Int J Clin Exp Med 2016;9(11):21270-21279
www.ijcem.com /ISSN:1940-5901/IJCEM0039219

Original Article

High ω-3/ω-6 ratio diet could alleviate blood-brain barrier dysfunction in rat model with traumatic brain injury

Jianguang Su[1*], Kai Quan[2*], Kun Fan[3], Jiaying Zhang[1], Jingfang Liu[1], Wei Hua[2], Ying Mao[2,4,5]

Departments of [1]Clinical Nutrition, [2]Neurosurgery, Huashan Hospital, Fudan University, Shanghai, China; [3]Institute of Biomedical Science, [4]State Key Laboratory of Medical Neurobiology, Institutes of Brain Science, [5]The Collaborative Innovation Center for Brain Science, Fudan University, Shanghai, China. [*]Equal contributors.

Received September 1, 2016; Accepted September 21, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: Objectives: Polyunsaturated fatty acids (PUFAs) have functional activity in many aspects, including brain trauma. Diets with different ratios of ω-3/ω-6 were applied to investigate the protective effect on delayed blood brain barrier (BBB) dysfunction after traumatic brain injury (TBI) in rat models. Methods: Moderate TBI was mimicked by Feeney's weight-dropping rat models. The effects of PUFA, with different ω-3/ω-6 ratios (1:1, 1:5, and 1:30) on BBB disruption, were examined by Evans blue (EB) dye varying from different time (day 3, 5, 7) after TBI. The corresponding change of 4-HNE and tight junction proteins were analyzed by immunofluorescence staining and western blotting technology. In the meantime, D-penicillamine (4-HNE inhibitor) was administrated to verify the role of 4-HNE in this event. Cognitive function was evaluated by Morris water maze test. Results: Delayed BBB disruption was observed in the injured brains. High ω-3/ω-6 ratios diet significantly reduced EB extravagation and 4-HNE level, and also enhanced the expression of tight junction proteins (ZO-1 and occludin). Inhibiting 4-HNE by administrating D-penicillamine could exert protective effects as well. In addition, high ω-3/ω-6 ratios diet could also promote the recovery of cognitive function after TBI. Conclusions: High ω-3/ω-6 ratio diet could alleviate delayed BBB dysfunction, which was associated with reduction of 4-HNE and enhancement of tight junction proteins. Besides, 1:1 might be a more suitable dietary ω-3/ω-6 ratio for TBI patient.

Keywords: Omega-3 fatty acids, traumatic brain injury, blood-brain barrier, tight junction proteins, 4-hydroxy-2-nonenal

Introduction

Traumatic brain injury (TBI) would cause secondary injury, brain edema, which is correlated with the disruption of blood-brain barrier (BBB) and abnormal permeability of allotropic proteins and water [1, 2]. Posttraumatic BBB dysfunction could be divided into immediate and delayed periods, and they will reach their peaks in 4-6 hours and 2-3 days respectively after TBI [1]. But the approaches to prevent delayed BBB disruption have been a focus concerned by humans for such a long time.

As we know, the supplement of ω-3 fatty acids (O3FAs) has been widely applied in various diseases, such as Alzheimer's disease, Parkinson's disease, and brain injury [3, 4]. The protective effect might be mediated by modulating multiple signaling pathways including NF-κB, PPAR, PI3K/Akt and MAPK [5, 6]. Recent studies have shown that dietary supplement of O3FAs could limit the BBB disruption in rat models with brain trauma [7], and could also reduce traumatic axonal injury [8]. In addition, it has been proved that endogenous conversion from ω-6 to ω-3 polyunsaturated fatty acids (PUFAs) in *fat-1* transgenic mice could alleviate the symptoms caused by spinal cord injury [9]. Clinical trials which is about the application of dietary O3FAs in TBI patients have been carried out (NCT01903525, NCT01814527). Posttraumatic inflammation, a probable cause of BBB disruption, could be suppressed by high O3FAs diet [10]. Furthermore, dietary ω-3/ω-6 ratio is also closely related to oxidative injury after TBI. 4-hydroxy-2-hexenal (4-HHE), an end-product of O3FAs peroxida-

Table 1. Ingredient composition of fat-free diet (g/kg)

Ingredient	Amount per kg
Casein	200.0
Methionine	3.5
Corn starch	448.0
Maltodextrin	100.0
Cane sugar	150.0
Cellulose	47.0
Mineral mixture	35.0
Calcium Carbonate	4.0
Vitamin mixture	10.0
Choline chloride	2.5

tion, could exert an anti-oxidative effect through activating Nrf2 pathway [11], while 4-hydroxy-2-nonenal (4-HNE), a metabolite of ω-6 fatty acids (O6FAs), may aggravate oxidative injury [12, 13].

However, excessive intake of O3FAs might inhibit platelet aggregation and raise the clinical concern for hemorrhagic complication [14]. Thus, the significance of O3FAs and optimal ratio of ω-3/ω-6 also need to be explored. In this study, we investigated the effect of O3FAs on secondary brain injury after TBI, and speculated that high ω-3/ω-6 ratio diet might protect posttraumatic BBB function by reducing oxidative stress. And it may serve as a potential therapy for brain edema after TBI.

Materials and methods

Establishment of moderate traumatic brain injury in rodent model

107 healthy male Sprague-Dawley (SD) rats weighing 250~300 g were from the Department of Laboratory Animal Science at Fudan University, and were housed in an optimal circumstance. TBI was mimicked by modified Feeney's weight-dropping method, as previously described [15]. In brief, rats were anesthetized with 10% chloral hydrate (0.35 ml/100 g) by intraperitoneal injection. After craniotomy, a right parietal bone window (diameter 5 mm) was made 3 mm behind the coronal suture and 2.5 mm beside the sagittal suture. The moderate TBI model was generated by a 20 g weight dropping from 30 cm height, and the impact diameter of the rats was 4 mm. The rats in sham group were only subjected to craniotomy without weight-dropping impact. All procedure and protocols were reviewed by Institutional Animal Care and Use Committee in Fudan University (20150650C003).

