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THE ANTIOXIDANT EFFECTS OF IPB-3S STRAIN *ORYZA SATIVA* BRAN EXTRACT TOWARDS MALONDIALDEHYDE AND GLUTATHIONE PEROXIDASE LEVELS IN RAT BRAIN INDUCED BY CARBON TETRACHLORIDE

D. Yoga Oktavianda [1] and R. Dwirini Gunarti [*2]

Faculty of Medicine [1], Universitas Indonesia, Indonesia.
Department of Biochemistry and Molecular Biology [2], Faculty of Medicine, Universitas Indonesia, Indonesia.

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Correspondence to Author:
R. Dwirini Gunarti

Faculty of Medicine, Universitas, Indonesia, Indonesia.

E-mail: rinairet@gmail.com

ABSTRACT: IPB-3S Strain *Oryza sativa* bran, a high-antioxidant food, was expected to protect the brain as a susceptible organ from oxidative damage. This study was conducted to analyze the effect of rice bran extract on malondialdehyde (MDA) and glutathione peroxidase (GSH) levels in the brain induced by carbon tetrachloride (CCl_4). A total of 24 Sprague-Dawley male rats was divided into 6 groups, including normal control (C1); negative control (C2); 150 mg/kg rice bran extract (I1); 150 mg/kg rice bran extract + CCl_4 (I2); 300 mg/kg rice bran extract (I3); and 300 mg/kg rice bran extract + CCl_4 (I4). The MDA levels were measured by Wills method, while GSH levels were measured by the Ellman method. The administration of low-dose (150 mg/kg) and high-dose (300 mg/kg) rice bran extracts significantly lowered MDA levels compared to control groups, both in the groups with and without the induction of CCl_4 ($p < 0.05$). Moreover, the administration of low-dose (150 mg/kg) also significantly increased GSH level ($p < 0.05$), contrary to the groups administered by high-dose (300 mg/kg) rice bran extracts, which relatively reduced GSH level. In conclusion, rice bran extract has been proven to increase antioxidants in rat brain tissue by reducing MDA levels and increasing GSH levels.

INTRODUCTION: In recent years, degenerative diseases in the world are increasing, including in Indonesia. Basic health research (Riskesdas) data in 2013 showed that the prevalence of degenerative diseases in Indonesia was high, including diabetes (2.1%), hypertension (9.5%), cancer (0.14%), coronary heart disease (1.5%), heart failure (0.3 %), and strokes (1.2%) [1].

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The increase in levels of free radicals in the human body, such as inflammation condition, pollutant exposure, radiation, or ischemic condition, was proven leading to degenerative disease, especially diseases related to vascular damage [2, 3]. Free radicals are compounds produced from the metabolic processes, namely the pro-oxidant reaction.

It causes oxidative damage in cell proteins and DNA, which forms lipid peroxide and malon-dialdehyde (MDA) [2, 3]. To suppress the effects of free radicals, the human body needs antioxidants. Glutathione peroxidase (GSH) is one of enzymatic antioxidants produced by human body which is an indicator for measuring free radical level [2, 3].

In addition, antioxidant is also contained in food, such as vitamin E, vitamin C, and plant flavonoid [2-4]. Besides, the exposure of carbon tetrachloride (CCl_4) induces the increase of free radical level, including in brain leading neuronal damage [4]. Moreover, the reduction of antioxidant levels was found in Alzheimer's disease, characterized by the decrease of GSH level [5, 6]. It was also shown in ischemic stroke patients [7]. Rice is the staple food in Indonesia. According to data in 2015, the amount of milled dry grain production in Indonesia increased from 2012 (69.06 tons) to 2014 (70.83 tons) [8]. Besides, IPB-3S strain *Oryza sativa* is an innovation that has been marketed and widely consumed in Indonesia since 2012 [9]. The advantages of this train were resistance to Rice Tungro Bacilliform Virus (RTBV), Rice Tungro Spherical Virus (RTSV), Blas disease (*Pyriculariaoryzae*) strain 033, and bacterial blight disease pathotypic III [9].

