Ketogenic Diet Modulates Neuroinflammation via Metabolites from *Lactobacillus reuteri* After Repetitive Mild Traumatic Brain Injury in Adolescent Mice

Dilirebati Dilimulati1 · Fengchen Zhang1 · Shuai Shao1 · Tao Lv1 · Qing Lu2 · Mengqiu Cao2 · Yichao Jin1 · Feng Jia1 · Xiaohua Zhang1

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Abstract
Repetitive mild traumatic brain injury (rmTBI) is associated with a range of neural changes which is characterized by axonal injury and neuroinflammation. Ketogenic diet (KD) is regarded as a potential therapy for facilitating recovery after moderate-severe traumatic brain injury (TBI). However, its effect on rmTBI has not been fully studied. In this study, we evaluated the anti-neuroinflammation effects of KD after rmTBI in adolescent mice and explored the potential mechanisms. Experimentally, specific pathogen-free (SPF) adolescent male C57BL/6 mice received a sham surgery or repetitive mild controlled cortical impacts consecutively for 7 days. The uninjured mice received the standard diet, and the mice with rmTBI were fed either the standard diet or KD for 7 days. One week later, all mice were subjected to behavioral tests and experimental analysis. Results suggest that KD significantly increased blood beta-hydroxybutyrate (β-HB) levels and improved neurological function. KD also reduced white matter damage, microgliosis, and astrogliosis induced by rmTBI. Aryl hydrocarbon receptor (AHR) signaling pathway, which was mediated by indole-3-acetic acid (3-IAA) from *Lactobacillus reuteri* (*L. reuteri*) in gut and activated in microglia and astrocytes after rmTBI, was inhibited by KD. The expression level of the toll-like receptor 4 (TLR4)/myeloid differentiation primary response 88 (MyD88) in inflammatory cells, which mediates the NF-κB pathway, was also attenuated by KD. Taken together, our results indicated that KD can promote recovery following rmTBI in adolescent mice. KD may modulate neuroinflammation by altering *L. reuteri* in gut and its metabolites. The inhibition of indole/AHR pathway and the downregulation of TLR4/MyD88 may play a role in the beneficial effect of KD against neuroinflammation in rmTBI mice.

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Dilirebati Dilimulati, Fengchen Zhang, and Shuai Shao have contributed equally to this work.

Yichao Jin
honam612@163.com; alexmason@sjtu.edu.cn

Feng Jia
projiafeng@163.com

Xiaohua Zhang
zxh1969@aliyun.com

Dilirebati Dilimulati
dlrabat@163.com

Fengchen Zhang
zhangfengchen1@gmail.com

Shuai Shao
proshaoshuai@163.com

Tao Lv
lvtao512@aliyun.com

Qing Lu
druqingsjtu@163.com

Mengqiu Cao
calomengqiu0@163.com

1 Department of Neurosurgery, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, No. 160 Pujian Road, Shanghai 200127, People’s Republic of China

2 Department of Radiology, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, No. 160 Pujian Road, Shanghai 200127, People’s Republic of China
Graphical abstract

Keywords Microglia · Astrogliosis · Aryl hydrocarbon receptor · Toll-like receptor · β-Hydroxybutyrate

Abbreviations

3-IAA  Indole-3-acetic-acid
AHR  Aryl hydrocarbon receptor
β-HB  Beta-hydroxybutyrate
CXCL1  Chemokine (C-X-C motif) ligand 1
CCI  Controlled cortical impact
DTI  Diffusion tensor imaging
ELISA  Enzyme-linked immunosorbent assay
FA  Fractional anisotropy
GFAP  Glial fibrillary acidic protein
HE  Hematoxylin and eosin
IL-1β  Interleukin β1
IRF-3  Interferon regulatory Factor 3
Iba-1  Ionized calcium-binding adapter molecule 1
KD  Ketogenic diet
MyD88  Myeloid differentiation primary response 88
MCT1  Monocarboxylate transporter 1
MD  Mean diffusivity
NF-κB  Nuclear factor kappa light chain enhancer of activated B cells
OCT  Optimal cutting temperature compound
PBS  Phosphate-buffered saline
rmTBI  Repetitive mild traumatic brain injury
TLR4  Toll-like receptor 4
TNF  Tumor necrosis factor

Introduction

Repetitive mild traumatic brain injury (rmTBI), also known as brain concussion, is a growing medical and economic problem worldwide, accounting for 70–90% of all traumatic
brain injury cases. rmTBI might increase the long-term risk for cognitive impairment and dementia, stroke, Parkinson disease, and epilepsy, and is associated with increased long-term mortality rates (Maas et al. 2017). Emerging evidence suggests that rmTBI is associated with histopathological changes such as astrogliosis, microglial activation, axonal injury, and phosphorylated tau immunoreactivity (Yu et al. 2018; Verboon et al. 2021). However, the exact effects of rmTBI on brain functions and the underlying pathological mechanisms remain elusive.

The pathophysiology of rmTBI is characterized by complex changes in cerebral energy metabolism. Normally, the brain uses glucose as the primary energy source; however, immediately after a traumatic injury, the metabolism in the brain switches to increased uptake and utilization of glucose relative to the rate of oxygen utilization. This altered metabolic state, called hyperglycemia, is characterized by uncoupling of glycolysis and oxidative phosphorylation and accumulation of lactic acid. This is followed by a prolonged period of cerebral glucose hypometabolism (Blanco et al. 2016), during which ketone bodies (KB) are used as the main alternative fuel in the brain (Prins et al. 2004; Morris 2005; Thau-Zuchman et al. 2021). Cerebral ketone metabolism is an important compensatory metabolic pathway that bypasses the early glucose metabolic derangements after TBI.

