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RESEARCH ARTICLE

Effects of chlorpyrifos on histopathological biomarkers of the freshwater teleost *Oreochromis niloticus*

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Abstract. An attempt was made to evaluate histopathological changes in the gills, liver, and kidney tissues of the freshwater teleost *Oreochromis niloticus* as biomarkers of chlorpyrifos toxicity. An experiment was conducted in glass aquaria with *O. niloticus* exposed for 28 days to sub-lethal concentrations of chlorpyrifos of 0.0 µg L^{-1} (control), 10 µg L^{-1}, and 20 µg L^{-1}. Chlorpyrifos caused distortions of the primary gill lamellae structure, the curling of secondary lamellae, desquamation, and epithelial hyperplasia on secondary gill filaments. Hepatocyte vacuolation and nuclear membrane necrosis were found in the liver. Additionally, the shrinkage of the glomeruli, thickening of Bowman's capsule, glomerular and tubular necrosis, intracytoplasmic vacuoles, and hyaline degeneration in the renal tubule were seen in the kidney of *O. niloticus* exposed to chlorpyrifos. No remarkable lesions were observed in the control group. The severity of lesions in the fish tissues was assessed using the standard semi-quantitative grading system. The degree of histopathological lesions in various fish tissues was found to increase with chlorpyrifos concentrations. Histopathological lesions appeared to be good biomarkers of chlorpyrifos toxicity.

Keywords: histopathology, biomarkers, tissues, organophosphate, fish

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Introduction

The use of chemical pesticides has become increasingly widespread in agriculture and households around the world in recent decades (Majumder and Kaviraj 2022). Organophosphates, which are employed not just as insecticides but also as acaricides, nematicides, herbicides, fungicides, and chemosterilants, and currently they account for 50% of global insecticide use (Casida and Quistad 2004). Despite the fact that most organophosphorus pesticides are considered to be less persistent, there have been instances of organophosphate residues having negative impacts on soil and aquatic biota (Bernabó et al. 2011, Das Gupta et al. 2011, Demetrio et al. 2014, Majumder and Kaviraj 2019, Raibeemol and Chitra 2020). Organophosphate is a strong neurotoxicant (Deb and Das 2013, Majumder and Kaviraj 2019) that works inside the body of organisms by attaching irreversibly to the serine hydroxyl group of acetylcholinesterase, rendering it inactive (Oruç 2010). When acetylcholinesterase is inhibited at neuromuscular junctions and cholinergic synapses, acetylcholine accumulates, disrupting nerve impulse propagation and interfering with nervous system energy consumption (Thompson and Richardson 2004, Da Cuna et al. 2011).

The study of histopathological biomarkers has long been considered a sensitive, rapid, and authentic tool in environmental monitoring because it permits looking into specific target organs that are responsible for vital functions (Bernet et al. 1999, Farhan et al. 2021). The chemical contaminants present in water bodies are most likely to cause pathologic lesions in different organs of fishes (Bernet et al. 1999). In fishes, gills remain in close contact with the surrounding aquatic environment and are essential for respiration, osmoregulation, and the excretion of nitrogenous waste products. Lesions in fish gills may reflect the extent of aquatic pollution (Stalin et al. 2019). Thus, gills are considered as the primary markers for aquatic pollution (Bernet et al. 1999). On the other hand, pesticides entering the body are eventually transported to the liver, bio-transformed and detoxified in the form of water-soluble substances, and finally excreted (Hodgson and Goldstein 2001). The liver is also one of the organs used as a promising biomarker of toxic injury and contaminant exposure (Stentiford et al. 2003). Similarly, the kidneys play an important role in the maintenance of homeostasis in an organism by regulating extracellular fluid volume and the acid-base balance (Bernet et al. 1999, Pal et al. 2012). Thus, any sort of assault by a pollutant can hamper their normal function and cause temporary or permanent disruption of body homeostasis.

