxical effects of the interaction of alcohol and triglycerides are brought into conjunction with alcohol concentrations and changes in genetic factors [38].

On the other hand, ethanol elevated LDL levels, thus changing the lipid metabolic profile. The index of atherosclerosis was also statistically higher in the alcoholic groups compared to non-alcoholic rats; however, whey supplementation suppressed this elevation. This suggests a protective effect of whey on atherosclerosis development during alcohol ingestion. The TC/HDL ratio in the alcoholic groups was lowest in the ethanol group supplemented with pumpkin seed oil, which can be considered as a protective. Our study showed that supplementation with whey and pumpkin seed oil modified the lipid metabolic profile in the specific lipid metabolic pathway and exerted protection in a different way. Moreover, some of the active pumpkin seed

oil ingredients, such as antioxidants, can by themselves be protective at the metabolic cellular levels. Ingestion of ingredients with different PUFA concentrations change the lipid profiles attributed to arachidonic acid synthesis and thus change the active lipid profile [39-41]. Studies have shown that the effect of whey at the triglyceride level is not expressed in people with a low body mass index [10] after intensive exercise [35].

Although studies [42,43] have revealed the cardioprotective effect of ethanol in the human population by increasing HDL cholesterol after chronic alcohol consumption, in our research, ethanol did not change the HDL cholesterol level. The reason for this result may be the decrease in the activity of the lecithin-cholesterol acyltransferase (LCAT) enzyme due to ethanol exposure [44]. The mechanisms by which alcohol use could alter lipoprotein distribution can be multifactorial. It was found [45,46] that alcohol intake is associated with lower cholesteryl ester transfer protein (CETP) activity, but the same results are not presented in other studies [47,48]. TaqIB, a variant of CETP that leads to lower CETP activity, influences the levels of HDL particles (HDL-P) and LDL particles (LDL-P), such as increases in large HDL-P and a shift from smaller to larger LDL-P [49]. The greatest increase in large HDL-P with alcohol intake was reported in a reference study [50], with a smaller increase in medium-sized HDL-P. Some investigators [51] have found that higher levels of small particle HDL-P are directly associated with higher risk of coronary heart disease, in contrast to the effect of larger HDL-P [52]. Since previous studies [53] noted a decrease in HDL cholesterol level in a diet with high amounts of carbohydrates in alcoholics, it seems that certain nutritional components of whey and pumpkin can be the reason for the absence of change in HDL cholesterol levels [54]. A meta-analysis study [10] showed that whey supplementation did not change the HDL-cholesterol level.

The levels of VLDL cholesterol in our experiment did not exhibit changes between the examined groups of animals. A study in humans [50] showed that during ethanol exposure, the levels of VLDL cholesterol change in a stepwise manner depending on the amount of consumed alcoholic beverages. In this study, increased consumption of alcohol tends to decrease the levels of VLDL cholesterol that can be a consequence of the alcohol-induced stimulation of lipoprotein lipase [55]. This suggests that ethanol consumption in our experiment was low, as in other studies. Studies have shown an increase in LDL cholesterol in chronic alcohol consumption [56]. Our results revealed that chronic consumption of alcohol elevated LDL levels. Supplementation with whey and pumpkin seed oil did not prevent LDL level elevation, but whey had a protective impact on the atherosclerosis index.

Consumption of ethanol leads to an increase in the availability of fatty acids in the liver, and therefore there is an increased likelihood of their storage in the liver, as well as increased release through lipoprotein. The endoplasmic reticulum is a site where lipid esterification and lipoprotein production take place. It is possible that the ethanol-induced proliferation of the smooth endoplasmic reticulum plays an important role in increasing the capacity of the liver to produce lipoproteins [57,58]. Studies [59,60] have shown that the combinations of certain food supplements and ethanol lead to the development of alcoholic liver disease. This is demonstrated by the combination of ethanol and corn oil rich in linoleic acid [59]. It was also reported that increased intake of PUFA in combination with alcohol promotes oxidative stress. However, our results have shown that pumpkin seed oil with alcohol changes lipid metabolism, but the reason for the increase in this class of lipoproteins is alcohol consumption. Pumpkin seed oil together with ethanol lowered the TC/HDL ratio, suggesting a protective effect that was obtained by subtle changes in lipid metabolism.

Taken together, our results showed that chronic daily consumption of alcohol produced changes in the liver lipid metabolic profile that led to decreased TG and increased the level of LDL and atherogenic risk. However, changes in biochemical parameters do not reveal significant liver damage, but LDH levels provide evidence that subtle pathohistological changes occurred in the liver, which were expressed in changed biochemical parameters. Furthermore, supplementation with pumpkin seed oil changed metabolism itself, suggesting that concomitant use of different supplements has to take into consideration the individual metabolic pathways. Supplementation with whey along the consumption of alcohol increased LDH levels and provided a protective effect on the athero-

sclerosis index. However, additional histopathological and biochemical aspects need to be investigated in order to provide a clearer picture of the prophylactic properties of whey and pumpkin seed oil supplementation on chronic ethanol damage.

CONCLUSIONS

Our study shows that daily consumption of whey and pumpkin seed oil had no effect on the ethanol-mediated increase in LDL and decrease in triglycerides. However, the supplements had some protective effects on the lipid status, the atherosclerosis index and on general health risk factors. Pumpkin seed oil intake itself changed the lipid metabolic profile of treated rats. However, pumpkin seed oil and whey with ethanol led to an elevation in LDH, suggesting potentially harmful effects.

Acknowledgments: The authors would like to acknowledge the professional assistance and support from the Community Health Centre "Medicus Universalis", Kruševac, Serbia, and the Vivarium of the Center for Biomedical Testing, Institute for Research and Development Galenika a.d., Belgrade, Serbia.

Author contributions: Snežana Janićijević Hudomal, Mirjana Mirić and Milica Mijović conceptualized and refined the research idea and created the research design; Ivan Radić, Stefan Veličković, Sonja Bulajić, Branislav Djerković, Miloš Mitić, Miroslav Adžić and Snežana Janićijević Hudomal performed the literature search; Bojan Joksimović and Duško Blagojević selected the statistical tests; Ivan Radić, Vojkan Nestorović, Vera Lukić and Nikola Tatalović collected and prepared the data; Bojan Joksimović, Nikola Tatalović and Duško Blagojević performed the statistical analyses; Ivan Radić, Stefan Veličković, Sonja Bulajić, Branislav Đerković and Miloš Mitić wrote the first draft of the manuscript; Miroslav Adžić, Duško Blagojević and Snežana Janićijević Hudomal wrote the second draft of the manuscript; Stefan Veličković and Nikola Tatalović edited the manuscript.

Funding: This work was funded by the Ministry of Education, Science and Technological Development, Republic of Serbia, Grant Nos. 173014 and III41029.

Conflict of interest disclosure: The authors declare that there is no conflict of interests.

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