Ketosis, or elevated plasma concentrations of KB, can be induced by a high-fat and low-carbohydrate ketogenic diet (KD) that induces a switch from glucose metabolism to fatty acid metabolism in the body (Prins et al. 2005; Mychasiuk and Rho 2017; Salberg et al. 2019). The classic KD is an isocaloric, high-fat, very low-carbohydrate, and normal-protein diet initially designed to treat patients with refractory epilepsy, particularly children (Koppel and Swerdlow 2018; Ding et al. 2021). Due to the positive effects of KD on refractory epilepsy, its use has been extended to treat a wide variety of diseases, such as Parkinson disease, Alzheimer disease, stroke, and moderate-severe TBI (Rho and Stafstrom 2012; McDonald and Cervenka 2018). Furthermore, KD can improve neurological function and increase the expression of monocarboxylate transporter 1 (MCT1) in the rat after TBI, in an age-dependent manner (Appelberg et al. 2009). Studies have shown that KB may mediate these benefits of KD. However, the exact mechanisms of beneficial effects of KD on TBI are not clear.

Gut microbiota (GM) plays an important role in the host physiology by impacting several metabolic and signaling pathways and neurological functions. The modulation of some of these pathways could be involved in the neuroprotection mediated by KD. It has been shown that KD alters the composition of GM in mice (Olson et al. 2018), and ketosis in humans is associated with the change in GM composition (Klein et al. 2016). Several studies have revealed that the GM can metabolize amino acid tryptophan into indole and its derivatives, that can act as aryl hydrocarbon receptor (AHR) ligands (Agus et al. 2018).

AHR is a ligand-dependent transcription factor that regulates a diverse spectrum of cellular functions by regulating gene expression in a ligand- and cell-type-specific manner and has roles in regulating immunity, stem cell maintenance, and cellular differentiation (Chen et al. 2019). The research on dietary tryptophan derived AHR ligands has identified novel interactions between the gut microbiome and central nervous system (CNS) inflammation.

L. reuteri is found as a gut symbiont in a number of mammalian species and has been described to participate in the transformation of dietary tryptophan into AHR agonists (Zelante et al. 2013). Interestingly, the abundance of L. reuteri is substantially altered in response to the KD (Olson et al. 2018). L. reuteri can use tryptophan as an energy source, producing the AHR agonist indole-3-acetic acid (3-IAA) as a metabolic product (Zelante et al. 2013). The microbial metabolites of tryptophan cross the blood–brain barrier and play a role in the inflammatory processes of the CNS by affecting AHR-driven mechanisms in microglia (Rothhammer et al. 2016). The latest research has also revealed that microglial AHR exerts both pro-inflammatory and anti-inflammatory effects in lipopolysaccharide-activated primary cultures of microglia, depending on the availability of exogenous AHR ligands (Lee et al. 2015). Therefore, the indole/AHR signaling pathway may play an important bridging role between the GM and neuroinflammation induced by rmTBI.

Microglial activation is one of the pathological changes occurring after rmTBI, and the mechanism of the regulatory effect of KD on microglial activation remains unknown. Among the variety of receptors involved in the signaling leading to microglial activation, an important contributor is toll-like receptor 4 (TLR4), which is mainly expressed on microglia. It has been reported that microglial pro-inflammatory function is activated by myeloid differentiation primary response 88 (MyD88)-dependent TLR4 signaling pathway after moderate-severe TBI (Zhang et al. 2018). Therefore, we hypothesized that KD would inhibit neuroinflammatory responses after rmTBI by modulating the MyD88-dependent TLR4 signaling pathway.

In this study, we evaluated the therapeutic potential of KD on rmTBI in adolescent mice and explored the underlying mechanisms of its anti-neuroinflammation effects.

Materials and Methods

Animals and Experimental Design

All procedures involving animals in this study were approved by the Animal Care and Experimental Committee.
of the School of Medicine of Shanghai Jiao Tong University (permit number: RJ2021-0203). A total of 90 specific pathogen-free (SPF), 3–4-week-old adolescent male C57BL/6 mice (10–12 g) were randomly divided into three groups: sham group fed with the standard diet (SCG), rmTBI group fed with the standard diet (TCG), and rmTBI group fed with KD (TKG). Mice were housed in individual cages in a temperature and humidity-controlled animal facility with a 12-h light/dark cycle. Mice were kept in the animal facility for at least 7 days before surgery, and they were given free access to food and water during this period.

The sample size was not predetermined by a statistical method, but our sample sizes are similar to those generally used in the field (Thau-Zuchman et al. 2021). Furthermore, sample sizes for mouse experiments were sufficient for normality, variance homogeneity and statistical analyses. Randomization and blinding (the experimenter being blind to treatment group) were undertaken in all animal experiments. Experimenter blinding was sufficient to control for selection bias.

