

Water-soluble quercetin modulates the choleresis and bile lipid ratio in rats

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Abstract. Water-soluble analogue of quercetin, corvutin is used in patients with myocardial infarction as blocker of 5-lipoxygenase. However, its effects on secretion, lipid content and physico-chemical properties of bile have not been understood yet. We investigated the effect of corvutin, applied in different doses, on the level of bile flow, the content of bile free and esterified cholesterol, phospholipids, triacylglycerols, and free fatty acids. In order to determine stability of the bile colloidal system, we examined the relationship between different lipid components. The rats were injected intraperitoneally with a bolus of corvutin. At doses of 2.5, 5, and 10 mg/kg, the latter increased bile flow and concentration of total cholates, as well as free fatty acids. Corvutin (5 mg/kg) elevated phospholipids and cholesterol content, but at a dose of 10 mg/kg it increased the concentration of bile cholesterol esters and triacylglycerols. Corvutin applied at doses of 2.5 and 10 mg/kg increased total cholates/cholesterol ratio, but at a dose of 10 mg/kg, the drug reduced cholesterol / esterified cholesterol ratio. The results suggest that corvutin exerts choleric effect and improves stability of bile colloidal system.

Key words: Water-soluble quercetin — Bile secretion — Bile lipid — Total bile acid — Lipid ratio

Introduction

Bile is an iso-osmotic electrolyte solution that is formed in the liver as a product of its secretory function. It mainly consists of water, in which there are organic and inorganic substances in suspension, dissolved or in equilibrium between both states. Bile contains bile acids (BAs) and numerous lipid components, including, phospholipids (PLs), free cholesterol, cholesterol esters, and to a lesser extent free fatty acids (FFAs) and triacylglycerols (TGs) (Alvaro et al. 1986). The liver actively eliminates cholesterol by secreting into bile either directly as free cholesterol or after its conversion into BAs, thereby regulating

its level in the body (Forker 1977). Therefore, cholesterol balance is achieved by modulating its esterification, biosynthesis, and excretion (Goldstein and Brown 1990).

Stability of the bile colloidal system is based on its micellar structure. BAs are the functional detergents which prevent cholesterol precipitation due to generation of micellar structures. They form aggregates with PLs derived from hepatocyte membranes, solubilize cholesterol and other insoluble organic compounds in bile to transport them from the liver to intestine (Weisberg 1984).

To evaluate the physical-chemical properties of bile, we assessed the ratios of its individual components rather than absolute values of concentrations. For example, we used the total bile acid/cholesterol ratio to analyze the colloidal stability of bile, which may be violated by oversaturation of bile with cholesterol or by reducing the level of BAs or PLs. An indicator of the potential predisposition of cholesterol to crystallization takes into account the role of PLs in stabilization of bile colloidal system. Cholesterol/cholesterol esters ratio was calculated to assess cholesterol esterification rate in hepatocytes (Moroz et al. 2009).

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In addition to their role in cholesterol homeostasis, BAs also induce bile flow, provide dispersion of dietary fats and facilitate their absorption in the small intestine. Fats are water-insoluble, unlike enzymes cleaving them, which are water-soluble molecules. Therefore, a prerequisite for hydrolytic cleavage of fats is their dispersion to form thin emulsion. Fat dispersion occurs as a result of interaction between several factors including BAs, FFAs, mono- and diglycerides, and proteins. Reduced amount of BAs in the bile causes a decrease in the intestinal absorption of fat and fat-soluble vitamins (Konturek 1980).

Nowadays, a lot of studies are focused on the search for natural remedies to prevent or treat human diseases. In the study, the flavonoid quercetin (3,5,7,3',4'-pentahydroxyflavone) was examined. The compound was selected because of its promising therapeutic properties that form the basis for potential benefits in maintaining overall health and disease resistance (Davis et al. 2009; Kelly 2011). According to the literature, quercetin demonstrates hepatoprotective effects by improving the functional status of the liver tissue at injuries of different origin (Tang et al. 2012; Lin et al. 2014). Herbal extracts containing quercetin show a marked choleric effect and an influence on bile composition (Filip and Ferraro 2003; Ofem et al. 2013; Hofmann et al. 2015).

It has been reported that effects of quercetin on the body can be different depending on the dose used (Choi et al. 2003; Marozieni et al. 2012). Quercetin, like most flavonoids, has low bioavailability to the body that limits the study of its effects *in vivo* (D'Archivio et al. 2010). In our previous studies, using corvitin, a water-soluble analogue of quercetin, we have shown a significant increase in tissue blood flow in the rat gastric mucosa and the liver (Vinogradova et al. 2012; Vovkun et al. 2014). In another study, we observed accelerated recovery of tissue blood flow in the pancreas, gastric mucosa and the liver of rats with acute pancreatitis following the treatment with corvitin (Vovkun et al. 2015). In addition, recently we have found that corvitin increases biliary content of both free and conjugated with amino acids BAs in rats depending on the tested doses (Vovkun et al. 2016). The impact of corvitin on the bile flow and biliary composition of lipids has not been investigated yet. To study this, we selected 5 half-hour samples of bile in rats. The scheme of experiment allowed us to estimate the effect of the test factor in time (in dynamics). In this way, we could determine the latency of corvitin-induced impact on the examined parameters and the time intervals when the drug had the greatest effect on certain parameters of choleresis.

