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Biochemical and Histopathology Analysis of Liver Damage in Hypercholesterolemic Rats Induced by Tomato Extract

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Abstract. Hypercholesterolemia is a condition caused by high cholesterol consumption. Tomatoes is well known has cholesterol-lowering effects. However, high consumption of tomato shall be concerned especially prooxidant potential that may damage the organ, especially in liver. This study aims to understand the effect of hypercholesterolemic and tomato administration to the rat's liver, which is monitored using aspartate transaminase (AST), alanine transaminase (ALT) and histological condition. A total 24 of 12-weeks-old male-rats divided into 4 groups, equally. The K1 as normal group consist of placebo-treatment rats; K2 group as hypercholesterolemia group induced with high-cholesterol diet and 2 ml of cholesterol; K3 group was hypercholesterolemia rats treated with 20 mg of atorvastatin; and the last is K4 group was cholesterol rats supplemented with 16 mg/ day of tomato extract. All samples were treated for 60 days. The highest levels of AST and ALT level was 76.39 U/L and 45.40 U/L, respectively, was found in K2. Then, K4 is not significantly different from K3 and K1 and significantly different from K2. The scoring results showed that all groups experienced damage in the form of parenchymal degeneration, fat degeneration and necrosis. From this study it can be concluded that tomato extract gives a protection to the liver from cholesterol-oxidation damage effect. The updating information about how tomato inhibite liver fattening. The liver condition probably can be considered as biomarker-related hypercholesterolemia and developed a diagnostic marker to prevent increases metabolic disorder in community.

Key words: hypercholesterolemia; liver; tomatoes

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INTRODUCTION

Cholesterol is needed to manage cell membranes structure, plasma lipoproteins, and produce sex-hormone. However, high lipid consumption contributes in increases blood-cholesterol level, which is then known as hypercholesterolemic condition (Ramírez et al., 2013). Then cholesterol is transported and accumulated in tissues lead cholesterol oxidation that triggers oxidative stress (Ooi et al., 2017). The hypercholesterolemia medication using statin type drug, including atorvastatin, reducing blood cholesterol levels by blocking the active site of hormone 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase during cholesterol synthesis (Antal et al., 2017; Bruder-Nascimento et al., 2019). The atorvastatin gives an improvement in lipid metabolism and protects kidney from damage in acute renal injuries (Ghelani et al., 2019). Although long-term medication of atorvastatin is related to some health cases, including human hepatotoxic (Clarke et al., 2016) by increasing oxidative-inflammation pathway (Zeng & Liu, 2019). Furthermore, atorvastatin and other statin group drugs may also contribute in increasing myopathy cases (Dixit & Icahn, 2018; Ramakumari et al., 2018) and muscle cells destruction (Camerino et al., 2016), therefore developing new and safer medication is needed to lowering cholesterol levels.

Tomato is a vegetable-fruit which is popular for culinary. It is also massively researched and proved reducing cholesterol and triglycerides levels (Cheng et al., 2017), and increase cholesterol-HDL levels (Thies et al., 2017). Tomato riches of antioxidant, including lycopene, vitamins C and E, vitamins A and β-carotene (Iswari & Susanti, 2016). However, consumption of a lot of antioxidants, shall be concerned because of it may transform into pro-antioxidants (Eghbaliferiz & Iranshahi, 2016; Ribeiro et al., 2018). Prooxidant is an oxidized-antioxidant caused by receiving exceed electron from free radical. It makes reverse reaction and increase the oxidation of phospholipids in cell membranes (Ribeiro et al., 2018). But, at the same time, tomato consumption may also contribute in lowering tissue and organ damage that caused by the hypercholesterolemia condition, that should be more analyzed as a part of prevention efforts.

Under hypercholesterolemia condition, the liver is the organ which responsible for managing lipid and oxidative stress condition, likely affected directly. The excess long-term supplementation of antioxidants in the body or high cholesterol level may cause oxidative stress, triggering histological changes in the

liver tissue. Therefore, this study aims to understand the liver histological view under hypercholesterolemia condition and the effect of tomato extract supplementation toward biochemical and histological biomarker analysis.