### Repetitive Mild Traumatic Brain Injury Mouse Model

Mice were subjected to deep anesthesia with 2% isoflurane (RWD Life Science Co., Ltd, R510-22, Shenzhen, China) and were placed in a stereotaxic frame in the prone position. After shaving the head, an incision was made along the midline of the scalp, and a self-made concave metal disc was adhered to the head. Controlled cortical impact (CCI) injury was induced using a PinPoint™ PCI3000 Precision Control Impactor™ (Hatteras Instruments, Cary, North Carolina, USA) with the following settings: a 2.5 mm impactor tip with a speed of 1.0 m/s, a depth of 1.5 mm, and a dwell time of 100 ms. SCG mice received the same anesthetic and surgical procedures without impaction. After the injury, the scalp was closed with sutures. Repetitive injuries were induced for a total of 7 times within a 24-h interval. Mice were allowed to recover on a warm carpet (37 ℃) until fully awake and active and then returned to their cages.

### Diet Interventions and Food Intake Studies

Mice in each group were fed with either the standard diet (Xie Tong Biotechnology Co., Ltd, XTKDCON, Jiangsu, China) or KD (Xie Tong Biotechnology Co., Ltd, XTKD01, Jiangsu, China). TKG mice were not preconditioned with KD and were put on a KD only after the CCI. The standard diet contained 10% protein, 80% carbohydrates, and 10% fat as (% kcal), and KD contained 10% protein and 90% fat (% kcal) as macronutrients. The caloric value of the standard diet and KD were 3.8 kcal/g and 6.7 kcal/g, respectively. In both the standard diet and KD, fat was derived from soybean oil and cocoa butter. Micronutrient content, fiber, and preservatives were matched on a per calorie basis. During experiments, mice had free access to the diets, which were placed in the food well of the cage-top wire lid. The composition of the control diet and KD are shown in Supplementary Tables 3 and 4.

Mice were placed in individual chambers with free access to pre-weighed diets and water. Food uptake was monitored by food weights. Measured values of food intake were normalized to body weight.

### Beam Walking

The goal of beam walking task was for the mouse to remain upright while traversing an elevated tapered beam (100 cm long, suspended horizontally 1 m above ground) from one end to the other to reach a safe, dark box. Animals were trained to walk on the beam before surgery to reinforce the goal of the task, and three trials were conducted per mouse, with one-minute breaks between the trials. Performance on the beam was quantified by measuring the time taken by the mouse to walk across the beam and the number of hind-leg foot slips that occurred during the task. The beam-walk balance test was performed on day 7 post-injury.

### Y-Maze Test

Spatial working memory was measured by the Y-maze test (Kraeuter et al. 2019). A Y-maze is a horizontal maze with three arms (50 cm in length and 10 cm in width) and walls (20 cm in height). The arms are symmetrically inclined to each other at 120°. Animals were set free for spontaneous movement throughout the Y-maze by placing them at the center of the maze. The mice typically like to explore a new arm of the maze, rather than returning to the one previously explored. An alternation sequence is the one when a mouse enters all three arms in a sequence without entering a single-arm twice in a row. A wrong alternation is when a mouse enters an arm two times in a row (Dowling and Allen 2018). The following equation was used to determine the percentage of the wrong alternations:

\[
\% \text{Wrong alteration} = \frac{\text{Number of wrong alternations}}{\text{Total arm entries} - 2} \times 100
\]

### Tissue Preparation for Light Microscopy

At 7d after rmTBI, mice were subjected to deep anesthesia with 2% isoflurane and perfused transcardially with 4% paraformaldehyde (Beyotime Biotechnology Co., Ltd, P-0099, Shanghai, China). The brains were removed, further fixed at 4 ℃ overnight in 4% paraformaldehyde, and then immersed in 30% sucrose/phosphate-buffered saline (PBS) (Beyotime Biotechnology Co., Ltd, C0221A, Shanghai, China) at 4 ℃ overnight. Specimens were mounted in the optimal cutting temperature compound (OCT) (Sakura Finetek Co.,...
There were six mice in each of the three groups. (Shitaka et al. 2011). Images of the injured cortex and ipsilateral hippocampus were captured at ×100 using a light microscope (Nikon Labophot; Nikon USA, Melville, NY). There were three mice in each of the three groups.

**Beta-hydroxybutyrate (β-HB), Glucose, Total cholesterol, Triglycerides, Total bilirubin, Biliverdin and Hemin Analysis in Plasma**

Blood samples were collected (n = 10/group) through intra-cardiac sampling, plasma was isolated by centrifugation (12,000×g for 5 min), and samples were prepared with EDTA anticoagulant tube. A ketone and glucose monitoring system (FreeStyle Optium Neo, Abbott, NJ, USA) was used to measure blood β-HB and glucose levels according to the manufacturer’s instructions. Total cholesterol (TCs) levels were determined using a cholesterol assay kit (10,007,640, Cayman Chemical Company Ltd., USA). Plasma triglycerides (TGs) were determined with the Triglycerides Assay Kit (TR0100; Sigma, St. Louis, MO, USA). Serum levels of total bilirubin, biliverdin, and hemin were determined with commercial ELISA kits by following the manufacturer’s instructions. Bilirubin (#B4126), biliverdin (#30,891), hemin (#MAK036), and brain β-HB (#MAK272) kits were purchased from Sigma-Aldrich Co., Ltd. (MO, USA).