The present study was aimed at examining effects of corvitin on the bile flow, biliary content of major lipid fractions, total BAs, and ratios between individual organic components of bile, applying different doses of corvitin as a regulatory substance which is promising for correction of external liver secretory function.

Materials and Methods

The work was performed in accordance with World medical assembly Declaration of Helsinki on Ethical Principles for Medical Research Involving Human Subjects, 1964; Declaration of Principles on Tolerance (28th session of UNESCO, 1995), Universal Declaration on Bioethics and Human Rights related to introduction of new biomedical technologies, accepted in 1997 in the city of Oviedo (Spain) and signed by parliament of Ukraine in 2002, Law of Ukraine No. 3447-IV "On the Protection of Animals from Cruelty". Before starting experiments, twenty four 12-week-old mature male Wistar rats, weighing 220–250 g were housed in a controlled environment ($22 \pm 2^\circ\text{C}$, relative humidity of 45–55%, 12-hour light/dark cycle), 6 animals *per* cage, with free access to food and water during the acclimatization period. Acute experiments were conducted after 18 hours of fasting. Rats were randomly divided into 4 groups of 6 animals each. The rats were anesthetized with natrium thiopentalum («Kyivmedpreparat», Ukraine, 6 mg/100 g, *i.p.*). Control animals (group I) were injected intraperitoneally with a bolus of vehicle (sodium chloride 0.9%, 0.1 ml/100 g), whereas rats from groups II, III, and IV received corvitin (2.5, 5, and 10 mg/kg, respectively) in the same manner. Corvitin was supplied by the PJSC SIC «Borshchahivskiy CPP» (Kyiv, Ukraine). The purity of test samples was more than 99%. Anesthetized rats were subjected to laparotomy, and the common bile duct was cannulated with a polyethylene catheter. All administrations were performed after an equilibration period of 30 min to stabilize bile flow rate. Secreted bile was collected every 30 min in the course of 2.5-hour experiment (30-min, 60-min, 90-min, 120-min and 150-min samples) by micropipette connected to a cannula, located in the bile duct. The level of bile secretion was calculated in μl *per* gram of body weight. Bile lipids were separated by the method of thin layer chromatography (Moroz et al. 2009). For this purpose, 0.1 ml of bile was added to 2.4 μl mixture of chloroform-methanol (2 : 1), and 0.5 ml acidified water (0.5 ml of concentrated sulfuric acid *per* 1 liter of double-distilled water) was added in 5 minutes. Samples were kept overnight for complete separation into two phases. The lower phase contained lipids. The extract was dried at 70°C . The dry residue was dissolved in 40 μl of chloroform-methanol mixture (3 : 1) and put on the plate as a thin strip. Chromatography was performed in a chamber, saturated by solvent vapor. The system of eluents included petroleum ether, diethyl ether, ether, icy acetic acid (30 : 10 : 0.2). Fractions of biliary lipids were stained with 5% phosphomolybdic acid in 96% ethanol, followed by heating at 100°C for 5 min. Identification of the major lipid fractions was performed using standards and color changes in molecular functional groups,

considering the relative mobility of fractions. Thin layer chromatography method was also used for separating free and conjugated BAs (Parchami-Ghazaei et al. 2010). For this purpose, 0.1 ml of bile was added to 1.9 ml of cold, extracted mixture of ethanol and acetone (1 : 3). Samples were kept cool (from -10 to 0°C) in a cold chamber for 25–30 minutes and then centrifuged for 10–12 min at 3000–4000 rpm. The extracts were then poured in conoid glass test tubes and dried at 37 – 40°C to get dry residues, which were dissolved in ethanol-water mixture (6 : 4, 50–100 μl). Then, 5–10 μl of samples were applied on the preliminarily washed and marked chromatography plates (15 cm, silica gel aluminium backed plates, Kavalier, Czech Republic). Changes in the content of free and conjugated BAs were determined in the system containing amyl ester acetic acid, toluole, butanole, acetic acid and water (3 : 1 : 1 : 3 : 1, respectively) in glass chromatography chambers. Chromatograms were stained five times by sprinkling dye from a glass fine-disperser pulveriser (15 ml icy acetic acid, 1 g phosphomolybdic acid, 1 ml sulphuric acid, and 5 ml of 50% trichloroacetic acid solution). Chromatograms were kept at 60 – 70°C for 5 min. The methods made it possible to separate mixture of bile lipids into the fractions: PLs, cholesterol, cholesterol esters, FFAs, TG and BAs into the following fractions: taurocholic acid, taurochenodeoxycholic acid + taurodeoxycholic acid, glycocholic acid, lycochenodeoxycholic acid + glycodeoxycholic acid, cholic acid, chenodeoxycholic acid + deoxycholic acid. The sensitivity of this method was 1–3 μg of lipids in the sample. The concentration of the test bile components was calculated in mg per 100 ml of bile. Quantitative determination of the levels of bile lipids and BAs was performed using a densitometer GP-920 (Shimadzu, Japan) under reflected light (λ 620 nm).

The results were processed by one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test. They were presented as mean \pm the standard error of the mean (SEM) and considered statistically significant at $p < 0.05$. Observed power ($\alpha = 0.05$) was 0.9. The most pronounced effect of corvitin was expressed as partial eta-squared (η_p^2). Values of η_p^2 were defined as the proportion of the effect + error variance that is attributable to the effect.

