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ORIGINAL ARTICLE

Prenatal Isoflurane Exposure Induces Developmental Neurotoxicity in Rats: the Role of Gut Microbiota

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Abstract

Fetal exposure to inhaled anesthetics, such as isoflurane, may lead to neurodevelopmental impairment in offspring. Yet, the mechanisms of prenatal isoflurane–induced developmental neurotoxicity have not been fully elucidated. Gut microbiota is a pivotal modulator of brain development and functions. While the antibiotic effect of isoflurane has been previously investigated, the relationship between prenatal isoflurane exposure and postnatal gut microbiota, brain biology, and behavior remains unknown. In the present study, we treated pregnant rats with 2% isoflurane for 4 h on gestational day 14. Their offspring were tested with novel object recognition task on postnatal day 28 (P28) to assess cognition. Fecal microbiome was assessed using 16S RNA sequencing. We also analyzed hippocampal expression of brain-derived neurotrophic factor (BDNF) in P28 rat brains. To further explore the role of gut microbiota on prenatal isoflurane–induced developmental neurotoxicity, we treated rats with mixed probiotics on P14 for 14 days and evaluated novel object recognition and hippocampal expression of BDNF on P28. Results indicate that prenatal exposure to isoflurane significantly decreased novel object recognition (novel object preference ratio: mean difference (MD) − 0.157; 95% confidence interval (CI) − 0.234 to − 0.080, $P < 0.001$) paralleled by diminished expression of hippocampal BDNF in juvenile rats. Prenatal exposure to isoflurane also significantly altered the diversity and composition of gut microbiota. Treatment with probiotics mitigated these changes in cognition (novel object preference ratio: isoflurane group vs. control group: MD − 0.177; 95% CI − 0.307 to − 0.047, $P = 0.006$; probiotic group vs. isoflurane group: MD 0.140; 95% CI 0.004 to 0.275, $P = 0.042$) and BDNF expression. Taken together, our findings suggest that gut dysbiosis may be involved in the pathogenesis of maternal isoflurane exposure–induced postnatal cognitive impairment. To determine the causal relationship between gut microbiota and cognition in prenatal anesthetic–induced developmental neurotoxicity, further studies are needed.

Keywords Isoflurane · Anesthetic neurotoxicity · Gut-brain axis · Gut microbiota

Introduction

With recent advances in medical and surgical technologies, more pregnant women are receiving semi-elective and emergent non-obstetric or open fetal surgery that requires general anesthesia. The developing fetal brain undergoes rapid and dramatic changes involving neurogenesis and synaptogenesis. More importantly, fetal brains are highly sensitive to environmental factors during this period. Whether maternal anesthetic administration can induce cognitive impairment in the fetus is a significant concern for clinicians. In fact, the United States Food and Drug Administration (2017) issued a warning that the repetitive or lengthy application of general anesthetic in pregnant women may affect the neurodevelopment of children's brains. A systematic review with meta-analysis found consistent results in 65 preclinical studies and concluded the presence of prenatal anesthesia–induced impairment of learning and memory in offspring of exposed laboratory animals (Bleeser et al. 2021). However, human studies are difficult to design and conduct. To our knowledge, as of this writing, there is only one published clinical observational study, which concluded that children exposed to anesthesia prenatally appear to present externalizing behaviors in childhood (Ing et al. 2021). Thus, the mechanism and

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treatment for prenatal exposure to anesthetic–induced developmental neurotoxicity are still not fully elucidated nor are the phenotype for anesthetic toxicity in humans established. While the question remains of whether results of animal studies are translatable to humans, animal research is an efficient and valuable way to identify the potential mechanism and treatment for prenatal anesthetic neurotoxicity. Moreover, due to the similarity in cytoarchitectonic organization between rodents and humans, rodents are considered a powerful model for studying developmental anesthetic neurotoxicity (Chinn et al. 2020).

Most previous studies have focused on the effect of maternal anesthesia on fetal neurologic function within the central nervous system (CNS). However, the development and homeostasis of the brain are intrinsically connected with those of the whole body. In particular, the function and microenvironment of the brain are substantially influenced by gut microbiota (the trillions of microorganisms that live in the gut) (Margolis et al. 2021). Disturbance of gut microbiota is associated with negative neuropsychiatric consequences, such as depression, anxiety, autism spectrum disorder, and postoperative cognitive dysfunction (Fung et al. 2017; Suganya and Koo 2020; Yang et al. 2018). This crosstalk between gut microbiota and the brain is known as the gut-brain axis (Mayer 2011).

Establishment and maturation of the gut microbiota are acknowledged to be crucial for healthy functioning of the immune and neuroendocrine systems and of metabolic activities. This process parallels neurodevelopment, which begins early in embryonic life and lasts through post-adolescence (O'Mahony et al. 2015; Thaiss et al. 2016; Cussotto et al. 2018; Tilg et al. 2020). Traditional concept holds that the prenatal period is one of sterility and intestinal microbiota is acquired shortly after delivery through mother-infant proximity and lactation (Escherich 1989; Harris and Brown 1927). However, Satokari et al. (2009) challenged this prevalent dogma by investigating the placenta and fetal meconium, concluding that the maternal microbes could transfer horizontally to the fetus in utero and may trigger an immune response and affect early development. Thus, although the exact timing of initial microbe exposure is controversial, it remains clear that prenatal and early postnatal microbial colonization of the gastrointestinal tract is a highly significant event, which could be pivotal in priming the immature immune system and brain development in healthy neonates. Moreover, external factors, such as prenatal stress and infection and even maternal diet, may lead to postnatal gut dysbiosis, which can be associated with neurodevelopmental disorders (Moya-Perez et al. 2017). For instance, Cho et al. (2020) showed that maternal antibiotic exposure in rats led to a lasting effect on the microbiota and brain in progeny. Moreover, maternal prebiotic supplementation has been shown to promote healthy gut microbiota and reduce anxiety in offspring (Hebert et al. 2021).

The antimicrobial effect of anesthetics has long been investigated. Isoflurane is a commonly used inhaled anesthetic for general anesthesia in various types of surgery. It has been found that isoflurane has activity against growth of both gram-positive and gram-negative bacteria and even multidrug-resistant pathogens (Batai et al. 1999). With the recent interest in the gut-brain axis, the effect of isoflurane on gut microbiota is an emerging area of investigation. Research on adult mice, found that a 4-h exposure to isoflurane decreased gut bacterial diversity (Serbanescu et al. 2019). Moreover, a study on neonatal rats exposed to isoflurane showed an altered gut microbiota by the time the animals were juveniles (Wang et al. 2019). However, how prenatal isoflurane exposure affects postnatal gut microbiota and brain biology and behavior remains unknown.