**Silver Staining**

Formalin-fixed OCT-embedded Sects. (40-µm-thick) were subjected to silver stain analysis to determine axonal damage. Silver staining was performed by using the FD Neuro-Silver Kit II (FD NeuroTechnologies, PK301, Ellicott City, MD) according to the manufacturer’s instructions but with some modifications. Densitometric analysis of silver staining was performed on 3 sections per mouse for corpus callosum. Images of the corpus callosum were captured at ×200 using a light microscope (Nikon Labophot; Nikon USA, Melville, NY) and were converted to grayscale with background subtraction. ImageJ software (NIH, Bethesda, MD) and were converted to grayscale with background subtraction. ImageJ software (NIH, Bethesda, MD) according to the manufacturer's instructions. Total cholesterol (TCs) levels were determined using a cholesterol assay kit (10,007,640, Cayman Chemical Company Ltd., USA). Plasma triglycerides (TGs) were determined with the Triglycerides Assay Kit (TR0100; Sigma, St. Louis, MO, USA). Serum levels of total bilirubin, biliverdin, and hemin were determined with commercial ELISA kits by following the manufacturer’s instructions. Bilirubin (#B4126), biliverdin (#30,891), hemin (#MAK036), and brain β-HB (#MAK272) kits were purchased from Sigma-Aldrich Co., Ltd. (MO, USA).

In vivo MRI studies were performed on a 7-Telsa Bruker Biospec 70/20 USR spectrometer (Bruker BioSpin Corporation, Billerica, Massachusetts, USA) 7 days after the induction of rmTBI. Animals were placed onto a cradle with a stereotactic head holder. Anesthesia was induced with 2% isoflurane via N2O/O2 (1:1) gas through a nose cone. Body temperature was maintained at 37.0 °C using warm air, monitored, and controlled via a rectal temperature probe (CWE model TC-1000, Ardmore, Pennslyvania, USA). Conventional MR scanning sequence included T2WI and diffusion tensor imaging (DTI).

A whole brain anatomical T2-weighted scan using a Rapid Acquisition with Refocused Echoes (RARE) sequence was performed with the following parameters: TR = 5500 ms, TE = 37 ms, RARE factor = 8, FOV = 2.5 × 2.5 cm, in plane resolution = 109 × 109 µm, 600 µm slice thickness, 45 slices, and 12 min acquisition time. The diffusion images were acquired with a spin-echo and echo-planar imaging (EPI) sequence between the olfactory bulb and the cerebellum.

The diffusion tensor images were obtained for fractional anisotropy (FA) and mean diffusivity (MD) using a weighted linear least squares method (Winston et al. 2016). Finally, a region-of-interest (ROI) analysis was performed in the corpus callosum using the RadiAnt DICOM Viewer.

**Immunohistochemical Staining**

Formalin-fixed OCT-embedded frozen sections (40 µm thick) were permeabilized with 0.3% Triton X-100 (Beyotime Biotechnology Co., Ltd, ST797, Shanghai, China) in PBS, followed by blocking for 4 h in 0.3% Triton X-100 with 10% donkey serum (Beyotime Biotechnology Co., Ltd, A7039, Shanghai, China). The sections were incubated in the primary antibody diluted in 0.3% Triton X-100 with 10% donkey serum for 48 h at 4 °C. After primary antibody incubation, the sections were washed in PBS and incubated in the secondary antibody diluted in 0.3% Triton X-100 with 10% donkey serum for 4 h at room temperature (RT). Sections were washed three times in PBS and incubated in Hoechst 33,342 (1:2,000; Thermo Fisher) for 15 min at RT for nuclear counterstaining. The sections were then mounted on microscope slides, sealed with clear nail polish, and stored at 4 °C for preservation. The negative control sections were incubated with only secondary antibodies. There were six mice in each of the three groups. The antibody information is listed in Supplementary Tables 1 and 2.

In our study, omission of the primary antibody and incubation of the sections only with secondary antibodies was used to confirm the specificity of the different secondary antibodies.
Immunofluorescence Microscopy Analysis

In order to assess microglial infiltration and astrogliosis, at least 6 selected microscope fields in hippocampus were observed in each group (×200 magnifications; Zeiss LSM880; Zeiss, Germany), and microglia or astrocytes present in these fields were statistically analyzed. Morphology of microglia and astrocytes were quantified using ImageJ software (NIH, Bethesda, MD) program as described previously (Sun et al. 2012). Furthermore, to observe the cellular localization and expression of AHR and RelB in microglia, randomly selected microscope fields were observed in each group (×400 magnifications; Zeiss LSM880; Zeiss, Germany). The negative control sections were observed in the same conditions. There were six mice in each of the three groups.

Western Blot Analysis

The hippocampus was harvested on day 7 after induction of rmTBI. The frozen brain samples were mechanically lysed in 20 mM tris (hydroxymethyl) aminomethane (Tris, pH 7.6), containing 0.2% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulphonyl fluoride, and 0.11 IU/mL aprotinin (all purchased from Sigma–Aldrich, Inc., St. Louis, MO, USA). The lysates were centrifuged at 12,000 × g for 15 min at 4 °C and the supernatants were collected. The protein concentrations in the supernatants were estimated by the Bradford method.

The proteins (20 μg/lane) were separated on 12% SDS polyacrylamide gels and electro-transferred onto a polyvinylidene difluoride membrane (Bio-Rad Lab, Hercules, CA). The membrane was blocked with 5% skim milk (Beyotime Biotechnology Co., Ltd, P0216, Shanghai, China) (w/v) in Tris-buffered saline with 0.1% Tween-20 (TBST) (Beyotime Biotechnology Co., Ltd, ST825, Shanghai, China) for 1 h at room temperature and incubated with primary antibodies diluted in TBST for 24 h. After the membrane had been washed three times in TBST, 15 min each time, it was incubated with the appropriate horseradish peroxidase-conjugated secondary antibody diluted in TBST for 1 h. The cross-reactive protein bands were visualized by enhanced chemiluminescence Western blot detection reagents (Millipore, Burlington, MA), and the results were quantified by Quantity One Software (Bio Rad, Hercules, CA, USA). The band densities were calculated as ratios of TLR4 and MyD88/β-tubulin in the lanes. There were six mice in each of the three groups. The antibody information is listed in Supplementary Tables 1 and 2.