In addition, rice bran, as a side product of rice processing, was proven as a high-antioxidant food [5, 12]. However, the utilization of rice bran in Indonesia was only used as animal feed [13]. Several studies had proven that rice bran contained antioxidant in the form of vitamin E (tocopherol and tocotrienol) and γ-oryzanol, which was able to prevent the onset of degenerative diseases, including diabetes mellitus, Alzheimer's disease, cardiovascular disease, and cancer [5-10]. It was also proven to have a hypolipidemic effect [12]. Furthermore, γ-oryzanol was also promoting hormones released in the brain, such as endorphins and norepinephrine [14]. Considering the benefits of rice bran for health, especially in antioxidant effect, this study aims to evaluate the antioxidant effect of IPB-3S Strain *Oryza sativa* Bran Extract on MDA and GSH levels in the rat brain induced by CCl_4.

MATERIALS AND METHODS: We conducted an experimental study in the Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia. Ethical consideration was approved by the Faculty of Medicine, Universitas Indonesia, Indonesia (Number: 514/UN2.F1/ETIK/2016). Based on Federer's formula [15] for sample size determination in animal studies, a total of 24 animal samples, in the form of the brain of Sprague-Dawley strain male rats aged 6-8 weeks with a weight of 150-250 g, were divided into six groups, including the normal control group (C1) without intervention; negative control group (C2) induced CCl_4 0.55 mg/kg; intervention group 1 (I1) given rice bran 150 mg/kg; intervention group 2 (I2) given rice bran 150 mg/kg and CCl_4 0.55 mg/kg; intervention group 3 (I3) given rice bran 300 mg/kg and intervention group 4 (I4) given rice bran 300 mg/kg and CCl_4 0.55 mg/kg.

Samples Intervention: Bran from IPB-3S strain *Oryza sativa* was extracted by grinding and sifting. Furthermore, with the addition of NaCl 0.9% 100 mL, 10 g of bran extract were dissolved and filtered. It was given orally once a day by using 10 mL syringe to the animal for a week based on the prescribed dose for each intervention group. Meanwhile, CCl_4 was induced once on the last day before surgery based on the dose determined for each group.

Brain Sampling: The animal samples were weighed after an intervention. Surgery was performed on 19th day by pulling and breaking the neck of the rats, then stretching by fixing all four extremities on the surgical board. The brain organs that had been taken were weighed and placed on 1.5 mL test tube, then stored in a freezer with a temperature of -20 °C.

Preparation of Solvent: We used Phosphate Buffer Saline (PBS) pH 7.4 as a solvent, obtained by mixing Na_2HPO_4, KH_2PO_4, NaCl, and distilled water. We added HCl and NaCl to neutralize pH. After the pH neutral, 50 mL distilled water was added to obtain PBS 0.05 M.

Preparation of Homogenate: The brain samples were cut into small pieces, weighed 180-220 mg. It was placed on test tube and added by PBS 1 mL gradually until it was mixed by using a micro-pestle. The mixture was centrifuged at a speed of 5000 rpm for 10 min. The supernatant was moved to the test tube and added by PBS 1 mL, then stored in the freezer.

Measurement of MDA Level: MDA levels were measured by using wills method [16]. The tubes were divided into 3 groups, consisted of blank, standard, and test, each Duplo. The blank tubes were filled by distilled water 400 µL and Trichloroacetic Acid (TCA) 20% 200 µL.

The standard tubes were divided into 5 tubes based on the concentration of Tetra Ethoxy Profane (TEP), including S1 (0.3125 nmol/L), S2 (0.625 nmol/L), S3 (1.25 nmol/L), S4 (2.5 nmol/L), and S5 (5 nmol/L). The test tubes were filled with distilled water 300 µL, brain sample 100 µL, and TCA 20% 200 µL. Each of the tubes was mixed by vortex until homogeneous, then centrifuged with the speed of 5000 rpm for 10 minutes in a temperature of 4^{o}C. The supernatant was taken and added by thiobarbituric acid (TBA) 0.67% 400 µL, then incubated in the boiling water (90 - 100^{o}C) for 10 minutes. Then, it was cooled at room temperature. Furthermore, the absorbance of the solution was read using a spectrophotometer at a wavelength of 530 nm (λ = 530 nm). The principle of MDA measurement was the color of the solution produced when MDA reacts with TBA at 100^{o}C. The absorbance data of each group will be calculated using a linear equation obtained from the measurement of standard tubes to measure MDA levels.

