Dear Editor,

Thank you very much for important comments and suggestions. We have modified manuscript accordingly. Changes are emphasized in marked version. Point-by-point responses and explanations are attached. The information requested by you in manuscript have been added at the position indicated by your comments in the text. English grammar of our manuscript has been checked by suggested American Journal Experts.

Thank you very much for this possibility.

Sincerely,

Stanislav Micuda

Reviewer 1

Without comments

Reviewer 2

Comments to Authors: The authors presented a carefully executed scientific study on the effect of resveratrol on biliary excretion in sham-operated and bile duct obstructed rats. Shown increase bile flow and activation transporters of bile acids and lipophilic compounds in normal rats when used resveratrol. Authors shown that resveratrol improves the morphological picture and disturbed molecular and biochemical indicators in bile duct obstructed rats.

1. It remains unclear the mechanism of this improvement. It is desirable to express the opinion of the authors on the mechanism of action of resveratrol on the improvement of the structural organization of the liver and excretion of bile acids, the bile flow in rats with obstructive bile duct. In fact, the cause of extrahepatic cholestasis is not excluded.

Reply: The protective effect of resveratrol on liver injury during bile duct obstruction in rats has been reported in several previous works (Ara et al., 2005; Kirimlioglu et al., 2006; Cenesiz et al., 2007; Muriel and Rivera-Espinoza, 2008; Chan et al., 2011; Wang et al., 2014b; Wang et al., 2015; Zhang et al., 2015; Tian et al., 2016). The protection was ascribed to an anti-oxidative and anti-inflammatory effects of resveratrol especially at early stages (1-7 days) after bile duct obstruction. However, previous study showed that the inhibitory effect of resveratrol on the expression of proinflammatory mediators may disappear since 7 day of bile duct obstruction (Chan et al., 2011). In agreement, we have also detected absence of changes in the expression of early and delayed phase cytokines. We detected only the reduced Platelet-derived growth factor receptor mRNA expression (Figure 6), which complies with the reduced bile duct proliferation in RSV-treated BDO animals. However, activation of liver inflammation and fibrosis is rather consequence than cause of the liver damage during obstructive cholestasis. The major factors activating these mechanisms are

cumulating toxic substances, especially bile acids. Thus, the most effective approach in prevention of liver injury is therefore to remove obstruction (which is not always possible like in inoperable biliary cancer or biliary atresia) or to reduce concentrations of cumulating toxic substances. In this situation, the significant reduction of bile acid concentrations in plasma of BDO rats by resveratrol may serve as even more effective hepatoprotective mechanism than its direct anti-inflammatory effect. The mechanism how resveratrol reduced BA plasma concentrations during obstructive cholestasis must be further analyzed. We excluded liver and renal pathways, which suggests reduced absorption of bile acids in intestine. Reduced absorption of bile acids after administration of resveratrol has indeed been described in two previous works, although not in obstructive cholestasis. These data together indicate that intestinal effect of resveratrol on bile acid absorption may significantly contribute to its hepatoprotective capabilities during different forms of cholestasis. Intestinal effects of resveratrol on bile acid homeostasis during obstructive cholestasis must be further studied, which is the major limitation of our study focused primary on liver mechanisms, especially those associated with bile production and secretion in healthy animals.

Last sentence of reviewer's comment indicated that the best way is to remove cause of obstruction. We fully support this approach. However, in certain situation like biliary atresia, advanced fibrotic changes, or inoperable tumors constricting bile duct is impossible to remove obstruction. Although no pharmacotherapy in such situation may fully prevent liver injury, suitable substance may slow progression of disease and delay onset of severe forms like cirrhosis. Important in this situation is to start therapy in early stages of the disease.

We have modified the respective paragraph of discussion. As mentioned in conclusion, our data points toward bile acid-reducing effect of RSV as a primary mechanism of hepatoprotection during obstructive cholestasis. We know that the effect of RSV in healthy animals is clinically more relevant, because resveratrol became widely used nutraceutical agent in population.

Minor comments:

2. In the introduction it is desirable to give more references to the presented data including articles from World Journal of Gastroenterology and World Journal of Hepatology.

Reply: We have added four appropriate citations to the manuscript.

