Comparing the Effects of Genistein, Silymarin, Lecithin on Improved Liver Necrosis Induced by Paracetamol Toxic Dose Administration in *Rattus novergicus* Wistar Strain

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ABSTRACT

Background: *Paracetamol, a widely used antipyretic and analgesic drug has been known for its side effect of liver toxicity resulting from free radical formation leading to necrotic hepatocytes. Oral genistein may reduce lipid peroxidation and increase total antioxidant capacity in liver. The present study was aimed to compare the effects of administering genistein, silymarin and lecithin on improved necrotic hepatocytes in Wistar rats fed with toxic dose of paracetamol.*

Method: *An experimental study was conducted at the Laboratory of Physiology and Anatomical Pathology, University of Brawijaya between May and September 2011. About 48 male rats were categorized into 4 groups. The first group was treated with 600 mg/kgBW of oral paracetamol. The other groups were treated with 600 mg/kgBW paracetamol and additional 2 mg/kgBW genistein, 50 mg/kgBW silymarin or 100 mg/kgBW lecithin. ALT, AST, bile acid, malondialdehyde (MDA) and glutation (GSH) levels were measured and centrilobular necrosis observed by histopathological examination. Data were analyzed statistically by ANOVA.*

Results: *AST and ALT level were significantly lower in genistein group ($p = 0.004$ and $p = 0.001$). The lowest bile acid level was found in the lecithin group ($p = 0.025$); while lowest MDA level was found in silymarin group ($p = 0.009$). The highest GSH level was found in lecithin group ($p = 0.001$). The lowest percentage of centrilobular necrosis was found in genistein group ($p = 0.001$).*

Conclusion: *Genistein, silymarin and lecithin supplementation improve liver necrosis induced by toxic dose of paracetamol. Among them, genistein is the most significant agent.*

Keywords: *genistein, silymarin, lecithin, paracetamol, hepatotoxicity*

ABSTRAK

Latar belakang: *Parasetamol, antipiretik analgesik yang banyak beredar di pasaran memiliki efek samping toksisitas hati akibat pembentukan radikal bebas yang menyebabkan nekrosis hepatosit. Pemberian genistein peroral mampu mengurangi peroksidasi lemak di hati dan meningkatkan kapasitas total antioksidan. Penelitian ini bertujuan untuk membandingkan efek pemberian genistein, silimarin, lesitin dalam memperbaiki nekrosis sel hati tikus Wistar yang diberi parasetamol dosis toksik.*

Metode: *Penelitian eksperimental dilakukan pada Laboratorium Fisiologi dan Patologi Anatomi, Fakultas Kedokteran Universitas Brawijaya sejak Mei hingga September 2011. Hewan coba sebanyak 48 tikus wistar jantan dibagi 4 kelompok. Kelompok I diberikan parasetamol dosis 600 mg/kgBB per oral. Kelompok II diberikan parasetamol 600 mg/kgBB + genistein 2 mg/kgBB. Kelompok III diberikan parasetamol 600 mg/kgBB + silimarin 50 mg/kgBB. Kelompok IV diberikan parasetamol 600 mg/kgBB + lesitin 100 mg/kgBB. Parameter yang diukur adalah kadar ALT, AST, asam empedu serta malondialdehida (MDA) dan glutation (GSH). Histopatologi hepatosit dilakukan untuk melihat nekrosis sentrilobuler. Analisa statistik menggunakan uji ANOVA dengan*

tingkat kepercayaan 95% atau α = 0,05.

***Hasil**: Kadar AST, ALT secara bermakna lebih rendah pada kelompok genistein (p = 0,004 dan p = 0,001). Kadar asam empedu terendah pada kelompok lesitin (p = 0,025). Kadar MDA terendah pada kelompok silimarin (p = 0,009). Kadar GSH tertinggi pada kelompok lesitin (p = 0,001). Persentase nekrosis sentrilobuler terendah pada kelompok genistein (p = 0,001).*

***Simpulan:** Pemberian genistein, silimarin dan lesitin memperbaiki nekrosis hati akibat parasetamol dosis toksik. Pemberian genistein paling bermakna dalam memperbaiki nekrosis sel hati.*