Several researchers documented the histopathological changes in different organs of freshwater fishes from organophosphate exposure: chlorpyrifos on *Cyprinus carpio* L. (Pal et al. 2012), *Anabas testudineus* (Bloch) (Velmurugan et al. 2020), *Channa punctatus* (Bloch) (Stalin et al. 2019); *Oreochromis mossambicus* (Peters) (Subburaj et al. 2020), *Pseudetroplus maculatus* (Bloch) (Raibeemol and Chitra 2020), *Heteropneustes fossilis* (Bloch) (Mishra et al. 2022), *Oreochromis niloticus* (L.) (Soum et al. 2022, Hossain et al. 2022); from dichlorvos on *Cirrhinus mrigala* (Hamilton) (Velmurugan et al. 2009); from monocrotophos on *C. mrigala* (Velmurugan et al. 2007); and from quinalphos on *C. carpio* (Chamarthi et al. 2014). However, the extent of histopathological alterations in different fish tissues may change with respect to species-specific variation in fishes because of differences in their tolerances of pollutants and variation in their structural organizations (Majumder and Kaviraj 2022).

Nile tilapia (*O. niloticus*) is a commonly cultured freshwater fish throughout Asia and Africa. It is considered a suitable model organism for toxicological research owing to its omnivorous feeding habits, fast growth rate, prolific breeding ability, hardy nature, ability to endure a wide range of climates, high market demand, and, above all, ease of maintenance in the laboratory (Majumder and Kaviraj 2019, Hossain et al. 2022). Chlorpyrifos is one of the most familiar organophosphates to farmers because it is cheap, widely available in different formulations, and is effective in small amounts (Hossain et al. 2022). In India, it is the second-largest selling organophosphorus agrochemical (Stalin et al. 2019). It has also been documented by several authors to be highly toxic to non-target organisms (Das Gupta et al. 2011, Demetrio et al. 2014, Majumder and Kaviraj 2019). Chlorpyrifos residues have been detected in surface waters (Chowdhury et al. 2012) and in farmed and wild fish (Sun and Chen 2008). The widespread presence of chlorpyrifos in the aquatic environment, as well as its potential to cause stress to aquatic organisms has prompted researchers to evaluate its effects on fishes and other aquatic organisms. In recent years, chlorpyrifos has been found to have negative effects on growth, blood counts, and on different organs of fishes (Majumder and Kaviraj 2019, Hossain et al. 2022). The objective of this study was to evaluate if the effects of chlorpyrifos can be assessed quickly by examining histopathological changes in the gills, liver, and kidney of Nile tilapia, *O. niloticus*.

Materials and methods

Test fish and chemical

O. niloticus fingerlings (total length 4.77±0.25 cm and weight 2.74±0.45 g) were obtained from a local fish hatchery (Naihati, W.B., India). They were

acclimatized to test conditions for two weeks before being used in the bioassay. The fish were fed a balanced diet with 30% crude protein throughout the acclimatization period. Dursban®, a commercial formulation (20% EC) of chlorpyrifos [O,O-diethyl O-3,5,6-trichloro-2-pyridinyl- phosphorothioate], was used as the test chemical.

Static bioassays

Bioassays were conducted in 15 L glass aquaria with five fish and 10 L of deep tube well water stored in an overhead tank (temperature 30 ± 3°C, pH 7.1 ± 0.1; free CO_2 3.42 ± 0.31 mg L^{-1}; dissolved oxygen 6.7 ± 0.2 mg L^{-1}; total alkalinity 125.75 ± 3.27 mg L^{-1} as $CaCO_3$; total hardness 142.33 ± 9.01 mg L^{-1} as $CaCO_3$) and five fish as per standard method (APHA 1995). The aquaria were set up in a randomized block design with three replicates for each of the three chlorpyrifos 20% EC treatments: 0, 10, and 20 μg L^{-1}, reflecting roughly 0, 25, and 50% of the 96 h LC_{50} values of chlorpyrifos 20% EC to *O. niloticus* (42 μg L^{-1}), respectively (Majumder and Kaviraj 2019). The entire experiment was continued for a period of 28 days. Fish excreta were removed from the aquaria daily. Moreover, 20% of the test medium was renewed every seven days with a pulse treatment of chlorpyrifos 20% EC at 20% of the initial nominal concentration. Continuous aeration was maintained to ensure adequate oxygen supply and to maintain ambient water quality in order to avoid a stressful scenario.