Grouping according to different dietary ratio of ω-3 to ω-6 fatty acids

Diet with different ratios of ω-3/ω-6 PUFAs were given to traumatic rats to investigate the effects on BBB disruption. Diet with a ω-3/ω-6 ratio of 1:30 was considered as low O3FAs diet, which is representative of western diet [4, 16]. Diet with a ω-3/ω-6 ratio of 1:5 was taken as intermediate O3FA diet, while 1:1 as high O3FAs diet [16, 17]. There were 5 groups in the study. Rats that were fed with low O3FAs diet after sham surgery were taken as control (SHAM group). Rats in low O3FAs group were fed with low O3FAs diet after TBI, and those in intermediate O3FAs group or high O3FAs group were fed with intermediate or high O3FAs diet. D-Penicillamine (PEN), which could bind to the carbonyl of 4-HNE as an inhibitor [18], was injected intraperitoneally (10 mg/kg per day) into TBI rats with low O3FAs diet to block 4-HNE (PEN group).

To address whether different ratio PUFAs diet could affect the BBB in a time related way, 36 rats were divided into two groups (low O3FAs group and high O3FAs group). In each group, 6 rats sacrificed respectively at day 3, 5, and 7 after TBI to quantify Evans blue (EB) extravasation. Then, the day 5 was chosen as representative time. Subsequently, 63 rats were divided into 5 groups and euthanized at day 5 to determine whether the protective effect on BBB disruption was dose-related or not, and to explore the role of 4-HNE in BBB function. The traumatic brains were prepared for EB quantification, western blot, and staining (Supplementary Figure 1). Besides the above, 8 rats were divided into two groups (low O3FAs group and high O3FAs group) to evaluate the cognitive function by Morris water maze test.

Dietary intervention after TBI

After TBI or sham surgery, rats in all groups were given a basic fat-free feed (**Table 1**). Oil mixture with different ω-3/ω-6 ratios were prepared beforehand by mixing fish oil (Mega-DHA 1000 mg capsules, containing 50% DHA and 20% EPA, Nature's Way, USA) and linoleic

Table 2. Composition of oil mixtures

ω-3/ω-6 Ratio	Fish oil	Linoleic acid
1:30	4%	96%
1:5	21%	79%
1:1	58%	42%

acid (95% purity, Shanghai Yuanye Bio-Tech, China) in different proportions (**Table 2**). And kept it free from light in cold storage. From the first day after injury till the day they were euthanized, rats in different groups were treated with corresponding oil mixture by gavage (5.5 ml/kg per day, amounting to DHA 1430 mg/kg and EPA 570 mg/kg for high O3FAs diet, DHA 530 mg/kg and EPA 210 mg/kg for intermediate O3FAs diet, DHA 100 mg/kg and EPA 40 mg/kg for low O3FAs diet per day).

Quantitative analysis of Evans blue (EB) dye

The integrity of BBB was quantified using EB dye extravasation [19]. EB dye (2% in saline, 4 ml/kg, Sigma-Aldrich) was injected into the internal jugular vein. Two hours after injection, the chest was opened, and the brain was perfused with saline. After decapitation, the brain was quickly removed and photographed. Brain tissues in the injured region were resected (about 5 mm × 5 mm × 3mm) and weighed for quantitative measurement. Tissues were homogenized in 1 ml 50% trichloroacetic acid, and centrifuged at 21000 g for 20 minutes. The supernatants were diluted with ethanol and measured on a spectrophotometer (λ=610 nm).

Hematoxylin eosin (HE) and immunofluorescence staining

Rat was perfused with saline and the brain was removed as mentioned above. Brain tissues in the injured region were cut into slices (10 μm) and were frozen using a Cryostat Microtome (CM1950, Leica, USA). Frozen sections were fixed with 4% paraformaldehyde for 10 minutes, stained with hematoxylin solution for 3 minutes, rinsed with hydrochloric acid alcohol for 10 seconds, and then stained with eosin solution for 2 minutes. After dehydrated and cleared, sections were mounted on slides with neutral gum and evaluated under microscope (DM2500, Leica, USA).

Frozen sections were fixed with ice-cold methanol (10 min), permeabilized with 0.3% Triton X-100 in PBS (10 min), and then blocked with 10% donkey serum (1 h). Sections were incubated with ZO-1 (1:100; Life Technologies), occludin (1:200; Abcam), or major facilitator superfamily domain-containing protein 2a (MFSD2A, 1:200; Abcam) polyclonal antibodies overnight at 4°C. Secondary donkey anti-rabbit IgG (Alexa Fluor 488-conjugated, 1:500; Abcam) was added into the staining system for 1 hour at room temperature in a dark chamber. Each treatment was followed by washing three times with PBS. The sections were mounted on slides with Vectashield containing DAPI, and visualized using a fluorescence microscope (DM2500, Leica, USA).

Western blotting technology

Brain tissues around the injured region (about 8 mm × 8 mm × 5 mm) were homogenized on ice in a sodium dodecyl sulfate (SDS) lysis buffer with sodium ortho-vanadate and phenylmethanesulfonyl fluoride (PMSF) protease inhibitor. Western blotting technology was performed with samples containing equal amount of total protein in a 10% SDS-PAGE gel, and 7.5% gel only for ZO-1. The separated proteins in the gel were then transferred onto a PVDF membrane. Nonspecific binding sites were blocked by incubating the PVDF membrane in Tris-buffered saline containing 0.1% Tween-20 and 5% non-fat milk (TTBS). Membranes were then incubated overnight at 4°C with 4-HNE (1:200; Abcam), ZO-1 (1:500; Life Technologies), Occludin (1:500; Abcam), or MFSD2A (1:1000; Abcam) antibodies, then each washed with TTBS three times for 10 minutes, and then incubated with Horseradish peroxidase-linked anti-rabbit IgG for 2 h at room temperature. After washing with TTBS, enhanced chemiluminescence was used to detect different protein levels. Images were analyzed by densitometry using PC Image J software (2.1.4.7 NIH, USA).