Enzyme-linked Immunosorbent Assay (ELISA) Analysis of Tumor Necrosis Factor (TNF), Interleukin-1 β (IL-1β), Chemokine (C-X-C motif) ligand 1 (CXCL1), Interferon Regulatory Factor 3 (IRF-3), and 3-IAA

On day 7 after rmTBI induction, mice were subjected to deep anesthesia using 2% isoflurane. The brains were quickly removed by dissection and kept over ice in the physiologic salt solution. The hippocampus specimens were separated, cut into small pieces, dispersed by repeated aspiration into a pipette tip, and suspended in 1 mL of physiologic salt solution with protease inhibitor (Beyotime Biotechnology Co., Ltd, P1006, Shanghai, China) in a test tube. Blood was collected by cardiac puncture, and the plasma was obtained by centrifugation (12,000 × g for 5 min). The colon was washed and flushed with PBS to remove luminal contents. Tissue samples were sonicated on ice in 10 s intervals at 20 mM in RIPA lysis buffer (Beyotime Biotechnology Co., Ltd, P0013B, Shanghai, China), and the homogenates were centrifuged at 7500 rpm for 20 min. The supernatants were used for measuring the concentrations of cytokines and chemokines with commercial ELISA kits by following the manufacturer’s instructions.

TNF (#JL10484), IL-1β (#JL18442), CCL1 (#JL42145), and IRF3 ELISA (#JL20345) kits were purchased from Jiang Lai Biotechnology Co., Ltd. (Shanghai, China). Quantification of 3-IAA in mouse was performed using an ELISA kit (abx150354, Abbexa Ltd., Sugar Land, TX, USA) according to a previously described method (Constante et al. 2021). There were six mice in each of the three groups.

DNA Extraction from Fecal Samples

Bacterial DNA from frozen fecal samples was extracted using a QIAamp DNA stool mini kit (Qiagen, Hilden, 51,504, Germany) according to the manufacturer’s instructions and stored at −20 °C until use. Nanodrop One spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to quantify the DNA concentration, and the quality of the extracted DNA was estimated by the ratio of absorbance at 260 and 280 nm.

Standard Curve for qPCR

The standard curve was constructed by plotting the threshold cycles (Ct) values against the log input extracted DNA from respective dilutions of bacterial suspension of a reference strain of L. reuteri (Jomehzadeh et al. 2020). Briefly, L. reuteri reference strain was grown in 5 mL of Man, Rogosa & Sharpe broth medium (MRS) (Huaikai microbial Co., Ltd, 027312, Guangdong, China) to an OD 600 nm of 0.6 on a Nanodrop
One spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and serially diluted to a final concentration range of $10^1$–$10^7$ CFU mL$^{-1}$. A 100μL of each dilution was plated on MRS agar and incubated under the microaerophilic condition for 48 h at 37 °C. Colonies were then enumerated and used for colony-forming unit extrapolation (CFU per milliliter). All experiments were performed at least in triplicate, and the average titer (CFU mL$^{-1}$) of three replicates was determined.

Community DNA from feces was extracted using a QIAamp DNA stool mini kit (Qiagen, Hilden, 51,504, Germany) as described above. By comparing the Ct values acquired to the standard curve, the number of cells of *L. reuteri* in the fecal samples were determined.

**Quantitative Real‑Time PCR**

As previously described, Real-time quantitative PCR was performed using *L. reuteri* specific primers (Jomehzadeh et al. 2020). Total bacterial DNA was applied as a template for qPCR. The qPCR reaction volume of 20 μL contained 2 μL of 50 ng/μL of DNA template, each of forward and reverse primer in a volume of 0.4 μL, 10 μL of SYBR Advantage Premix, and nuclease-free water added to obtain the final volume of 20 μL. Initial denaturation at 95 °C for 30 s were followed by 40 cycles at 95 °C for 10 s, annealing at 60 °C for 30 s and extension at 70 °C for 15 s, and final elongation at 72 °C for 5 min. All experiments were performed in triplicates. There were six mice in each of the three groups. The following *L. reuteri* specific primer pair was used: Lreu-1: 5′-CAGACAATCTTTGATTGTAG-3′; Lreu-4: 5′-GCTTGTGGTTGGCTCTTC-3′.

**Statistical Analysis**

All data are presented as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, San Diego, USA). All data were subjected to one-way ANOVA followed by Tukey’s test. Statistical significance was inferred at $P < 0.05$. Normality was checked by Shapiro–Wilk test, and homogeneity of variance was checked by Brown-Forsythe test in multiple groups. The results showed that the variance was similar. The results of the tests for normality and variance homogeneity are shown in Supplementary Tables 5 and 6.

**Results**

**A Stable Repetitive Mild Traumatic Brain Injury Mouse Model Was Established**

The rmTBI mouse model was induced with the CCI device. All mice were then subjected to dietary interventions. One week later, all mice were subjected to behavioral tests and experimental analysis (Fig. 1A). The hematoxylin & eosin (HE) staining of neurons and T2-weighted imaging (T2WI) were performed in our study. The general view and HE staining of the brains showed that rmTBI did not induce acute brain damage, including contusion and hemorrhage (Fig. 1B). Furthermore, no acute brain damage was observed in the brains of SCG and TCG mice in T2WI, suggesting that the impacts did not induce a moderate or severe brain injury (Fig. 1C).