Histological studies

At the end of the 28-day exposure, three fish from each experimental group were sampled and euthanized with 3% urethane. The gills, liver, and kidney samples were collected and fixed in aqueous Bouin's fixative overnight for histological studies. Before fixation, gill tissues were decalcified with 5% HNO_3 in 70% alcohol. The tissues were washed in distilled water after fixation, dehydrated in a graded ethanol series, and cleared in xylene. Before being embedded in paraffin blocks, the tissues were infiltrated with paraffin wax for 1.5 hours at 56–58°C. A rotary microtome was used to cut serial transverse sections of tissue blocks at a thickness of 5 μ. Tissue sections were stretched on Mayer's albuminized glass slides. The sections were deparaffinized with xylene and brought to distilled water through a descending series of ethanol and stained with Delafield's Hematoxylin and Eosin stain (Bradbury 1969). After being dehydrated through an ascending ethanol series, the sections were cleared with xylene, finally mounted with DPX (Dibutylphthalate Polystyrene Xylene), and examined under a light microscope.

Semi-quantitative scoring

The severity of lesions in the fish tissues was categorized according to the semi-quantitative scoring approach of Peebua et al. (2006): (-) no histopathological lesions in any field on the slides; (+) mild histopathological lesions found in < 25% of the fields on the slides; (++) moderate histopathological lesions found in >75% of the fields on the slides; and (+ + +) all fields of the slides showed severe histopathological lesions. An importance factor from 1 (minimal pathologic importance) to 3 (marked pathologic importance) was assigned to each change following Bernet et al. (1999).

Results

Histopathological alterations on the gills, liver, and kidney tissues of control and chlorpyrifos treated *O. niloticus* are presented in Figs. 1, 2, and 3. Semiquantitative scoring of lesions of the gills, liver, and kidney tissues of *O. niloticus* after chlorpyrifos exposure for 28 days is shown in Table 1.

Histopathological changes in gills

The normal structure of each gill consists of primary lamellae with a row of secondary lamellae on either

Table 1
Histopathological lesions in different tissues of Oreochromis niloticus exposed to chlorpyrifos

Histopathological lesions	Reaction Pattern	Importance factor	C	CPF1	CPF2
Gills					
Epithelial hyperplasia	P	1	-	++	++
Epithelial hypertrophy	P	1	-	+	++
Lamellar disorganization	R	1	-	+	++
Epithelial lifting	R	1	-	+	++
Rupture of lamellar epithelium	R	2	-	++	+++
Rupture of pillar cells	R	2	-	+	++
Epithelial necrosis	R	3	-	+	++
Hemorrhage	C	1	-	+	++
Liver					
Necrosis of hepatocytes	R	3	-	+	++
Pyknotic nucleus	R	2	-	+	++
Fatty vacuolization	R	1	-	++	++
Kidney					
Glomerular shrinkage	R	2	-	++	++
Expansion of space inside Bowman's capsule	P	2	-	+	++
Nuclear hypertrophy	R	1	-	+	++
Hyaline degeneration	R	2	-	++	++

Histopathologic lesions: (-) absent, (+) mild (< 25% of fields), (++) moderate (> 75% of fields), (+++) severe (all fields). Reaction Pattern: C: circulatory disturbances; R: regressive changes and P: progressive changes, Importance factor: 1 = minimal; 2 = moderate and 3 = marked pathological importance; C: Control; CPF1: Chlorpyrifos 10 μg L^{-1}; CPF2: 20 μg L^{-1}