Morris water maze test

The cognitive function of TBI rats was assessed by Morris water maze test. Before test, different ratio of ω-3/ω-6 PUFAs were offered to the TBI rats for 14 days, and were still offered during the days when they were tested. In order to visualize the movement of white rats, a black circular swimming pool (150 cm in diameter and 45 cm in height) was filled with water (20-22°C) and an appropriate amount of

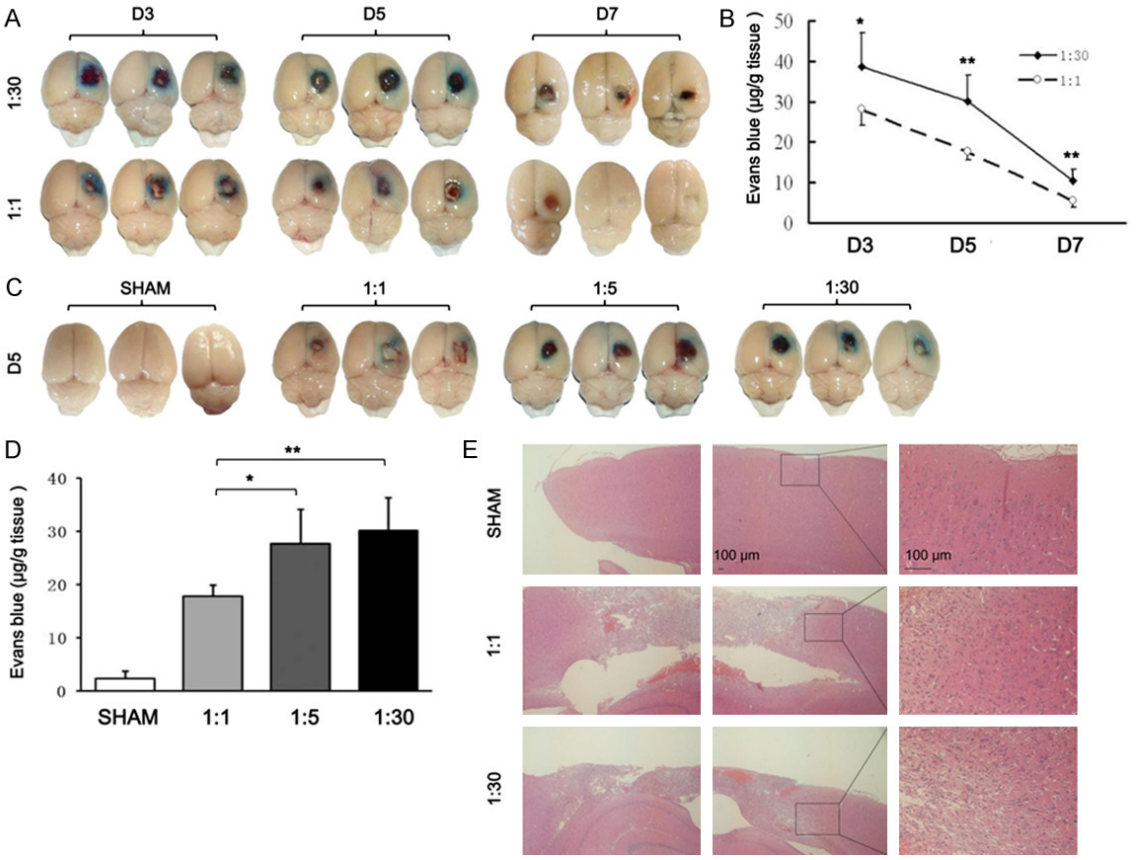


Figure 1. The effects of high O3FAs diet on EB extravasation after TBI. A, B. The gross appearance of brains and EB quantification showed that the BBB disruption was significantly alleviated in high O3FAs (1:1) group at 3, 5 and 7 days after TBI attack. C, D. High O3FAs diet reduced EB extravasation in a dose-related way at day 5 after injury. E. Histological panels showed at 5 days, around the areas that neuron cells and vascular structure on the injury regions of the cerebral cortex after TBI, brain edema was less intense in high O3FAs group (scale bar=100 μm, *P<0.05 and **P<0.01).

carbon ink. The pool was divided into four quadrants, and a black platform was placed in the target quadrant (2 cm below the water surface) with markers in different shapes to provide clues. During the navigation trial, the rats were trained 4 times a day (starting at 10:00 a.m.) for 5 consecutive days. For each trial, a rat was given a maximum of 60 seconds to find the hidden platform (the only escape route). If the rat failed to reach the platform in 60 seconds, it would be guided to the platform and stay for 10 seconds. The time of escape latencies was recorded. Rats were allowed to have a 30-minute rest after final navigation training. Then in the immediately followed spatial probe trial, the platform was removed, and the swimming paths were monitored by a camera. The time spent in the target quadrant and the time crossing the platform within 60 seconds were calculated.

Statistical analysis

The statistical analyses were done with SPSS 16.0 (IBM, USA). Quantitative variables were shown as mean ± SD. Data were subjected to t-test or one-way ANOVA test. Statistical significance was achieved at $P<0.05$.

Results

High O3FAs diet has protective effect on TBI

TBI models were established successfully in 103 SD rats, except 3 died during the surgical procedure and 1 died from excessive bleeding on the second day after surgery. Most rats after TBI had mild dyskinesia of left limbs and different levels of dullness or irritableness. No epilepsy or coma was observed. Brain injury and edema were more extensive in