METHODS

This study was used a post-test only randomized control group design model. As many as 24 healthy male-white-rats weighing 160-170 grams homogeneous and 12-weeks-old. The rats were acclimatized for a week in cages sized 50×40 cm, humidity 68%, with temperature 28 °C and light exposure for 12 hours. During acclimatization, rats were given standard feed and drink ad libitum. The rats were then divided into 4 groups randomly and equally. The first group was a normal control group (K1) or healthy mice given placebo. In the K2 group or negative control, rats were fed high cholesterol feed (Matos et al., 2005) and induced with 2 ml of cholesterol until hypercholesterolemia and placebo. The K3 group or positive control group, was the hypercholesterolemic rats given 20 mg/ KgBW of atorvastatin, while the K4 group was the hypercholesterolemic rats given with 16 mg/ KgBW of tomato extract. The treatments were carried out every day for 60 days.

Table 1. Composition of a high cholesterol diet (g / 1000g)

Components	Weight (g)
Casein	240
Corn Stalk	299.6
Soya Oil	250
Cholesterol	10
Colin	0.4
Mineral	10
Vitamin	20
Cellulose	80

Tomato extract was conducted using 30 kg of fresh tomatoes which steamed for 15 minutes at 120 °C, then crushed using a blender and macerated using petroleum ether. After being macerated, the tomatoes were put in the oven at 40-50 °C to dry. After dried, the tomatoes were blended become coarse powder, then sieved using a sieve No.100, the powder obtained from the sieve was weighed 500 mg and suspended with water.

The rat's blood was taken in the 61st day using a microhematocrit through the retro-orbitalis plexus as much as 3 ml and collected in a tube. The collected blood was then allowed to stand in the room for half an hour and then centrifuged at 8000 rpm for 5 minutes to obtain the serum. The serum was put in a vial tube as much as 1.8 ml, and used to measure aspartate transaminase (AST) and alanine transaminase (ALT) concentration using the Diagnosis System (DiaSys) GmbH (Holzheim, Germany), with spectrophotometry techniques. Each test using 10-100 µL and following manufacturer's protocol.

After rats' blood collection, they were sacrificed by dislocating the neck, and the liver was aseptically collected and removed to the 4% formalin solution. The liver was then used for histological-slide analysis then observed under a microscope at five different views, 20 cells were randomly counted and each cell's score was assessed by the Manja Roenigk histopathological scoring model (Ramachandran & Kakar, 2009). Types of liver damage that were observed included necrosis, parenchymal degeneration, and hydrophic degeneration.

The biochemical in histological data was analysis using Saphiro-Wilk's test for normality followed by analysis using One-way ANOVA with a confidence level of 95%, then continue with analysis between treatment groups with the least significant difference (LSD), with a confidence level of 95%. The histological data obtained in the form of hepatic cell scores were then statistically analyzed using the Kruskal Wallis's test and further tested to determine the differences between groups using the Mann Whitney statistical test.

Table 2. Criteria for liver histopathological assessment (Ramachandran & Kakar, 2009)

Cell destruction type	Criteria	Score
Normal (N)	Normally hepatocyte	1
Parenchymatous degeneration (PD)	The steatosis condition which makes hepatocyte looks cloudy swelling, with yellow-looks cholesterol within cells	2
Hydropic degeneration (HD)	the hepatocyte cells absorb too much water. The cells look bigger than other hepatocyte but the color is same	3
Necrotic (Nc)	Dead cells, no nucleus inside or the nucleus appears but the membrane cell is not clear	4

Note : scoring was conducted by multiplying the number of cells with the damage category. Based on these criteria, the possible minimum score is 100, if in normal conditions and possible maximum score is 400 for cells necrotic condition.

RESULTS AND DISCUSSION

In this research, the tomato effect on lowering AST and ALT as an indicator of liver damage under hypercholesterolemia condition was first recorded. Increasing cholesterol consumption affects internal metabolism to use excesses exogenous cholesterol as the main source of cholesterol synthesis. It is needed for metabolism processes, including hormones production and cell membrane synthesis. The implications of this condition are, 1) endogenous cholesterol will be distributed and deposited in the body tissues, including blood vascular that triggers atherosclerosis; 2) accumulation of acetyl-CoA (as a main compound in cholesterol synthesis) converted into triglyceride that accumulated as lipid droplets in hepatocyte (Mato et al., 2019). The massive lipid droplets potentially build up oxidation on unsaturated fatty acid that damages hepatocyte (Choi et al., 2017) and triggers liver cell necrosis. This damage can be observed from the high levels of AST and ALT in the blood serum and the presence of fatty symptoms in the liver cells. The average score of AST and ALT levels in all rat groups were normally and homogeny distributed, also the mean score was also significantly different (Table 3).

Table 3. Average blood AST and ALT levels in rats.