In the present study, we observed the effects of isoflurane exposure in pregnant rats during the midgestational period on cognition paralleled by the diversity and bacterial community structure of gut microbiota in juvenile offspring rats. Within the brain, we evaluated the expression of brain-derived neurotrophic factor (BDNF) in the hippocampus. BDNF is a vital regulator of neural development and function. In developing circuits, BDNF mediates neuronal differentiation and growth as well as synapse formation, maturation, plasticity, and higher cognitive functions (Park and Poo 2013). Previous studies found that anesthetics, such as ketamine and methadone, administered to rats during the prenatal period led to their offspring presenting cognitive impairment accompanied by BDNF downregulation (Lum et al. 2021; Zhao et al. 2014). Studies have also shown that inhaled anesthetic, such as sevoflurane, administered to pregnant rats induced long-lasting learning and memory dysfunction in their pups that was associated with BDNF inhibition (Wu et al. 2018, 2020). Thus, dysregulated expression of BDNF in prenatal anesthesia–induced developmental neurotoxicity is likely an important pathological change within the brain. Furthermore, we also investigated whether postnatal probiotic supplementation could attenuate isoflurane-induced neurotoxicity through the targeting of the gut-brain axis.

Materials and Methods

Animals

This study was conducted in accordance with animal care guidelines and approved by the Ethics Committee of the Peking University Health Science Center (LA2020052).

Gestational day 7 (G7) pregnant specific-pathogen-free (SPF) Sprague Dawley rats were purchased from Beijing Vital River Experimental Animal Technology Co., Ltd. (Beijing, China) and housed under 12:12 light:dark cycle (20 ± 2 °C, humidity 50 ± 10%) conditions with ad libitum feeding.

Isoflurane Exposure

Pregnant rats were administered isoflurane on G14. The rats were placed in a plexiglass chamber connected to a vaporizer. Rats in the isoflurane group were exposed to 2% isoflurane plus 50% O_2 for 4 h. A total gas flow rate of 6 L was used to maintain a steady state of anesthetic gas and prevent accumulation of expired CO_2 within the chamber. The control rats were exposed to the same conditions for 4 h but without isoflurane. Spontaneous breathing and skin color of rats were observed every 3 min. The oxygen, carbon dioxide, and anesthetic agent fractions were monitored continuously using a gas analyzer (Datex-Ohmeda, Madison, WI, USA). During anesthetic exposure, the environmental temperature was maintained at 37 ± 0.5 °C. We performed another study to observe the blood gas indices and glucose of G14 pregnant rats exposed to 2% isoflurane for 4 h. As the results revealed, there were no marked differences in blood gas indices and glucose between G14 pregnant rats exposed to isoflurane or 50% O_2 (see Appendix in the Supplementary Material).

Experimental Protocol

The schematic of the study is presented in Fig. 1.

Cohort A

Twelve pregnant rats (G14) were randomly assigned to two groups: control group ($n = 6$) and isoflurane anesthesia group ($n = 6$). On postnatal day 28 (P28), the healthy male offspring (17 in the control group and 19 in the isoflurane group) were tested using novel object recognition (NOR) task. After the NOR task, all offspring rats were euthanized and fresh fecal samples were collected immediately from the terminal rectum of each animal into individual sterile microcentrifuge tubes and stored at − 80 °C. In addition, brain tissues were harvested and hippocampi were extracted and stored at − 80 °C.

Cohort B

Twelve pregnant rats (G14) were randomly assigned to two groups: control group ($n = 4$) and isoflurane anesthesia group ($n = 8$). On P14, healthy male offspring of the isoflurane-exposed dams were randomly divided into isoflurane group ($n = 13$) and probiotic group ($n = 13$). For the probiotic group, a commercial probiotic mixture (Golden Bifid; Inner Mongolia Shuangqi Pharmaceutical Co., Ltd., Huhehaote, China) composed of no less than 0.5×10^7 cfu/0.5 g *Bifidobacterium longum*, 0.5×10^6 cfu/0.5 g *Lactobacillus bulgaricus*, and 0.5×10^6 cfu/0.5 g *Streptococcus thermophiles* was administered to the animals. Probiotics were first dissolved in sterile water, given in a volume of 4.0 mL/kg body weight and administered at 2.0 g/kg dose intragastrically for 14 days until P28 (Yu et al. 2019). Rats in the control and isoflurane groups received only intragastric administration of sterile water in the same volume and period as the probiotic group. On P28, all rats were tested using the NOR task. After the procedure, all rats were euthanized, the brain

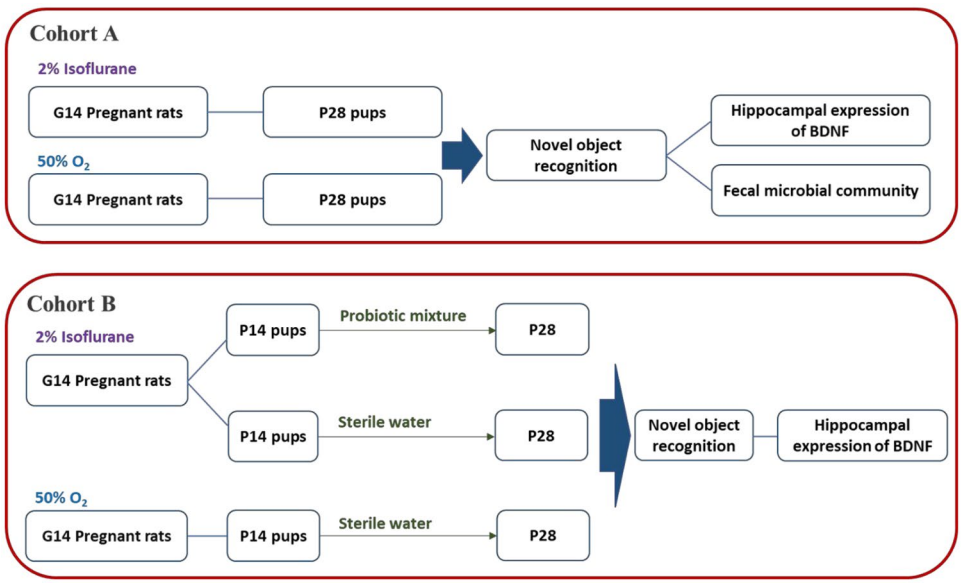

Fig. 1 Study schematic

tissues were harvested, and hippocampi were extracted and stored at − 80 °C.

