

Fecal Microbiota Transplantation Alleviated Cerebral Ischemia Reperfusion Injury in Obese Rats

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This study aimed to investigate whether fecal microbiota transplantation (FMT) provides protection for stroke injury in obese patients. Rats were fed high-fat diet (HFD) for 4 weeks and subjected to middle cerebral artery occlusion (MCAO). After FMT for 30 days, body weight, serum total cholesterol and triglyceride levels, neurological score, brain water content, and cerebral infarction volume were measured. Brain reactive oxygen species, superoxide dismutase and malondialdehyde were detected and the levels of Bcl-2, Bax and cleaved caspase-3 were examined. Rats fed with HFD had higher body weight and higher serum total cholesterol and triglyceride levels. Neurological score was lower, brain water content and cerebral infarction volume were higher in obese rats following MCAO, but FMT improved neurological deficit. Moreover, oxidative stress was enhanced in obese rats following MCAO, but FMT attenuated oxidative stress. Brain Bcl-2 level was lower while Bax and cleaved caspase-3 levels were higher in obese rats following MCAO, but FMT attenuated oxidative stress. In conclusion, FMT increased brain Bcl-2 level and decreased Bax and cleaved caspase-3 levels. In conclusion, FMT attenuated cerebral ischemic injury in obese rats and the beneficial effects of FMT may be mediated by the attenuation of oxidative stress and apoptosis in the brain.

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Introduction

Cerebrovascular disease is associated with high morbidity and mortality, and causes big burden for health care (Li et al. 2021). Ischemic cerebrovascular disease is a typical cerebrovascular disease and reperfusion is commonly used for the treatment. However, ischemia/reperfusion can cause brain injury and lead to several complications such as limb dysfunction, epilepsy and mental disorders (Tian et al. 2021; Jurcau and Simion 2021). Notably, obesity is a main risk factor of ischemic stroke and accumulating evidence suggests that the susceptibility to ischemic brain damage substantially increases in animal models with concomitant obesity (Tu et al. 2011; Yawoot et al. 2021). Therefore, it is important to develop novel treatment methods for cerebral ischemia reperfusion injury in obese patients.

Probiotics are microorganisms that are beneficial to the host by maintaining the integrity of intestinal flora, reducing bacterial translocation and preventing infection (Barbosa and Vieira-Coelho 2020; Judkins et al. 2020). It was reported that cerebral ischemic injury induced the changes in intestinal flora and promoted inflammation (Singh et al. 2016). Fecal microbiota transplantation (FMT) has become an effective gut microbiota intervention approach for the treatment of a variety of diseases (Vendrik et al. 2020). A recent study reported that FMT could alleviate cerebral ischemia reperfusion injury in aged mice (Feng et al. 2022). However, the effects of FMT on obese stroke

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patients remain unclear. Therefore, in this study we aimed to investigate whether FMT provides protection for cerebral ischemia reperfusion injury in obese patients. We established middle cerebral artery occlusion (MCAO) model in obese rats and examined the changes in cerebral ischemia reperfusion injury.

Materials and Methods

Animals

The use of all animals was in accordance with the relevant provisions of Experimental Animal Ethics Committee of Kunming Medical University. The adult male Sprague-Dawley (SD) rats (weight 230-270 g) were provided by the Animal Laboratory Center of Kunming Medical University (Kunming, China). All rats were housed in controlled conditions (21-22°C and 12 h light-dark cycle), with free access to food and water. The rats were fed with normal diet and high fat diet (HFD, with 43% of total calculated energy from lipids, 21% of total calculated energy from protein and 35% from carbohydrates), respectively. After 4-week feeding of normal diet or HFD, the rats with ordinary diet were subdivided into two groups: sham group and MCAO group. Rats in MCAO group were subjected to thread bolt procedures to establish MCAO model as described previously (Jia et al. 2021). Rats in sham group did not receive thread bolt and other operations. The rats with HFD were subjected to thread bolt procedures to establish obese MCAO model, and then subdivided into two groups: obese MCAO group and FMT group. Rats in obese MCAO group were fed with saline as control after MCAO, while rats in FMT group were treated with fecal microbiota from young rats via enema for 30 consecutive days as described previously (Du et al. 2021). Briefly, fresh stool samples were collected from young rats housed under specific pathogen-free conditions and immediately suspended in sterile water to 200 mg/ml. The suspension was centrifuged at 2,000 rpm for 10 min and the supernatant was collected as fecal suspension. Obese rats with MCAO were fixed in a supine position and sterilized catheters were inserted into the anus up to 5-8 cm. Next, 5 mL of fecal suspension or equal volume of sterile saline was injected into the colon of obese rats through the catheters and maintained for 3 min. These procedures continued for 30 days. The rats were killed by cervical dissociation at the end of experiments.

Measurement of serum lipid levels

About 2 mL of blood were taken from the hearts of the rats and centrifuged at 3,000 r/min for 10 min to separate the serum. Total cholesterol (TC) and triglyceride (TG) levels in the serum were measured using CX4 automatic biochemistry analyzer (Beckman Coulter, Fullerton, CA, USA).