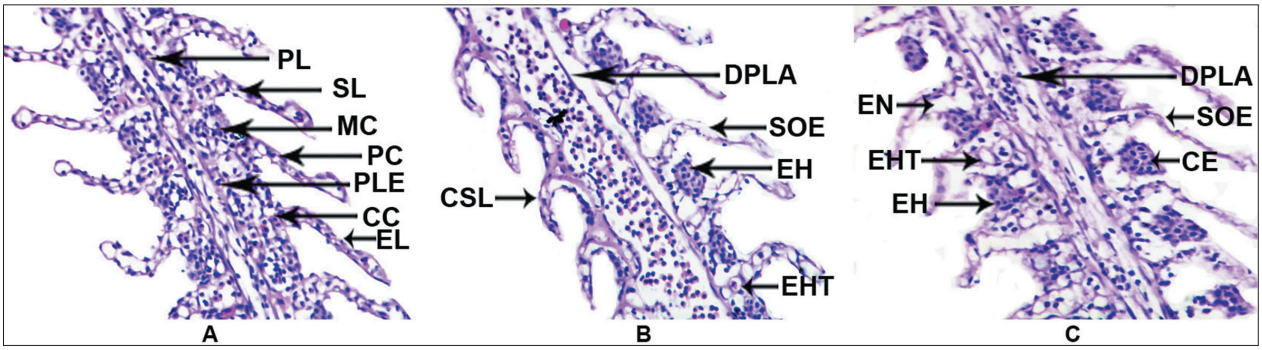

Figure 1. Histology of gills of *Oreochromis niloticus* exposed to chlorpyrifos. **A**. gill of control *O. niloticus* showing primary (PL) and secondary gill lamellae (SL) with normal mucous cells (MC), chloride cells (CC), and pillar cells (PC) with epithelial linings of primary (PLE) and secondary lamellae (EL). **B**. gill of *O. niloticus exposed* to 10 μg L^{-1} chlorpyrifos for 28 days showing epithelial hyperplasia (EH), epithelial hypertrophy (EHT), disorganization of primary lamellar architecture (DPLA), sloughing of outer epithelium of secondary lamellae (SOE). **C**. gill of *O. niloticus* exposed to 20 μg L^{-1} chlorpyrifos for 28 days showing increased epithelial hyperplasia (EH), hypertrophy (EHT), clumped epithelial cells (CE) at the base of secondary lamellae, epithelial necrosis (EN), disorganization of primary lamellar architecture (DPLA), and sloughing of outer epithelium of secondary lamellae (SOE). H & E; X 400.

side. In control fish, squamous epithelial cells covered the surface of primary and secondary lamellae. Prominent mucous cells and blood capillaries were seen in primary lamellae. Secondary lamellae showed extensive vascularization covered with a thin layer of epithelial cells separated by pillar cells. The interlamellar spaces appeared normal (Fig. 1A). At low chlorpyrifos concentrations (10 μg L^{-1}), curling of secondary lamellae, sloughing of epithelial lining, epithelial hyperplasia, and increasing interlamellar space on primary gill filaments were all seen in *O. niloticus* (Fig. 1B). Fish treated with higher chlorpyrifos concentrations (20 μg L^{-1}) showed necrosis on primary gill lamellae, hemorrhage, secondary gill lamellar hypertrophy, rupture of gill epithelium and pillar cells, clumped epithelial cells

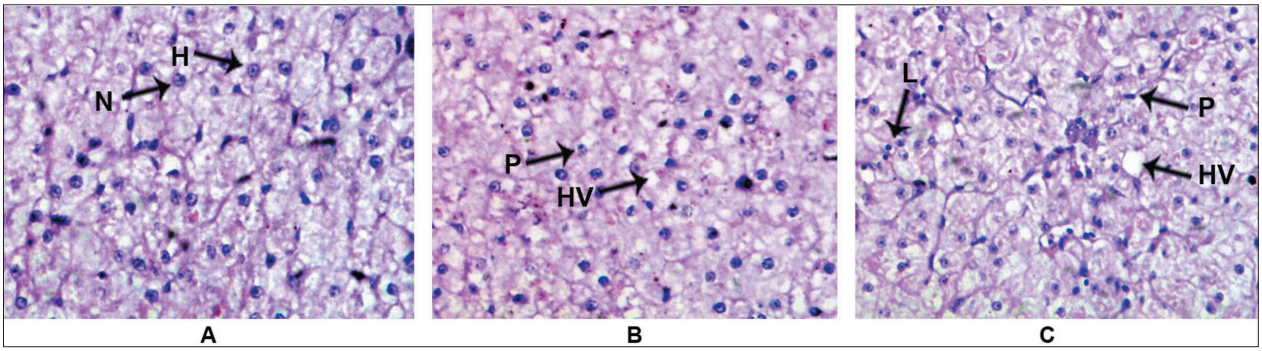

Figure 2. Histology of liver of *Oreochromis niloticus* exposed to chlorpyrifos. **A**. liver of control *O. niloticus* showing polygonal hepatocytes (H) with spherical nucleus (N) and cytoplasmic glycogen and fat droplets. **B**. liver of *O. niloticus* exposed to 10 μg L^{-1} chlorpyrifos for 28 days showing vacuolation of hepatocytes (HV) and pyknotic nuclei (P). **C**. liver of *O. niloticus* exposed to 20 μg L^{-1} chlorpyrifos for 28 days showing increased vacuolation of hepatocytes (HV), pyknotic nuclei (P), and leukocyte infiltration (L). H & E, X 400.