Results

Effect of corvitin on bile flow rate in rats

Figure 1 shows that corvitin (2.5, 5, and 10 mg/kg) evokes an increase in bile flow rate in most half-hour bile samples during 2.5 hours of experiment compared to the results obtained in control rats. Corvitin-induced secretory response of the liver was observed no sooner than 30 minutes after

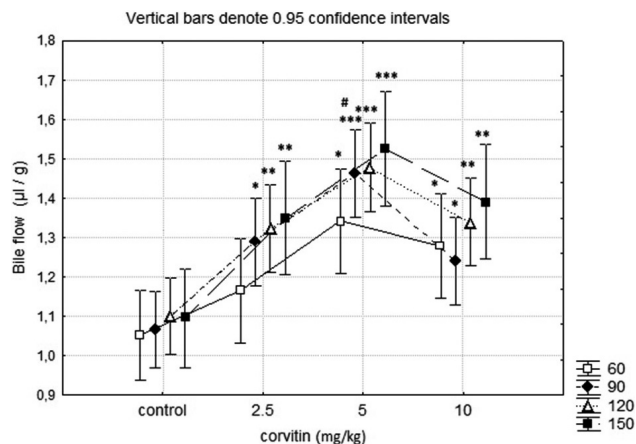


Figure 1. The volume of secreted bile induced by corvitin in different periods of experiment. Bile samples were collected every 30 min during 2.5 hours of the experiment. The data are shown as the means \pm 95%CI; $n = 6$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. control group; # $p < 0.05$ corvitin 2.5 mg/kg vs. corvitin 5 mg/kg; observed power (at α 0.05) = 0.97; partial eta-squared (η_p^2) = 0.44.

administration. In particular, corvitin (2.5 mg/kg) significantly increased bile flow rate in the 90-min sample by 20.6% ($p < 0.05$), in the 120-min sample by 20.0% ($p < 0.01$), and in the 150-min sample by 22.7% ($p < 0.01$). Doubling dose of corvitin caused more intense bile secretion compared to the previous group. In particular, bile flow rate exceeded that in the control group in the 60-min sample by 24.1% ($p < 0.05$), in the 90-min sample by 36.4% ($p < 0.001$), in the 120-min sample by 34.5% ($p < 0.001$), and in the 150-min sample by 39.1% ($p < 0.001$). Corvitin (10 mg/kg) significantly increased the volume of secreted bile in the 60-min, 90-min, 120-min, and 150-min samples by 21.9% ($p < 0.05$), 15.9% ($p < 0.05$), 21.8% ($p < 0.01$), and 26.4% ($p < 0.01$), respectively. The maximum secretory response of the liver to corvitin injections (5 mg/kg) was observed in 2 hours after its administration (1.53 ± 0.04 $\mu\text{l/g}$) compared to effects of its injections at doses of 2.5 and 10 mg/kg (1.35 ± 0.07 $\mu\text{l/g}$; $p < 0.01$ and 1.39 ± 0.07 $\mu\text{l/g}$; $p < 0.01$), respectively (Fig. 1).

Effect of corvitin on total BAs secretion in rats

In rats treated with various doses of corvitin the content of BAs was increased in bile samples in comparison with changes in control animals, mainly in the second half of the experiment. In different tested groups we observed about a similar effect of flavonoid on the concentrations of total BAs. For example, corvitin (2.5 mg/kg) increased the content of total BAs. At that, no significant changes were found in the 30-min, 60-min, and the 90-min samples, whereas the content of total BAs increased in the 120-min

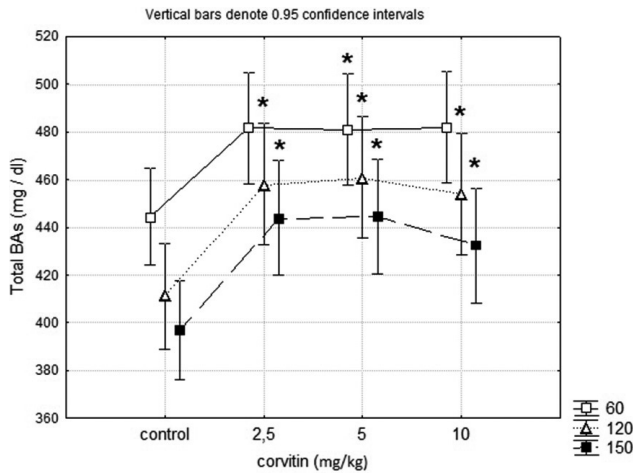


Figure 2. The total BAs level in corvitin-treated rats (2.5, 5, 10 mg/kg). The data are shown as the means ± 95%CI; $n = 6$; * $p < 0.05$ vs. control group; observed power (at alpha 0.05) = 0.95; $\eta_p^2 = 0.41$.

sample by 11.4% ($p < 0.05$), and in the 150-min by 11.9% ($p < 0.05$). Corvitin (5 mg/kg) increased the content of BAs in the 60-min, 120-min, and the 150-min of the experiment by 8.2% ($p < 0.05$), 12.1% ($p < 0.05$), and 12.0% ($p < 0.05$), respectively. Corvitin (10 mg/kg) caused a slight increase in the level of total BAs in the 120-min and the 150-min samples by 10.4% ($p < 0.05$) and 9.0% ($p < 0.05$), respectively (Fig. 2).