Page 6 – citations: (Roma et al., 2008; Brandoni et al., 2012)

Page 7 (Heeboll et al., 2014; Ferramosca et al., 2017)

3. The activity of ALT and AST are indicators of damage to cell membranes, but not signs of cholestasis

Thank you for this comment. We agree that activity of transaminases are indicators of hepatocellular damage reflecting integrity of cell membranes. In this context, the major

indicators of cholestasis were meant plasma levels of bile acids (most sensitive), and cholesterol.

Reply: The sentence in results section has been changed.

The article is described one cholephilic compound - azithromycin. At the same time, from the text should be that used the multiple substrates ("cholephilic compounds").

Reply: As cholephilic substances we meant molecules excreted primary into bile. Except for azithromycin (the substrate for Mdr1/Mrp2), we evaluate bile acids (model substrates for Bsep), and glutathione (model substrate for Mrp2). Quantification of biliary excretion of these substances therefore reflects the changes in activities of major ABC transporting proteins contributing to biliary excretion of drugs. Such information has therefore implication for estimation of changes in pharmacokinetics of substrates of these transporters in subject using oral resveratrol.

The name of paragraph in results has been changed in order to make the meaning more specific. These molecules are named as cholephiles in abstract and we added specification to methods.

Reviewer 3

Comments to Authors: The authors have presented an interesting study on resveratrol and its effects on biliary secretion in cholestatic and controls rats. The authors showed that administration of resveratrol induces an increase in bile flow and in rats with obstructed biliary tracts RSV attenuated histological and biochemical signs of biliary cirrhosis, including reduction in bile acid concentrations in plasma and fibrotic markers in the liver.

Specific comments:

1. Although the authors make it clear why they chose the dose of 10 mg/kg they should have performed experiments with lower doses as well in order to show that there is a dose-dependent effect of RSV.

Reply: Excessive experimentation and dose selection was performed in previous studies (Ara et al., 2005; Cenesiz et al., 2007; Chavez et al., 2008). Doses lower than 10 mg/kg orally were not effective. We therefore tested directly this dose and upon receiving promising results from preliminary study with several animals, we continued to full-scale study. This dose corresponds with recently recommended dosage of resveratrol in humans, which have increased over the time of our study to minimum 250-500 mg per day. Thus, doses lower than 10 mg/kg would not bring the relevant information applicable to clinically used supplementary doses of a drug.

The sentence in methods for dose justification has been modified accordingly.

2. How these data/results compare with the findings from administration of UDCA for example?

Reply: In a few works when resveratrol has been compared with UDCA such as in experimental nonalcoholic fatty liver disease (Ali et al., 2016), α-naphthyl-isothiocyanate-induced acute cholestasis (Wang et al., 2014a), or intrahepatic cholestasis of pregnancy (Chen et al., 2016) the hepatoprotective effect or resveratrol seemed to be slightly superior. Nevertheless, similar data in obstructive cholestasis are missing. The reason may be the fact that while resveratrol repeatedly demonstrated hepatoprotection after bile duct ligation, UDCA may worsen obstructive cholestasis because it promotes formation of bile infarcts (Barone et al., 2004; Fickert et al., 2013). Similar effect of UDCA was also seen in animal model of primary sclerosing cholangitis (Fickert et al., 2002). Thus, it can be summarized that despite multiple hepatoprotective molecular mechanisms of UDCA (extensively reviewed (Roma et al., 2011; Beuers et al., 2015)), it is not suitable for combination or as a reference in research of obstructive cholestasis.

Due to lack of data from references directly comparing effect of resveratrol and UDCA during obstructive cholestasis and existence of possible harmful mechanisms of UDCA during bile duct obstruction, we suggest not to modify manuscript with such information.

3. In Materials and Methods it is unclear which parts of the liver were used for mRNA, protein and paraffin sections? Were always the same parts used for specific analysis?

Reply: Yes, for all three analyses were used sections from the median lobe of the liver; in all animals the same parts.

New sentence explaning this information has been added to the methods.

4. Figure 2, what does mRNA % of control means? What is control?

Reply: Reviewer perhaps meant Figure 1 (Figure 2 represents bile flow and biliary excretion). All figures presenting mRNA and/or protein expression use samples from saline treated sham rats as a reference (control = 100%).

The sentence explaining situation has been added to Figure 1 caption. Captions of Figure 4, 5 and 6 have been modified.

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