**KD Elevated Serum β‑HB, Brain β‑HB, Plasma TC and Decreased Serum Glucose, Plasma TG After rmTBI**

β-HB and glucose levels were measured in serum with ketone and glucose monitoring system. It is well-established that KD is associated with an induction of higher ketone levels and low glucose levels (Olson et al. 2018). Significantly increased serum β-HB level, brain β-HB level and decreased serum glucose level were detected in TKG mice, compared with the SCG and TCG mice (Fig. 2A, B, C). As shown in Fig. 2D, no significant change in food intake were observed. Moreover, we observed that KD-treated mice did not gain as much bodyweight as other groups did (Fig. 2E).

TG, TC, total bilirubin, biliverdin and hemin levels were measured in plasma with commercially available kits, respectively. Considerably reduced plasma TG level and increased plasma TC level were detected in TKG mice, compared with the SCG and TCG mice (Fig. 2F, G). Significantly increased serum total bilirubin was observed in TCG and TKG mice, compared with the SCG mice. Although serum levels of biliverdin and hemin were rose modestly after rmTBI, there were no significant differences between groups (Supplementary Fig. 1). These results indicate the presence of ketotic state in mice fed with KD.

**rmTBI Caused Neurobehavioral Dysfunction, Which Was Ameliorated by KD**

The neurobehavioral assessments were performed on 7th day post-injury. The balance and motor coordination of mice were assessed using the beam walk test. Average crossing time and hind-leg foot slips significantly increased in TCG.
mice, as compared with SCG mice. Moreover, KD decreased average crossing time and hind-leg foot slips in TKG mice compared with TCG mice (Fig. 3A, B). Moreover, the memory dysfunction as assessed by the Y-maze test indicated that rmTBI caused significant impairment of spatial working memory in TCG mice, compared with that in SCG mice; TKG mice showed significantly improved memory performance in the Y maze test (Fig. 3C). These observations indicate that KD treatment could ameliorate neurobehavioral dysfunction caused due to rmTBI.

**rmTBI Caused Axonal Injury, Which Was Alleviated by KD**

Silver staining revealed abnormalities of white matter in the brains of TCG and TKG mice. The density of silver staining in the corpus callosum of TCG mice on 7th day post-injury showed significant axonal damage compared with that in SCG mice. However, there was a significant reduction of corpus callosum silver staining density in TKG mice (Fig. 4A, C).

FA and MD are the most commonly used DTI-derived metrics, which are believed to reflect overall white matter health. On 7th day after rmTBI, TCG mice showed increased FA and MD values in the corpus callosum compared with those in SCG. Meanwhile, TKG mice showed decreased FA and MD values, as compared with TCG mice (Fig. 4B, D and E). Thus, axonal damage on 7th day after rmTBI, was significantly alleviated by KD.

**rmTBI Led to Microglial Activation and Astrogliosis, Which Were Inhibited by KD**

rmTBI is followed by microglial activation and other neuroinflammatory responses. Change in microglial activation was evaluated by the number of Iba-1 positive cells in hippocampus by immunohistochemical staining. We found that the number of Iba-1 positive cells was dramatically increased in TCG mice, as compared with SCG mice. Meanwhile, the number of Iba-1 positive cells were decreased in TKG mice, as compared with TCG mice (Fig. 5A, B).

Immunohistochemical staining of astrocyte marker glial fibrillary acidic protein (GFAP) was used to evaluate astrogliosis in hippocampus. Mice in TCG had significantly increased number of GFAP-positive cells, as compared with SCG mice, and mice in TKG had fewer GFAP-positive cells, as compared with TCG mice (Fig. 5C, D).

The morphological analyses of hippocampal microglia and astrocytes showed a shortening of microglia processes, an elongating of astrocytes processes, and no change of cell body size after rmTBI. Meanwhile, we also found considerably morphological changes of hippocampal microglia and astrocytes in KD-fed mice (Supplementary Fig. 2). These
observations indicate that rmTBI caused microglial activation and astrogliosis, which were suppressed by KD.

**rmTBI Activated *L. reuteri* Mediated Indole/AHR Signaling Pathway, Which Was Inhibited by KD**

Standard curve obtained by plotting the average Ct values against the estimated log_{10} CFU for *L. reuteri* is shown in Fig. 6A. The amounts of *L. reuteri* (Log_{10} CFU/gram of feces) were determined in mice feces with qRT-PCR. The results showed that the amount of *L. reuteri* (Log_{10} CFU/gram of feces) in TCG mice was markedly increased compared with that in SCG mice. KD reduced the amount of *L. reuteri* in TKG mice, compared with that in TCG mice (Fig. 6B).

To determine the role of indole/AHR pathway after rmTBI, we measured the levels of 3-IAA with ELISA, a potential endogenous AHR ligands in serum, large intestines, and brains in mice. The results showed that the levels of 3-IAA levels in TCG mice were markedly increased compared with those in SCG mice. Moreover, KD decreased the levels of 3-IAA in TKG mice (Fig. 6C-E). There were no significant differences in the levels of 3-IAA between SCG and TKG mice.

