

**Effects of Moringa Leaf Extract and Moderate Intensity Exercise on Histopathological Appearance and Autophagy Gene Expression**

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Abstract

The prevention of liver disease could be conducted through preventive strategies, such as antioxidants and exercise. Moringa leaf extract has substances that could act as antioxidant, while exercise is also known protecting the liver from disease by changing hepatic metabolism and autophagy. This study was aimed at investigating the effect of moringa leaf extract and moderate intensity exercise on histopathological appearance and autophagy gene expression of wistar rat liver. The method used in this study was animal experiment using 24 male wistar rats divided into 4 groups, including control group, moringa group, exercise group, and moringa + exercise group. Moringa leaf extract was given in a low dose (5.7 mg/kgW) per oral, 5 times a week, for 4 weeks. Meanwhile, the 20 m/minute treadmill exercise was given for 30 minutes per day, 5 times a week, for 4 weeks. Results showed a significant change on histopathological scoring in exercise group ($p=0.011$) compared to control group. Increased autophagy gene expression was found in moringa + exercise group compared to control group (LC3 0.90 fold; p62 0.87 fold). In summary, this study presented that moderate intensity exercise induced changes on histopathological appearance of wistar rat liver that might be associated with physiological inflammation. Moringa, with its antioxidant properties, combined with increased autophagy might improve histopathological changes in moringa + exercise group.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), a disease characterized by microvesicular steatosis in histopathological findings, is highly related with obesity, type 2 diabetes mellitus, and dislipidemia, or better known as metabolic syndrome (Petaja and Yki-Jarvinen, 2016). NAFLD could lead to liver fibrosis and hepatocellular carcinoma (Dhamija et al., 2019). Currently, NAFLD prevalence in Asia has the same percentage as in Western countries, which is around 25 %, along with increasing numbers of hepatocellular carcinoma and end-stage liver disease secondary to NAFLD (Fan et al., 2017). Patients with NAFLD also experience reduction of quality of life compared to healthy people (Golabi et al., 2016; Samala et al., 2020). Research showed that patients with NAFLD reported 18-20% more days when they could not perform daily activities (Golabi et al., 2016). Even without liver fibrosis, NAFLD patients have the worst quality of life compared to other patients with chronic medical illnesses, while body composition, as a modifiable risk factor, is significantly related to quality of life in NAFLD patients (Samala et al., 2020).

Studies have demonstrated some non-modifiable and modifiable risk factors for NAFLD occurrence. Familial predisposition, ethnicity, gender, and age are non-modifiable risk factors, while obesity, insulin resistance, hyperlipidemia, intestinal microbiota and oxidative stress, and metabolic syndrome are modifiable risk factors for NAFLD (Iqbal et al., 2019; Benedict and Zhang, 2017). The most prominent risk factors for NAFLD are obesity, metabolic syndrome, and insulin resistance, while advanced age (>50 years), men gender, Hispanic and Caucasian ethnics are also important risk factors that must be taken into account (Iqbal et al., 2019).

Physical exercise has been known as a way to prevent NAFLD by increasing numerous expression of metabolism related genes and decreasing liver fat level (Townsend et al., 2019; Medrano et al., 2020). Exercise causes muscle damage, which induces phagocyte infiltration causing biomolecules damage as a final process of oxidative stress (Kawamura and Muraoka, 2018). Fortunately, our bodies also have mechanisms to respond to such conditions, one of which is called autophagy process.

Autophagy is a body mechanism for delivering

cytoplasmic materials from both inside or outside the cell to lysosome for degradation. There are three types of autophagy, including macroautophagy, microautophagy, and chaperone-mediated autophagy; macroautophagy is then referred as autophagy (Galluzzi et al., 2017; Mizushima et al., 2010). Several studies showed that the increment of autophagy gene expression aligns with regular physical excersises (Tarawan et al., 2019; He et al., 2012). Autophagy also has roles in metabolism of cholesterol and triglycerides (Zhang et al., 2018).