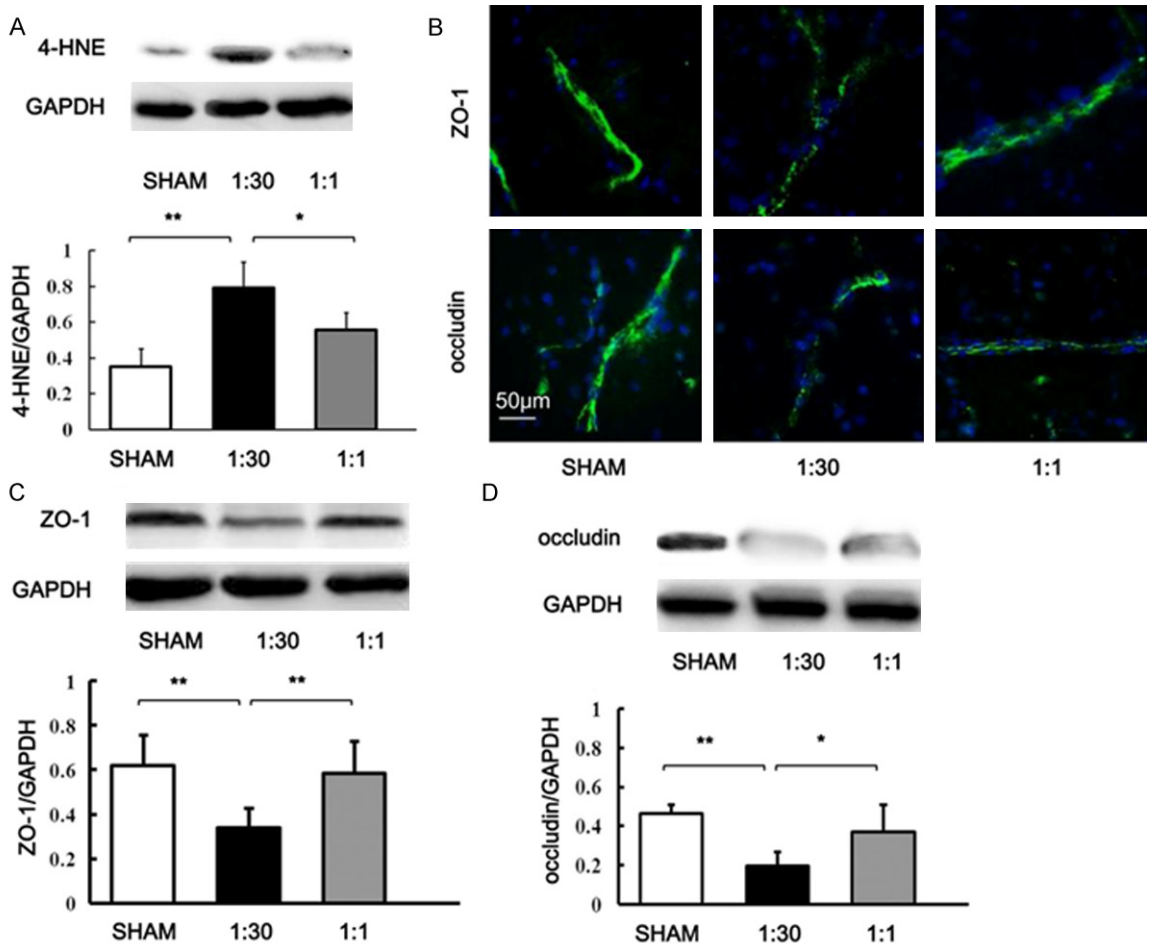


Figure 2. The effects of high O3FAs diet on 4-HNE level and the expression of tight junction proteins in injured brains. (A) Western blotting technology showed that 4-HNE level significantly decreased in high O3FAs (1:1) group at 5 days after TBI. (B) Immunofluorescence staining (scale bar=50 μm) and (C, D) Western blotting technology showed that ZO-1 and occludin expressions in injured tissues significantly enhanced in high O3FAs group.

low O3FAs group (**Figure 1A**). EB extravasation decreased gradually from day 3 to day 7 after traumatic injury (**Figure 1B**). The reduction of EB extravasation was more significant in high O3FAs group than that in low O3FAs group at day 3 ($P<0.05$), day 5 ($P<0.01$) and day 7 ($P<0.01$). At day 5, the decrease of EB extravasation in rats in high O3FAs group was more notable compared with that in intermediate O3FAs group ($P<0.05$) and in low O3FAs group ($P<0.01$), but there was no significant difference in EB extravasation between intermediate O3FAs group and low O3FAs group ($P>0.05$) (**Figure 1C**, **1D**). In addition, the adjacent brain edema was obviously attenuated at day 5 in high O3FAs group by HE staining, which is characterized by vascular degeneration and intercellular space dilation in the surrounding tissues of hemorrhage (**Figure 1E**). These data indicated that high O3FAs diet had the most significant protective effect on post-traumatic BBB dysfunction.

High O3FAs diet could reduce 4-HNE and enhance the expression of tight junction proteins

As 4-HNE could affect the expression and function of tight junction proteins [13], ZO-1, occludin, and MFSD2A were investigated in different groups. Compared with SHAM group, rats in low O3FAs group showed higher 4-HNE level at day 5 ($P<0.01$). Meanwhile, there was a significant decrease of 4-HNE in high O3FAs group compared with low O3FAs group ($P<0.05$), which indicated that high O3FAs diet could reduce the production of 4-HNE after TBI (**Figure 2A**). The expression of ZO-1 ($P<0.01$) and occludin ($P<0.01$) decreased significantly

