The seluang fish (Rasbora spp.) diet to improve neurotoxicity of endosulfan-induced intrauterine pup's brain through of oxidative mechanism

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Original Article

The seluang fish (*Rasbora spp.*) diet to improve neurotoxicity of endosulfan-induced intrauterine pup's brain through of oxidative mechanism

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SUMMARY

Epidemiological evidence suggested the relationship between pesticide exposure and increased risk of autism. In autism, there is a change in the production of neurotransmitters and oxidative stress. Seluang fish has high protein that has not been investigated for its effects on autism. This study aimed to determine the effect of seluang fish on neurotransmitters and oxidative stress in endosulfan-induced rats intrauterine. This study used pup's rat induced by endosulfan intrauterine. The pregnant rats divided into 2 groups were K0 without endosulfan induced and K1 with endosulfan induced. After 4 weeks, 8 pup's rats of K0 and K1 were sacrificed for blood and brain samples, while 8 pup's rats were given a standard diet (P1) and fed a Seluang fish diet (P2) respectively for 4 weeks. Then examined the parameters of serum and brain serotonin, brain H_2O_2, SOD, and MDA levels. The results showed that K1had increased brain H_2O_2 and MDA levels, whereas brain and serum serotonin levels and brain SOD decreased significantly compared to K0. Group P1 and P2 showed decreasing brain H_2O_2 and MDA levels significantly than K1, brain serotonin levels increased significantly than K1, whereas serum serotonin

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levels were not significantly different ($p = 0.483$). Group P1 and P2 were not different significantly in the serum and brain serotonin, and brain SOD levels. The brain H_2O_2 ($p < 0.001$) and MDA levels ($p < 0.001$) were different significantly. We concluded that seluang fish diet was able to repair oxidative stress in the brains of rats due to endosulfan induction during pregnancy.

1. Introduction

Autism is a neurological development disorder that has characteristics of behavioral disorders and communication deficits [1]. The prevalence of autism is approximately 6,5-6, for every 1000 people [2]. Until now the mechanism of autism has not been clearly understood. Autism is considered a multifactorial disorder influenced by genetics, immunology and environmental factors including oxidative stress. A recent study in pregnant women exposed to pesticides showed that there was a correlation between organochlorine exposure and the incidence of autism during the period of nervous system organogenesis. Other studies also showed the close relationship between exposure to organophosphate insecticides; chlorpyrifos and a form of autism incidence. Some types of pesticides cause dysregulation of the nervous system such as organochlorine, organophosphate, and Carbamate. This epidemiological evidence strengthens the relationship between pesticide exposure and increased autism risk [3].

A number of neurochemical studies linked autism to neurotransmitter formed from the amino acid tryptophan. Adamsen et al. proved that there is a correlation between Autism Spectrum Disorder (ASD) and low serotonin levels in brain fluids [4].

In addition neurotransmitter alteration autism also caused by oxidative stress. The effects of oxidative stress on mitochondrial function in individuals with ASD suggest that there is an imbalance between the formation of ROS and the mechanism of antioxidant defense in its organ systems [5]. In addition, there is also a decrease in catalase and SOD activity in erythrocytes [6,7]. Increased oxidative damage occurs on mitochondrial DNA protein showed a decrease in antioxidant capacity and increased oxidative stress in the brains of patients with autism may cause dysfunction in autistic patients [8–10].

In patients with autism, we concern about nutritional intake. There are several compounds in foods that are not recommended for consumption by people with autism, such as gluten. However, there has been a lack of research on the effect of fish consumption, especially seluang for the symptoms of autism. Fish is a source of nutrition including essential amino acids, essential fatty acids, high vitamins and minerals, especially fish with whole meat and bones are consumed. Seluang fish (*Rasbora* spp.) is a river fish that is known and widely consumed by the people of South Borneo and is included as endemic fish of Borneo and Sumatra. The nutrient content per 100 g of seluang fish includes protein content of 40% w/w and fatty acid DHA 1.04% w/w. The previous study from Triawanti et al. reported that seluang fish could improve nutritional status in rats after malnutrition [11]. A study by Yunanto et al. proved that white rats fed with seluang fish had better spatial memory and higher PPARγ and PPARα expressions compared to rats fed by standard feed [12]. Triawanti et al. study had proven that seluang fish can improve the oxidative stress condition of the brain after malnutrition [13].