Previous study also showed an increased autophagy gene expression and protein levels that might be associated with histopathological changes and lipid metabolism in Wistar rat liver after 8 weeks of moderate and high intensity exercises (Gunadi et al., 2020). Some studies, investigating 30-60 minutes moderate aerobic exercise for 3-5 days per week, have represented a relative reduction of intrahepatic lipid (Keating et al., 2012). Recent studies conducted moderate intensity exercise also stated similar result (Abdelbasset et al., 2020). Other studies stated the superiority of moderate intensity exercise compared to low intensity exercise (Nath et al., 2020), while a study stated an equal effect of moderate intensity exercise compared to low and high intensity exercises (Keating et al., 2015). For those reasons, authors decided to choose moderate intensity exercise for this study.

Other known method to prevent NAFLD is increasing the number of antioxidants, such as flavonoid and antosianidin (Ferramosca et al., 2017). Some herbs are known to contain such substances, one of which is Moringa leaf (Vergara-Jimenez et al., 2017; Kou et al., 2018). Moringa is widely known in Indonesia as kelor, which is also called as a magical plant because of its various benefits, including as medicinal plants, cosmetics, and food ingredients. From a survey, the majority of respondents claim the superiority of moringa as herbal medicine that are safe to consume, has no side effects, and have a similar efficacy to non-herbal medicines (Irawan and Patricio, 2017). Moringa is also known to have a hepatoprotective effect againts carbon tetrachloride and acetaminophene induced liver toxicity and an effect in decreasing total cholesterol and triglycerides levels (Nanjappaiah and Hugar, 2012; Bais et al., 2014; Atta et al., 2018). Recent studies had not determined the exact roles of autophagy and moringa leaf extract consumption on liver lipid metabolism. Thus,

this study aimed to find out the effect of Moringa leaf extract consumption and moderate intensity exercise on Wistar Rat liver histopathological changes and autophagy gene expressions.

METHODS

Animals

Male Wistar rats (8 week old; body weight 210 ± 10 g; $n = 24$) were divided into 4 groups, including control group, intervention group 1 (taking moringa extract orally, once per day for 4 weeks), intervention group 2 (performing 30 minutes treadmill running, 5 times a week, for 4 weeks), and intervention group 3 (taking moringa extract orally and performing 30 minutes treadmill running, 5 times a week, for 4 weeks).

Male wistar rats were chosen to reduce confounding factors, for example hormones interplay. The rats were given standard chow diet and housed at a room temperature with 12 hours of light and dark cycles every day. We conducted all procedures based on the use and care of laboratory guidelines (Council, 2011). The protocol of the experiment was approved by the Research Ethics Committee of the Faculty of Medicine, Universitas Kristen Maranatha-Rumah Sakit Immanuel Bandung No 098/KEP/VII/2020.

Treadmill Training Protocol

Before the intervention, for 3 weeks, the subjects were given time to adapt to the environments (2 weeks) and to the exercise procedures (1 week). A 20 m/min treadmill speed for 30 minutes, 5 times a week, for 4 weeks, was used to conduct moderate intensity exercise based on lactate threshold (Gunadi et al., 2020; Lesmana et al., 2016). Non-training rats served as the sedentary control ($n=6$), other groups were exercise group ($n=6$) and exercise + moringa leaves extract group ($n=6$). At the last day, several minutes after the last intervention, subjects were sacrificed. The livers were obtained and weighed. A portion (1 g) was taken for autophagy gene expression, while other portions were prepared for making histopathological slides.

Plant Extract and Dose Selection

Moringa leaf extract powder was obtained from Central Java. We used a low dose of moringa leaf ex-

tract powder, 5.7 mg/kg for each rat, per oral, 5 times a week, for 4 weeks. At the end of this study, the rats were terminated. The liver was extracted for histopathology examination and semiquantitative PCR.

Hematoxylin and Eosin (H&E Staining)