***Kata kunci:** genistein, silimarin, lesitin, parasetamol, hepatotoksisitas*

INTRODUCTION

Liver injury caused by drugs or chemical substance is an increasing health problem. Drug- or toxin-induced hepatotoxicity is defined as a liver injury of any stages caused by toxic substances or drugs.[1] The manifestation of hepatocytes injury is very extensive, starting from the asymptomatic increased activity of transaminase enzymes, reversible condition to severe liver disease such as the fatal fulminant acute liver failure.[2] Indonesia, there is no precise incidence about drug or chemical substance toxicity in liver. Current estimation suggests an incidence 1 in 10,000 patients.[3]

Acetaminophen or paracetamol is one of common drugs in the market with a relatively limited therapeutic and toxic dose. Overdose may cause severe hepatotoxicity or even liver failure, which may appear as centrilobular necrosis of hepatocytes in experimental animals and human.[4,5] *N-acetyl-p-benzoquinone-imin* (NAPQI) is a toxic metabolite of paracetamol that causes hepatocyte damage.[6] Intracellular steps critical for liver cells death include mitochondria dysfunction, formation of reactive oxygen species (ROS) and peroxynitrite.[7] Our preliminary study found that the toxic dose of paracetamol for the present study was 600 mg/kgBW on 12 hours following the administration of paracetamol, on which centrilobular necrosis of hepatocytes may occur up to 100%.

Hepatoprotective properties are extremely necessary since the liver performs including as the controller of body metabolism and provide protection especially against drug and toxin.[3,8] Lecithin and silymarin have been proven in some studies that they may protect liver cells against the destructive effects of drugs.[9] Genistein, one of active substances among isoflavones, has been clinically proven as phytoestrogen with antioxidant properties. Its effect on liver has been studied recently.[10,11] Some studies have demonstrated the protective effect of oral genistein, which may reduce peroxidation of lipid in liver and increase the antioxidant capacity.[12,13] The present study was aimed to compare the effects of administering genistein, silymarin and lecithin on improved necrotic hepatocytes in Wistar rats fed with toxic dose of paracetamol.

METHOD

An experimental study with post test control group was conducted using 48 male rats (*Rattus novergicus Wistar strain*) aged 2.5-3 months, weighing between 150-250 g. Ill rats or rats dying during the study were excluded with exception of resulting from treatment. The study was performed at the Laboratory of Physiology and Anatomical Pathology, Faculty of Medicine, University of Brawijaya between May and September 2011, which had obtained approval from the Ethics Committee of Health Study at Faculty of Medicine, University of Brawijaya.

The material used were 500 mg paracetamol (acetaminophen) tablets dissolved in carboxy-methyl cellulose, standard experimental animal fed (PARS), silymarin (Sigma, USA), lecithin (Sigma, USA), genistein (Sigma, USA). The reagents used for evaluating parameters of the study were rat GSH assay kit (Biogenix, USA), bile acid assay kit (MD Bio, USA), MDA assay kit (MD Bio USA).

The experimental animals were randomly selected and divided into four major groups. The first group received oral paracetamol at dose of 600 mg/kgBW; the second had 600 mg/kgBW paracetamol + 2 mg/kgBW genistein; the third received 600 mg/kgBW paracetamol + 50 mg/kgBW silymarin; and the fourth had 600 mg/kgBW + 100 mg/kgBW lecithin. The rats were subsquently terminated in 12 hours following the treatment. The measured parameter were levels of AST, ALT, bile acid, malondialdehyde (MDA) and glutathione (GSH). Histopathological profiles of

hepatocytes were evaluated using hematoxylin eosin (HE) staining to observe centrilobular necrosis. The measurement of ALT, AST and bile acid levels were performed enzymatically using serums of wistar rats.

Interpretation of serum ALT and AST results were performed by using spectrophotometric diagnostic kits (Sysmex®); while the measurement of bile acid was referred to the rat total bile acid assay kit. GSH level were measured using GSH assay kit with sample specimens obtained from liver tissues. Measurement of MDA level was performed by using the MDA assay kit with sample specimens, which were also obtained from liver tissue. Histopathological examination of the hepatocytes was conducted using HE staining. Specimens were examined under microscope equipped by digital camera with 40 x and 100 x magnifications. Paracetamol-induced liver damage was observed by identification of necrotic zone 3 on hepatocytes, hydrophyc alteration accompanied with accumulation of fat and infiltration of inflammatory cells. The percentage of centrilobular necrosis of zone 3 hepatocytes was calculated based on 10 area of randomly observed slides on 400 x magnification. Data were analyzed by one way ANOVA test analysis with the level of 95% confidence interval or $\alpha = 0.05$; and when there was any significant difference, post-hoc analysis was performed using least significant difference (LSD) test.