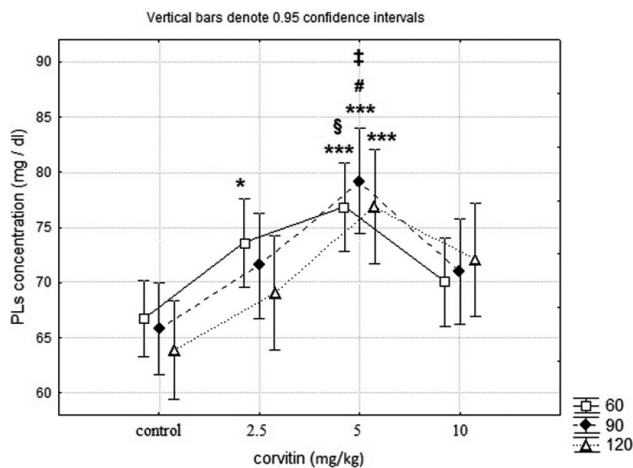


Figure 3. The effect of corvitin on bile PLs concentrations in rats. Secreted bile was collected each half-hour during 2.5 hours of the experiment. The data are shown as the mean ± 95%CI; $n = 6$; * $p < 0.05$; *** $p < 0.001$ vs. control group; # $p < 0.05$ corvitin 2.5 mg/kg vs. corvitin 5 mg/kg; § $p < 0.05$ corvitin 5 mg/kg vs. corvitin 10 mg/kg; observed power (at alpha 0.05) = 0.99; $\eta_p^2 = 0.59$.

Effect of corvitin on bile lipid secretion in rats

To determine the effect of corvitin on biliary lipid composition, we examined the types of lipid molecules in the liver bile. Corvitin (5 mg/kg) modified the concentration of bile PLs, and its effect was observed no earlier than 30 minutes after the introduction, compared with control value. At that, PL level in the 60-min, 90-min and the 120-min samples increased by 15.3% ($p < 0.001$), 20.4% ($p < 0.001$), 20.3% ($p < 0.001$), respectively. Corvitin (2.5 mg/kg) increased PL level by 10.3% ($p < 0.05$) in the 60-min bile sample. The stimulatory effect of 10 mg/kg flavonoid on bile PL was not observed. Comparison of the results between different groups showed that the maximum biliary PL concentration when applying corvitin (5 mg/kg) was observed in the 90-min sample of bile (79.2 ± 2.7 mg/dl versus 71.5 ± 2.2 mg/dl for 2.5 mg/kg of the drug; $p < 0.05$) (Fig. 3).

Biochemical analysis of bile showed that compared with control data only a dose of corvitin of 5 mg/kg was effective to increase the level of bile cholesterol in the 90-min, 120-min, and the 150-min samples by 16.5% ($p < 0.05$), 17.1% ($p < 0.05$), and 16.9% ($p < 0.05$), respectively. The response of the liver to corvitin was observed 1 hour after injection. Corvitin (2.5 and 10 mg/kg) did not cause any significant changes in cholesterol content compared with control animals. The cholesterol concentration difference between samples was minimal (Fig. 4).

To investigate the effect of corvitin on cholesterol ester excretion, rats were treated with this flavonoid in the doses of 2.5, 5 and 10 mg/kg. It should be noted that the concentration of cholesterol esters increased significantly only in response to

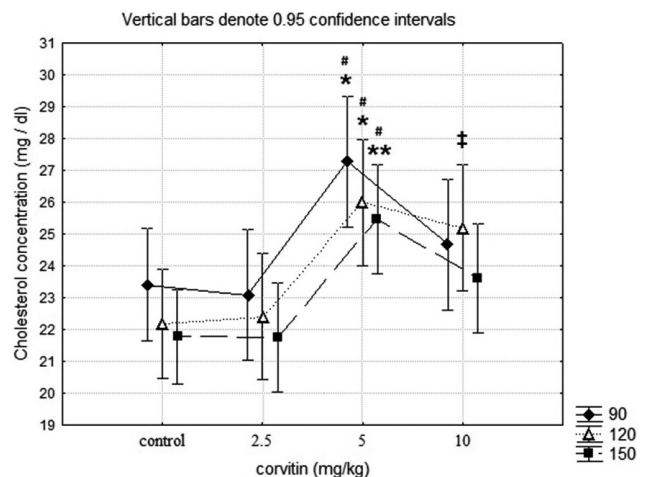


Figure 4. Cholesterol concentration in the bile after the corvitin treatment. The data are shown as the means ± 95%CI; $n = 6$; * $p < 0.05$; ** $p < 0.01$ vs. control group; # $p < 0.05$ corvitin 2.5 mg/kg vs. corvitin 5 mg/kg; ‡ $p < 0.05$ corvitin 2.5 mg/kg vs. corvitin 10 mg/kg; observed power (at alpha 0.05) = 0.98; $\eta_p^2 = 0.46$.