Novel Object Recognition

The rats were habituated to the testing room and test arena (60 cm × 60 cm × 40 cm) for 15 min 1 day prior to the exploration phase. Twenty-four hours later, rats were placed into the box and allowed to freely explore two identical objects for 5 min. Rats that spent less than 30 s exploring both objects were excluded from further analysis. Twenty-four hours after the exploration phase, one of the familiar objects was replaced by a novel object with different colors and shapes. Rats were placed into the test box and allowed to freely explore these objects for 5 min. The arena and objects were cleaned with 70% ethanol after each trial. Time rats spent exploring each object was recorded with two stopwatches by two investigators blind to the rat group allocations. The novel object preference ratio was calculated as the ratio of time spent exploring the novel object to the total time spent exploring the familiar and novel objects.

Quantitative PCR Analysis for Messenger RNA Expression of BDNF

Total RNA was extracted from the whole hippocampus using TRIzol reagent (Thermo Fisher Invitrogen Life Technologies, Waltham, MA, USA). Isolated RNA was reverse transcribed into complementary DNA (cDNA) with RevertAid First Strand cDNA Synthesis Kit (Servicebio, Wuhan, China). Quantitative polymerase chain reaction (qPCR) was performed using designed primers (BDNF: F(5′-*GTGTGACAGTATTAGCGAGTGGG*-3′) and R(5′-*ACGATTGGGTAGTTCGGCATT*-3′); glyceraldehyde 3-phosphate dehydrogenase (GAPDH): F(5′-*CTGGAGAAACCTGCCAAGTATG*-3′) and R(5′-*GGTGGAAGAATGGGAGTTGCT*-3′)) and SYBR® Green Master (Roche Bioscience, Indianapolis, IN, USA) for 95 °C for 10 min and 40 cycles, then at 95 °C for 15 s and 60 °C for 60 s. Relative gene expression for BDNF was calculated relative to the expression of GAPDH messenger RNA (mRNA).

Western Blot Analysis for Protein Expression of BDNF

The homogenate hippocampal proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein concentrations of samples were determined using the bicinchoninic acid (BCA) protein assay. Fifty micrograms of proteins per protein sample was electrophoresed on a polyacrylamide gel. The blots were immunoreacted with anti-BDNF (1:3000; Servicebio) and anti-β-actin (1:1000; Servicebio) antibodies. Then, the density of the protein bands was quantified by AlphaView Software 3.4 (ProteinSimple, Santa Clara, CA, USA).

High-Throughput Sequencing of Fecal Microbial Community and Data Analysis

DNA from feces was extracted by a commercially available stool DNA kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. Purity and quality of the genomic DNA were checked on 0.8% agarose gels. Then, amplification of 16S rRNA gene was performed with the universal primers 338F (*ACTCCTACGGGAGGCAGCAG*) and 806R (*GGACTACHVGGGTWTCTAAT*), which target the V3–V4 hypervariable region. Deep sequencing of all samples was performed on a MiSeq platform using Illumina Analysis Pipeline Version 2.6 (Illumina, San Diego, CA, USA). After screening the raw data by excluding low-quality reads, the qualified reads were separated using the sample-specific barcode sequences and trimmed with Illumina Analysis Pipeline Version 2.6. The datasets were analyzed using QIIME 1 (Caporaso et al. 2010). Sequences at a similarity level of 97% were clustered into operational taxonomic units (OTUs), to generate rarefaction curves and to calculate diversity indices. All sequences were classified into different taxonomic groups using the Ribosomal Database Project Classifier tool (Wang et al. 2007). To evaluate α diversity of the gut microbiome, Chao1 and PD_whole_tree indices were calculated. Chao1 index was calculated to measure the number of OTUs that were present in the sampling assemblage and to reflect the microflora richness (Chao et al. 2002). PD_whole_tree index was calculated as the sum of the branch lengths of the phylogenetic tree constructed from all species for a given sample (Faith 1994). Both these indices were compared using the unpaired *t* test. For assessment of the beta diversity, principal coordinate analysis (PCoA) based on the unweighted UniFrac distances was performed. For the detection of significance of PCoA between groups, permutational multivariate analysis of variance (PERMANOVA) was employed. PERMANOVA is a permutation-based extension of multivariate analysis of variance and is usually used as a significance test for the beta diversity of microbiome data (Kelly et al. 2015). Further, the characterization of microbial features differentiating the fecal microbiota specific to different taxonomic types was identified using linear discriminant analysis (LDA) coupled with effect size (LEfSe) (Segata et al. 2011). LEfSe is an algorithm for exploring high-dimensional biomarkers. For the LEfSe analysis, the significance threshold for the analysis of variance (ANOVA) was set at 0.05. The threshold logarithmic LDA score for discriminative features was 3.0.

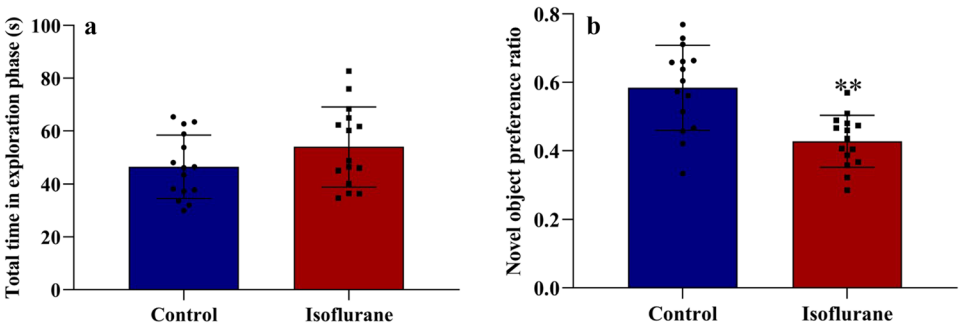

Fig. 2 Prenatal isoflurane exposure and impaired cognition. Data are presented as mean ± SD. **a** Total exploration time of total objects during the exploration phase. **b** Novel object preference ratio of the recognition phase. Unpaired *t* test ($n = 15$/group). $^{**}P < 0.01$