Measurement of GSH Level: We used Ellman method for measuring GSH level [17]. The tubes were divided into 3 groups, consisted of blank, standard, and test, each Duplo. Blank tubes were filled by distilled water. Standard tubes for GSH were divided into standard 1 (1 µL), standard 2 (2 µL), standard 3 (4 µL), standard 4 (5 µL), and standard 5 (10 µL). Each test tubes were filled by sample 50 µL Furthermore, each tube was added by TCA 5% 200 µL. Then, it vortexed until homogenous and centrifuged at a speed of 500 rpm for 10 min in a temperature of 40 ^{o}C. The supernatant from each tube was taken 1.5 mL and moved into tube 2 mL. PBS pH 8.0 1800 µL was added in each tube, followed by DTNB 25 µL. It was incubated in the dark with room temperature for 60 min. The absorbance was read using a spectrophotometer at a wavelength of 420 nm (λ = 420).

Data Analysis: The results of absorbance were collected and processed using Microsoft Excel 2013 to determine the linear equation and determinant coefficient that would be used to measure MDA and GSH levels. The data were analyzed using Statistical Product and Service Solution (SPSS) version 20. Statistical difference of MDA and GSH levels between each group was known by using One-way Anova test with p-value < 0.05 considered to be significant.

RESULTS:

Characteristics of Sample: Animal weight before the intervention was ranged from 150 - 250 g. The results showed that there was no significant difference in weight between each of the intervention groups (p > 0.05). However, the data in **Table 1** shows that there was a decrease in weight in all intervention groups. The average weight is shown in **Table 1**, both before and after the intervention. **Table 1** also shows the average brain weight. The average weight of experimental brains in this study was 1.5 g. Furthermore, the brain weight used for MDA and GSH measurements ranges from 180- 20 mg.

TABLE 1: SAMPLE CHARACTERISTICS

	Groups					
	C1	**C2**	**I1**	**I2**	**I3**	**I4**
	Body Weight (g)					
Before intervention	205.00 ± 47.69	233.75 ± 37.05	201.25 ± 22.50	221.25 ± 17.96	203.75 ± 29.26	196.25 ± 28.68
After intervention	198.75 ± 19.31	220.00 ± 38.51	178.75 ± 29.54	207.50 ± 16.58	196.25 ± 22.86	183.75 ± 30.92
Difference	-6.67 ± 24.66	-13.75 ± 4.79	-22.50 ± 12.58	-13.75 ± 6.29	-7.50 ± 11.90	-12.50 ± 9.57
Brain weight (g)	1.460 ± 0.188	1.587 ± 0.102	1.527 ± 0.110	1.552 ± 0.074	1.517 ± 0.149	1.380 ± 0,205
Ratio of brain weight/body weight	0.00734	0.00737	0.00869	0.00753	0.00776	0.00758

C1: normal control; C2: negative control - induced by CCl_4; I1: 150 mg/kg rice bran extract; I2: 150 mg/kg rice bran extract + CCl_4; I3: 300 mg/kg rice bran extract; I4: 300 mg/kg rice bran extract + CCl_4.

MDA Level of Brain: MDA standard curve was needed to calculate the MDA level, which was obtained by using the Wills method. The standard absorbance was used to make the MDA standard curve, to find out the linear equation (y = ax + b) and the determinant coefficient (R^2). The following

is the MDA standard curve **Fig. 1**. The absorbance was the value at the y-axis in the linear equation of the MDA standard curve. We found out the x-value as MDA level for each sample. In addition, sample dilution also affects the absorbance results. In this study, sample dilution was carried out 4 times. Therefore, the MDA level formula was x = ((y + 0.0058) / 0.0477) x (4/sample weight) in units of nmol/mg tissue. The MDA levels of each group were 0.012053562 nmol/mg (C1), 0.012605571 nmol/mg (C2), 0.006114272 nmol/mg (I1), 0.006261227 nmol/mg (I2), 0.007726532 nmol/mg (I3) and 0.009012678 nmol/mg (I4). The mean of MDA levels in each group was significantly different in **Fig. 2**.