Neurological score measurement

At the end of experiments, neurological score of the

rats in each group was measured in a blind manner with an 18-point scoring system as described previously (Xing et al. 2008). The higher the score, the better neurological function.

Brain water content measurement

After the rats were killed, the brain tissues were dissected from the rats and weighed for wet weight, and then dried in a 105°C oven to constant weight and weighed. The water content of the brain tissues was calculated based on wet weight and dry weight.

Triphenyl-tetrazolium chloride (TTC) staining

Cerebral infarction volume of the rats in each group was measured based on TTC staining. Briefly, the brain tissues were cut into 1 mm thick coronal sections. The sections were then stained with TTC at 37°C for 20 min. Next, the sections were fixed in 4% paraformaldehyde for 24 h, and then the images were taken for stained sections for further analysis. ImageJ software was used to calculate cerebral infarction volume.

Measurement of oxidative stress markers

The brain tissues were homogenized by using a homogenizer on ice, and then centrifuged at 3,000 r/min for 10 min at 4°C. The supernatants were collected, and protein concentration was determined by bicinchoninic acid kit. The contents of reactive oxygen species (ROS), superoxide dismutase (SOD) and malondialdehyde (MDA) were measured using commercial kits (Nanjing Jiancheng Bioengineering, Nanjing, China) according to the instructions.

Western blot analysis

The supernatants from homogenized brain tissues were electrophoresed and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% nonfat milk, and then incubated with primary antibodies for Bcl-2, Bax, cleaved caspase-3 and β -actin (all from Proteintech, Shanghai, China) at 4°C overnight. After washing with Tris-buffered saline-Tween 20 buffer (TBST), the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Proteintech) at room temperature for 1 h. The membranes were then washed with TBST and the bands were visualized with electrochemiluminescence kit (Abcam, Cambridge, MA, USA). The intensity of the bands was analyzed with ImageJ software.

Statistical analysis

All measurement data were expressed as mean \pm standard deviation (SD) and analyzed with SPSS 17.0 software. The comparisons among multiple groups were analyzed by one-way analysis of variance (ANOVA). P < 0.05 was considered statistically significant.

Results

FMT reduced neurological deficit and brain water content in obese rats with MCAO

To verify that rats were obese after feeding with HFD, we measured body weight of the rats in each group. The results showed that body weight in rats fed with HFD was significantly higher than that of rats fed with normal diet (Fig. 1A). Biochemical analysis confirmed that serum TC and TG levels in rats fed with HFD were significantly higher than those of rats fed with normal diet (Fig. 1B). These data demonstrated that HFD induced obesity in the rats.

To evaluate the effects of FMT on cerebral ischemia

reperfusion injury in obese rats, we measured neurological score. As expected, rats in sham group showed no neurological deficit, while rats in MCAO group showed neurological deficit, indicating that we successfully established MCAO model. Moreover, obese rats showed severe neurological deficit after MCAO compared to rats with normal weight. However, FMT significantly reduced neurological deficit in obese rats with MCAO (Fig. 2A).

Next, we measured brain water content in rats in each group. The results showed that brain water content was the lowest in sham group, was higher in MCAO group, and was the highest in obese MCAO group. However, brain water content was significantly lower in FMT group than in obese MCAO group (Fig. 2B). Collectively, these data suggest





A. Body weight of the rats in each group. Sham group (Sham), sham-operated rats; MCAO group (MCAO), rats subjected to middle cerebral artery occlusion (MCAO); obese MCAO group (MCAO + Obese), obese rats fed with high fat diet (HFD) and subjected to MCAO; FMT group (MCAO + Obese + FMT), obese rats fed with HFD, subjected to MCAO and treated with fecal microbiota transplantation (FMT). B. Serum total cholesterol (TC) and triglyceride (TG) levels of the rats in each group. Data were shown as mean \pm SD (n = 6). [#]P < 0.01 compared to Sham and MCAO groups.



Fig. 2. Neurological score and brain water content in the rats. A. Quantitative analysis of neurological score. B. Brain water content in rats in each group. Data were shown as mean \pm SD (n = 6). *P < 0.05.

that obesity could aggravate cerebral ischemia reperfusion injury while FMT could alleviate cerebral ischemia reperfusion injury, especially in obese rats.

FMT reduced cerebral infarction volume in obese rats with **MCAO**

To confirm that FMT could alleviate cerebral ischemia reperfusion injury in obese rats, we examined cerebral infarction in rats in each group. TTC staining showed almost no cerebral infarction in sham group, and obvious cerebral infarction in MACO group. Cerebral infarction was severe in obese MACO group compared to MACO

group, and was less severe in FMT group compared to MACO group and obese MACO group (Fig. 3A).

Comparison of cerebral infarction volume in each group showed that cerebral infarction volume was the smallest in sham group, was larger in MCAO group, and was the largest in obese MCAO group. However, cerebral infarction volume was significantly smaller in FMT group than in obese MCAO group (Fig. 3B). Taken together, these results indicate that obesity could aggravate cerebral infarction while FMT could alleviate cerebral infarction, especially in obese rats.