We made 2- μ m thick sections stained with hematoxylin and eosin for histopathology examination using microscope (LEICA ICC50) with 100x and 400x magnification. Blind analysis was completed by a pathologist expert. The evaluation of the slides was administered using scoring system that had been used in our previous study. Level of congestion/sinusoidal dilatation, cloudy swelling/injury, and inflammation were evaluated. For the level of congestion or sinusoidal dilatation, we used this scoring: 0 = No congestion/sinusoidal dilatation, 1 = Mild congestion or centrilobular (zone III) sinusoidal dilatation, 2 = Moderate congestion or centrilobular (zone II) sinusoidal dilatation, 3 = Severe congestion or centrilobular (zone I) sinusoidal dilatation. For the level of cloudy swelling/hepatic injury, we used this scoring: 0 = No cloudy swelling/hepatic injury, 1 = Mild cloudy swelling/hepatic injury (zone III), 2 = Moderate cloudy swelling/hepatic injury (zone II), 3 = Severe cloudy swelling/hepatic injury (zone I). For the severity of the inflammation, we used this scoring: 0 = No hepatic inflammation, 1 = Mild hepatic inflammation or periportal inflammation, 2 = Moderate hepatic inflammation or periportal and intraparenchymal inflammation, 3 = Severe hepatic inflammation or periportal and intraparenchymal inflammation with bridging necrosis (Prapatsorn et al., 2010; Gunadi et al., 2020). Geometric representations of a hepatic lobule (Zone I, II, III) and a schematic representation of a sinusoid, as well as the corresponding zonation associated with lipid metabolism in the liver, are shown in Figure 1 (Gunadi et al., 2020; Trefts et al., 2017).

RNA Extractions and Semi-quantitative PCR

According to manufacturer instructions, we performed RNA extraction from frozen liver tissues using TRIsure reagent (Bioline, United Kingdom). Then, we measured the concentration and purity of the RNA using spectrophotometry at 268/280 nm absorbance (M200 Pro, Tecan, Morrisville, NC). To conduct semi-quantitative PCR, we used The One Step RT PCR Kit (Bioline, United Kingdom). Housekeeping gene GAPDH was used in this study. BluePad Detection sys-

tem was used to visualize the gels, then the PCR bands were quantified using Image J. The list of primer sequences is provided in Table 1.

mogeneity tests on the data. We found a normal distribution and homogenous. In the differences of liver histopathology scoring, we used One Way Anova Test and

Table 1. Primers Used for Semi Quantitative-PCR Analysis

Gene Symbol	Primer Sequence (5' to 3')		Product Size (bp)	References
	Upper Strand: sense	Lower Strand: antisense		
LC3	GGTCCAGTTGTGCCTTTATTGA	GTGTGTGGGTTGTGTACGTCG	153	(Yin et al., 2013)
p62	CTAGGCATCGAGGTTGACATT	CTTGGCTGAGTACCACTCTTATC	116	(Kowalik et al., 2016)
GAPDH	GTTACCAGGGCTGCCTTCTC	GATGGTGATGGGTTTCCCGT	177	(Wang et al., 2017)

Design

In this study, the authors chose animal experimental study with completely randomized design for grouping the animals. The assessments were conducted one time only, at the end of the experiments (posttest design).

Data Analysis

We analyzed the data using SPSS 20.0 by conducting normality and homogeneity tests before comparing the differences among the groups using One Way ANOVA, followed by post hoc Bonferroni if the data were homogenous or Games-Howell test if the data were not homogenous. Histopathological slides were determined using Hepatic Lobules Zonation Scoring System to determine the vasodilatation of liver sinusoids, cloudy swelling, and inflammation process. The non-parametrical results were analyzed by Kruskal Wallis, with Mann Whitney post hoc test.

RESULT

Effects of Moringa and Treadmill Exercise on Body and Liver Weight

In this study, we found no differences on body weight before (284.29 ± 2.57) and after the treatment (289.38 ± 2.50) and liver weight after treatment (10.15 ± 0.29) in all groups.]

Effects of Moringa and Treadmill Exercise on Liver Histopathology

To examine the effects of moringa and training on liver histopathology, we performed normality dan ho-

Table 2. Effects on Liver Histopathology Scoring

Groups	Liver Scoring \pm SEM	N	F	p
Control	1.17 ± 0.48	6		
Moringa	2 ± 0.26	6	4.397	0,016*
Exercise	3 ± 0.37	6		
Moringa + Ex	1.83 ± 0.31	6		

* = significant ($p < 0,05$)

found significant differences as shown in Table 2. We performed post hoc Bonferroni test to know the differences among the groups and found a significant difference between exercise group and control group ($p=0.011$). Meanwhile, in other groups, we found no significant differences (Table 2).

For the details of liver scoring, we found differences presented in these tables and figures.