at the base of secondary lamellae, sloughing of gill epithelium, and secondary lamellar shortening (Fig. 1C). Most of the changes in *O. niloticus* gill tissue after chlorpyrifos exposure for 28 days were found to be regressive changes except for epithelial hyperplasia and hypertrophy, indicative of progressive changes, and gill hemorrhage, which indicated circulatory changes. All the gill lesions in both of the chlorpyrifos-exposed groups were common, but the severity of the lesions differed depending on the gradient of chlorpyrifos concentrations.

Histopathological changes of the liver

Normal polygonal hepatocytes with a central spherical nucleus were seen in the control fish. Hepatocytes that were enlarged with glycogen or neutral fat were the most common. The distribution of sinusoids between hepatocytes became irregular (Fig. 2A). Hepatocytic vacuolation and nuclear membrane deformation were clearly apparent in the liver of *O. niloticus* at lower chlorpyrifos concentrations (10 μg L^{-1}) (Fig. 2B). *O. niloticus* subjected to a higher concentration of chlorpyrifos (20 μg L^{-1}) displayed cloudy swelling and vacuolation of hepatocytes, pyknotic nuclei, fatty infiltration, hepatocyte degradation, and necrosis in some portions of the liver (Fig. 2C). All the changes in the hepatic tissue of *O. niloticus* exposed to chlorpyrifos were of the regressive type.

Histopathological changes of the kidney

In control fish, the kidney consisted of numerous renal tubules and Bowman's capsule with well developed glomeruli. The columnar epithelial cells in the proximal renal tubules had a brush border. No brush borders were found in distal tubules or collecting ducts (Fig. 3A). *O. niloticus* from the group treated with the low chlorpyrifos concentration (10 μg L^{-1}) (Fig. 3B) showed distortion of the glomerulus, expansion of the space between the glomerulus and Bowman's capsule, and tubular necrosis in a few portions (Fig. 3B). In addition to these changes, *O. niloticus* treated with a higher chlorpyrifos concentration (20 μg L^{-1}) showed intracytoplasmic vacuoles in the renal tubular epithelium, hyaline degeneration of the tubular epithelium, and dilation of the tubular lumen (Fig. 3C). Regressive changes of a moderate importance factor were noted in the kidney tissue of *O. niloticus* exposed to chlorpyrifos; the exception was the space expansion inside Bowman's capsule, which is of the progressive type.

Discussion

Gill morphology has long been recognised as a valuable indicator of aquatic pollution because of its huge surface area and direct contact with the external environment (Al-Ghanbousi et al. 2012, Xing et al. 2012). Light microscopic observation of the gills