Statistics

Data were expressed as the mean ± standard deviation (SD). For cohort A, differences were compared using the unpaired *t* test. For cohort B, one-way ANOVA followed by Tukey's post hoc test were performed. The effective difference of novel object preference ratio between groups was quantified as the mean difference (MD) and 95% confidence interval (CI). $P < 0.05$ was regarded as statistically significant. Statistical analysis was carried out using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

Results

Cohort A

Prenatal Exposure of Isoflurane Decreases Novel Object Recognition in Juvenile Rats

Six offspring rats (2 in the control group and 4 in the isoflurane anesthesia group) were excluded due to insufficient time spent on total objects during the exploration phase. For offspring that were included in the exploration phase (15 in the control group and 15 in the isoflurane group), there was no significant difference in the time spent on total objects between the two groups. Compared with the control rats, the novel object preference ratio was significantly decreased in the rats of isoflurane group (MD − 0.157; 95% CI − 0.234 to − 0.080, $P < 0.001$) (Fig. 2).

Prenatal Exposure of Isoflurane Reduces Expression of Hippocampal BDNF in Juvenile Rats

Results from qPCR indicated that mRNA expression of hippocampal BDNF was significantly lower in the isoflurane group ($P < 0.01$) compared with the control group (Fig. 3a). Similarly, protein expression of BDNF in the hippocampus was also significantly decreased in rats exposed prenatally to isoflurane ($P < 0.01$) (Fig. 3b).

Gut Microbiome Composition in Rats Prenatally Exposed to Isoflurane Differs Significantly from That of Control

The microbial diversity and species richness as measured with alpha diversity showed differences between the control and isoflurane groups. Compared with the control group, both Chao1 and PD_whole_tree indices were significantly

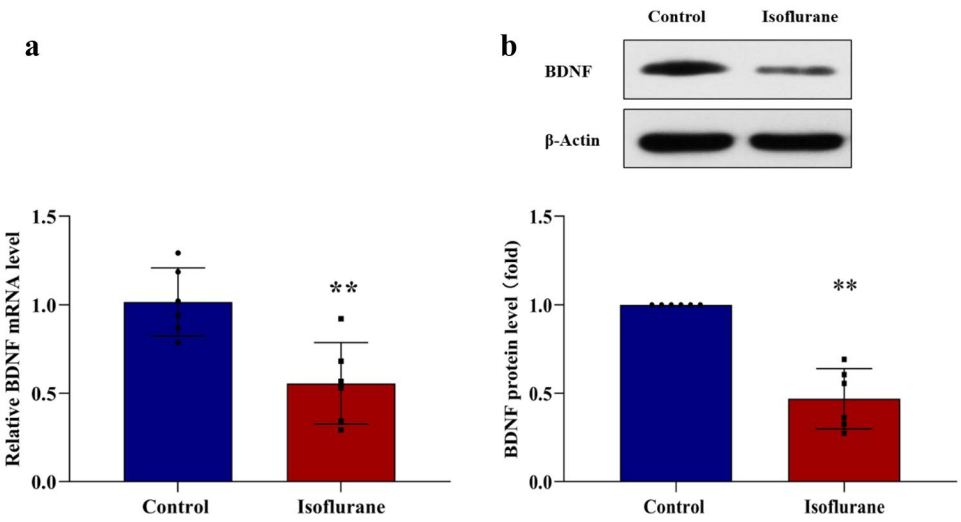

Fig. 3 Hippocampal expression of BDNF was decreased by prenatal isoflurane exposure. Data are presented as mean ± SD. **a** mRNA expression of BDNF ($n = 6$/group). **b** Protein expression of BDNF ($n = 6$/group). Unpaired *t* test, $^{**}P < 0.01$

lower in the isoflurane group (Fig. 4a). Beta diversity between the two groups was examined by PCoA, which revealed that gut microbiota of juvenile rats exposed prenatally to isoflurane were significantly distinct from controls (Fig. 4b).

We employed the LEfSe algorithm to identify the differences in microbial composition between the isoflurane and control groups. LEfSe analysis demonstrated that a total of 21 features had significantly different abundances between the two groups (Fig. 5). At the class level, the microbiota of rats in the isoflurane group was characterized by a preponderance of *Negativicutes*. At the order level, the microbiota of isoflurane-exposed rats was characterized by a preponderance of *Selenomonadales*. At the family level, the microbiota of rats in isoflurane group was characterized by a preponderance of *Porphyromonadaceae* and *Acidaminococcaceae* whereas the microbiota of control rats was characterized by a preponderance of *Peptostreptococcaceae*. At the genus level, compared to the control group, the abundances of the *Erysipelatoclostridium*, *Parabacteroides*, *Ruminiclostridium_6*, *Phascolarctobacterium*, and *Prevotellaceae_NK3B31_group* were significantly higher and the abundances of the *Prevotella_9*, *Romboutsia*, *Ruminococcus_1*, *Alloprevotella*, *Clostridium_sensu_stricto_1*, *Tyzzerella*, *Coprococcus_2*, *Rikenellaceae_RC9_gut_group*, and *Lachnospiraceae_UCG_001* were significantly lower in feces of prenatally isoflurane-exposed rats. In addition, at the species level, compared with the control rats, animals exposed prenatally to isoflurane showed a significantly increased abundance of *Lactobacillus vaginalis* but decreased abundance of *Bacteroides fragilis*.

