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ORIGINAL ARTICLE

Larval distribution, migratory pattern and histological effects of *Toxocara canis* in *Rattus norvegicus*

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Abstract The common dog roundworm *Toxocara canis* can infect other animals and humans which may act as their paratenic and accidental hosts, respectively. Larvae do not further develop to adult worms in these hosts. Instead, they undergo migration to various body organs, causing the neglected parasitic disease known as toxocariasis. Although rats are considered as potential paratenic hosts of the parasite, there are only few studies which utilized *Rattus norvegicus* (Sprague–Dawley strain) for experimental infections involving toxocariasis. This study aimed to determine whether *T. canis* could establish in Sprague–Dawley rats artificially infected with 500 *T. canis* embryonated eggs and if the animals can be used as animal models for toxocariasis. Following squash method and tissue digestion, larvae were recovered from the lungs, liver and brain of the infected rats. Furthermore, gross examination of organs revealed macroscopic lesions and hemorrhages in the lungs and brain. Microscopically, accumulation of inflammatory cells, thickening of alveoli lining and destruction of bronchial walls and hepatic necrosis were observed. This study showed that *T. canis* has established in Sprague–Dawley rats and could serve as a model for *Toxocara* infection studies.

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Introduction

Toxocariasis is a zoonosis caused by the roundworms of cats and dogs, *Toxocara cati* and *T. canis*, respectively (Fan et al. 2004). The parasite is not only present worldwide in its definitive host; it also occurs in different animal hosts, including humans (Strube et al. 2013).

Unembryonated eggs are passed by infected dogs into the environment during defecation. Optimal temperatures and humidity allow these eggs to embryonate, making them infectious to final, paratenic and accidental hosts (Fan et al. 2004). Transmission to humans normally occurs through accidental ingestion of embryonated eggs from contaminated soil (Khademvatan et al. 2013), from food contaminated with eggs or from ingestion of parasitized tissues of paratenic hosts. Upon ingestion, embryonated eggs hatch into larvae which may migrate to different tissues for months or even years, causing inflammatory reactions in the affected organ (Roldán and Rubinsky-Elefant 2012).

Although many cases of toxocariasis in humans are asymptomatic, this disease may manifest clinically in four forms based on the organs and tissues affected, and these systemic infections may be attributed to the size of inoculum and the host response against migrating larvae (Pawlowski 2001). These are visceral larva migrans, ocular larva migrans, neurological toxocariasis, and covert toxocariasis (Magnaval et al. 2001; Despommier 2003; Roldán et al. 2010).

Several experimental animals have been used to study *T. canis* larval migration in paratenic hosts. Rodents, most especially mice, are often used in these studies as they

usually harbor the parasites, may become reservoirs of infection for other possible hosts and show a migratory pattern and distribution of *Toxocara* sp. like that in humans (Janecek et al. 2014). On the other hand, rats are relatively overlooked animal models for observing larval migration as well as pathological and behavioral changes caused by *T. canis* (Strube et al. 2013). This study aimed to determine the migratory pattern and pathological changes caused by *T. canis* larvae in tissues *R. norvegicus* (Sprague–Dawley strain).

Materials and methods

Ethical considerations

Rats were maintained and infected according to accepted standards of laboratory animal management and experimentation (AVMA 1993, as cited by Cardillo et al. 2009). The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of the Philippines Los Baños with assigned protocol number 2015-0051.

Inoculum preparation

Adult female *T. canis* worms were collected from naturally infected dogs following treatment with pyrantel or piperazine. The female worms were dissected longitudinally and their uteri were isolated and squeezed to obtain the eggs (Alcântara-Nevez et al. 2008). The eggs were then placed in 2% formalin and incubated for 35 days until embryonation. Larval development in eggs was observed and confirmed through microscopy (Havasiová-Reiterová et al. 1995). The embryonated eggs were then washed three times with normal saline to remove formalin, followed by successive sedimentation by centrifugation (Cardillo et al. 2009).

Artificial infection

Twenty-four male *Rattus norvegicus* (Sprague–Dawley strain), aged 5–8 weeks were used in the study. Seven groups of three rats were randomly selected for artificial infection while the remaining group was designated as the controls. Rats were placed under deprivation diet 12 h before inoculation, and then were artificially infected with approximately 500 embryonated *T. canis* eggs orally using a silicone-coated syringe. A small amount (0.5 ml) of distilled water was also delivered to the rats to wash off the remaining eggs in the syringe. The remaining group of three rats served as the uninfected control and received 1 ml distilled water in a similar manner (Cardillo et al. 2009; Pineda and Ramos 2012).