Group	AST (U/L)	P value	ALT (U/L)	P value
K1	40.456 ± 2.776^{a}	0.000	29.536 ± 1.125^{a}	0.000
K2	76.385 ± 2.246^{b}		45.395 ± 1.177^{b}	
K3	47.335 ± 1.911^{c}		33.096 ± 1.722^{c}	
K4	48.226 ± 1.048^{c}		32.526 ± 1.149^{c}	

Note: the letters (a-c) indicate significant difference of one-way ANOVA test, at the significance level (α) = 0.05 or confident level = 95%.

The lowest AST and ALT level were recorded in K1 and significantly different with other groups. Then the AST and ALT level both in K3 and K4, is higher than normal rats but significantly different bellow K2. The high concentration of AST and ALT in blood probably indicates liver damage. Because both biomarkers originally found in hepatocyte, and will be released into blood circulation when necrosis happen in the hepatocyte. Furthermore, the application of atorvastatin and tomato extract able to reduce AST and ALT secretions which mean there were able to reduce liver cell damages.

High cholesterol intake generates fatty acid metabolism and deposition, it bonds with radical oxygen species (ROS) and forms lipid peroxidation (Agmon & Stockwell, 2017). The free radical and oxidized lipid destroy the cell's membrane structure by wide spreading the oxidation of poly-unsaturated fatty acid in phospholipid bilayer. Destructed cell's membrane makes several conditions, including necrosis and permeability lost. The cell's membrane is important as entrance regulator for water, organic materials and minerals to enter the cell (Agmon & Stockwell, 2017; Otunola et al., 2010). The condition losses the cell permeability increase cell's turgidity and liquid flow in and out, which is damaging the cell (Kloska et al., 2020). In the liver, high cholesterol diet contributes may contribute in hepatocyte destruction and increase the AST and ALT serum levels (Otunola et al., 2010). Based on the observation, increased concentration of AST and ALT relates to the histological conditions in all groups (Figure 1).

The histological picture in the K1 group shows the structure of normal liver cells, in the form of polygonal cells, well-defined cell membranes and homogeneous red cytoplasm. In contrast to the K2 group, which showed that many rat liver structures were damaged in the form of hydropic degeneration, parenchymal degeneration, and necrosis. Groups K3 and K4 also experienced liver cell damage. The damage was in the form of hydropic degeneration, parenchymal degeneration, and necrosis, but the numbers were lower than the K2 group. The amount of damage in each group was then averaged. The results of the average damage are presented in Table 4.

In other hand, the occurred damage also caused by lipid peroxidation of unsaturated fatty acids mostly found in the sinusoids and hepatocyte of K2 group. The damaged sinusoid in K2 group was shown by widening the gap between sinusoids part caused by fatty acid degeneration formation of fatty vacuoles which will create empty spaces in the sinusoids caused by an increase in fatty acids in intracytoplasmic accumulation (Ramachandran & Kakar, 2009).

The results of normal cell scoring and liver damage showed histological changes in high cholesterol diet group and the treatment group. In this research, all of the hepatocyte shown there were damage, but most of the normal cells was observed in K1, and severe condition was found in K2 group. The atorvastatin or tomato extract supplementation are able to

reduce the destruction effect of hypercholesterolemia, which observed in group K3 and K4, respectively (Table 4).