Cohort B

Administration of Probiotic Mixture Improves Prenatal Isoflurane–Induced Cognitive Decline in Juvenile Rats

No rats died during the study period. Four offspring rats (2 in the isoflurane group and 2 in the probiotic group) were excluded due to insufficient time spent on total objects

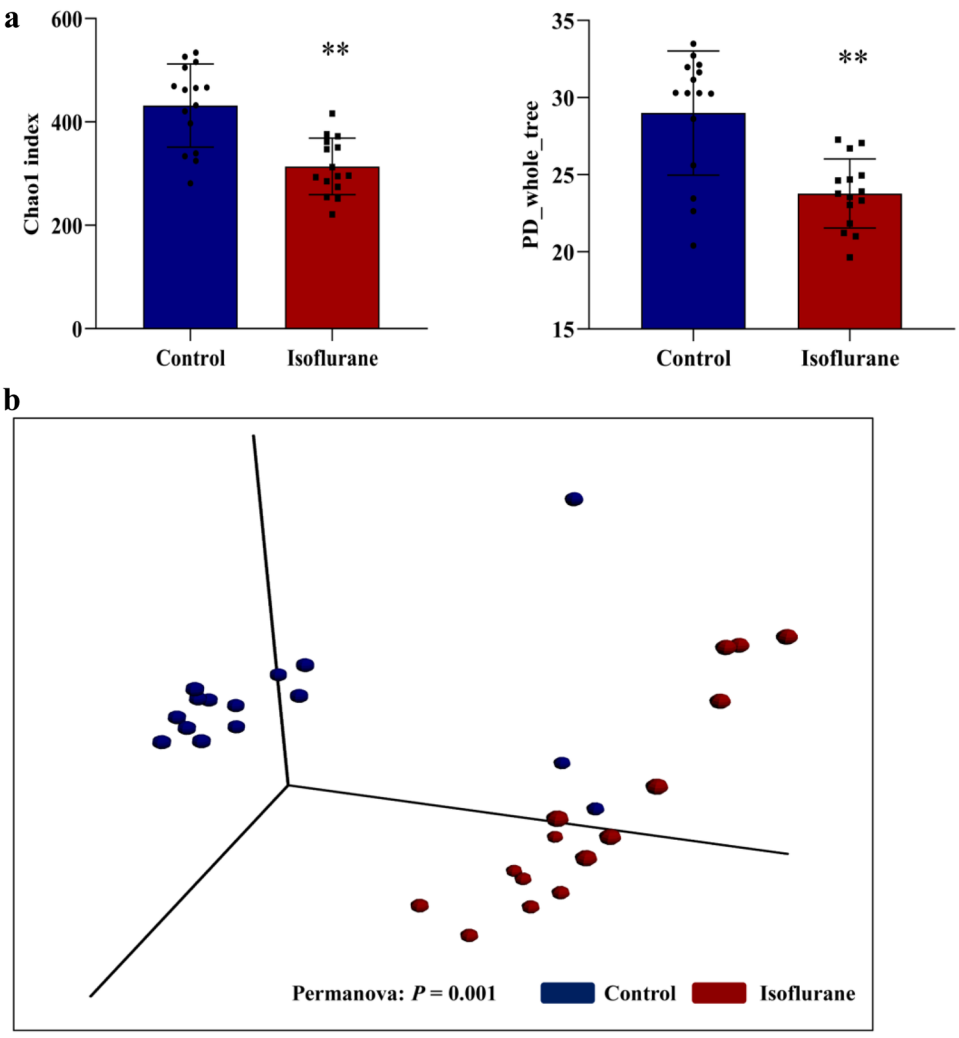

Fig. 4 Diversity of microbiota showed a significant difference between the control and anesthesia groups. **a** Alpha diversity of the fecal microbiome was compared using Chao1 and PD_whole_tree ($n = 15$/group). Unpaired t test, $^{**}P < 0.01$. **b** Beta diversity of the fecal microbiome was compared using principal coordinate analysis ($n = 15$/group). PERMANOVA, $P = 0.001$

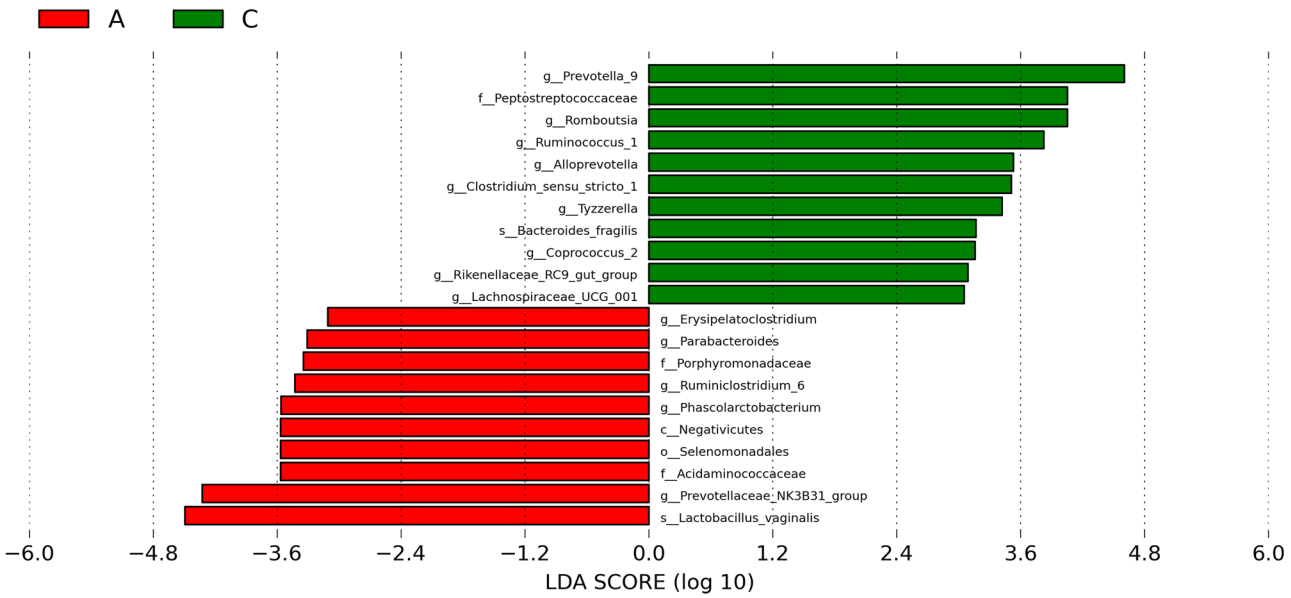

Fig. 5 Significant difference in the gut microbiome of rats, as revealed by LDA analysis based on OTU-characterized microbiomes ($n = 15$/group). A, anesthesia group; C, control group

during the exploration phase. For offspring that were included (11 in the isoflurane group and 11 in the probiotic group), compared with the control group ($n = 13$), the novel object recognition ratio was significantly decreased in the isoflurane animals (MD − 0.177; 95% CI − 0.307 to − 0.047, $P = 0.006$). Rats that received probiotics showed significantly improved cognition compared with isoflurane rats (MD 0.140; 95% CI 0.004 to 0.275, $P = 0.042$) (Fig. 6).