This study aimed to prove that the seluang fish can affect the secretion of neurotransmitters and oxidative stress in the brain tissue of pulp induced by endosulfan intrauterine.

2. Methods

This study had been received approval from the ethics committee of the Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, Indonesia (Number 468/KEPK-FK UNLAM/EC/2017). This study was an experimental study with posttest-only with control group design.

2.1. Materials

The materials used in this study were white rats (*Rattus norvegicus*), pup's brain, distilled water, deionized water, standard rat feed (comfeed PARS 53% (12% water, 11% protein, 4% fat, 7% fiber, 8% ash, 1.1% calcium, 0.9% phosphorus, antibiotics, 53% coccidiostat), 23.5% wheat flour, 23.5% water), endosulfan, rat TNF-α ELISA kit, rat HSP70 ELISA kit, ether, phosphate buffer saline (PBS) pH 7, 200 μL 100% TCA, 100 μL 1%, sodium thiobarbiturate, 250 μL HCl 1 N, EDTA, dichromate, glacial acetate, H_2O_2, olive oil, adrenaline, sodium bicarbonate (Na_2CO_3) and potassium iodide (KI).

2.2. Animal procedure

2.2.1. Acclimatization

Adult female and male rats were kept for 1 week before being treated to provide the same physical and psychological conditions. During maintenance, white rats were given the same distilled water and food sufficiently.

2.2.2. Endosulfan induction

After a one-week acclimatization period, female rats were injected by PMSG and HCG in accordance with estrous cycle. Female rats were mated with male rats from the same strain. 1 female was mated to 1 male. After mating, female rats were individually placed in a polypropylene cage. Female rats those had been positively pregnant were weighed and distributed randomly divided into 2 groups. The control group (K2) without endosulfan induction while the treatment group (K1) was induced by endosulfan with a dose of 1 mg/kg BW.

Endosulfan was given by dissolving it in olive oil and administered orally during 21 days of pregnancy. After the female rats gave birth, endosulfan treatment was terminated. The pups were left to suckle on their mothers. When the pups had reached four weeks of age, 8 pups from each group were terminated and brain tissues were taken to measure levels of MDA, SOD, and H_2O_2 of the brain. The blood was collected to measure serotonin and GABA level. Then, 16 another pups that induced by endosulfan divided into two groups: P1 was given standard diet, and P2 was given seluang fish diet for 4 weeks. Then, all of the pups were terminated and brain tissues were taken to measure levels of MDA, SOD, and H_2O_2 of the brain. The blood was collected to measure serotonin and GABA level.

2.3. Preparation of seluang diet

Fresh seluang fish was grilled to obtain raw fish porridge. Then the porridge was steamed with steam heat for 1 h and dried with oven until the moisture content is about 8%. Next, regrinding to remove clots or bone particles. After being dried granules, the fish powder is made into pellets and used as rat feed.

2.4. SOD, H_2O_2, and MDA levels assay from brain homogenate

The brain was pounded with mortar at room temperature and added with 1 mL of PBS pH 7.4 until it became liquid. Then taken 5 mL and centrifuged at 8000 rpm for 20 min. The supernatant was then taken for measurement of H_2O_2, MDA, and SOD.

2.4.1. Measurement of brain SOD levels

Incubation was performed on 3 ml of a solution containing 0.05 M Na_2CO_3, 0.1 M EDTA pH 10.2. Furthermore, the solution was added 100 μL brain homogenate and 100 μL adrenaline with (3.10^{-4}) BM

189 M. Initial absorption measurements (A_0) was performed with a spectrophotometer at 480 nm wavelength. After that, the sample was incubated for 5 min at 30 °C and got the absorbance (A_1).

2.4.2. Measurement of brain H_2O_2 levels

Measurement of H_2O_2 was using a spectrophotometer. At first, making a standard curve. A total of 20 μmol H_2O_2 was added with 2 ml of dichromate:glacial acetic acid (1:3) mixture. Then the mixture was heated in boiling water for 10 min. Then the cooled mixture was measured for absorbance at a wavelength of 570 nm. The same procedure was done for 40,60,80,100,120, 140,160 and 180 μmol H_2O_2. A graph was made between the absorbance on the Y-axis with levels of H_2O_2 on the X-axis to obtain a linear equation.