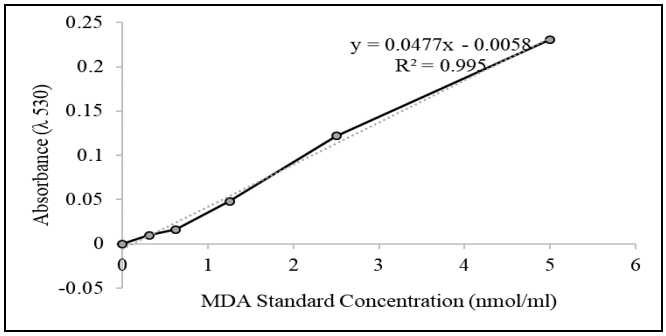

FIG. 1: MDA STANDARD CURVE

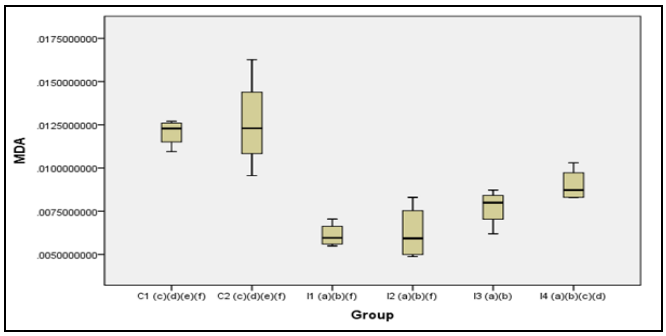

FIG. 2: MDA LEVEL BASED ON INTERVENTION GROUP

C1: normal control; C2: negative control - induced by CCl_4; I1: 150 mg/kg rice bran extract; I2: 150 mg/kg rice bran extract + CCl_4; I3: 300 mg/kg rice bran extract; I4: 300 mg/kg rice bran extract + CCl_4. The (letters) next to the group shows a significant difference ($p < 0.05$) between groups: (a) compared to C1; (b) compared to C2; (c) compared to I1; (d) compared to I2; (e) compared to I3; and (f) compared to I4. The administration of rice bran extracts significantly reduced MDA levels, both in the dose of 150 mg/kg and 300 mg/kg ($p < 0.05$). However, the administration of low-dose (150 mg/kg) tended to be better than the high-dose. In addition, this condition occurred either with or without the induction of CCl_4.

GSH Level of Brain: GSH standard curve was needed to calculate GSH level, which was obtained by using the Ellman method. The standard absorbance was used to make the GSH standard curve. The following is the GSH standard curve **Fig. 3**. We found out the x-value as GSH level with the formula, x = ((y - 0.0083)/0.034) x (sample weight). The means of GSH levels in each group were 0.00187441 µL (C1), 0.001766632 µL (C2), 0.002683065 µL (I1), 0.002459008 µL (I2), 0.002659722 µL (I3) and 0.001068627 µL (I4). The results showed that some of the groups were significantly different each other, **Fig. 4**. Compared to the control groups, the administration of low-dose (150 mg/kg) rice bran extracts significantly increased the production of GSH level ($p < 0.05$). In contrast, the administration of high-dose (300 mg/kg) rice bran precisely decreased the GSH level, especially if it was also induced by CCl_4.