RESULTS

Of 48 wistar rats in the study, none was excluded. On the measurement of bile acid level, we found some extreme data, which was excluded during statistical analysis. General characteristics of each study parameter are presented in Table 1, with $p < 0.05$ was considered as significant.

Measurement of AST level at 12th hours after treatment demonstrated that the highest level was found in group I receiving only toxic dose of paracetamol (Figure 1). There was lower AST level in the group receiving lecithin, silymarin and genistein compared to the group I. Thus, when we compared to other groups, the lowest AST level was found in group II ($p = 0.004$). By using post hoc analysis with LSD, there was evidence that genistein and silymarin supplementation brought significant difference on AST level compared to the group receiving only toxic dose of paracetamol ($p = 0.001$ and $p = 0.024$). However, no significant difference was found between genistein and silymarin group ($p = 0.17$).

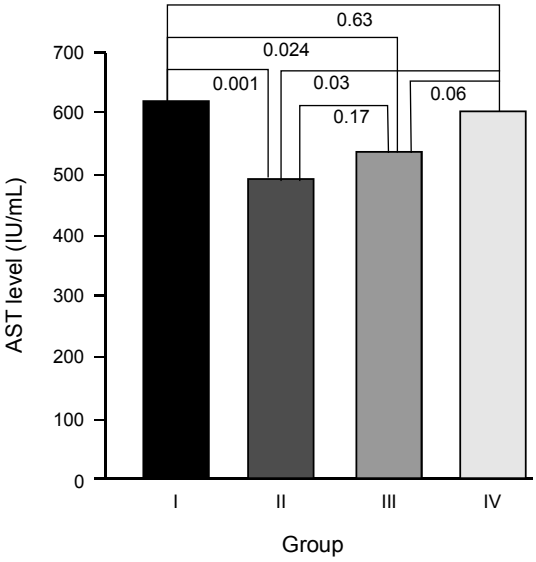

Figure 1. Comparison of AST level among various treatment groups

Table 1. Results of various study parameter measurement in each treatment group

Parameter	Group I n = 12	Group II n = 12	Group III n = 12	Group IV n =12	p*
AST level (U/L)	604.5 ± 48.7 (543.9–665.1)	472.1 ± 78.6 (376.9–567.2)	520.2 ± 33. 9 (478.1–562.3)	587.8 ± 44.2 (532.9–642.7)	0.004
ALT level (U/L)	623.3 ± 43.8 (568.8–677.7)	455.2 ± 46.6 (397.4–513.1)	577.5 ± 42.6 (524.5–630.4)	558.3 ± 39.7 (508.9–607.6)	0.001
Bile acid level (mmol/L)	36.5 ± 15.6 (11.5–61.4)	19.5 ± 4.5 (13.86–25.03)	9.13 ± 5.7 (2.09–16.17)	11.2 ± 8.9 (-2.9–25.4)	0.025
MDA level (nmol/mg)	113.5 ± 0.9 (112.3-114.6)	111.9 ± 2.0 (109.4-114.5)	30.4 ± 2.5 27.3-33.6	104.8 ± 19.4 (80.8-128.9)	0.009
GSH level (µmol/mg tissue)	652.3 ± 231.9 (364.2–940.1)	2519.2 ± 314.9 (2128.1–2910.3)	2654.2 ± 306.1 (2274.1-3034.3)	2672.5 ± 275.3 (2330.6–3014.4)	0.001
Necrotic hepatocyte (%)	92.4 ± 2.3 (89.5–95.2)	45.8 ± 6.1 (38.2–53.4)	58.0 ± 15.7 (38.5–77.5)	49.8 ± 7.98 (39.9–59.71)	0.001

*ANOVA test; statistical analysis was showed in mean ± SD (95% CI); ALT: alanine transferase; AST: aspartate aminotransferase; GSH: glutation; MDA: malondialdehyde

Measurement of ALT level at 12th hours after treatment showed that the highest level was found in group I receiving only toxic dose of paracetamol (Figure 2). ALT level was lower in the groups receiving silymarin, followed by lecithin group and finally the genistein group. Hence, group II demonstrated the lowest ALT level compared to other groups (p = 0.001). Based on post hoc analysis, there was evidence that genistein and lecithin supplementation had significant difference on ALT level compared to the group receiving only toxic dose of paracetamol (p = 0.001 dan p = 0.031). Moreover, there was significant difference between genistein and lecithin group (p = 0.002).