Preparation of test solution was made with a total of 1 ml of brain homogenate was added 5 ml of PBS pH 7.4. A mixture of 1 ml was taken and added to 2 ml of dichromate:acetate (1:3) mixture and then wrapped in aluminum foil for 30 min. The mixed solution was heated using a water bath for 10 min at 100 °C. The solution was cooled to room temperature. The solution was then transferred into the cuvette and measured its absorbance using UV-VIS at a wavelength of 570 nm.

2.4.3. Measurement of brain MDA levels

From the last procedure, 200 μL supernatant was taken for measurement of MDA levels. The first thing to do was making MDA standard curve. As many as 0.05 μM MDA standard added 1 mL of distilled water, then placed in Eppendorf tube. Thereafter, 100 μL of 100% TCA, 100 μL sodium thiobarbituric 1%, and 250 μL HCl 1 N were added respectively. Then heated at 100 °C for 20 min, and centrifuged 3500 rpm for 10 min. Subsequently, 450 μL supernatant was taken and the distilled water added to 3500 μL. Then read with the spectrophotometer with a maximum wavelength of 540 nm. The same thing was done to 0.025, 0.0125, 0.00625, 0.003125 and 1.56×10^{-5} μM MDA. Then making graphs for the relationship between absorbance on the Y-axis and MDA levels on the X-axis to obtain a linear equation.

2.5. Serotonin's brain and serum assay

The measurement method refers to the rat Serotonin ELISA Kit (Novateinbio, USA). The materials and standard reference were placed at room temperature. As much as 100 μL standard reference, blank standard, samples were dissolved in dilution and placed into the well and then incubated for 3 h at room temperature. The suspense washed with PBS and washed up to 4 times. The conjugates were filled into well as much as 200 μL. The suspensions were washed with washing buffer up to 4 times then 200 μL substrate solution was added to well and incubated 30 min at room temperature. The stop solution was added to every well and was read for 30 min at a 540 nm wavelength.

2.6. Data analysis

The data were collected and tabulated. We used a comparative analysis for this study. The data were tested using Shapiro Wilk for normality test and Levene for homogeneity test. If the data were normally distributed and homogenous, Anova Test would be performed with a 95% confidence level.

3. Results

Blood serotonin levels were presented in Fig. 1. The ANOVA test results showed no significant difference in blood serotonin levels between all groups ($p = 0.483$). It indicated that seluang diet had not been able to increase blood serotonin levels significantly.

Brain serotonin levels were presented in Fig. 2. The Kruskal Wallis test results showed significant differences between all groups ($p = 0.000$). In the seluang diet group, there was a significant decrease in brain serotonin levels compared to K0, P1, and P2. After standard feed (P1) and the seluang diet (P2) treatment, there was an increase in brain serotonin levels even though they were not as high as in the control group (K0). Mann Whitney's test showed there were significantly different between K0 with all

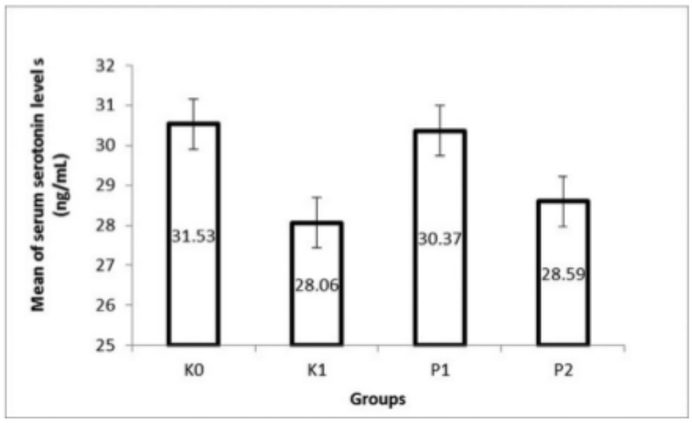

Fig. 1. Serum serotonin levels of the experimental rats. (K0 = normal; K1 = endosulfan induced; P1 = endosulfan induced + standard diet; P2 = endosulfan induced + seluang fish diet. $P = 0.483$).