Probiotics Mitigated Prenatal Isoflurane–Induced Downregulated Expression of Hippocampal BDNF in Juvenile Rats

Our data showed that expression of BDNF in the hippocampus of rats in the isoflurane group was significantly lower than that of the control group ($P < 0.01$). However, intragastric administration of probiotics effectively inhibited the decreased level of BDNF in the hippocampus induced by prenatal isoflurane exposure ($P < 0.01$) (Fig. 7).

Discussion

In the present study, we found that rats exposed prenatally to isoflurane exhibited a significant decrease in novel object recognition paralleled by diminished expression of hippocampal BDNF. Prenatal exposure to isoflurane also markedly altered the diversity and composition of gut microbiota. Postnatal treatment with probiotics mitigated these changes in cognition and BDNF expression.

By translating neurodevelopment across human and laboratory animals, the rat at G14 is equivalent to the human fetus

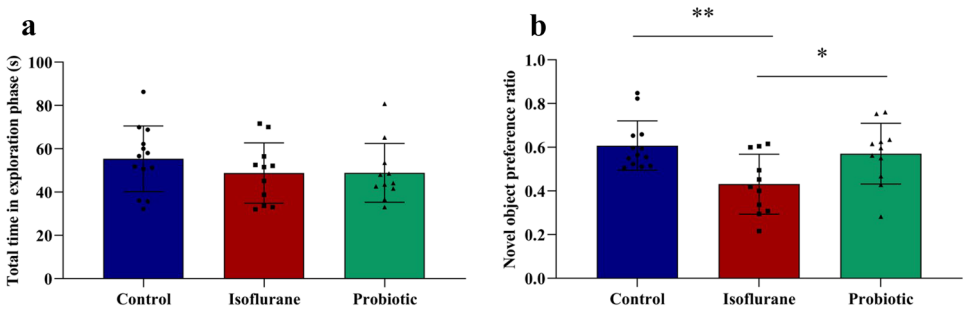

Fig. 6 Probiotics significantly ameliorated the prenatal isoflurane–induced cognitive decline. Data are presented as mean ± SD. **a** Total exploration time of total objects during the exploration phase. **b** Novel object preference ratio of the recognition phase. One-way ANOVA, Tukey's post hoc test ($n = 13$ in the control group, $n = 11$ in the isoflurane group, $n = 11$ in the probiotic group). *$P < 0.05$, **$P < 0.01$

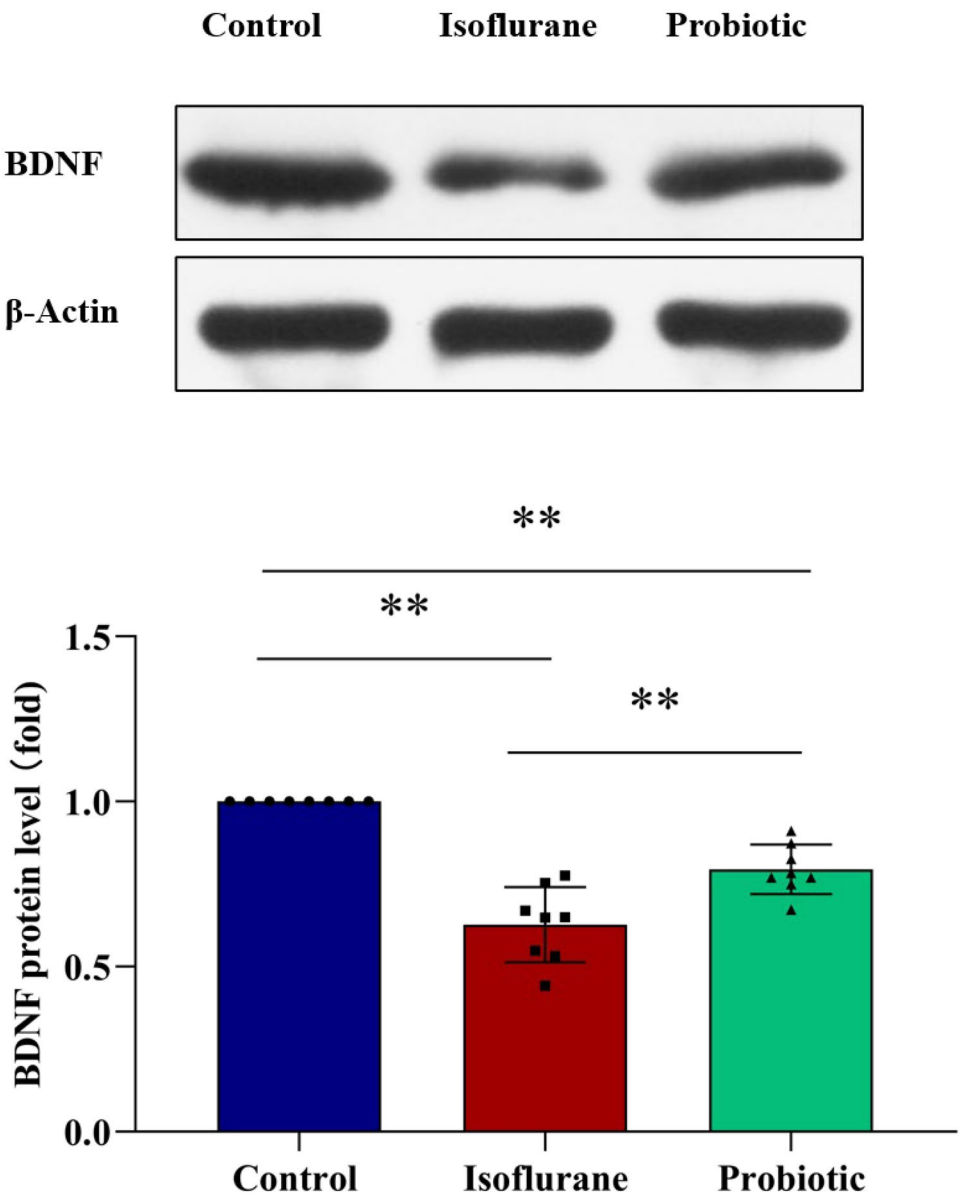

Fig. 7 Probiotics significantly increased the prenatal isoflurane–induced reduction of the expression of BDNF in the hippocampus. Data are presented as mean ± SD. One-way ANOVA, Tukey's post hoc test ($n = 8$/group). $^{**}P < 0.01$