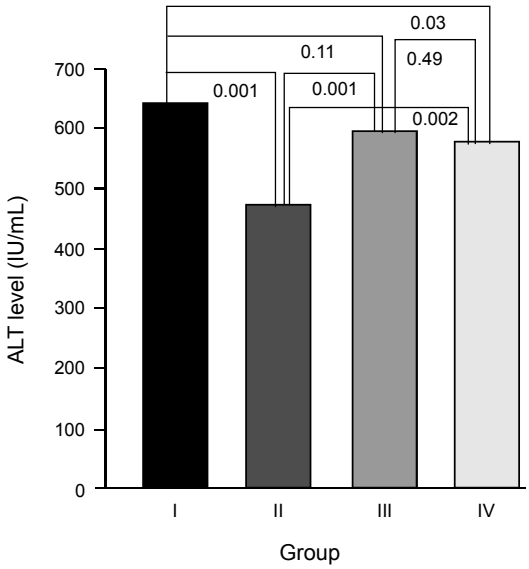

Figure 2. Comparison of ALT level among various treatment groups

Measurement of bile acid level at the 12th hour indicated the highest level in group I, i.e. the group that received toxic dose of paracetamol (Figure 3). Lower levels were found respectively in the group receiving genistein, followed by silymarin and the last was lecithin group. Consequently, group IV had the lowest bile acid level among other groups (p = 0.025). The post hoc analysis indicated that silymarin and lecithin supplementation had significant differences regarding bile acid level compared to the group receiving only toxic dose of paracetamol (p = 0.03 and p = 0.05). However, there was no significant difference between silymarin and lecithin groups (p = 0.9).

Measurement of tissue MDA level at the 12th hour demonstrated the highest value in group I, the group which received toxic dose of paracetamol (Figure 4). Lower levels of MDA were measured in the group receiving genistein, followed by lecithin and finally silymarin. Based on the post hoc analysis, silymarin supplementation had provided evidence of the most significant difference on MDA level among all groups (p = 0.009).

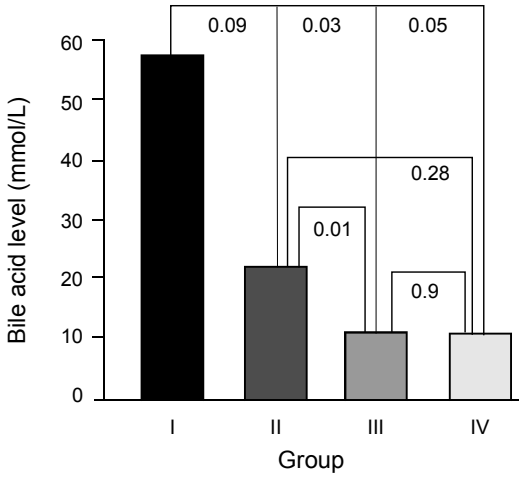

Figure 3. Comparison of bile acid level among various treatment groups

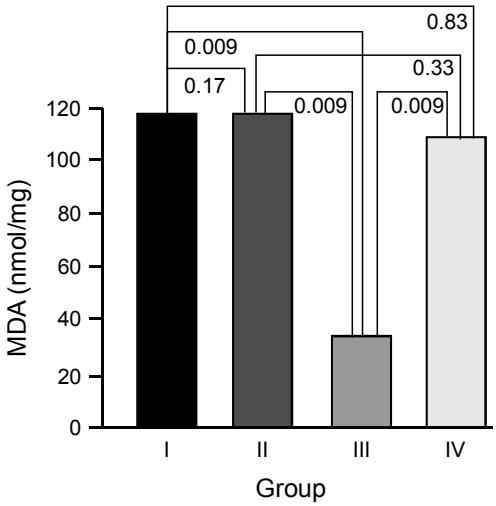

Figure 4. Comparison of MDA level among various treatment groups

When measuring the level of GSH, a natural antioxidant inside the cell, we found that at the 12th hour, it showed the lowest level in group I, i.e. the group receiving toxic dose of paracetamol (Figure 5). Higher GSH levels were consecutively found in the group receiving genistein, followed by the silymarin and finally the lecithin group. As the result, group IV had the highest GSH level compared to other groups (p = 0.000). The post hoc analysis provided evidence that genistein, silymarin and lecithin supplementation were equally significant in increasing GSH level compared to the group receiving only toxic dose of paracetamol (p < 0.001). However, there was no group more significant among those three (p > 0.05).