Table 3. Effects on Liver Histopathology Scoring

Groups	Control	Moringa	Exercise	Moringa + Ex
Control		NS	*	NS
Moringa			NS	NS
Exercise				NS
Moringa+Ex				

Table 4. Primers Used for Semi Quantitative-PCR Analysis

Scoring	Control			
	0	1	2	3
Congestion/ Sinusoidal Dilatation	3 (50%)	3 (50%)	0	0
Cloudy Swelling/ Injury	6 (100%)	0	0	0
Inflammation	4 (66.7%)	0	2 (33.3%)	0

In control group, we found 50% level 0 and 50 % level 1 of congestion/sinusoidal dilatation, 100% level 0 of cloudy swelling/injury, and 66.7% level 0 and 33.3% level 2 of inflammation.

Table 5. Liver Scoring in Moringa Group

Scoring	Control			
	0	1	2	3
Congestion/ Sinusoidal Dilatation	1 (16.7%)	5 (83.3%)	0	0
Cloudy Swelling/ Injury	6 (100%)	0	0	0
Inflammation	0	5 (83.3%)	1 (16.7%)	0

In moringa group, we found 16.7% level 0 and 83.3% level 1 of congestion/cloudy swelling, 100% level 0 of cloudy swelling/injury, and 83.3% level 1 and 16.7% level 2 of inflammation.

Table 6. Liver Scoring in Exercise Group

Scoring	Control			
	0	1	2	3
Congestion/ Sinusoidal Dilatation	0	6 (100%)	0	0
Cloudy Swelling/ Injury	3 (50%)	3 (50%)	0	0
Inflammation	0	3 (50%)	3 (50%)	0

In exercise group, we found 100% level 1 of congestion/cloudy swelling, 50% level 0 and 50% level 1

of cloudy swelling/injury, and 50% level 1 and 50% level 2 of inflammation.

Table 7. Liver Scoring in Moringa + Exercise Group

Scoring	Control			
	0	1	2	3
Congestion/ Sinusoidal Dilatation	3 (50%)	3 (50%)	0	0
Cloudy Swelling/ Injury	6 (100%)	0	0	0
Inflammation	0	4 (67.7%)	2 (33.3%)	0

In moringa + exercise group, we found 50% level 0 and 50 % level 1 of congestion/sinusoidal dilatation, 100% level 0 of cloudy swelling/injury, and 67.7% level 1 and 33.3% level 2 of inflammation.

Effects of Moringa and Treadmill Exercise on Autophagy Gene Expression

After four weeks of treatments, we found a significant decrease of LC3 in moringa + exercise group (0.90 fold) compared to control group and a significant decrease of p62 gene expressions in moringa + exercise group (0.87 fold) as shown in Table 9-10 and Figure 2. These results might show an increase of autophagy in groups that were given moringa per oral and moderate intensity of treadmill exercise, but there was no difference in autophagy gene expression in other treatments (moringa or exercise) compared to the control group.

The data had a normal distribution, homogenous for p62 but non homogenous for LC3 gene expression. Therefore, we used One Way Anova test for both gene expressions, continued with post hoc Games-Howell for LC3 gene expression and Bonferroni for p62 gene expression.

In Figure 2, we presented PCR band of LC3, p62, and GAPDH as the result from semiquantitative PCR (Figure 2A). The result of post hoc test showed significant differences between LC3 ($p=0.038$) and p62 ($p=0.024$) gene expressions between moringa+exercise group compared to control group (Figure 2B).