Collection of samples

Groups of three rats were anaesthetized and euthanized at 3, 5, 8, 10, 15, 30 and 60 days post infection (DPI). The control group was also euthanized at 60 DPI. The following organs and tissues were collected: brain, liver, lungs, kidneys and skeletal muscles (masseter, pectoral, pelvic and limb muscles) (Taira et al. 2013). Each sample was divided into two halves, one half for artificial digestion and the other half for histological examination (Cardillo et al. 2009). All organ samples subjected to artificial digestion were completely digested.

Processing of samples

Brain samples obtained from the rats were observed for presence of larvae through squash method. Each brain sample was divided into small portions for more efficient and thorough observation. Each portion was squashed thinly between a glass slide and a coverslip and observed under 100 × and 400 × magnification. The other organs were cut into small pieces and placed in digestive solution (pepsin, 5 g; 37% HCl, 10 ml in 1000 ml distilled water). The mixture was digested using a magnetic stirrer (IKA® CMAG HS 7) set at 37 °C to recover the remaining larva. Sedimental liquid was poured in a centrifuge tube and centrifuged for 2 min at 1500 rpm. The sediment was successively washed using normal saline solution until the supernatant was clear. The supernatant was poured out, leaving only the sediments, which was observed under compound light microscope to check for the presence of larvae at 100 × and 400 × magnification.

Gross and histological examination

Organs were first examined macroscopically at necropsy. The organs were then placed in neutral buffered 10% formalin for fixing and processed for histological sectioning. Briefly, tissues were dehydrated with increasing grade of ethanol and embedded in paraffin wax. Tissue sections were cut at 5 µm using microtome. These sections were stained using haematoxylin and eosin (Cardillo et al. 2009). Processed slides were viewed under compound light microscope at 100x and 200x magnification and analyzed for possible pathological changes.

Statistical analysis

Mean number of larvae per organ, recovery rate as the percentage of total larvae recovered (%), and organ

distribution (%) of larvae were used to present data on larval counts. Shapiro–Wilk test of normality was used to verify the distribution of data on larval counts at different DPI. Data which were not normally distributed were analyzed using Kruskal–Wallis test to determine larval recovery and migration patterns into different organs or tissues at different time points. Differences were significant if $p < 0.05$.

Results

Larval migration

Total larval recovery was low at only up to 0.6%. Larvae were recovered from the brain, liver and lungs from the 5 to 15 DPI while no larvae were recovered from the kidneys and muscles of the infected rats (Figs. 1, 2). The number of larvae recovered from the lungs ($X^2 = 4.115$, $p = 0.661$), liver ($X^2 = 4.930$, $p = 0.553$), brain ($X^2 = 4.926$, $p = 0.553$), kidneys ($X^2 = 0.000$, $p = 1.000$), and muscles ($X^2 = 0.000$, $p = 1.000$) revealed no significant differences among post-inoculation days. Furthermore, larvae were not also recovered from any tissues of the control rats.

Pathological observations

Severe haemorrhages were visible on the surface of lungs at 5 and 8 DPI. Small areas of lung consolidation were also observed in 15 and 30 DPI but these appeared occasionally. Starting at 3 DPI, all infected rats displayed inflammatory reactions in the lungs characterized by enlargement of alveoli and thickening of alveolar septa, accumulation of inflammatory cells which are composed of lymphocytes, eosinophils, neutrophils and macrophages (Fig. 3b). There was also occurrence of hemorrhagic areas on 3–15 DPI. Many bronchi displayed destruction of epithelium, while a few exhibited mucus secretions in the lumen on 10 and 15 DPI (Fig. 3c).