Calculation on the percentage of necrosis was performed simultaneously with the evaluation of liver histopathological examination. The percentage

of centrilobular necrosis of zone 3 hepatocytes was calculated based on 10 area of randomly observed slides on 400 x magnification at the 12th hour. The highest percentage of hepatocyte damage was found in group I that was the group receiving only toxic dose of paracetamol. Moreover, lower percentage was consecutively found in the group receiving silymarin, followed by the lecithin group and the last was genistein group (Figure 6). Consequently, group II showed the lowest percentage of necrosis compared to the other (p = 0.001).

Figure 7 demonstrated histopathological results of liver tissue obtained from each group and examined at the 12th hour. Severe hepatocyte damage was found in the group that received only toxic dose of paracetamol. In genistein and lecithin group, the hepatocyte structure was fine; although some cells were necrotic. In the group receiving silymarin, irregular structure of liver cells was found along with vacuolization in several sites.

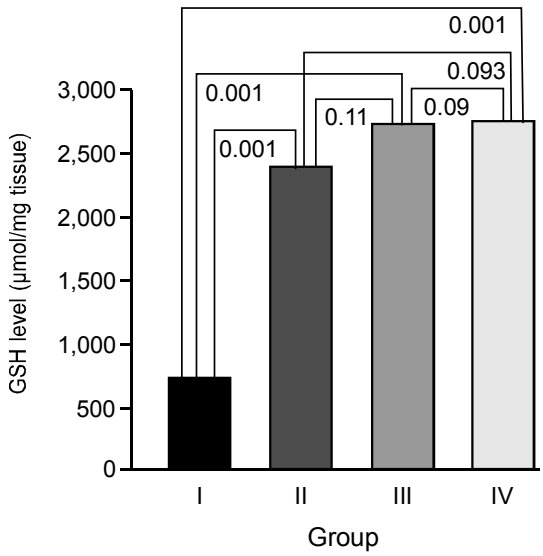

Figure 5. Comparison of glutation (GSH) level among various treatment groups

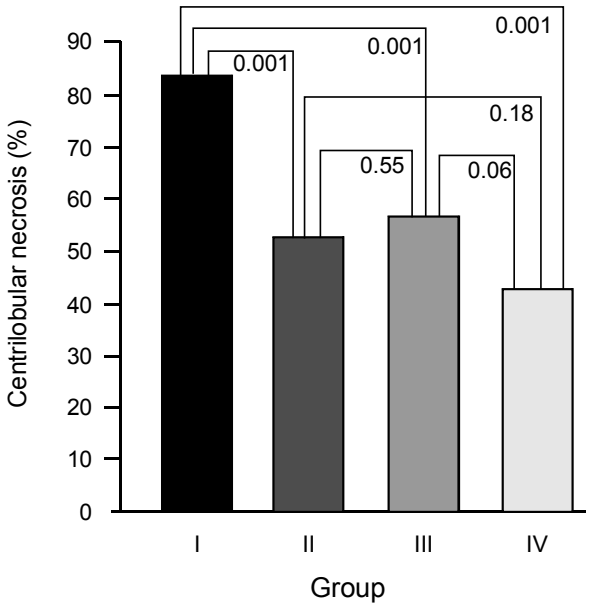

Figure 6. Comparison on percentage of centrilobular necrosis of zone 3 hepatocytes among various treatment groups