in the second trimester (Clancy et al. 2001, 2007) when both species show similar neurodevelopmental profiles (Clancy et al. 2007; de Graaf-Peters and Hadders-Algra, 2006). In people, most non-obstetric surgeries and fetal interventions are performed in this period (Goodman 2002; Tran 2010; Pisano et al. 2020; Favero et al. 2021). A concentration of isoflurane of at least 1.5 minimum alveolar concentration (MAC) is usually required to facilitate uterine quiescence and minimize the risk of preterm labor (Chai et al. 2019). Mazze et al. (1985) demonstrated that 2% isoflurane represented 1.5 MAC in the pregnant rodent. Thus, in the present study, G14 pregnant rats exposed to 2% isoflurane were used to mimic anesthesia in pregnant women during the second trimester. The exposure durations in previous studies that investigated prenatal isoflurane exposure–related developmental neurotoxicity were 1 to 8 h (Bleeser et al. 2021). Moreover, the FDA warning (United States Food and Drug Administration 2017) states that "Published studies in pregnant animals and young animals have shown the use of general anesthetic and sedation drugs for more than 3 h caused widespread loss of nerve cells in the brain." Thus, in our study, we exposed the pregnant rats to isoflurane for 4 h. The NOR task is preferentially used to assess visual and spatial short-term memory and particularly amenable to young animals because it relies on the rodent's innate preference for exploring novel over familiar stimuli and it is free from response contingencies and requires no pretraining (Antunes et al. 2012; Reger et al. 2009). Proficient NOR task performance emerges between weaning and postnatal day 29 (P29) (Reger et al. 2009). Therefore, P29 is the time point operationally defined as the initiation of the juvenile

period when rats are able to exhibit robust object recognition after a longer interval of 24 h compared with adult (P50 +) rats. Huang et al. (2018) found that exposure to isoflurane at 2% concentration for 3 h in pregnant rats resulted in damage to cognition in offspring. Consistent with this, we found that the NOR task performance of juvenile rats (P30) prenatally exposed to 2% isoflurane was significantly decreased compared with that of the control group.

Increasing evidence indicates that the gut microbiota strongly impacts brain development and behavior (Lozupone et al. 2012). Perturbations to microbial exposure perinatally may alter gut microbiota and lead to long-term neural consequences (Gars et al. 2021). In our previous study (Wang et al. 2019), we found that the diversity and composition of microbiota in neonatal rats exposed to isoflurane were altered by the time the animals were juveniles. Similar results were shown in the sevoflurane-induced developmental neurotoxicity mice model (Liu et al. 2021). In our current study, together with cognitive impairment, the composition of gut microbiota was significantly altered in prenatally isoflurane-exposed juvenile rats. Alpha diversity measures the number and richness of microbial taxa within a sample. Maintaining the richness and diversity of gut microbiota and abundance of dominant bacteria is crucial for health (Kriss et al. 2018). Elevated richness and diversity of gut microbiota are considered hallmarks of a healthy gut ecosystem and linked to microbiota stability and resilience to perturbation (Backhed et al. 2012; Lloyd-Price et al. 2016). In our study, species richness and diversity were estimated using the Chao1 estimator and PD_whole_tree index, respectively. Our data showed that both Chao1 and PD_whole_tree indices were markedly decreased in the isoflurane group. This indicated that the species and the number of gut microbes were fewer in the offspring of rats exposed prenatally to isoflurane than in non-exposed animals. Beta diversity was measured using PCoA, which assessed the differences in community composition between the samples. Our results revealed significant switching in overall composition of gut microbiota between prenatally isoflurane-exposed rats and controls. These data suggest that prenatal exposure to isoflurane induced overall alteration in the diversity and structure of intestinal flora.

We further employed the LEfSe analysis to detect the variation in taxa of gut microbiota, which could provide direct evidence of the role of specific bacteria in the physiologic process. LEfSe analysis revealed that for rats prenatally exposed to isoflurane, a total of 21 specific gut microbes were significantly altered. Elevated abundance of *Lactobacillus vaginalis* was observed in the isoflurane rats. *Lactobacillus* species are generally identified as beneficial bacteria (Azad et al. 2018). However, it is important to note that different strains can have very specific effects. Moreover, their effects may vary in health and disease and in different disease states. Various strains of *Lactobacillus* have been found increased in persons with Parkinson's disease (PD) (Hasegawa et al. 2015; Hill-Burns et al. 2017; Petrov et al. 2017). Hence, an increase in some detrimental *Lactobacillus* species such as *Lactobacillus vaginalis* may be related to isoflurane-induced cognitive impairment. Our results further showed that the use of a probiotic mixture containing *Lactobacillus bulgaricus* could improve prenatal isoflurane–induced cognitive impairment, indicating that this is probably a beneficial *Lactobacillus* species. In fact, Molina et al. (2021) found that administration of *Lactobacillus bulgaricus* promotes neuronal health and enhances neural immunity. Thus, it is worth to note the effects and mechanisms of action of various bacterial strains in isoflurane-related neurotoxicity.

We also found reduced abundances of *Lachnospiraceae* UCG-001 and *Prevotella_9* in the isoflurane group rats. Decreased abundance of *Lachnospiraceae* has been discovered in neurologic diseases such as PD (Keshavarzian et al. 2015; Unger et al. 2016). In the antibiotic-induced neurotoxicity animal model, decreased abundance of *Lachnospiraceae* was found to significantly correlate with behavioral changes (Guida et al. 2018). Most *Lachnospiraceae* are short-chain fatty acid (SCFA) producers (Hill-Burns et al. 2017). SCFAs are key mediators of host-microbe crosstalk and have beneficial effects on various neurologic disorders (Caracciolo et al. 2014; Gerhardt and Mohajeri, 2018). Thus, decreased abundance of *Lachnospiraceae* may be associated with neurotoxicity induced by prenatal isoflurane exposure.