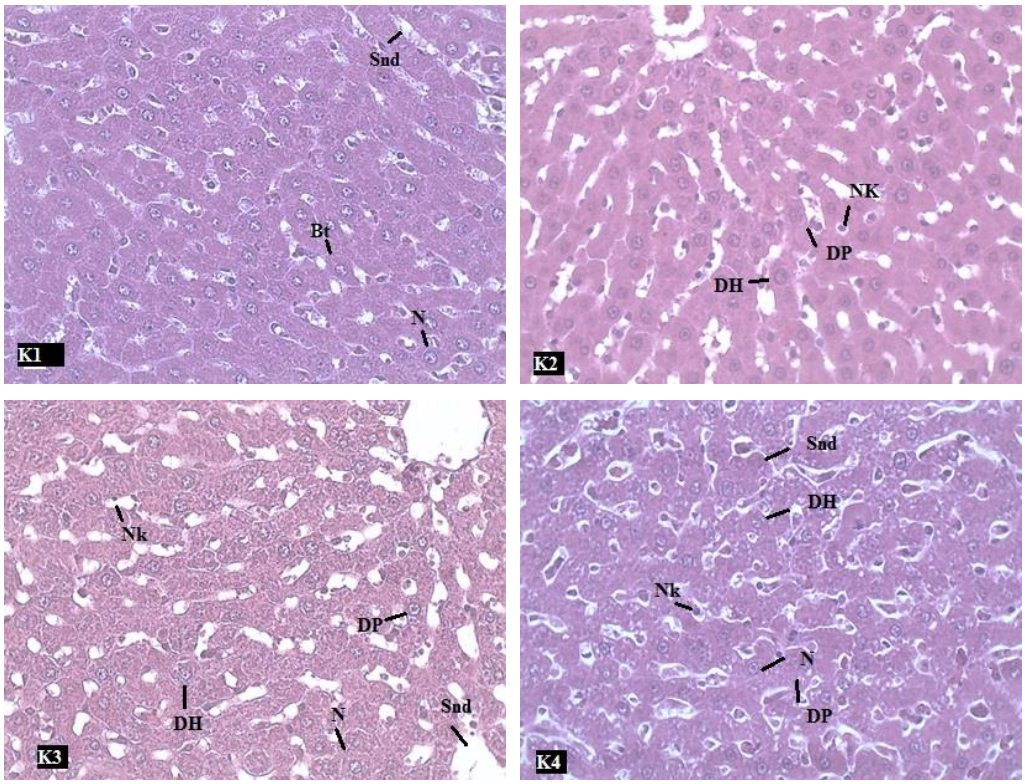

Figure 1. Post-treatment liver histology. Normal (N) hepatocyte cells are polygonal and have a clear nucleus and clear margins. The damage that occurs in hepatocytes is parenchyma degeneration (DP), hydropic degeneration (DH) and necrosis (Nk). Snd: Sinusoid, Bt: Margin. HE dye, Microscope Observation 400X Magnification, 1 Bar: 50µm.

Table 4. The percentage of scoring of rats' histological liver damage level.

Groups	Hepatocyte damage (%)				Average total score
	N	PD	HD	Nc	
K1	69.3	18.33	2.67	9.67	152.7[a]
K2	13.00	21.33	27.33	38.33	291.0[b]
K3	67.66	18.66	4.33	9.33	155.3[a]
K4	67.66	18.66	4.00	9.33	154.3[a]

Note: the letters (a-c) indicate significant difference of one-way ANOVA test, at the significance level (α) = 0.05 or confident level = 95%.

The consumption of atorvastatin shown has no effect in increasing AST and ALT levels, significantly (Table 3). The atorvastatin works as a competitive inhibitor to HMG-CoA reductase which inhibits the transformation of HMG-CoA into mevalonate (Fried, 2008), in cholesterol synthesis mechanism. Mevalonate reduction decreases cholesterol production and diminish steatosis risk in hepatocyte. Furthermore, same effect was found in K4 group, the tomato extract supplementation was able to inhibit hepatocyte destruction from free radical damage impact. The mechanism may involve the regulation of gene expression and free radical-scavenging action.

The tomato extract supplementation in K4 (Table 3), was inhibited the excesses production of the liver-damage biomarkers. The liver cell damage was observed in mild condition, shown by the low destructed cells compared to the K2 (Figure 1 & Table 4). The tomato's bioactive contents, including carotenoid, especially lycopene, are activators for hepatocyte's nuclear receptors, including liver X receptor (LXR), heterodimer retinoid acid receptor (RAR)-retinoid X receptor (RXR) and peroxisome proliferator-activated receptor (PPAR) (Elias et al., 2019). All of the receptor mainly acts in cell proliferation, cholesterol sensor, participates in lipid regulation, and cholesterol metabolism (Ratneswaran et al., 2017),

then both RAR and RXR are a pair receptor which work together in many cell regulation pathways (Lobo et al., 2010). The LXR-RXR heterodimer binding regulates several genes involved in the synthesis, absorption, excretion for cholesterol homeostasis, and lipoprotein metabolism (Xu et al., 2018). PPAR belongs to the nuclear receptor (NR) type II family, a group that includes the non-steroidal NR that forms obligate heterodimers with RXR. These heterodimeric receptors have functional domains for binding to DNA and ligands. PPARs bind to recognition sequences in the promoter region of their target genes and act directly to regulate gene transcription (Hiebl et al., 2018)