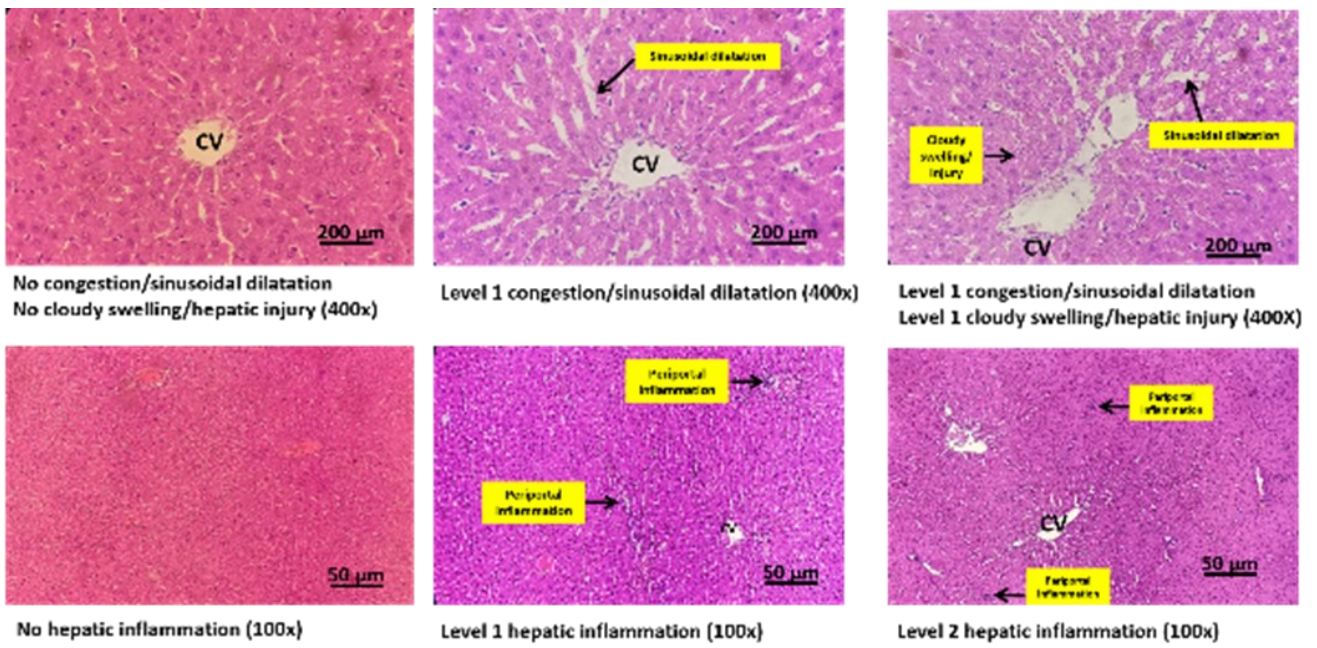


Figure 1. Histopathological Appearances based on Congestion/Sinusoidal Dilatation, Cloudy Swelling/Injury, and Inflammation

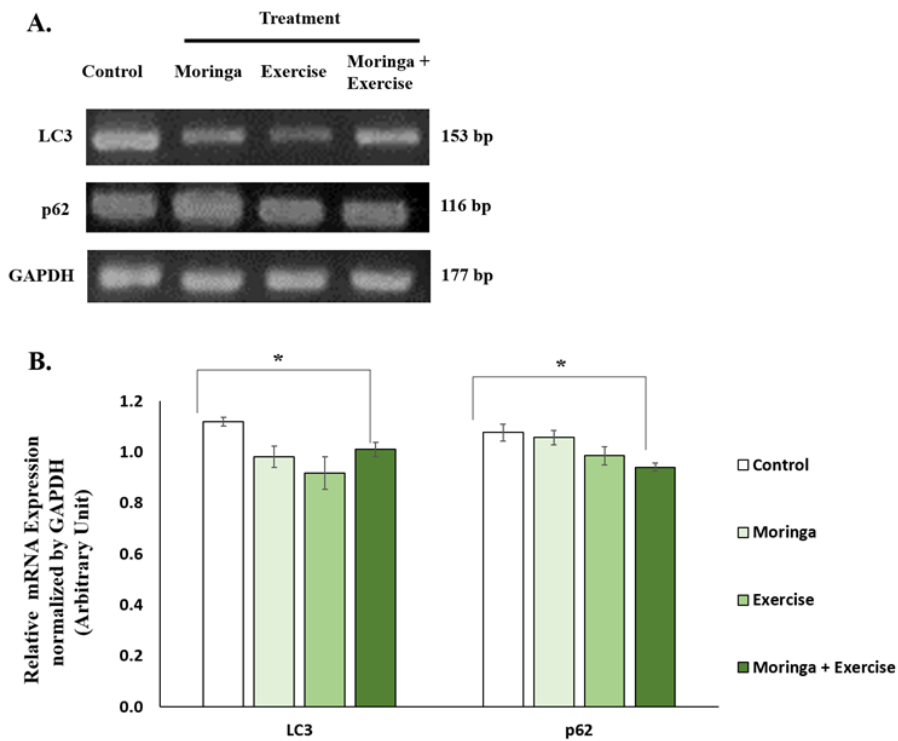


Figure 1. Autophagy Gene Expression after Treatments

Table 8. LC3 Relative Ratio after Treatments

Groups	LC3 Relative Ratio \pm SEM	N	F	p
Control	1.119 \pm 0.02	6		
Moringa	0.982 \pm 0.04	6	4.188	0,019*
Exercise	0.917 \pm 0.06	6		
Moringa + Ex	1.010 \pm 0.03	6		