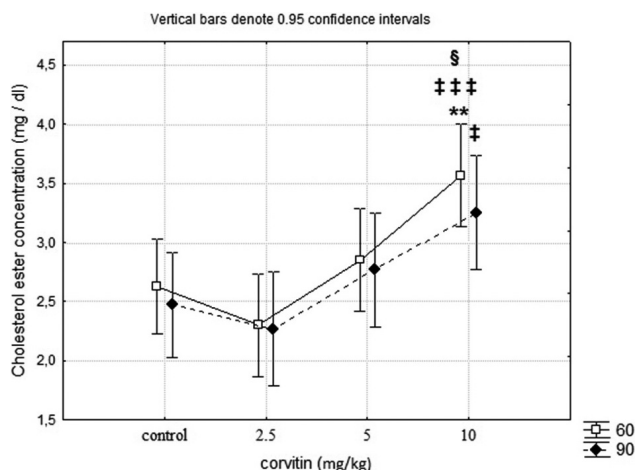


Figure 5. Cholesterol ester concentration in rats bile under the effect of corvitin. The data are shown as the means \pm 95%CI; $n = 6$; $** p < 0.01$ vs. control group; $\$ p < 0.05$ corvitin 5 mg/kg vs. corvitin 10 mg/kg; $\ddagger p < 0.05$; $\ddagger\ddagger\ddagger p < 0.001$ corvitin 2.5 mg/kg vs. corvitin 10 mg/kg; observed power (at alpha 0.05) = 0.99; $\eta_p^2 = 0.63$.

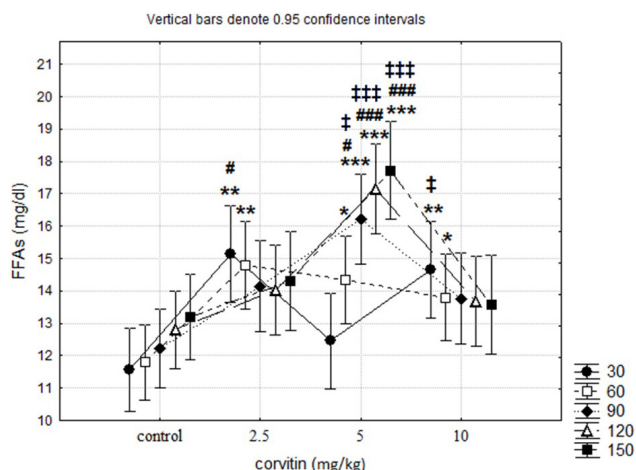


Figure 6. Changes in the level of bile FFAs after applying corvitin. The data are shown as the means \pm 95%CI; $n = 6$; $* p < 0.05$; $** p < 0.01$; $*** p < 0.001$ vs. control group; $\# p < 0.05$; $\#\#\# p < 0.001$ corvitin 2.5 mg/kg vs. corvitin 5 mg/kg; $\ddagger p < 0.05$; $\ddagger\ddagger\ddagger p < 0.001$ corvitin 5 mg/kg vs. corvitin 10 mg/kg; observed power (at alpha 0.05) = 0.99; $\eta_p^2 = 0.6$.

10 mg/kg corvitin not earlier than 30 minutes after injection. The changes were detected in the 60-min and in the 90-min samples by 35.7% ($p < 0.01$) and 31.6% ($p < 0.05$), respectively. That is, the response of bile secretion to the tested drug lasted 1 hour. The maximum concentration of cholesterol esters was observed in the 60-min sample of bile (Fig. 5).

The applications of corvitin (at all tested doses) also increased the biliary levels of FFAs in comparison with control data. In the rats treated with 2.5 mg/kg flavonoid, we observed an augmentation of biliary FFA concentration in the 30-min and the 60-min samples by 30.9% ($p < 0.01$) and 25.4% ($p < 0.01$), respectively. 5 mg/kg corvitin increased the bile FFA level in the samples from the 60-min to 150-min by 21.4% ($p < 0.05$), 32.6% ($p < 0.001$), 34% ($p < 0.001$), and 34.2% ($p < 0.001$), respectively. In response to 10 mg/kg of corvitin, the FFAs concentration increased in the 30-min by 26.7% ($p < 0.05$) and in the 60-min sample by 16.9% ($p < 0.05$). Among all groups, the effect was most prolonged at corvitin dose of 5 mg/kg with maximum response in the 150-min sample of bile (17.7 ± 0.5 mg/dl vs 14.3 ± 0.9 mg/dl for 2.5 mg/kg of the drug; $p < 0.01$ and 13.6 ± 0.8 mg/dl for 10 mg/kg of the drug; $p < 0.001$) (Fig. 6).

The content of TGs in bile exceeded significantly the control value in the 60-min sample by 28.5% ($p < 0.05$) exclusively under the influence of 10 mg/kg corvitin (Fig. 7).

Effect of water-soluble quercetin on the ratio of bile lipid components

Potential ability of cholesterol to crystallization (cholesterol/BAs+PLs) after applying all tested doses of corvitin remained

at the control level. This result demonstrates that in this case the balance of micelle-forming components is kept, and thus the stability of bile colloidal system remains unchanged. In animals treated with 2.5 mg/kg corvitin, the increased ratio of total cholates/cholesterol within the whole experiment was found, indicating improved conditions for preserving stability of bile colloidal system. In particular, in the 30-min, the 60-min, 90-min, 120-min, and 150-min samples of bile, the ratio exceeded the control values by 19.8% ($p < 0.05$),