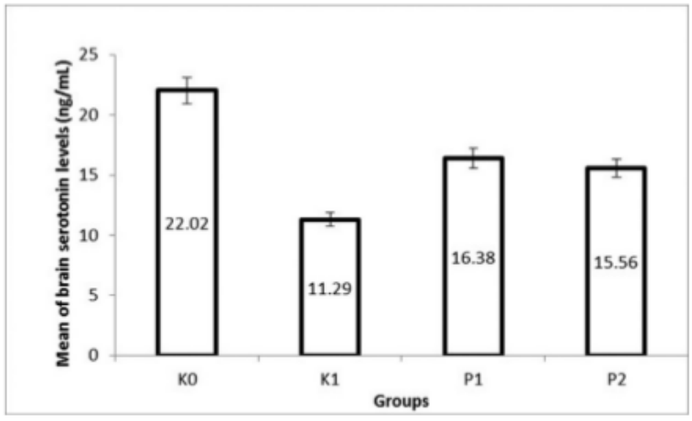

Fig. 2. Brain serotonin levels of the experimental rats. (K0 = normal; K1 = endosulfan induced; P1 = endosulfan induced + standard diet; P2 = endosulfan induced + seluang fish diet. $P = 0.000$).

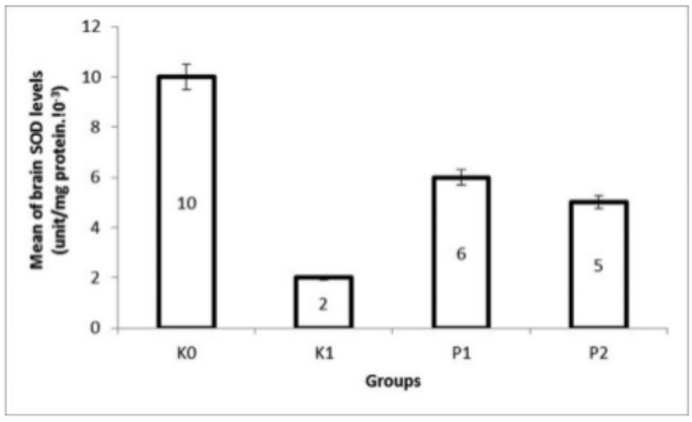

Fig. 3. SOD levels of the experimental rat's brain. (K0 = normal; K1 = endosulfan induced; P1 = endosulfan induced + standard diet; P2 = endosulfan induced + seluang fish diet. $P = 0.000$).

groups, K1 with P1 and P2, while between P1 and P2 there were no significant differences. It can be concluded that seluang diet can increase serotonin levels in rats induced by endosulfan intrauterine.

3.1. Oxidative status

Brain SOD levels were presented in Fig. 3. Test of Kruskal Wallis statistics showed that there were significant differences in brain SOD levels between all groups ($p = 0.000$). The results of further tests showed a significant difference between endosulfan-induced group *vs* without endosulfan. This was caused by exposure to endosulfan which was an oxidant makes the body unable to produce endogenous antioxidants, so the brain SOD levels of the endosulfan-induced group are lower than those without endosulfan. After the standard diet (P1) and seluang diet (P2) treatment, there was a significant increase in SOD levels from K1. It indicated that the seluang diet was able to increase the levels of SOD enzymatic antioxidants and can counteract superoxide radicals.

Levels of H_2O_2 were presented in Fig. 4. The ANOVA test results showed significant differences in H_2O_2 levels between all groups ($p = 0.000$). Post-hoc analysis tests showed significant differences between group without endosulfan and groups induced by endosulfan. The group induced by endosulfan has a higher level of brain H_2O_2 because exposure to endosulfan includes large oxidant compounds so that the body was not able to form antioxidants. After being given the standard diet (P1) and seluang diet (P2) there were significant decreases in H_2O_2 levels compared to the K1 group. It showed that the seluang diet can reduce H_2O_2 levels due to endosulfan induction.