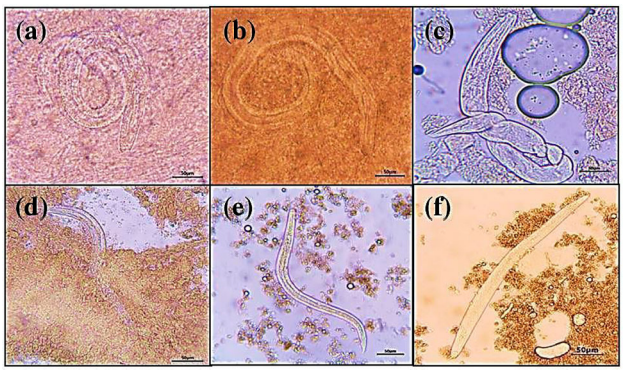

Fig. 1 *Toxocara canis* larvae recovered from **a**, **b** brain, **c**, **d** liver and **e**, **f** lungs of infected rats by squash method or artificial tissue digestion

In the brain, a macroscopic lesion was observed in 1/2 rats at 60 DPI. Microscopically, infected rats presented variable-sized cavities filled with blood starting at 8 DPI (Fig. 4a). Trapped larvae were also seen in some of the brain section (Figs. 4b, c). However, no evident surrounding inflammatory response was elicited by these larvae.

No macroscopic differences were noticed in the liver of all uninfected and infected rats. Liver of uninfected rats showed the normal histological structure of the hepatic tissue, composed of arranged cords of hepatocytes, sinusoidal capillaries, central vein and portal triad while microscopic observation of the liver of infected rats revealed areas of inflammatory infiltrates at 10 and 15 DPI (Fig. 5b, d). Additionally, 1/3 infected rats on day 15 PI showed degeneration and necrosis of hepatocytes (Fig. 5b).

Lastly, the kidneys and the muscles did not present any macroscopic alterations for both control and infected rats. Histological sections of kidneys and muscles observed in both uninfected and infected rats are characterized by normal structure of glomerular capillaries surrounded by renal capsules and muscle fibers although some kidney sections of infected rats displayed congestion brought about by inflammatory infiltrates, destruction of kidney corpuscle structure and hemorrhage (Fig. 6b).

Discussion

The migratory route of *T. canis* larvae has been observed in a variety of experimental animals by other authors, the mouse being the most commonly utilized (Strube et al. 2013). According to Abo-Shehada and Herbert (1984), after penetrating the intestinal wall, larvae first enter a hepato-pulmonary phase where larvae reach the liver, and then the lungs. From there, larvae are distributed throughout the body. Myotropic-neurotrophic phase of migration marks the end of the visceral phase, when larvae already accumulate into the brain and muscles. In the present study, hepato-pulmonary and neurotrophic phases of *T. canis* larval migration in Sprague–Dawley rats have been observed, larvae being recovered from the liver, lungs and brains of the infected rats. However, the difference can be noticed in terms of the organs first invaded by the larvae. The larvae were first observed in the lungs before they were observed in the liver, as opposed to the usual route in mice which is from the liver to the lungs. A review by Othman (2012) stated that there are many disputes concerning the migratory route and accumulation of *T. canis* larvae in different organs of experimental animals. To reconcile these arguments, it was just assumed that the

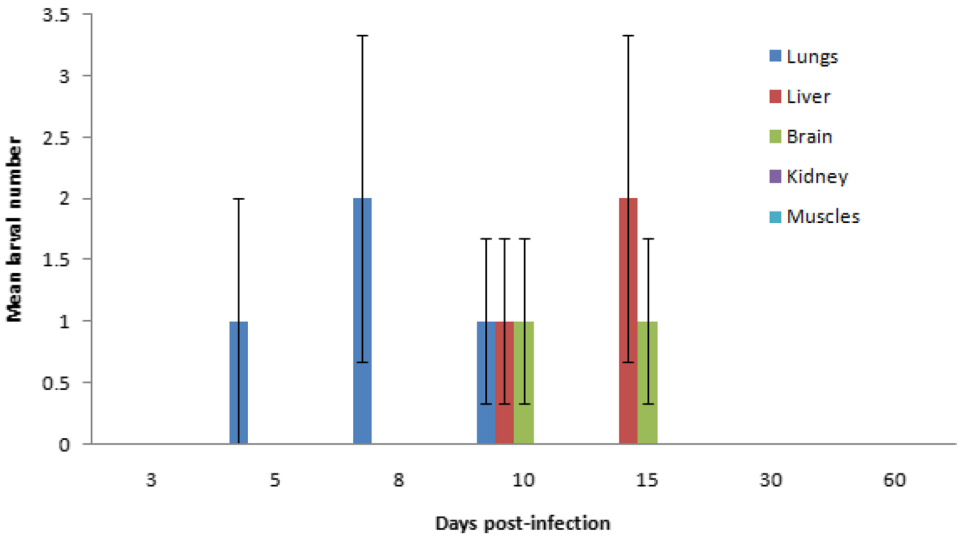

Fig. 2 Mean number of *T. canis* larvae recovered from different organs of rat infected with 500 *T. canis* embryonated eggs