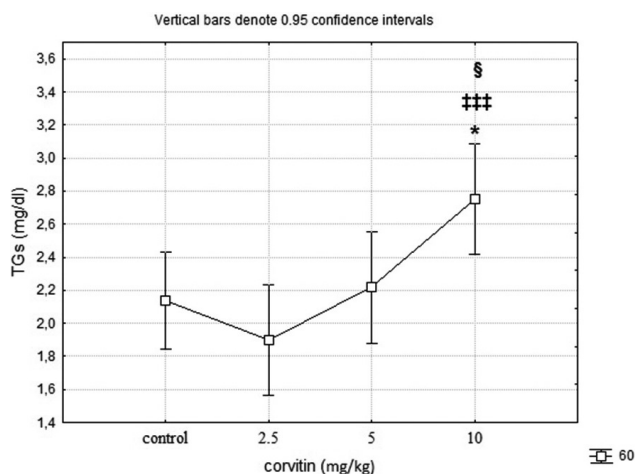


Figure 7. Effects of corvitin on biliary TGs level in rats. The data are shown as the means \pm 95%CI; $n = 6$; $* p < 0.05$ vs. control group; $\$ p < 0.05$ corvitin 5 mg/kg vs. corvitin 10 mg/kg; $\ddagger\ddagger\ddagger p < 0.001$ corvitin 2.5 mg/kg vs. corvitin 10 mg/kg; observed power (at alpha 0.05) = 0.98; $\eta_p^2 = 0.46$.

16.4% ($p < 0.05$), 17.1% ($p < 0.01$), 15.2% ($p < 0.05$), and 15.9% ($p < 0.05$), respectively (Table 1). Corvitin (5 mg/kg) didn't alter the ratio in any of the groups, while 10 mg/kg of the drug increased total cholates/cholesterol ratio by 15.2% ($p < 0.05$) in the 60-min bile sample only ($F = 2.25$, $\eta^2 = 0.43$). The ratio cholesterol/cholesterol ester was reduced by corvitin at a dose of 10 mg/kg, since this group of animals demonstrated an increase in the content of cholesterol esters in deciliter of bile up to 35.7% at unchanged concentration of cholesterol itself. In particular, in the 30-min, 60-min and 90-min samples, the ratio decreased by 25.5% ($p < 0.01$), 30.3% ($p < 0.01$), and 10.3% ($p < 0.01$), respectively ($F = 3.97$, $\eta^2 = 0.54$ indicates a large effect of corvitin). Thus, ratios of total cholates/cholesterol and cholesterol/cholesterol esters achieved statistically significant changes with η^2 ranging from 0.43 (the smaller effect of total cholates/cholesterol) to 0.54 (the larger effect of cholesterol/cholesterol ester).

Discussion

This is the first report on the effect of corvitin on secretion of bile and its individual lipid components. In this study,

we found that corvitin increased the intensity of the bile secretion during the 2.0 hours of the experiment. To the best of our knowledge, there is no sufficient data regarding choleric effect of purified quercetin, however, it has been reported that the herbal extracts containing different amounts of this flavonoid stimulate the secretion of bile (Ofem et al. 2013). According to our results, the use of corvitin (5 mg/kg) is more effective for increasing bile flow rate than a dose of 2.5 or 10 mg/kg. In all tested groups treating with corvitin resulted in the growth of total BAs concentration in bile. These results are consistent with the data obtained by Zhang et al. (2016) about quercetin-induced increase in the levels of total BAs in rats. It has been recently found that quercetin isolated from the seeds of black beans activates BA secretion in mice (Chavez-Santoscoy et al. 2014). It is important to note that there is a tight coupling between the level of BAs and secretion of bile lipids in all animal species (Cohen et al. 1999). An increase in bile BAs content in all experimental groups of animals is a clearly positive result of the study because it indicates possible improvements of emulsifying efficiency of bile by corvitin. It is known that BAs play a role of fat emulsion stabilizer. Their presence in the intestine

Table 1. Effect of corvitin (2.5, 5 and 10 mg/kg) on ratio of concentration of total cholates and cholesterol, cholesterol and total cholates+phospholipids and cholesterol and cholesterol esters in rats bile

Group	samples	total cholates/cholesterol	cholesterol/total cholates+phospholipids	cholesterol/cholesterol ester
Control	30-min	16.7 ± 0.4	5.0 ± 0.17	10.6 ± 0.6
	60-min	17.1 ± 0.65	4.9 ± 0.2	9.9 ± 0.5
	90-min	17.5 ± 0.8	4.6 ± 0.3	9.7 ± 0.5
	120-min	17.8 ± 0.9	4.7 ± 0.26	9.6 ± 0.5
	150-min	17.6 ± 0.8	4.7 ± 0.2	9.2 ± 0.5
Corvitin (2.5 mg/kg)	30-min	20.0 ± 0.8*	4.4 ± 0.16	9.4 ± 0.5
	60-min	19.9 ± 0.5*	4.4 ± 0.1	10.3 ± 0.4
	90-min	20.5 ± 0.6** [#]	4.2 ± 0.15	9.7 ± 0.4
	120-min	20.5 ± 0.6** [‡]	4.2 ± 0.1	8.6 ± 0.3
	150-min	20.4 ± 0.6** [‡]	4.3 ± 0.1	8.6 ± 0.3
Corvitin (5 mg/kg)	30-min	18.2 ± 1.0	4.8 ± 0.25	9.7 ± 1.1
	60-min	17.9 ± 0.7	4.8 ± 0.16	10.4 ± 1.5
	90-min	17.5 ± 0.6	4.9 ± 0.14	11.3 ± 2.0
	120-min	17.6 ± 0.4	4.8 ± 0.09	11.9 ± 2.2
	150-min	17.5 ± 0.3	4.9 ± 0.08	11.2 ± 1.8
Corvitin (10 mg/kg)	30-min	18.7 ± 0.6	4.7 ± 0.15	7.9 ± 0.6
	60-min	19.7 ± 0.4*	4.4 ± 0.09	6.9 ± 0.5** [‡]
	90-min	18.6 ± 0.6	4.6 ± 0.15	7.7 ± 0.4
	120-min	17.7 ± 0.9	4.8 ± 0.23	8.6 ± 0.4
	150-min	17.8 ± 0.9	4.7 ± 0.25	8.7 ± 0.4