* = significant ($p < 0,05$)**Table 9.** p62 Relative Ratio after Treatments

Groups	LC3 Relative Ratio \pm SEM	N	F	p
Control	1.077 \pm 0.03	6		
Moringa	1.057 \pm 0.03	6	4.570	0,014*
Exercise	0.985 \pm 0.04	6		
Moringa + Ex	0.940 \pm 0.02	6		

* = significant ($p < 0,05$)

DISCUSSION

In this study, we found significant differences of histopathology scoring in Wistar rat livers after treadmill exercise and moringa treatments. Moringa might serve as a good scavenger to eradicate free radicals, for example nitric oxide. Parts of moringa plants, such as leaves, roots, and seeds, have important medicinal properties and antioxidants components, such as alkaloids, tannins, phenolic, and flavonoids (Bais et al., 2014; Kou et al., 2018). Our study showed no differences of liver scoring between moringa and moringa + exercise groups compared to control group. This result was in line with another study that proved that a low dose of moringa has antioxidant activity (El Rabey et al., 2018).

From the result of autophagy gene expression, we found a significant increase of autophagy gene expression showed by a decrease of LC3 dan p62 in moringa + exercise group compared to control group. Autophagy, which refers to macroautophagy, is an important mechanism to degrade damaged organelles in an autophagosome to produce new substances for cytoplasmic material, as a process to maintain cellular homeostasis (Frake and Rubinsztein, 2016). Autophagy is a highly dynamic process, influenced by exercise, nutrient starvation, ROS, cancer, aging, and other factors

(Frake and Rubinsztein, 2016; Tarawan et al., 2019; Cordani et al., 2019).

Exercise is a potential inducer of autophagy in liver, both in acute and chronic exercises (Kwon et al., 2020; Pi et al., 2019). Exercise has been known as a preventive strategy against NAFLD, because exercise can protect the liver by inducing molecular processes to help regulate hepatic metabolism, mainly lipid metabolism (Trefts et al., 2015). Our previous study showed that moderate treadmill exercise induced autophagy in liver, that might be associated with lipophagy (Gunadi et al., 2020). In lipophagy, lipid droplets are degraded through activation of autophagic proteins, one of which in LC3 (Kloska et al., 2020; Martinez-Lopez et al., 2016). While p62 is a substrate degraded in an autophagy process, including lipophagy in liver. P62 acts as a signaling protein for various pathway, for example mTOR, and also has a physiological role in the liver associated with obesity (Manley et al., 2013).

Antioxidant potential of moringa might have a connection with autophagy process in liver, but in this study, moringa extract alone did not induce autophagy gene expression. This result might show that a low dose of moringa extract did not influence autophagy. However, based on histopathological result in moringa + exercise group compared to control group, we hypothesized that antioxidant property of moringa might protect the liver from physiological inflammation caused by exercise. Future study needs to be performed to investigate the effect of a higher dose of moringa extract on autophagy of wistar rat liver.

CONCLUSION

In summary, we presented that moderate intensity exercise alone induced liver histopathological changes, while moringa + moderate intensity exercise had no significant difference compared to control group, indicating that physiological inflammation caused by exercise might be neutralized by the antioxidant property of moringa extract. The histopathological changes were supported by the increase of autophagy gene expression in moringa + moderate intensity exercise group compared to control group. The most influenced group in this research was moringa + moderate intensity exercise group, because exercise in this group did not induced physiological inflammation, which suggested that

moringa protected the liver through its antioxidant properties, accompanied by autophagy process activated by oxidative stress to protect the cell from apoptosis. Further research needs to be performed for examining the modulation of autophagy by moringa and exercise, with different doses of moringa and different type, duration, and intensity of exercise.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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