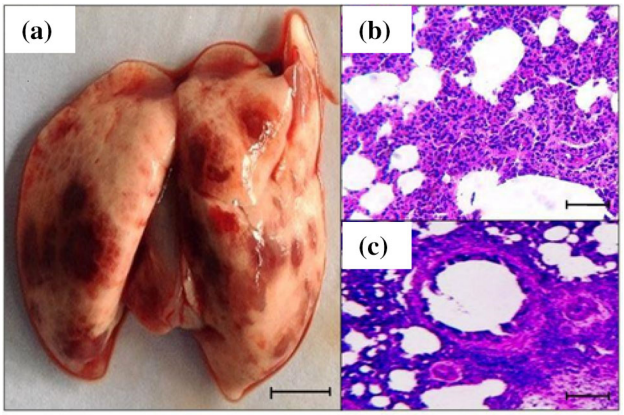

Fig. 3 Macroscopic and histological changes in the lungs of *T. canis*-infected rat. **a** Hemorrhagic lesions in the lungs of rat; scale bar = 4 mm. **b**, **c** Air sacs separated by thick alveolar lining and destroyed epithelium of a bronchiole respectively. H&E staining; scale bar = 50 µm

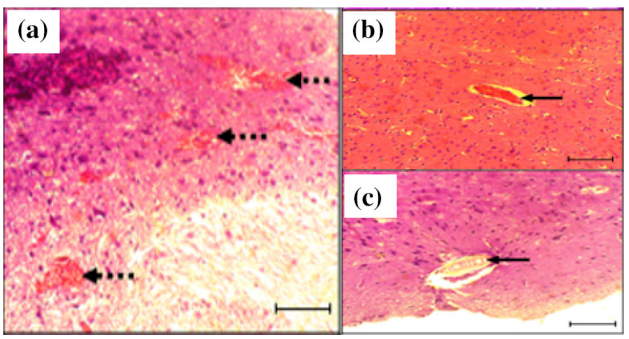

Fig. 4 Histological changes in the brain of *T.canis*-infected rat. **a** Visible hemorrhages in the brain on day 8 PI (dotted arrows), **b**, **c** *T. canis* larvae in the brain (solid arrows). H&E staining; scale bars = 50 µm

larvae undergo continuous larval migration and redistribution which depends on the paratenic host, and not just a

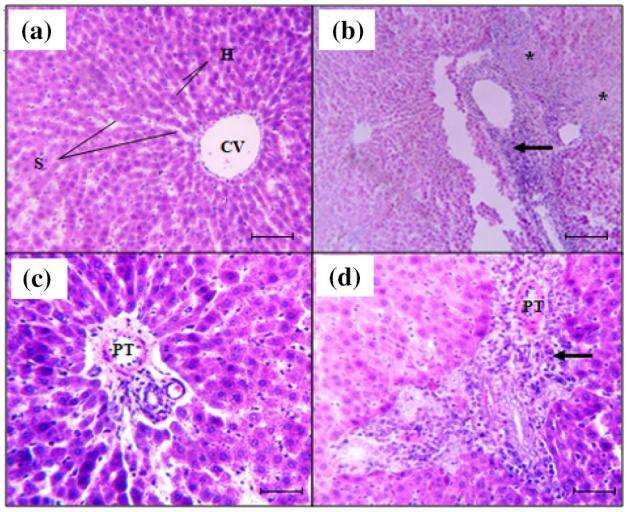

Fig. 5 Histological changes in the liver of *T. canis*-infected rat. **a**, **c** Liver of uninfected rat showing the normal histological appearance of liver parenchyma and portal triad, **b**, **d** inflammatory cell aggregates (black arrows) and necrosis of hepatocytes (asterisks) H&E staining; scale bar = 50 µm; CV = central vein, PT = portal triad, H = hepatocytes, S = sinusoids