Data are represented as mean ± SEM; $n = 6$; * $p < 0.05$; ** $p < 0.01$ significant difference compared with control group; [#] $p < 0.05$ corvitin 2.5 mg/kg compared with corvitin 5 mg/kg; [§] $p < 0.05$ corvitin 5 mg/kg compared with corvitin 10 mg/kg; [‡] $p < 0.05$ corvitin 2.5 mg/kg compared with corvitin 10 mg/kg.

decreases the surface tension of lipid droplets, which promotes the formation of very fine and stable emulsion particles with a diameter of about 0.5 microns. This leads to a tremendous increase in the surface contact of lipase with an aqueous solution. Thus, the more BAs amount, the thinner emulsion of fat, and the better and faster they split lipase. This is especially true of conjugated BAs, amphiphilic molecules of which determine their surface-active properties (Hofmann and Mysels 1992). In our previous studies, we have found that corvutin increases the content of conjugated BAs in rat bile, especially tauro-conjugates (Vovkun et al. 2016).

In the present study, we have shown that corvutin stimulates the secretion of bile PLs, most strongly at a dose of 5 mg/kg. PLs are essential components of cell membranes, plasma, and bile. In all mammalian species, including humans, bile PLs are a mixture of lecithin molecules, 95% of which is phosphatidylcholine. Lecithins designed for secretion into bile are fully synthesized *de novo*. PLs in concert with BAs are basic organic solutes of bile, playing a crucial role in cholesterol and dietary lipid solubilization. The presence of the phosphoric acid residue in the PL molecule makes it a “solvent” for not only cholesterol but also for other hydrophobic compounds. Not only BAs but PLs and cholesterol are amphiphilic molecules. In this regard, in an aqueous medium, such as bile, these compounds cannot exist in a monomolecular form, therefore they form lamellar or micellar structures (Kawamoto et al. 1980). Formation of micelles facilitates dispersion and effective absorption of fats and fat-soluble vitamins in the small intestine. The ratio of mixed micelles and vesicles depends on bile composition and concentration of BAs.

Oversaturation of bile with cholesterol is observed either in case of its overproduction or PLs and BAs deficiency in the liver (Marzolo et al. 1990). We believe that increased biliary concentration of PLs and total BAs in rats treated by corvutin (especially at a dose of 5 mg/kg) is an important result of the present study. In humans and animals, the biological role of PLs is diverse, but they are of particular importance for the liver. Their deficiency results in fatty infiltration of the liver, disorders of bile formation and secretion, an increase in cell membrane permeability, and dysfunction of hepatocytes (LaMont et al. 1992). In case of abnormal PLs synthesis, fatty acids with glycerol form a neutral fat that is accumulated in hepatocytes, displacing other components (Rossmeislet al. 2014). Another very important role of PLs is delayed collagen synthesis and increased collagenase activity. Collagen triggers the replacement of the epithelial tissue by a serous tissue, but PLs exert antifibrotic effect (Sahebkar et al. 2013). Our results suggest the effect of corvutin on the PLs content in rat bile of animals receiving the drug at doses of 2.5 and 5 mg/kg. We assume that corvutin-induced increase in the synthesis of PLs by hepatocytes is a positive factor

not only for stabilization of bile colloidal system, but also for prevention of fat accumulation in the liver tissue and fibrosis development.

The source of cholesterol in the body is food, but most of it is synthesized *de novo* by hepatocytes of the liver (Ott and Lachance 1981). Cholesterol may be removed from the liver either through the bloodstream or through bile (Kawamoto et al. 1980; Goldstein et al. 1990). The synthesis of BAs from cholesterol is a complex multienzyme process in which insoluble and uncharged cholesterol molecule is converted to BA molecule which, when ionized, is amphiphilic, membrane-dissolved, and water-soluble detergent (Cohen et al. 2009). Excessive accumulation of cholesterol in hepatocytes leads to liver damage. Cholesterol accumulation in the mitochondria causes their dysfunction, resulting in increased production of reactive oxygen species and the start of apoptosis (Arguello 2015).

We have found that corvutin increases the concentrations of both BAs and cholesterol in bile. This result can be explained by the activation of cholesterol metabolism in the liver under the influence of the flavonoid. However, corvutin increases the concentration of cholesterol only at a dose of 5 mg/kg, while the content of BAs grows at using all tested doses of the drug. It is likely that under normal conditions, the impact of corvutin on BA synthesis from cholesterol is stronger than the synthesis of cholesterol itself. Zhang et al. (2016) have reported that quercetin facilitates the conversion of cholesterol into BAs and increases cholesterol excretion in bile. In our opinion, corvutin-mediated regulation of cholesterol homeostasis may be the result of modulating the activities of key enzymes involved in cholesterol metabolism in the liver. On the other hand, increased production of BAs stimulates the synthesis of cholesterol *de novo* and its secretion into bile.