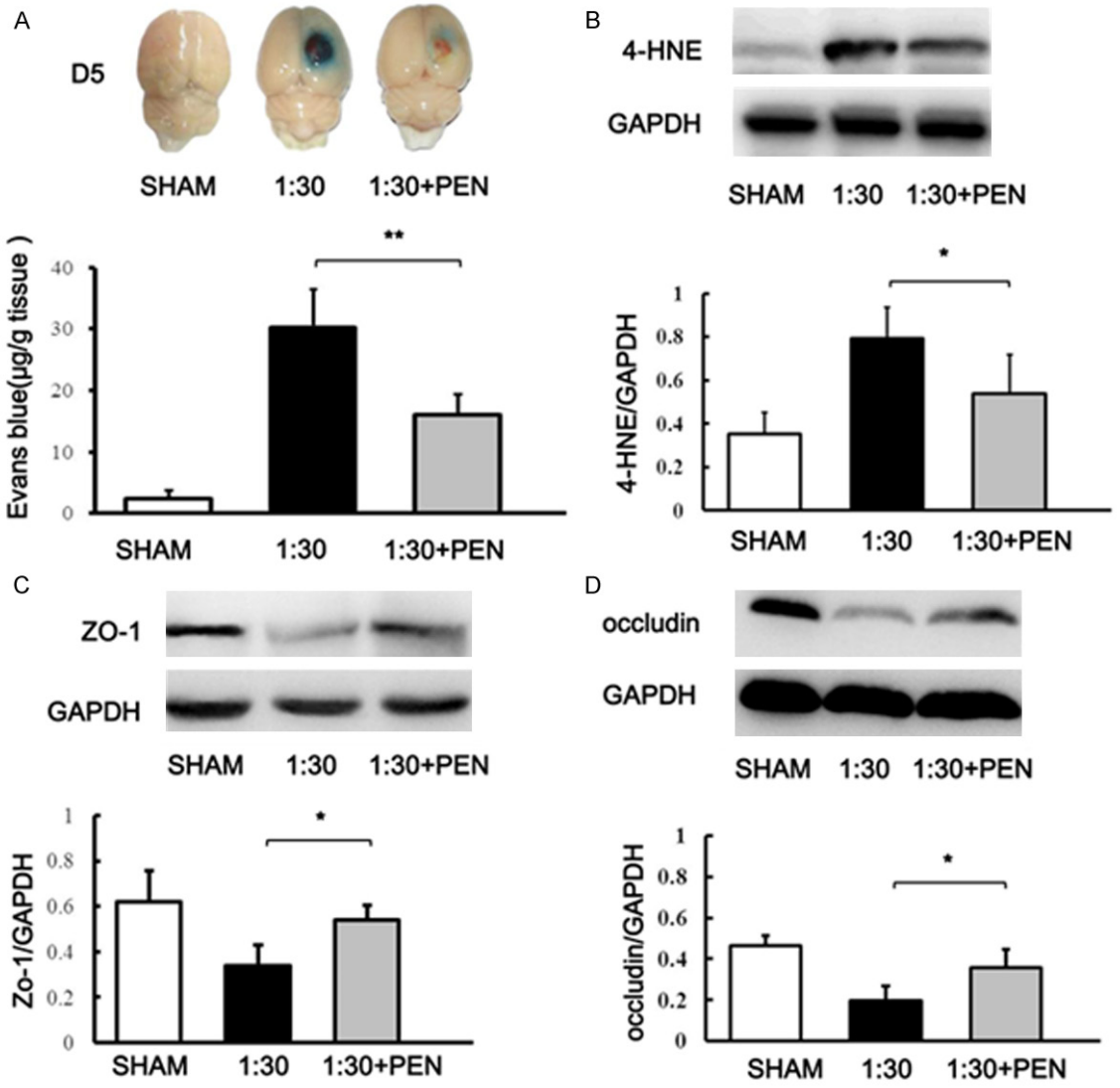


Figure 3. Inhibiting 4-HNE by D-penicillamine could alleviate BBB disruption by enhancing the expression of tight junction proteins. A. The gross appearance and EB quantification of the injured brain showed that D-penicillamine could attenuate BBB dysfunction at day 5 after TBI. B-D. Western blotting showed that 4-HNE level in injured tissues was significantly decreased, and the expressions of ZO-1 and occludin were dramatically enhanced after D-penicillamine treatment (n=6 in each group).

in low O3FAs group at day 5 compared with those in SHAM group by immunofluorescence staining (**Figure 2B**) and western blotting (**Figure 2C**, **2D**). Similarly, compared with low O3FAs group, a significant increase of ZO-1 ($P<0.01$) and occludin ($P<0.05$) expression was observed in high O3FAs group. The expression of MFSD2A showed no statistical difference between the groups (Supplementary Figure 2). So, MFSD2A might not be the downstream target for high O3FAs diet as demonstrated here. All these data indicated that high O3FAs diet, which produced less 4-HNE, could enhance the expression of tight junction proteins.

4-HNE inhibitor could protect posttraumatic BBB function

4-HNE has been considered to be one of the biomarkers for oxidative stress [20]. D-Penicillamine (4-HNE inhibitor) was injected into TBI rats with low O3FAs diet to reduce brain 4-HNE level, and then BBB function and tight junction protein expression were examined at 5 days after TBI. A significant decrease of 4-HNE

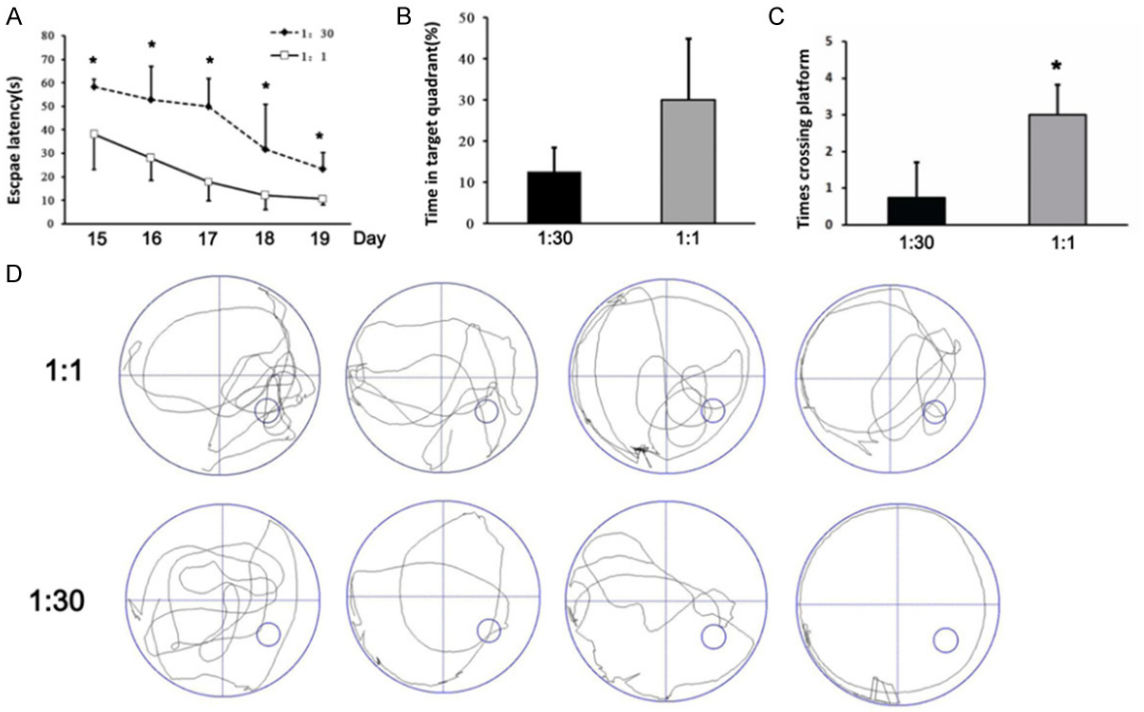


Figure 4. The effects of high O3FAs diet on cognitive function after TBI. A. Place navigation trails showed that escape latency in high O3FAs group was significantly reduced. B-D. Spatial probe trials showed that longer time was spent in target quadrant, and the numbers of crossing the platform in high O3FAs group was increased, according to the trajectory recording.