simple one-way process from the intestine to the liver, lungs and then to the brain and muscle. Hence, it can be concluded that the deviation in the migration of the larvae in the rat from the above mentioned usual migratory route is normal since migration routes and predilection sites of *Toxocara* spp. are host species dependent (Strube et al. 2013). Moreover, it was possible that instead of passing to the hepatic portal system to rapidly invade the liver, the larvae might have reached the lung first through the thoracic duct and the heart, thus bypassing the liver and then eventually entering the systemic circulation again to be distributed to other organs, one of which is the liver (Abo-Shehada and Herbert 1984; Abo-Shehada et al. 1984).

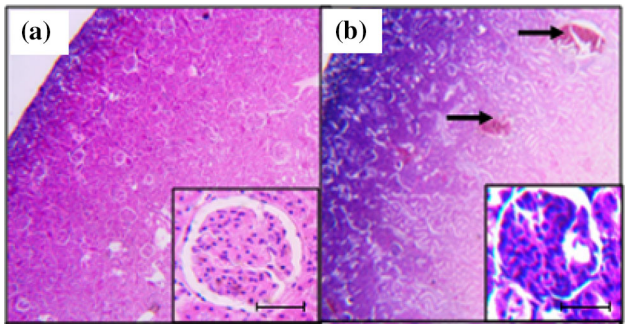

Fig. 6 Histological changes in the kidneys and muscles of *T.canis*-infected rat. **a** Kidney of uninfected rat showing the normal histological appearance of kidney. **b** Kidney of infected rat on 15 DPI showing haemorrhages (black arrows) and irregular glomerular morphology (inset)

The results of the study is similar to that of Zibaei et al. (2010) using a dose of 2500 *T. cati* eggs. There were no larvae recovered from the kidney and muscles of the rats. Furthermore, less larval recovery was also observed in the liver than the lungs at 5 DPI, as with the present study. On the other hand, the results of this present study did not coincide with some authors such as Lescano et al. (2004) who reported a high number of larvae in the lungs at 5 DPI in rats (Wistar strain) inoculated with the same dose of *T. canis* embryonated eggs, and Santos et al. (2009) who also found a high concentration of larvae in the said organ of rats (Wistar strain) as early as 3 DPI. Both authors also reported an increase in larval recovery in the brain starting at 15 DPI and lasting to 30 DPI, while this was not the case in the present study. Persistence of larvae in the muscles in rats and mice during the entire observation period was also noted (Lescano et al. 2004; Cardillo et al. 2009; Santos et al. 2009; Janecek et al. 2014). Other organs such as the kidneys contributed to the total recovered larvae based on the findings of Lescano et al. (2004) and Santos et al. (2009).

Overall, the present study revealed recovery rates of up to 0.6% which was low compared to previous studies involving *T. canis* migration in experimental animals (Janecek et al. 2014; Havasiová-Reiterová et al. 1995). Several factors have been considered as possible reasons for this. Generally, a small infection dose like the one used in the present study leads to a low percentage of larvae (Camparoto et al. 2008). This claim was supported by Havasiová-Reiterová et al. (1995) who used varying doses of *T. canis* eggs in mice and obtained 10–25.7% larval recoveries at doses of 5 or 7 eggs respectively, but a 36.4% larval recovery at a dose of 1000 eggs, showing a directly proportional dependence between larval burdens and infective dose. Furthermore, a dose of approximately 1000–5000 *Toxocara* eggs administered to chicken, gerbils and mice resulted to larval recovery rates of up to 46.5% (Gargili et al. 1999; Janecek et al. 2014; Zibaei et al. 2010). Moreover, Kayes and Oaks (1976) stated that there exists a dose–response effect during *T. canis* larval migration in mouse where larger inoculum dose resulted to heavier *T. canis* infection which was not the case as the present study using a rat model. Furthermore, less recovery rate of larvae could be explained because many embryonated eggs could have passed unhatched through the feces, compared with rodent model studies in which *Toxocara* eggs were given previously decorticated (Zibaei et al. 2010; Resende et al. 2015). This was done to mimic the natural state by which *Toxocara* eggs were ingested from the environment. Other organs or tissues such as the intestines, eye, spleen, heart, salivary glands and genital organs that were not included in the current study were demonstrated by other authors to harbor migrating larvae of the parasite, thereby affecting larval recovery (Abo-Shehada and Herbert 1984; Takayanagi et al. 1999; Santos et al. 2009; Taira et al. 2013; Sommerfelt et al. 2014).