Bile, besides free cholesterol, contains cholesterol esters and FFAs which are a soluble form of cholesterol removal. We have found that the level of cholesterol esters in rat bile is substantially increased only after applying corvutin at a dose of 10 mg/kg. It is possible that the largest dose of corvutin increases the activity of acyl CoA:cholesterol acyl transferase (ACAT) enzyme. The latter catalyzes the process of cholesterol esterification, transforming highly hydrophobic cholesterol molecule in a less hydrophobic form (Suckling and Stange 1985).

Fatty acids of animals, both free and those that are part of the lipid molecules, perform energy and plastic function. A certain amount of fatty acids has membrane origin, while others are secreted from hepatocytes. A small amount of fatty acids facilitates the release of both BAs and cholesterol (Mingrone et al. 1990). An interesting and controversial result of the work is a significant increase in the concentration of FFAs in bile under the influence of all tested corvutin doses. It is known that increasing the

concentration of fatty acids in the blood is usually due to the high content of epinephrine, for example under stress or starvation. On the contrary, reduced blood level of fatty acids (hypolipidemia) is observed in hypothyroidism or after insulin administration (Menshikov 1987). Possibly, amplified hepatic biosynthesis of fatty acids under the influence of corvitin is a preparatory phase to further activation of esterification processes in the liver which are required for the formation of PLs, TGs and more hydrophilic cholesterol esters.

This hypothesis is confirmed by the data obtained in rats treated with corvitin at a dose of 10 mg/kg. In particular, we observed significantly higher level of cholesterol esters in rat bile after intraperitoneally administered corvitin (by 35.7%) compared to the rats not treated or treated with smaller doses of the tested drug. Under similar conditions, an increase in TGs content by 28.5% was observed. However, metabolic conversion under the influence of 10 mg/kg corvitin was less effective in bile formation, since bile flow rate was lower than it was at corvitin dose of 5 mg/kg. Some researchers have demonstrated that quercetin modulates hepatic lipogenesis, which is manifested in lowering of liver TGs amount and preventing fatty liver in mice. The authors have emphasized that different doses of quercetin have a variety of effects. In particular, Ying et al. (2013) have reported that quercetin effectively reduces the amount of liver TGs, collagen and the size of fat particles only if used in high doses. Small doses of the drug evoke a weak and short-term effect. The properties of bile and its ability to perform inherent physiological functions largely depend not only on the concentration of its individual components, but also on their ratio. Therefore, to describe the characteristics of bile in the rats, we used different indexes and ratios. For example, we calculated the following ratios: total cholates/cholesterol (cholato-cholesterol coefficient), cholesterol/BAs+PLs (an indicator of the potential predisposition of cholesterol to crystallization), and cholesterol/cholesterol esters (an indicator of cholesterol esterification). Normally, cholato-cholesterol coefficient is greater than 10. The decline of the index indicates a violation of colloidal stability due to oversaturation of bile with cholesterol or reducing the level of BAs. We have found that the treatment with 2.5 and 10 mg/kg corvitin results in a certain increase in cholato-cholesterol ratio. These doses of flavonoid do not alter the concentration of cholesterol, but increase the amount of total BAs in bile. In animals treated with 5 mg/kg corvitin, the cholato-cholesterol coefficient does not exceed the control values, because in this group, the contents of both total cholates and free cholesterol increased. However, we do not consider this result as the evidence of disturbed detergent properties of bile, since in this group of rats, we observed an increase in both the bile outflow

and amount of PLs and FFAs in it. These bile components promote solubilization of cholesterol in mixed micelles and vesicles and its subsequent removing into the small intestine. An indicator of the potential predisposition of cholesterol to crystallization does not differ from that of the control rats in all tested groups. Reduced cholesterol/cholesterol esters ratio was observed only when corvitin was used at a dose of 10 mg/kg. This result is associated with a significant increase in the content of bile cholesterol ester after application of the tested drug in this experimental group of animals. It indicates that hepatic cholesterol esterification is activated under the influence of corvitin, which reduces lithogenicity of bile.

Thus, the data obtained in acute experiments have revealed that corvitin modulates external function of the rat liver by increasing the amount of secreted bile, enhancing the conversion of cholesterol into BAs, and facilitating excretion of cholesterol and its esters from the liver into bile. Corvitin also stimulates the synthesis of PLs and FFAs in hepatocytes and their secretion in bile. All these processes contribute to improving colloidal properties of bile and enhancing its ability to form mixed micelles and solubilize cholesterol. Corvitin-induced increase in the concentrations of biliary BAs and FFAs can improve the dispersion properties of bile, and therefore the digestion of fat in the small intestine. The ratio of bile lipid components after corvitin application points to stabilization of colloidal system of bile. Corvitin does not affect the potential ability of cholesterol to crystallization, keeping balance of micelle-forming components of bile and preventing the development of gallstones. These data support the possibility of clinical application of corvitin for correction of disturbed bile secretion and qualitative improvement of bile composition.

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