Mechanistically, AHR can modulate neuroinflammation via competitive binding with RelB. Therefore, we further investigated the expression and nuclear localization of AHR and RelB in microglia and astrocytes on 7th day after rmTBI, using immunofluorescent staining (Vogel and Matsumura 2009; Chen et al. 2019). Immunofluorescence analysis revealed that TCG mice showed a marked increase
in the expression of AHR/RelB and co-localization in the nucleus of Iba-1 positive microglia and GFAP positive astrocytes, as compared with SCG mice. In TKG mice, compared with TCG mice, the AHR/RelB co-localization was more pronounced in the cytosol than in the nucleus (Fig. 7A). The negative control images have been added to supplementary materials (Supplementary Fig. 3).

CCL1 and IRF3 are involved in the neuroinflammation after rmTBI. Thus, the levels of CCL1 and IRF3 in hippocampus were determined with ELISA. The results showed that the levels of CCL1 and IRF3 in TCG mice were markedly increased compared with those in SCG mice. KD attenuated the expressions of CCL1 and IRF3 in TKG mice (Fig. 7B, C). Collectively, these results suggest that L. reuteri mediated indole/AHR signaling pathway might participate in the pathological development of rmTBI by modulating inflammatory activation and KD played an important role in suppressing L. reuteri mediated indole/AHR signaling pathway.

**rmTBI Activated the TLR4/MyD88 Mediated NF-κB Pathway, Which Was Inhibited by KD**

Expression levels of TLR4 and MyD88 in hippocampus were measured with Western blotting. Increased levels of TLR4 and MyD88 were observed in the hippocampus of mice in TCG on 7th day post-rmTBI, compared with those in SCG mice. Moreover, KD decreased levels of TLR4 and MyD88 in TKG mice (Fig. 8A-C and Supplementary materials).

TNF and IL-1β are involved in neuroinflammatory responses after rmTBI. Hence, the levels of TNF and IL-1β in hippocampus were determined with ELISA. Our results revealed that the expression levels of TNF and IL-1β in TCG mice were markedly increased compared with those in SCG mice. KD attenuated the expressions of TNF and IL-1β in TKG mice, compared with those in TCG mice (Fig. 8D, E). Taken together, these findings suggested that rmTBI activated the TLR4/MyD88 mediated NF-κB pathway, which was inhibited by KD.

**Discussion**

It was found that rmTBI induced by CCI caused axonal damage, microglial activation and astrogliosis, and impaired neurological functions. KD given immediately post rmTBI in adolescent mice significantly increased the blood β-HB levels and led to functional neurological benefits. KD, compared with control diet, inhibited the microgliosis, astrogliosis, and pro-inflammatory activation after rmTBI. Furthermore, rmTBI also activated TLR4/MyD88 mediated AHR pathway, which were attenuated by KD.

Spatial reference and working memory are mainly hippocampus-based (Garrett et al. 2020). CA1 subcircuit-dependent spatial working memory and reference memory...
are commonly impaired in brain-damaged patients (Ameen-Ali et al. 2015). Earlier studies indicated that selective removal of parvalbumin interneurons from the CA1 region of the hippocampus induced selective alterations in spatial working memory (Murray et al. 2011). Consequently, the hippocampal CA1 region was selected for further investigation in our study. Moreover, the Y-maze task is particularly used to evaluate the attention and spatial working memory of mice because of their natural ability to find an escape through small holes (Walrave et al. 2016). Here we show that experimental rmTBI causes behavioral abnormalities, including motor coordination and spatial memory impairment. Meanwhile, KD ameliorated rmTBI-induced neurobehavioral dysfunction.

rmTBI triggers axonal injury, which is associated with post-traumatic neurobehavioral impairment. Silver staining and DTI are two reliable approaches for the estimation of axonal damage after rmTBI. Several studies have confirmed that acute axonal injury in various white matter regions in rmTBI is indicated by argyrophilic structures and significant silver uptake compared to sham (Winston et al. 2016). Of all regions, the corpus callosum may have the most prominent and persistent course of silver staining abnormalities at 7 days (Hylin et al. 2013). FA and MD are the most commonly used DTI-derived metrics, which are believed to reflect overall white matter health, maturation, and organization (Hofstetter and Assaf 2017). In this study, results of silver staining and DTI showed significant axonal injury 7 days post-rmTBI. The increased intensity of silver staining in the injured mice correlated with their impaired motor coordination, as assessed by the beam walking test. We found that KD could ameliorate axonal damage and improve motor coordination in mice with rmTBI.

KD protects myelinated axons by increasing myelination that could improve axonal energy support (Stumpf et al. 2019). TBI-induced changes in cerebral glucose metabolism occur as a series of neurochemical events (Prins and Matsumoto 2016). When cerebral metabolism of glucose is compromised following brain injury, ketones can fulfill the energy requirements of the brain cells. Classical KD, with high-fat and low-carbohydrate content, can increase ketone levels and act as alternative substrates for all cell types. These KB stimulate mitochondrial metabolism and increase its metabolic efficiency, reduce the production of reactive oxygen species, and supply up to 70% of the energy required for brain function (Thau-Zuchman et al. 2021). Therefore, KD may be beneficial for the treatment for axonal damage following rmTBI.