was detected after D-Penicillamine administration, which resulted in a significant decrease in EB extravasation (P<0.01, **Figure 3A**), compared with low O3FAs group (P<0.05, **Figure 3B**). In the meantime, a significant increase of ZO-1 (P<0.05) and occludin (P<0.05) was observed in PEN group (**Figure 3C**, **3D**). These data suggested that the protective effect of high O3FAs diet was correlative to the reduction of 4-HNE.

High O3FAs diet could accelerate the recovery of cognitive function after TBI

The effect of high O3FAs diet on cognitive function after TBI was evaluated with Morris water maze test. In place navigation trial, no significant difference in swimming speed was observed between high O3FAs and low O3FAs groups (245.9 ± 20.2 mm/s *vs* 206.7 ± 28.5 mm/s). ANOVA analysis revealed a significant difference between the two groups, as high O3FAs group exhibited shorter escape latency (P<0.05, **Figure 4A**). In spatial probe trial, the rats in high O3FAs group spent longer time in the target quadrant compared with low O3FAs group, although the difference was insignificant (**Figure 4B**). Nevertheless, compared with low O3FAs group, a significantly increased number of platform crossings was observed in high O3FAs group (P<0.05, **Figure 4C**, **4D**). These data suggested that high O3FAs diet could improve the spatial learning and memory function after TBI.

Discussion

Brain edema is one of the major risk factors for high mortality after TBI, and the integrity of BBB is critical to reduce posttraumatic edema. In this study, we demonstrated the protective effect of high O3FAs diet on delayed BBB dysfunction after moderate TBI, and this effect of high O3FAs diet on tight junction proteins was associated with 4-HNE reduction.

In our study, delayed posttraumatic BBB dysfunction was observed in rat models, and it was gradually recovered from 3 to 7 days after TBI. We have also identified that dietary supplementation of O3FAs can inhibit the post-

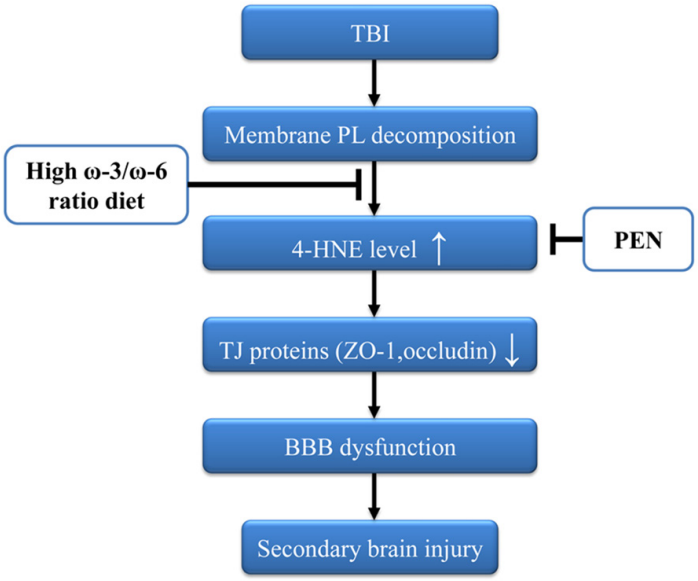


Figure 5. The proposed pathways involved in this study, secondary BBB dysfunction might partly result from 4-HNE accumulation which could alter the expression and function of the tight junction proteins. Reducing 4-HNE production through administration of high O3FAs diet, or inhibiting 4-HNE by D-penicillamine could result in enhanced expression of tight junction proteins and improved BBB function after TBI.

traumatic BBB disruption, ameliorate the secondary injury and improve the outcomes of neurologic function. More importantly, our finding indicated that the modern dietary ratio of ω-3/ω-6 (1:20~50) might not be beneficial for TBI patients, and diet with a high ω-3/ω-6 ratio (1:1) could be helpful.

Our results are consistent with previous reports [7, 21, 22], while the detailed mechanism and optimal dietary ratio of ω-3 to ω-6 PUFAs need to be investigated further on clinical application. The mechanism for delayed BBB disruption has been summarized that it might originate from increased permeability with aberrant expression and distribution of tight junction proteins, and augmented transport of pinocytosis vesicles across the BBB [1]. Since high O3FAs diet showed a protective effect on delayed BBB dysfunction, we speculate that the metabolites of ω-3 or ω-6 PUFAs might affect tight junction proteins or pinocytosis-related proteins. 4-HHE, the end-product of peroxidation of O3FAs, has been demonstrated to protect brain injury by upregulating heme oxygenase-1 through the Nrf2 pathway [11]. On the contrary, 4-HNE, which is a metabolic product of O6FAs and contains a carbonyl group, may cause protein carbonylation and alter its function [18, 23]. Our finding showed that high O3FAs diet resulted in the decrease of 4-HNE and the increase of tight junction proteins in the injured brain, as 4-HNE has been reported to affect endothelial cell permeability by modulating tight junction proteins [12, 13]. Our results also revealed that the inhibition of 4-HNE by administration of D-penicillamine after low O3FAs diet could increase the tight junction proteins. Therefore, we inferred that the protective effect of high ω-3/ω-6 ratio diet might partly result from the reduce of the production of 4-HNE (**Figure 5**). MFSD2A, which is found to be a transporter for DHA [24], could regulate BBB function by suppressing transcytosis [25]. However, MFSD2A might not be the downstream target for high O3FAs diet as was demonstrated here.