MDA levels were presented in Fig. 5. Kruskal Wallis test results showed some significant differences in brain MDA levels among all groups ($p = 0.000$). The MDA level of the endosulfan induced brain group was higher than endosulfan induced groups. The group induced by endosulfan and followed by standard diet and the seluang diet had lower brain MDA levels than those with endosulfan induction. It meant that the seluang diet can reduce oxidative stress in the brains of pulps due to the induction of endosulfan intrauterine.

4. Discussion

Exposure to endosulfan during pregnancy and lactation in female rats can affect the neurotransmitter; γ-aminobutyric acid (GABA), glutamate, serotonin and dopamine [14,15]. These findings suggested that neurotransmitter disorders may cause neurobehavior disorders caused by endosulfan exposure [15].

Serotonin is a monoamine compound; a neurotransmitter at nerve cell synapse. Fig. 1. Showed that blood serotonin levels in the group exposed to endosulfan were lower than the control, it

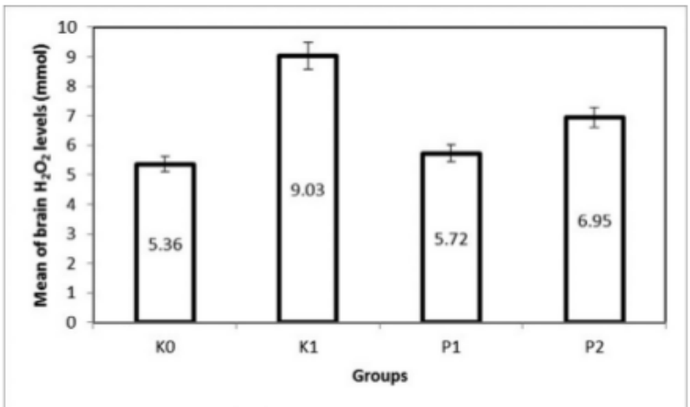

Fig. 4. H2O2 levels of the experimental rat's brain. (K0 = normal; K1 = endosulfan induced; P1 = endosulfan induced + standard diet; P2 = endosulfan induced + seluang fish diet. $P = 0.000$).

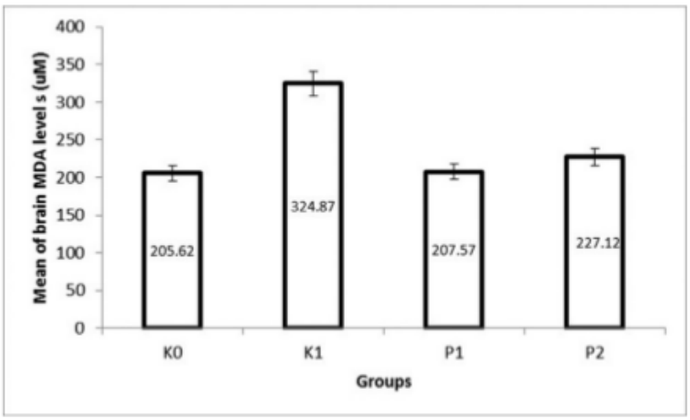

Fig. 5. MDA levels of the experimental rat's brain. (K0 = normal; K1 = endosulfan induced; P1 = endosulfan induced + standard diet; P2 = endosulfan induced + seluang fish diet. $P = 0.000$).

demonstrated the process of nerve cell synapse from rats and parents exposed to endosulfan were not good and would affect brain function. One of the disorders of neurotransmitter synaptic nerve cells is autism in children. A Study from Adamsen et al. [4] explained that serotonin levels in cerebrospinal fluid in autism patients were lower than normal. In this study, the application of endosulfan in pregnant mice made the production of serotonin in the brains of children born decrease. Thus, exposure to endosulfan in pregnant rats may represent model children with autism rats. Brain serotonin levels in the rat's group given the seluang diet increased higher than those in the endosulfan group alone and did not differ greatly from the group fed standard feed. This indicated that the seluang diet can increase brain serotonin levels so that the function of signal transmission to the brain can run better. Seluang contains various amino acids needed to synthesize various brain proteins including serotonin.

The mechanism of brain disorders due to endosulfan induction may be related to oxidative stress. Endosulfan exposure can cause oxidative stress in humans as well as rats [16,17]. Endosulfan can trigger systemic toxicity, reactive oxygen species (ROS) and lipid peroxidation, which can be seen with the increase of malondialdehyde (MDA) [18,19]. The brain consists of phospholipids in the cell membranes so that if damage occurs, it can make some brain disorders [20,21].