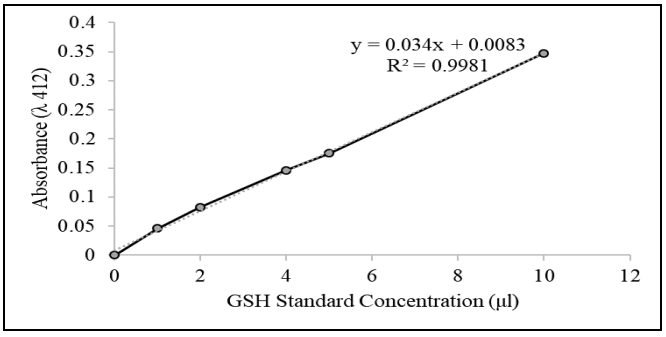

FIG. 3: GSH STANDARD CURVE

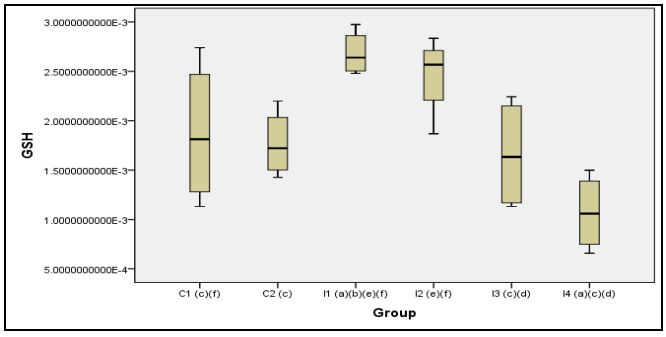

FIG. 4: GSH LEVEL BASED ON INTERVENTION GROUP

C1: normal control; C2: negative control - induced by CCl_4; I1: 150 mg/kg rice bran extract; I2: 150 mg/kg rice bran extract + CCl_4; I3: 300 mg/kg rice bran extract; I4: 300 mg/kg rice bran extract + CCl_4. The (letters) next to the group shows a significant difference ($p < 0.05$) between groups: (a) compared to C1; (b) compared to C2; (c) compared to I1; (d) compared to I2; (e) compared to I3; and (f) compared to I4.

DISCUSSION: Based on the results of this study, all groups experienced weight loss after the intervention. However, weight loss tended to be greater in the intervention groups than the control group. According to the results of Hongu *et al.* (2014), consumption of rice bran significantly reduced LDL and total cholesterol. It also proved that a balanced diet supplemented with rice bran consumption for 8 weeks could reduce body weight around 4.7 kg in overweight and obesity adults [15]. Justo *et al.* (2012) also proved that rice bran was able to reduce blood pressure, triglycerides, cholesterol, and blood glucose levels by increasing insulin sensitivity in obese rats [16].

In addition, rice bran was proven to contain antioxidants, including γ-oryzanol and vitamin E. According to Parrado *et al.* (2003), antioxidant enzymes, such as catalase and glutathione peroxidase, were relatively low in the brain, whereas levels of oxidized substrates were relatively high in the brain, such as polyunsaturated fatty acids (PUFAs) and catecholamines [17].

The Effect of Induction of Carbon Tetrachloride (CCl_4) in Rat Brain: The results of this study indicate that the induction of CCl_4 relatively increased MDA level and decreased GSH level. It was in line with Mahmoud *et al.* (2013) and Soliman *et al.* (2011), proving CCl_4 induction increased MDA and decreased GSH in the brain, impairing neuron function [4, 18]. According to Ritesh *et al.* (2015), oxidative stress in the brain due to CCl_4 induction varied depending on the area of the brain, including midbrain (62%), hindbrain (105%), and forebrain (159%) [19].

According to U. S. EPA, CCl_4 is metabolized by CYP2E1 enzymes into several metabolites, including trichloromethyl radicals and trichloro-methyl peroxy-radical. These two metabolites are highly reactive oxygen species (ROS), which can cause cell damage by covalently binding to cellular macromolecules to form nucleic acids, proteins, and lipid additions. Peroxy-radical trichloromethyl can damage PUFAs on cell membranes, forming fatty acid-free radicals. CCl_4 is fat-soluble that can penetrate cell membranes, distributed, and stored in several organs, such as the liver, brain, kidney, and heart, then rapidly absorbed by the liver and brain [20].

The Effects of Rice Bran Extract towards MDA level in Rat Brain: The results of this study showed that the administration of rice bran extract, both low-dose (150 mg/kg) and high-dose (300 mg/kg), with or without the induction of CCl_4, significantly reduced MDA levels of the brain ($p < 0.05$) compared to either normal or negative control. It was in line with the results of the studies by Parrado *et al.* (2003) and Sengupta *et al*. (2014), which showed a decrease in MDA levels in rats fed rice bran compared to controls [17, 21].

Rice bran extract contains antioxidant compounds in the form of γ-oryzanol and tocopherol (vitamin E), which affect the regulation of lipid metabolism and inhibit oxidative damage. According to Parrado et.al (2003), the content of γ-oryzanol in rice bran was 0.25 + 0.10 mg/g [17]. The study by Sengupta *et al.* (2014) in Wistar rats proved that the administration of rice bran oil decreased lipid peroxidation induced by sodium arsenite for 14 days. Without administration of sodium arsenite, rice bran oil also significantly reduced MDA levels of brain tissue [21]. Similarly, the study by Parrado et *al.* (2003) in Wistar rats proved that water-soluble oryzanol enzymatic extract (WSOEE) derived from rice bran had antioxidant effects by absorbing peroxyl radicals and inhibiting lipid peroxidation due to the induction of cumene hydroperoxide. The product of lipid peroxidation, malondialdehyde (MDA), which was significantly reduced by WSOEE [17].