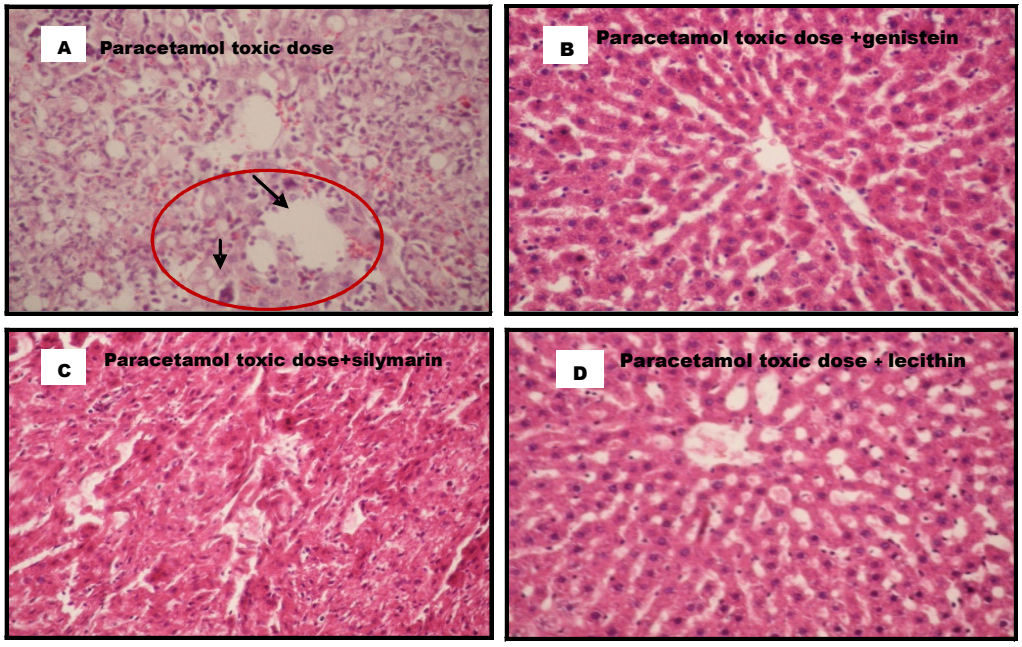

Figure 7. Histopathology of liver tissue demonstrating swollen liver cells (vacuolization), burst, pale staining cytoplasm (A), a relatively fine structure of hepatocytes (B and D) and hepatocyte structures that turn into irregular structure and develop vacuolization (C)

DISCUSSION

Base on the present study, we found that the type of hepatotoxicity effect on liver cells induced by toxic dose of paracetamol is necrotic hepatocellular; which is characterized by centrilobular necrosis, increased AST and ALT level due to inflammatory process and oxidative stress of the hepatocytes. Acetaminophen-induced hepatotoxicity specifically causes zone 3 necrosis with submasive (bridging) or massive (panacinar) necrosis in severe cases.[14,15] Histopathological observation of liver tissues in the present study also showed necrosis of zone 3 along with mitochondria swelling, which appeared as large vacuolas and some were observed as ruptured vacuola (vacuolation).

The preliminary study indicated that liver necrosis has been observed since initial administration of paracetamol and it reached 100% at the following 12th hour. Ruepp et al, detected minor changes under the electron microscoped at 15 minutes after dosing with 500 mg/kg acetaminophen. The changes started in the centrilobular zone and they observed increasing severity and distribution with time.[16]

Sixty minutes after the dosing, mitochondrial dilatation was observed as vacuolation under the light microscope. The mitochondria were swollen and aggregated.[16,17] Similar findings, which were also observed in the centrilobuler zone, were reported by Heinloth et al, who performed 150 mg/kg acetaminophen exposure in rats. No ultrastructural alteration was found both in the control group or in rats treated with 50 mg/kg acetaminophen.[18,19]

Liver necrosis was observed in all groups with the highest percentage found in the control group, which receiving only toxic dose of paracetamol. It indicates that genistein, silymarin and lecithin supplementation does not provide protection against acute drug-induced liver injury. Therefore, the term "hepatoprotectors" that has been widely used for substances to protect the liver should be considered further.

In the group treated with silymarin, there was microscopic extensive necrosis although in smaller number compared to the control group. Such finding may be observed due to poor oral bioavailability of silymarin as it has hydrophilic properties causing difficulties to penetrate intestinal epithelium; unless when given in large dose or by intravenous route. The second possibility is the receptors of silymarin are located extracellular, which cause it unable to prevent intracellular paracetamol-induced oxidative stress. Third possibility includes slow onset of action for silymarin; therefore, the effect of silymarin on improving liver cell may not be observed at 12th hours after treatment.

In genistein and lecithin groups, we observed less extensive necrosis compared to the control group. The lowest percentage of liver cell necrosis was found in genistein group. Genistein is active substances found in lipophilic isoflavone, which is absorbed from the small intestine to circulation and has intracellular action as antioxidant.[12] Its role as antioxidant could be explained *in vitro* through the mechanism of polyphenol activity scavenging free radical, which promotes inhibition of lipid peroxidation and nitric oxide production.[13] The above mentioned basic concept may explain our study results that we found lower AST and ALT level, as well as lower percentage of necrosis and higher GSH level in the group treated with genistein compared to the control.