High O3FAs diet has protective effects on posttraumatic BBB, however, excessive O3FAs in diet might inhibit platelet aggregation [14]. In the meantime, O6FAs are essential for cells, but too much intake of O6FAs might be detrimental for the repairing of disrupted BBB. Appropriate ratio of ω-3 to ω-6 PUFAs is quite important, and the most suitable one is 1:1 according to this study. However, there are several limitations in this study. First, the protective effect of high ω-3/ω-6 ratio PUFAs on BBB might result from other potential mechanisms. Secondly, ω-3 and ω-6 PUFAs both include several kinds of fatty acids, which have their own individual biological properties and functionalities. Further studies should focus on specific fatty acids rather than general classes.

In conclusion, our results provide novel evidence that high O3FAs diet could alleviate the delayed BBB dysfunction after moderate TBI. Taken the economy and feasibility, appropriate ω-3/ω-6 ratio diets are still recommended as posttraumatic nutritional support, and 1:1 might be a suitable dietary ratio for TBI patient. This protective effect may partly depend on the reduction of 4-HNE and the enhancement of tight junction proteins in BBB.

Acknowledgements

This work was funded by the Scientific Research project supported by Huashan Hospital, Fudan University (2013QD04). The authors' contributions were as follow: S.J. and Q.K. developed the TBI animal models, performed EB dye and immunofluorescence staining, and drafted the manuscript. F.K. did western blot. H.W. and M.Y. guided the experiments and revised the manuscript. Z.J. participated in the statistical analysis, and L.J. was involved in the study design. We would like to thank Professor Yang Guoyuan and the staff from Med-X Research Institute of Shanghai Jiaotong University for their collaborative support. We also thank Wang Xiaowen and Nelson Nellie for proofreading the manuscript.

Disclosure of conflict of interest

None.

Address corresponding to: Dr. Wei Hua, Department of Neurosurgery, Huashan Hospital, Fudan University, No. 12 Middle Wulumuqi Road, Shanghai 200040, China. Tel: +86 021-52887240; E-mail: doctor.huawei@gmail.com; Dr. Jingfang Liu, Department of Clinical Nutrition, Huashan Hospital, Fudan University, No. 12 Middle Wulumuqi Road, Shanghai 200040, China. Fax: +86 021-52888530; E-mail: ljfhuashan@hotmail.com

References

[1] Chodobski A, Zink BJ and Szmydynger-Chodobska J. Blood-brain barrier pathophysiology in traumatic brain injury. Transl Stroke Res 2011; 2: 492-516.

[2] Lin Y, Pan Y, Wang M, Huang X, Yin Y, Wang Y, Jia F, Xiong W, Zhang N and Jiang JY. Blood-brain barrier permeability is positively correlated with cerebral microvascular perfusion in the early fluid percussion-injured brain of the rat. Lab Invest 2012; 92: 1623-1634.

[3] Janssen CI and Kiliaan AJ. Long-chain polyunsaturated fatty acids (LCPUFA) from genesis to senescence: the influence of LCPUFA on neural development, aging, and neurodegeneration. Prog Lipid Res 2014; 53: 1-17.

[4] Lewis M, Ghassemi P and Hibbeln J. Therapeutic use of omega-3 fatty acids in severe head trauma. Am J Emerg Med 2013; 31: 273, e275-278.

[5] Wellhauser L and Belsham DD. Activation of the omega-3 fatty acid receptor GPR120 mediates anti-inflammatory actions in immortalized hypothalamic neurons. J Neuroinflammation 2014; 11: 60.

[6] Draper E, Reynolds CM, Canavan M, Mills KH, Loscher CE and Roche HM. Omega-3 fatty acids attenuate dendritic cell function via NF-kappaB independent of PPARgamma. J Nutr Biochem 2011; 22: 784-790.

[7] Russell KL, Berman NE, Gregg PR and Levant B. Fish oil improves motor function, limits blood-brain barrier disruption, and reduces Mmp9 gene expression in a rat model of juvenile traumatic brain injury. Prostaglandins Leukot Essent Fatty Acids 2014; 90: 5-11.

[8] Bailes JE and Mills JD. Docosahexaenoic acid reduces traumatic axonal injury in a rodent head injury model. J Neurotrauma 2010; 27: 1617-1624.

[9] Lim SN, Gladman SJ, Dyall SC, Patel U, Virani N, Kang JX, Priestley JV and Michael-Titus AT. Transgenic mice with high endogenous omega-3 fatty acids are protected from spinal cord injury. Neurobiol Dis 2013; 51: 104-112.

[10] Calder PC. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr 2006; 83: 1505S-1519S.

[11] Zhang M, Wang S, Mao L, Leak RK, Shi Y, Zhang W, Hu X, Sun B, Cao G, Gao Y, Xu Y, Chen J and Zhang F. Omega-3 Fatty Acids Protect the Brain against Ischemic Injury by Activating Nrf2 and Upregulating Heme Oxygenase 1. J Neurosci 2014; 34: 1903-1915.

[12] Mertsch K, Blasig I and Grune T. 4-Hydroxynonenal impairs the permeability of an in vitro rat blood-brain barrier. Neurosci Lett 2001; 314: 135-138.

[13] Usatyuk PV, Parinandi NL and Natarajan V. Redox regulation of 4-hydroxy-2-nonenal-mediated endothelial barrier dysfunction by focal adhesion, adherens, and tight junction proteins. J Biol Chem 2006; 281: 35554-35566.

[14] Bays HE. Safety considerations with omega-3 fatty acid therapy. Am J Cardiol 2007; 99: 35C-43C.

[15] Feeney DM, Boyeson MG, Linn RT, Murray HM and Dail WG. Responses to cortical injury: I. Methodology and local effects of contusions in the rat. Brain Res 1981; 211: 67-77.