Antioxidant effects in rice bran have the potential to prevent and treat chronic diseases related to high free radicals, such as atherosclerosis, neuro-degeneration, and cancer. According to Bhatia *et al.* (2016), rice bran extract inhibited inflammatory factors (PGE2) and free radicals, which were potential as a nutraceutical for prevention of microglia dysfunction, including Alzheimer's disease [22]. The study by Wang *et al*. (2014) in high-lipid diet rats also showed that the aqueous enzymatic extract from rice bran (AEERB) likely reduced MDA levels in hyperlipidemia rats [23].

Besides, MDA levels in rat brains given high-dose (300 mg/kg) rice bran extract were significantly higher than those given low-dose (150 mg/kg), either with or without CCl_4 induction ($p < 0.05$). It

showed that low-dose (150 mg/kg) rice bran was more effective than high-dose (300 mg/kg).

The Effect of Rice Bran Extract towards GSH level in Rat Brain: According to the results of this study, the level of GSH was likely similar between normal and negative control groups. Besides, there was a significant increase of GSH level in the group given low-dose (150 mg/kg) rice bran extract compared to normal and negative controls ($p < 0.05$). In contrast to the group given high-dose (300 mg/kg) rice bran extract, which significantly reduced GSH level in the brain, especially if added with the induction of CCl_4 ($p < 0.05$).

The study by Minatel *et al.,* (2016) stated that γ-oryzanol from rice bran extract had the effect of cholesterol-lowering, anti-inflammatory, anti-cancer, and anti-diabetic. It also stated that the distribution of γ-oryzanol mainly in the brain [24]. Furthermore, Sengupta *et al.* (2014) proved that there was a significant increase in the level of glutathione reductase (GR) and GSH in rat brain tissue after the administration of rice bran oil, both in the groups with and without the induction of sodium arsenite ($p < 0.05$) [21].

In contrast, the study by Wang *et al*. (2014) showed that there no significant difference of GSH levels between the Wistar rat groups given a normal diet, hyperlipidemic diet, or AEERB ($p > 0.05$) [23]. It also showed that the group given AEERB tended to have lower GSH levels compared to a normal diet. It possibly due to the low level and slow metabolism of antioxidant enzymes in the brain, including GSH, which inhibited the recovery process of cumulative oxidative damage [23].

Limitation: The sample size was needed to be added, even though this study had fulfilled the minimum standard of sample size. The conditions of animal care place might be uncomfortable for animals triggering the distress that affected the physiological response of animals. Furthermore, storage conditions might also influence the results of this study. The MDA and GSH measurements of all samples were not carried out simultaneously in one day, which might affect the results. Further, research was needed to determine the range of optimal doses to increase endogenous antioxidant activity, in the form of clinical trials in animals as well as humans. It was also needed to determine the antioxidant effects of rice bran focused on a certain degenerative disease. Moreover, it was needed to find out other ingredients in rice bran which potentially able to improve health. Development of rice bran products as food or drink can also be carried out along with socialization to the public about the benefit of rice bran consumption as a healthy food.

CONCLUSION: The induction of CCl_4 tended to increase MDA levels and decrease GSH levels in the brain compared to uninduced ones. The administration of low-dose rice bran extract (150 mg/kg) and high-dose (300 mg/kg) reduced the MDA levels significantly, either in groups induced by CCl_4 or not. Meanwhile, the increase of GSH levels was only seen in the groups administered by low-dose rice bran (150 mg/kg), contrary to the high-dose rice bran (300 mg/kg), which decreased GSH levels. Thus, this study proved that rice bran extract had antioxidant component which was able to reduce MDA levels and increase GSH levels in rat brain, despite the low-dose rice bran extract was more effective than high doses.

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CONFLICTS OF INTEREST: The authors declare no conflicts of interest.

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