Although the role of *Prevotella* spp. in the incidence of diseases is debated, decreased abundance of *Prevotella* has been found in many neurodegenerative diseases (Heintz-Buschart et al. 2018). *Prevotella* is also linked with inflammatory conditions (Dillon et al. 2016). Xu et al. (2020) demonstrated that probiotic administration improves immunity, protein metabolism, and antioxidative ability in piglets, paralleled by increased relative abundance of *Prevotella_9*. Furthermore, our results showed that the abundances of *Bacteroides fragilis* and *Clostridium_sensu_stricto_1* were significantly decreased in the isoflurane exposure group. Such changes have also been observed both in the PD rat model and in people with PD and Alzheimer's disease (Bedarf et al. 2017; Fu et al. 2009; Zhang et al. 2020).

BDNF is a member of the neurotrophin family and a central regulator of synaptogenesis and persistence of long-term memory consolidation (Bekinschtein et al. 2008). Exposing the developing fetus to anesthetics may lead to cognitive decline in postnatal life through affecting the BDNF system in the hippocampus. For example, Yan et al. (2004) found that offspring of rats prenatally exposed to cocaine exhibited decreased BDNF expression. Similarly, ketamine administered to rats during the prenatal period led to their offspring presenting cognitive impairment accompanied by BDNF downregulation (Zhao et al. 2014).

Other studies have revealed that exposure to sevoflurane in pregnant rats induced long-lasting learning and memory dysfunction in their pups that was associated with BDNF inhibition (Wu et al. 2018, 2020). Consistent with these findings, in our study, we found that maternal exposure to isoflurane resulted in long-term decreased expression of BDNF in parallel with impaired cognition. These data demonstrate that reduced BDNF within the brain is associated with prenatal isoflurane–induced cognitive decline. Furthermore, we treated the offspring with mixed probiotics and evaluated their novel object recognition ability and hippocampal expression of BDNF. Our data showed that postnatal probiotic supplementation could significantly attenuate prenatal isoflurane–induced cognitive defects and downregulation of BDNF expression in the hippocampus of offspring rats. Increasing evidence suggests that BDNF is highly involved in the communication between gut microbiota and the brain. Dysregulation of BDNF is considered a hallmark of a disturbed gut-brain axis (Hoban et al. 2016). In addition, germ-free mice exhibit significant reductions in BDNF levels in the brain and cognitive dysfunction compared with specific-pathogen-free mice (Sudo et al. 2004). More importantly, Bercik et al. (2011) showed that gut microbiota can directly regulate central BDNF expression free from the influence of the autonomic nervous system, gastrointestinal neurotransmitters, or inflammation. Therefore, our findings appear to support the therapeutic effect of probiotics on prenatal isoflurane exposure–induced developmental neurotoxicity in juvenile rats by upregulating expression of BDNF through the gut-brain axis.

The present study has limitations. First and foremost, our results only suggest that the gut microbiota was involved in prenatal isoflurane–induced neurotoxicity and do not indicate that the behavioral outcomes were actually caused by alteration in the gut microbiota of the offspring rats. Second, we did not investigate the mechanism and process of prenatal isoflurane–induced postnatal gut dysbiosis. Hypothetically, plausible explanations include the following: (1) Maternal flora was altered by isoflurane exposure, and vaginal delivery exposed the fetus to fecal and vaginal bacteria. Then, intimate contact, such as lactation, licking, and grooming, transferred maternal microbiota to the pup after birth; (2) isoflurane traversed the placenta and directly altered fetal gut microbiota before birth; (3) isoflurane undermined maternal gut microbiota balance during pregnancy resulting in the release of atypical metabolites, thus affecting the fetal or neonatal intestinal environment, which, in turn, influenced the origin and formation of offspring gut microbiota before or after birth. These possibilities may occur independently or likely codependently. Hence, it is difficult to ascertain the precise process by which prenatal isoflurane exposure affects gut microbiota in juvenile rats. Third, we employed an experimental animal model by which most research is conducted to explore the effects of prenatal inhaled anesthesia exposure on the developing brain. Yet, in clinical practice, it is rare that pregnant women receive anesthesia alone without surgery or other procedures. Hence, we would not expect equivalent results between animal and human studies. In addition, our study only included male pups to eliminate the interference of sex. However, as a biologic variable in anesthesia-induced developmental neurotoxicity, some studies revealed that male and female animals may exhibit different outcomes in behavioral, molecular, cellular, and genetic domains (Cabrera et al. 2020). Therefore, we cannot draw similar conclusions regarding prenatal isoflurane exposure–induced gut microbiota dysbiosis in female rats.

In conclusion, to the best of our knowledge, our study is the first to reveal that prenatal exposure of rats to a clinically similar concentration of isoflurane may induce changes in gut microbiota, decrease hippocampal expression of BDNF, and lead to associated cognitive impairment in offspring. Postnatal administration of probiotics mitigated these detrimental effects. These findings suggest that in rats, gut dysbiosis may be involved in the pathogenesis of maternal isoflurane exposure–induced postnatal cognitive impairment in offspring. To determine the causal relationship between gut microbiota and cognition in prenatal anesthetic–induced developmental neurotoxicity, further studies are needed. Even so, attention should be paid to administration of anesthetics during pregnancy with a view to understanding the interactions between commensal bacterial species and the developing brain.

Abbreviations ANOVA: Analysis of variance; BCA: Bicinchoninic acid; BDNF: Brain-derived neurotrophic factor; CI: Confidence interval; CNS: Central nervous system; FDA: Food and Drug Administration; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; LDA: Linear discriminant analysis; LEfSe: Linear discriminant analysis effect size; MAC: Minimum alveolar concentration; MD: Mean difference; NOR: Novel object recognition; OTUs: Operational taxonomic units; PCoA: Principal coordinate analysis; PERMANOVA: Permutational multivariate analysis of variance; qPCR: Quantitative polymerase chain reaction; SCFA: Short-chain fatty acid; SD: Standard deviation; SPF: Specific-pathogen-free.

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Author Contribution Li-Kuan Wang helped conceive and design the study, acquired the data, analyzed and interpreted the data, and drafted and critically revised the manuscript. Xu-Dong Yang helped conceive and design the study, analyzed and interpreted the data, and drafted the manuscript. Dan Zhou helped in the animal allocation and data analysis. Tong Cheng helped conceive and design the study and analyzed the data. Xiang Zhang helped design the study. Hai-Yin Wu helped

conceive and design the study, analyzed and interpreted the data, wrote and critically revised the manuscript, and approved the final version to be submitted.

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Declarations

Conflict of Interest The authors declare no competing interests.

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