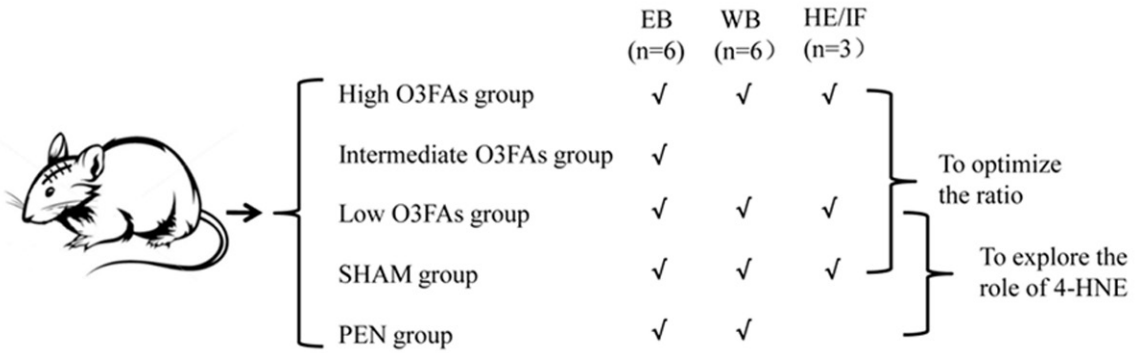
[16] Simopoulos AP. Evolutionary Aspects of Diet: The Omega-6/Omega-3 Ratio and the Brain. Mol Neurobiol 2011; 44: 203-215.

[17] Simopoulos AP. Human Requirement for N-3 Polyunsaturated Fatty Acids. Poult Sci 2000; 79: 961-970.

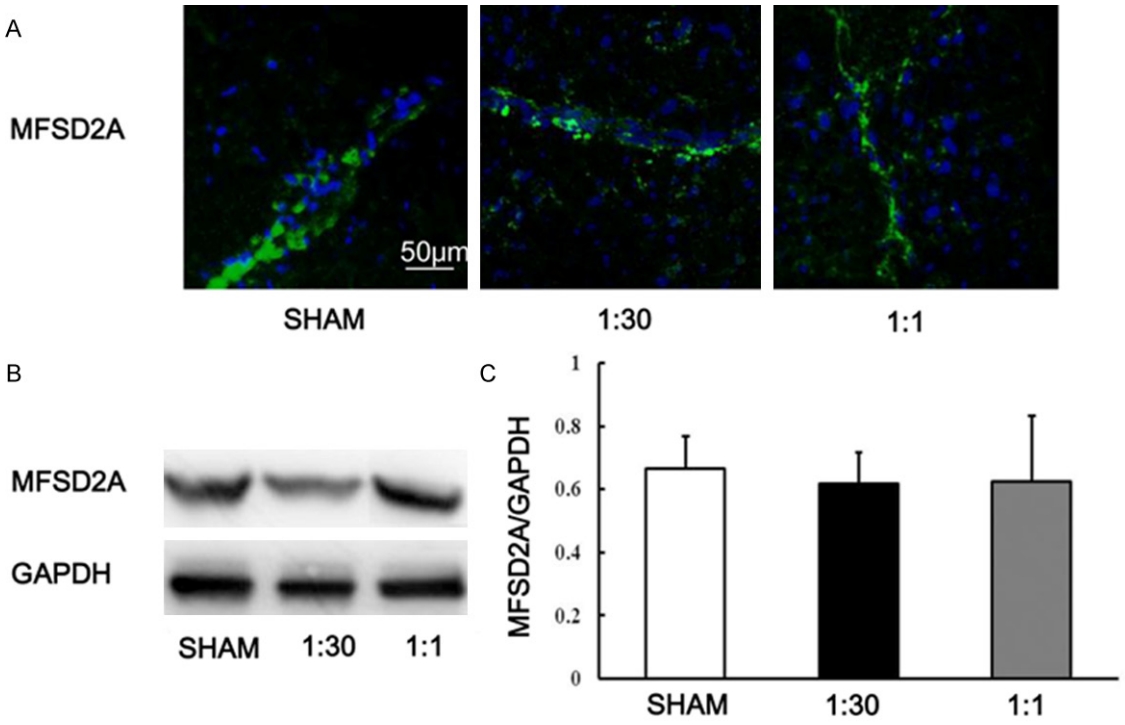
[18] Hall ED, Vaishnav RA and Mustafa AG. Antioxidant therapies for traumatic brain injury. Neurotherapeutics 2010; 7: 51-61.

[19] Baskaya MK, Dogan A, Rao AM and Dempsey RJ. Neuroprotective effects of citicoline on brain edema and blood-brain barrier break-

down after traumatic brain injury. J Neurosurg 2000; 92: 448-452.
[20] Zhong H and Yin H. Role of lipid peroxidation derived 4-hydroxynonenal (4-HNE) in cancer: focusing on mitochondria. Redox Biol 2015; 4: 193-199.
[21] Barrett EC, McBurney MI and Ciappio ED. omega-3 fatty acid supplementation as a potential therapeutic aid for the recovery from mild traumatic brain injury/concussion. Adv Nutr 2014; 5: 268-277.
[22] Mills JD, Bailes JE, Sedney CL, Hutchins H and Sears B. Omega-3 fatty acid supplementation and reduction of traumatic axonal injury in a rodent head injury model. J Neurosurg 2011; 114: 77-84.
[23] Andringa KK, Udoh US, Landar A and Bailey SM. Proteomic analysis of 4-hydroxynonenal (4-HNE) modified proteins in liver mitochondria from chronic ethanol-fed rats. Redox Biol 2014; 2: 1038-1047.
[24] Nguyen LN, Ma D, Shui G, Wong P, Cazenave-Gassiot A, Zhang X, Wenk MR, Goh EL and Silver DL. Mfsd2a is a transporter for the essential omega-3 fatty acid docosahexaenoic acid. Nature 2014; 509: 503-506.
[25] Ben-Zvi A, Lacoste B, Kur E, Andreone BJ, Mayshar Y, Yan H and Gu C. Mfsd2a is critical for the formation and function of the blood-brain barrier. Nature 2014; 509: 507-511.



Supplementary Figure 1. The design and grouping of rats on the experiments.



Supplementary Figure 2. Immunofluorescence staining (A) and western blotting technology (B, C) showed that there was no significant difference in the expression of MFSD2A protein in injured tissues among 1:30, 1:1 and SHAM groups (scale bar=50 μm).