Astrocytes are crucial for brain metabolism and are involved in many processes such as regulating glucose metabolism, participating in fatty oxidation (Gzielo et al. 2019). Under KD conditions, astrocytes mitochondria may spare glucose utilization, do not increase their insulin

![Fig. 4](image-url)
sensitivity, and provide trophic support to neurons (Koppel et al. 2021). Meanwhile, astrocytes are highly sensitive to a wide range of injuries. Astrogliosis is also a lingering impact seen in mice following rmTBI. Axonal damage and the related release of inflammatory cytokines by activated microglia can induce astrogliosis (Davenport et al. 2016). Our investigation showed that rmTBI caused reactive astrogliosis, and KD could attenuate astrocytes reactivity. We hypothesize these outcomes may be primarily related to KD-induced metabolic changes. Thus, modulation of metabolism in astrocytes might be a potential therapeutic target for rmTBI.

Studies on KD have shown that a fat-rich and carbohydrate-poor diet can inhibit microglial activation after moderate-severe TBI (Thau-Zuchman et al. 2021). Studies examining the mechanisms of therapeutic effects of KD on rmTBI are lacking. In this study, we have demonstrated that rmTBI caused neuroinflammation, which was ameliorated by KD. In addition to reducing the number of activated microglia and astrocytes, KD may also directly affect the pro-inflammatory function of inflammatory cells (Guan et al. 2020). This negative regulatory effect of KD may play an important role in reducing neuroinflammation mediated by inflammatory cells after rmTBI. Therefore, further explorations were conducted to elucidate underlying mechanisms of KD-regulated neuroinflammatory activation.

Since inflammatory damage is the key pathophysiological feature of rmTBI, we chose microglial activation and astrogliosis as the focus in our research to study the mechanisms underlying the beneficial effects of KD on rmTBI. Multiple molecular pathways, such as STAT and nuclear factor-κB (NF-κB) are involved in the regulation of neuroinflammation (Qin et al. 2012; Kobayashi et al. 2013; Tanaka et al. 2015). Previous studies have reported that moderate-severe TBI activated the TLR4/MyD88 mediated NF-κB pathway in microglial cells (Zhang et al. 2018). Our results show that KD could suppress the expression of TLR4 and MyD88 and negatively modulate the level of the inflammatory cytokines TNF and IL-1β 7 days after rmTBI. However, the mechanism by which KD regulates the TLR4/MyD88 mediated NF-κB pathway after rmTBI is not clear.

AHR could be activated by numerous exogenous compounds. Indole and its derivates such as 3-IAA and indole-3-propionic acid (3-IPA) have been identified as high-affinity
endogenous ligands to the AHR (Gao et al. 2020). GM can impact levels of various neuroactive molecules in the peripheral nervous system and the brain (Vuong et al. 2017). Tryptophan, an essential amino acid in the diet, can be converted to 3-IAA by some bacteria of genus *Lactobacillus* and *Bifidobacterium* through aromatic amino acid aminotransferase and indole lactic acid dehydrogenase–dependent pathways (Cervantes-Barragan et al. 2017; Gao et al. 2020). Moreover, it has been reported that KD altered the GM and significantly decreased *L. reuteri* in mice fed with KD (Olson et al. 2018). However, whether *L. reuteri* mediated indole/AHR signaling pathway is involved in the regulation of KD on inflammatory activation after rmTBI was unclear.

Mechanistically, activated AHR translocates into the nucleus, interacts with RelB, and occupies RelB/AHR-response elements (RelB/AHRE) of promoters, resulting in activation of downstream target genes, like CCL1, IRF3 (Vogel and Matsumura 2009). It has been reported that AHR signaling potentially mediates post-stroke gliosis and ischemic brain injuries (Chen et al. 2019). In this study, we have shown that *L. reuteri*-produced tryptophan metabolite, 3-IAA, may be associated with neuroinflammation. Our study found that there were reduced co-localization of AHR/RelB in the nucleus of inflammatory cells and lower level of CCL1 and IRF3 in mice fed with KD, post-rmTBI. Thus, *L. reuteri* may participate in the modulation of KD on inflammatory activation after rmTBI.
inflammatory activation after rmTBI through indole/AHR signaling.

**Conclusion**

Axonal damage, microglial activation and astrogliosis induced by rmTBI could be ameliorated in adolescent mice by giving KD immediately after rmTBI. KD significantly increased blood β-HB levels and provided functional neurological benefits. *L. reuteri* mediated indole/AHR and TLR4/MyD88 mediated NF-κB signaling pathways in the hippocampus were inhibited after KD intervention, which indicated a suppressed neuroinflammation related to microglial activation and astrogliosis. Our study provides insights into the potential mechanisms of beneficial effects of KD in rmTBI in adolescent mice.
Limitations

The exact mechanism by which KD affects the synthesis of indole compounds in the GM remains unclear. Inhibition of the indole/AHR pathway in inflammatory cells is the next logical step for confirmation of the role of GM produced indole metabolites in mediating the inhibitory effect of KD on neuroinflammatory responses. Several studies have reported age-related differences in the neuroprotection provided by KD and MCT1 expression after TBI (Appelberg et al. 2009). We have chosen adolescent mice for this study, further studies are required to verify the beneficial effect of KD on adult mice with rmTBI.

Supplementary Information

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Author Contributions

DD, FZ and YJ designed the experiments. DD, FZ and SS performed and analyzed most experiments with the help of TL, MC, QL. DD and FZ were major contributors in writing this manuscript. FJ and XZ participated in the discussions and revised the manuscript. YJ supervised the entire project and was responsible for finalizing and submitting the manuscript. All authors read and approved the final manuscript.

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Data Availability

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.
Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical Approval Animal protocols were approved by the Animal Care and Experimental Committee of the School of Medicine of Shanghai Jiao Tong University.

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