In the present study, we also found higher bile acid level, especially in the control group receiving toxic dose of paracetamol only. Increased bile acid indicates cholestasis that may occur due to administration of toxic dose paracetamol. However, it is different from the theory proposed by Farrel et al, suggesting that paracetamol-induced hepatotoxicity is dose dependent with acute onset and characteristic signs of significant increased ALT level without or very small amount of increased bile acid or alkaline phosphatase.[20] Further studies is necessary to evaluate the type of paracetamol-induced liver injury, whether it is hepatocellular or mixed. Different bile acid level between the treatment and control group suggests that there is inhibiton for the development of cholestasis due to genistein, silymarin and lecithin supplementation. Consequently, supplementation of those three substances may inhibit the process of cholestatic liver.

Increased tissue MDA level in the group receiving toxic dose of paracetamol indicats that there is oxidative stress process, which appeared as lipid peroxidation, one of mechanisms for paracetamol-induced hepatotoxicity. It provides evidences for theory and concepts on free radical that mediates paracetamol-induced hepatotoxicity and it may become the reason of using antioxidant for patients with acute liver injury.

The group treated with silymarin had lower MDA level, which may be associated with the antioxidant mechanism of silymarin that inhibits lipid peroxidation.[21] It is different from the concept of lipid peroxidation by phosphatidylcholine; therefore, in lecithin group we found high MDA level. There are some possibilities including less adequate dose in the

group treated with lecithin since the lecithin dose was determined through literature study or the lecithin itself may have specific metabolism pathway.

Baghdasaryan et al, performed dietary manipulation containing lecithin and demonstrated that it did not affect phospholipid content and the composition in liver and serum bile acid. Abnormal phospholipid metabolism in hepatocytes is not major or pathological factor for drug-induced hepatotoxicity.[22] The MDA level in genistein group also had no significant difference compared to the control group. The antioxidant mechanism of genistein may not through inhibition of lipid peroxidation, but through other pathway that should be studied further.

Paracetamol-induced hepatotoxicity reduced GSH level in the control group. When the GSH level is low, unconjugated NAPQI can bind covalently to protein, causing altered intracellular homeostasis that may lead to cell necrosis. It is assumed that NAPQI may disturb calcium influx into mitochondria that causes apoptosis and necrosis due to the formation of free radicals, hydroxyl, nitrite and nitrate radicals.[23]

Lecithin supplementation increases higher GSH level compared to other substances. It is assumed that its mechanism of action includes inhibition of lipid peroxidation, which restrain oxidative stress and minimize centrilobular hepatic necrosis.[9,24] Consistent with this study results, silymarin as antioxidant has been reported to be effective for maintaining GSH level in the liver cells and it also may increase cellular GSH.[25,26] An experimental study conducted by Muriel on paracetamol-induced acute intoxication in rats and simultaneously treated with silymarin, suggested protective effect of silymarin through reduced lipid peroxidation and increased GSH level.[7] Genistein supplementation that has a role as antioxidant can suppress oxidative stress due to high level of NAPQI and induces the synthesis of intracellular GSH.

Aneja et al, performed an animal experimental study to evaluate the role of genistein in acute hepatotoxicity induced by carbon tetrachloride.[27] The study demonstrated that genistein supplementation prior to the treatment may increase intracellular GSH level and being brought to normal level after treatment. The mechanism may include increased GSH synthesis enzymes such as *c-glutamylcysteine* synthetase and GSH synthetase, which are key enzymes in GSH biosynthesis.[27,28]

Kuzu et al, also conducted similar study with acute liver injury model induced by carbon tetrachloride, but with repeated genistein supplementation. Their results suggested that repeated dose of genistein may reduce inflammation and necrosis of the liver. It is assumed to be associated with inhibition of lipid peroxidation and inhibiton on nitric oxide (NO) production by macrophage stimulated by lipopolysaccharides (LPS) or interferon (IFN).[13]

CONCLUSION

Genistein, silymarin and lecithin supplementation may improve liver necrosis caused by toxic dose of paracetamol. Genistein is better than silymarin and lecithin in improving necrosis of the liver cells. It is expected that our study may become the basic reason for utilizing drugs that may improve liver function in patients with hepatotoxicity and may offer assistance to determine government policies on its supplies at health care facilities.

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