Extensive macroscopic hemorrhages were observed in the lungs of the infected rats similar to what was reported by Cardillo et al. (2009) and Janecek et al. (2014) in mice infected with either *T.canis* or *T. cati* respectively and with *T. cati* in pigs (Sommerfelt et al. 2014). Immunological aberrations, especially the presence of inflammatory cells and destruction of airways coincide with those observed by other authors in various animals including mice, chicken, rats and pigs (Pinelli et al. 2006, 2007; Cardillo et al. 2009; Oryan et al. 2009; Al-Quraishi 2013; Sommerfelt et al. 2014; Resende et al. 2015). According to Bowman et al. (1987) and Pinelli et al. (2006), the presence of persistent pulmonary inflammation, eosinophilia and increased IgE production in the absence of *Toxocara* larvae is due to high levels of circulating antigens of the parasite that remain in the circulation even after infection.

In the brain, few lesions were observed in the rat as opposed to Cardillo et al. (2009) and Janecek et al. (2014) who reported several superficial hemorrhagic foci that appeared on the brain of mice during the first week of infection. These lesions may have been brought by bleeding and injuries inflicted by larvae that penetrated out of arteries on the brain surface (Bisseru 1969) Evidence of active larval migration in the brain which include areas of hemorrhages and larval sections with no cellular reaction around them were detected histologically. These findings were similar with those of Bisseru (1969) and Resende et al. (2015) who also found very mild inflammatory response in the brain. According to Bisseru (1969), *T. canis* larvae do not accumulate intentionally in the brain yet are unable to leave and are trapped in the organ because of their size that approximate those of the brain arteries. Interestingly, the brain is considered as a site of "immunological privilege" wherein larvae are not or rarely

enclosed by the inflammatory cells compared in other organs. This less immune reactivity may explain the neuroaffinity of *T.canis*. Furthermore, the brain serves to act as a reservoir of the parasite and the usual occurrence of the larvae in the brain of its host is presumed to play an important source of infection to other animals and humans, the rat acting as a paratenic host (Dunsmore et al. 1983).

Oryan et al. (2009) and Bisseru (1969) found white multifocal nodules, representing larval encystment in the liver of infected chickens and mice respectively. Moreover, granuloma formation around or close to these encysted larvae have been frequently noted by Demirci et al. (2006). These observations, though, were not observed in the present study. Histologically, foci of inflammatory cells and necrosis of liver cells were usually seen among hepatocytes and surrounding portal tracts coinciding with the findings of other authors (Cardillo et al. 2009; Janecek et al. 2014; Sommerfelt et al. 2014). According to Bisseru (1969), the pathological lesions indicate active larval migration in the liver.

Kidneys and the muscles of infected rats present absence or few changes compared to the controls, whereas in other studies, congestion brought about by inflammatory infiltrates were seen in the kidneys, while degenerated muscle fibers and aggregation of neutrophil and eosinophil were observed between muscle fibers in infected rodents (Cardillo et al. 2009; Al-Quraishi 2013). Concentration of *Toxocara spp.* larvae in the kidneys and muscles of experimental animals seemed to have a direct proportionality with time, which is increasing larval recovery from the kidneys and muscle later post infection (Taira et al. 2013).

In the present study, larvae were recovered from various organs of the infected rats. This indicates that *T. canis* had undergone body migration in the animal. The presence of migrating larvae in selected organs of the infected rats and the observed gross and histological changes in the tissues attributed to these migrating larvae confirm that the Sprague–Dawley rats could act as paratenic host for *T. canis* and can be used as animal model for toxocariasis. Further studies may be done to verify if same observations hold true in other strains and species of paratenic hosts and explore factors that might affect larval migration of the parasite.

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Author contributions Conceived and designed the experiments: VGVP, KKRL, CMB; Performed the experiments: KKRL, CMB; Data analysis: KKRL; Contributed reagents/materials/analysis tools: VGVP, CMB, KKRL; All authors participated in writing the final paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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