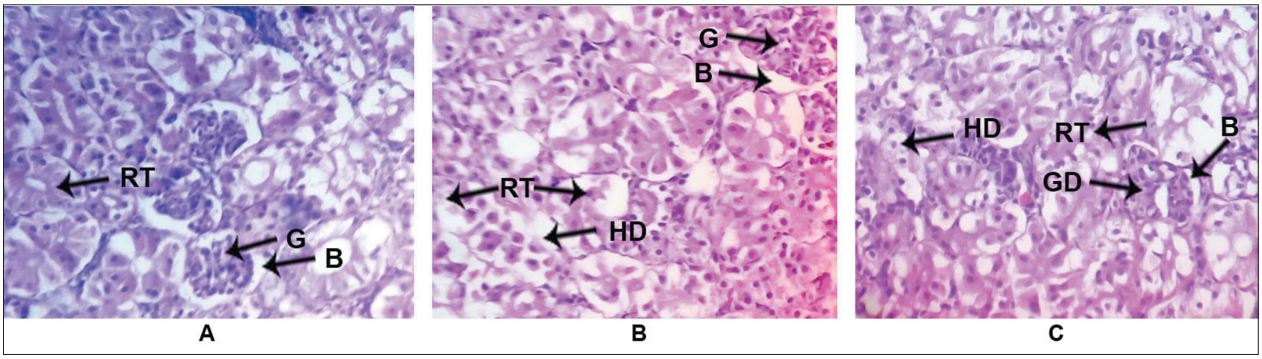

Figure 3. Histology of kidney of *Oreochromis niloticus* exposed to chlorpyrifos. **A**. Kidney of control *O. niloticus* showing normal glomerulus (G), Bowman's capsule (B) and normal renal tubules (RT). **B**. Kidney of *O. niloticus* exposed to 10 μg L^{-1} chlorpyrifos for 28 days showing distortion of the glomerulus (G), expansion of the space between the glomerulus and Bowman's capsule (B), and hyaline degeneration in the renal tubule (RT). **C**. Kidney of *O. niloticus* exposed to 20 μg L^{-1} chlorpyrifos for 28 days showing distortion of the glomerulus (GD), thickening of Bowman's capsule (B), intracytoplasmic vacuoles, and hyaline degeneration (HD) in the renal tubular epithelium (RT). H & E, X 400.

of *O. niloticus* treated with chlorpyrifos in the present study demonstrated increased interlamellar space in the primary lamellae, epithelial hypertrophy and hyperplasia, epithelial lifting, secondary lamellar fusion, general necrosis, and edema in some portions. Similar histopathological changes in gills from chlorpyrifos exposure were reported by Pal et al. (2012), Xing et al. (2012) and Hossain et al. (2022). Epithelial lifting and hyperplasia may increase the distance between toxicants present in the external environment and the vascular area of gills. Consequently, it acts as a primary defense strategy to prevent toxicants from entering the gills. Similarly, secondary lamellar fusion is thought to be a stress-protective response in fish (Borg and Trombetta 2010). Richmonds and Dutta (1989) opined that pesticide-induced necrosis and epithelial cell rupture can result in hypoxia and respiratory failure. The collapse of pillar cells and the rupture of blood vessels, culminating in the release of blood via the outward pushing lamellar epithelium, is one of the most obvious chlorpyrifos-induced histopathological alterations in the gill (Pal et al. 2012).

The liver is regarded as the organ responsible for detoxification as it can break down metabolically toxic chemicals coming from a certain level, but if this level is exceeded, then the toxic chemicals adversely affect the liver's regulating mechanism and ultimately lead to structural changes in the it (Cengiz et al. 2017). Thus, histopathological changes in the liver provide valuable information about the health status of the stressed fish. The most notable histopathological changes of hepatic cells in the current study included cloudy swelling and vacuolation of hepatocytes, necrosis, hepatocyte dissociation, and fatty infiltration. Similar changes in the liver were also observed by Pal et al. (2012) and Hossain et al. (2022). Singh and Singh (2008) and Ayoola and Ajani (2008) revealed a loss of balance between the rate of synthesis and the rate of release of substances within hepatocytes, which is indicative of fatty degeneration (Singh and Singh 2008, Ayoola and Ajani 2008).

Notable histopathological changes found in *O. niloticus* in the present study, such as glomerular shrinkage, increased space between the glomerulus and Bowman's capsule, cytoplasmic vacuoles in the renal tubule, and dilation of tubular lumen resemble the effects of chlorpyrifos in *C. carpio* (Pal et al. 2012). Pesticide pollution affects the kidney indirectly through the circulatory system. High concentrations of chlorpyrifos may lead to glomerular degeneration that ultimately affects filtration rates in fishes (Pal et al. 2012). Hypertrophy seen in the tubular epithelium of fishes may possibly be due to hyperactivity to overcome chlorpyrifos-induced stress. Intoxication and degenerative processes can result in

necrosis in the renal tubule, which could lead to metabolic abnormalities in fishes (Subburaj et al. 2020).

Conclusion

The results of the present microscopic examination clearly indicate that chlorpyrifos in minute concentrations can cause histopathological alterations in different vital organs in the fish and ultimately have some adverse effects on fish physiology. In addition, a direct correlation was observed between the concentration of pesticide and the severity of histopathological alterations in the fish. Therefore, histopathological changes can be used as excellent biomarkers for early detection of insecticide pollution in aquatic ecosystems. Proper precautions should be included in pesticide monitoring programs and before the application of this pesticide in the environment.

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Conflict of interest. The author declares no conflicts of interest.

Ethical Statement. Animal care was done in accordance with the University of Kalyani's animal care protocols.

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