Tomato's lycopene also regulates PPARα which plays a role in the induction of genes related to fatty acid re-uptake, lipoprotein anabolism, and fatty acid degradation through β-oxidation in hepatocytes (Desmarchelier & Borel, 2017). Consumption of a high-fat diet actually has an impact on decreasing PPARα activity which has an impact on fat accumulation in the liver (Elias et al., 2019). Meanwhile, the PPARγ expression increased in response to excess lipid input. This may be related to the role of PPARγ in droplet formation for lipid storage in hepatocytes. The accumulation of lipids is dominated by triacylglycerols and fatty acids, in sufficient quantities to significantly increase the lipid content of cell membranes (Agmon & Stockwell, 2017). An increase in phospholipids and a decrease in membrane cholesterol can turn the membrane more fluid, ensuring mobility of flip-flop rotation between phospholipid molecules, but decreasing protection against oxidation. Chronic increase in lipid accumulation, especially cholesterol, has an impact on increasing ROS which causes autophagy (Ghosh et al., 2018). Prooxidants in the form of Car-OO$^{\bullet}$ expand the occurrence of lipid peroxidation which destroys the unsaturated fatty acid chains forming the phospholipid membrane and increases the fluidity of the phospholipid bilayer (Zheng-Hong, 2019). A membrane that is too liquid is more susceptible to exposure to free radicals causing higher oxidation.

Lycopene modulation also affects the activation of the PPAR signaling pathway which then increases the formation of PPARγ, RXR-α, and RXR-β (Lowe et al., 2018). In addition, tomato extract increases lipid metabolism by increasing ApoA4 and inhibiting fatty acid synthase (FASN) gene expression in the liver (Zhao et al., 2020). However, the antioxidant properties of Carotenoids can turn into prooxidants when the amount exceeds the body's requirements. As a free-radical scavenger, carotenoids react with radical oxygen species (ROS) or reactive nitrogen species (RNS) in three different mechanisms: 1) adduct formation, 2) electron transfer, and 3) allylic hydrogen abstraction (Barros et al., 2018). The interaction with free radicals is reversible in the presence of Vitamin C. During prooxidant conditions, carotenoid compounds are antagonistic to the phospholipid bilayer, and changes in chemical properties to certain ROS groups and the effect of pH. Changes in chemical properties occur due to oxidative stress as a result of lipid peroxidation in a high $^1O_2$ environment (Kawata et al., 2018). Based on its chemical structure, the formation of carotene-peroxyl (Car-OO) radicals will more easily occur in carotenoids with cyclic ends such as β-carotene because the number of double bonds is lower than lycopene (Desmarchelier & Borel, 2017; Ribeiro et al., 2018). Although it is likely that prooxidants will occur when ROS / RNS has increased massively, the contribution of tomato carotenoids, especially lycopene is the main protection that promotes endogenous antioxidant defenses through Nrf2-KEAP1 pathway and $^1O_2$ quenching (Elias et al., 2019).

Apart from carotenoids, tomatoes contain various other important bioactive compounds such as vitamin C, vitamin E and flavonoids (Iswari & Susanti, 2016). The vitamin C as an antioxidant functions to bind singlet oxygen preventing oxidation reactions and reverse the condition of prooxidants back into antioxidants (Pacier et al., 2015). The content of the vitamin C in tomatoes prevents chain reactions that occurs after the vitamin E capturing peroxyl radicals and producing tocopherol radicals (Grosso et al., 2013). The flavonoids in tomatoes also play important role in counteracting free radicals by donating hydrogen atoms to free radicals. Atomic donors serve to slow down the rate of autoxidation by converting lipid radicals into stable forms (Treml & Šmejkal, 2016).

The understanding of hepatocyte and liver tissue conditions during hypercholesterolemia and tomato extract treatment depicts a new preventive and curative approach in liver fattening. The implication of this research, it is more understandable that the liver fattening and destruction caused by ROS in hypercholesterolemia condition can be prevent by the tomato consumption. Then, the updating information about how tomato inhibited liver fattening and liver condition probably can be considered as biomarker-related hypercholesterolemia and developed a diagnostic marker to prevent increases metabolic disorder in community. In the next improvement it is still needed advance research in mapping cells, tissues and organs destruction in hypercholesterolemia condition as a holistic prevention.

CONCLUSION

High cholesterol intake for long period induction has triggered hypercholesterolemia condition and

increased the AST and ALT level higher than normal or supplemented rats (K3 and K4). Besides that, both supplementation of 20 mg/ KgBW/ rats of atorvastatin or 16 mg/ KgBW/ day of tomato extract had lower AST and ALT concentration compared to the hypercholesterolemia rats. It can be concluded that tomato extract supplementation may affect especially in reducing liver fattening and inhibiting hepatocyte destruction. The induction of 16 mg/ KgBW/ day of tomato extract has significantly prohibited AST and ALT from increase upper of the its concentration in K2 or hypercholesterolemia condition.

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