In this study, seluang diet was also able to repair damage to brain cells due to exposure to endosulfan through an oxidative mechanism. This was demonstrated by an increase in SOD levels, a decrease in H_2O_2 and MDA. Brain cells damaged by oxidative stress regenerate and eventually are able to secrete serotonin in sufficient quantities to function as a neurotransmitter.

The SOD has a role to neutralize radical oxygen. SOD will help oxygen radical reaction to H_2O_2. If the SOD level is low, the body cannot neutralize the radical oxygen present in the body due to exposure to endosulfan, oxidative stress occurs widely. Other indicators that show that exposure to intrauterine endosulfan can cause oxidative stress in the brains of rats born are levels of H_2O_2 brain. If oxygen radicals are high, the functional SOD will neutralize it to H_2O_2 so that the level of H_2O_2 will also increase. H_2O_2 may result in a chain lipid peroxidation reaction that produces MDA; a marker of lipid damage.

Other meta-analyses showed that in the blood of ASD patients there was a decrease in levels of reduced glutathione (27%), GPx (18%), methionine (13%) and cysteine (14%) and increased concentrations of oxidized glutathione (45%) compared to the control group [22]. In the brain, glutathione is the most abundant antioxidant in the cell and a decrease in the thiol group is characteristic of several neurodegenerative diseases. Glutathione plays a role as a reducing agent and antioxidant [23].

This study showed that seluang diet was able to improve oxidative stress caused by endosulfan induction. Seluang fish contains amino acids cysteine and DHA fatty acids. The amino acid content of cysteine which has a thiol group in seluang fish can act as an antioxidant. This had been proven by a

study from Triawanti et al. [13] malnourished rats given seluang fish showed improvement in oxidative stress conditions. The MDA level decreased, and the enzymatic antioxidant activity was improved. Cysteine levels which act as antioxidants in Seluang fish are 0.32 mg/100 g. Seluang fish also contain glycine and glutamate [12]. In *in vitro*, cysteine plays a role in the binding of various types of free radicals, one of which is OH•, whereas *in vivo*, cysteine together with glycine and glutamate are precursors of glutathione enzymes; enzymes that play a role in reducing oxidation of ROS [24].

Seluang fish also contains DHA with levels of 1.04 g/100 gr [12]. Docosahexaenoic acid (DHA) is one of the unsaturated fatty acids and is an essential fatty acid, which cannot be made by the body so it should be obtained from the outside. DHA is one of the many omega-3 fatty acids found in fish and fish oil. DHA is an essential fatty acid that is needed by the brain because brain cells do not have the enzyme Δ6-desaturase which functions in the synthesis of DHA. DHA can act as an antioxidant [25]. Several studies show that the DHA diet increases the expression of mRNA catalase and glutathione peroxidase (GPx) in the muscles, brain, and liver. DHA inhibits the neurodegenerative process by increasing antioxidant activity [26].

The ability of the diet to neutralize oxidative stress due to endosulfan induction followed by its ability to overcome neurotoxicity was shown by the increase in brain serotonin levels. This indicated the provision of seluang diet did not aggravate the neurotoxicity caused by induction endosulfan can even repair it.

5. Conclusion

According the data, we concluded that endosulfan exposure in pup's brain can trigger some oxidative stress and neurotransmitter disorders. The seluang fish diet can increase brain and serum serotonin level, SOD level and decrease H_2O_2 and MDA level in endosulfan-induced pup's brain. Seluang fish diet could improve and didn't aggravate neurotoxicity that caused by endosulfan-induced pups.

Declarations

Ethics approval and consent to participate

This research had been received approval from the ethics committee No. 468/KEPK-FK UNLAM/EC/VII/2017 of the Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, Indonesia.

Conflict of interests

None of the authors had any conflict of interests to declare.

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Authorship

Triawanti formulated the research questions, designed the study, nutritional and biochemical consultant, and carried it out. Meitria Syahadatina Noor and Didik Dwi Sanyoto contributed in experimental laboratory procedure. Hendra Wana Nur'amin contributed in statistical analysis and manuscript. All authors contributed in writing the article and approved it before submission.

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