Fig. 3. Cerebral infarction volume in the rats. A. Triphenyl-tetrazolium chloride (TTC) staining of the brain tissues. B. Quantitative analysis of cerebral infarction volume. Data were shown as mean \pm SD (n = 6). *P < 0.05.

FMT attenuated oxidative stress in the brains of obese rats with MCAO

To reveal the mechanism by which FMT could alleviate cerebral ischemia reperfusion injury in obese rats, we examined oxidative stress markers in the brains of the rats in each group. The results showed that SOD level was the highest in sham group, was lower in MCAO group, and was the lowest in obese MCAO group. However, SOD level was significantly higher in FMT group than in obese MCAO group (Fig. 4A). In contrast, ROS and MDA levels were the lowest in sham group, were higher in MCAO group, and were the highest in obese MCAO group. However, ROS and MDA levels were significantly lower in FMT group than in obese MCAO group (Fig. 4B, C). Taken together, these results indicate that obesity could aggravate oxidative stress in the brain of rats following MCAO, while FMT could alleviate oxidative stress following MCAO, especially in obese rats.

FMT inhibited apoptosis in the brains of obese rats with MCAO

It is known that oxidative stress causes tissue injury by inducing apoptosis (Hu et al. 2021). Therefore, we detected apoptosis markers Bcl-2, Bax and cleaved caspase-3 in the brains of the rats in each group by western blot analysis (Fig. 5A). Densitometry analysis of the bands showed that Bcl-2 level was the highest in sham group, was lower in MCAO group, and was the lowest in obese MCAO group. However, Bcl-2 level was significantly higher in FMT group than in obese MCAO group (Fig. 5B). In contrast, Bax and cleaved caspase-3 levels were the lowest in sham group, were higher in MCAO group, and were the highest in obese MCAO group. However, their levels were significantly lower in FMT group than in obese MCAO group (Fig. 5B). Collectively, these results suggest that obesity promotes apoptosis in the brain of rats following MCAO, while FMT inhibits apoptosis following MCAO, especially in obese rats.



Fig. 4. The contents of superoxide dismutase (SOD), reactive oxygen species (ROS) and malondialdehyde (MDA) in the brain tissues.

A. Quantitative analysis of SOD level in the brain tissues. B. Quantitative analysis of ROS level in the brain tissues. C. Quantitative analysis of MDA level in the brain tissues. Data were mean \pm SD (n = 6). *P < 0.05.



Fig. 5. Protein levels of Bcl-2, Bax and cleaved caspase-3 in the brain tissues. A. Representative blots for the detection of Bcl-2 (BCL-2), Bax (BAX) and cleaved caspase-3 (C-Casp3) in the brain tissues. β-actin was presented as control. B. Densitometry analysis of the levels of Bcl-2, Bax and cleaved caspase-3 in the brain tissues. Data were mean ± SD (n = 6). *P < 0.05.</p>

Discussion

It is known that obesity is associated with morbidity and mortality of cerebrovascular disease. High plasma cholesterol level is a risk factor for ischemic stroke (Iso 2021). Therefore, it is important to understand the link between obesity and stroke. Interestingly, recent studies suggest that HFD may cause the abnormality of intestinal flora and contribute to ischemic stroke via the activation of inflammation reactions (Sampson et al. 2016; Singh et al. 2016; Rothhammer et al. 2018). A recent study showed that FMT could restore the abnormality of intestinal flora and attenuated ischemic stroke injury in aged mouse model (Feng et al. 2022). However, the efficacy of FMT on ischemic stroke in obese population remains unclear.

In this study, we established MCAO model in HFDinduced obese rats and compared neurological dysfunction in the model with or without treatment with FMT. Based on the measurements of such indexes as neurological score, brain water content and cerebral infarction volume, we showed that cerebral ischemia reperfusion injury was worse in obese rats compared to normal weight rats after MCAO. However, FMT significantly improved neurological score while it reduced brain water content and cerebral infarction volume in obese rats with MCAO. These results indicate that obesity could aggravate cerebral ischemia reperfusion injury but the vicious effects of obesity on stroke could be alleviated by FMT.

Next, we investigated how FMT alleviated cerebral ischemia reperfusion injury in obese rats. Based on the measurements of oxidative stress indexes such as SOD. ROS and MDA, we found that oxidative stress was worse in obese rats compared to normal weight rats after MCAO. However, FMT significantly attenuated oxidative stress in obese rats with MCAO. These data are consistent with previous findings that obesity enhances oxidative stress in a variety of diseases including stroke (Colak and Pap 2021). However, FMT could effectively attenuate oxidative stress in rat model of stroke. Furthermore, we examined whether FMT could inhibit oxidative stress induced apoptosis in the brains of obese rats with MCAO. Based on the detection of apoptosis markers Bcl-2, Bax and cleaved caspase-3, we found that obesity enhanced apoptosis of brain tissues following ischemic injury, but FMT inhibited apoptosis of brain tissues in obese rats following ischemic injury. The activation of apoptosis following ischemic injury involves neuroinflammatory response (Wimmer et al. 2018). Therefore, further studies are needed to examine whether FMT inhibits inflammatory signaling pathways to provide protection for ischemic stroke in obese population. In addition, the safety of FMT for the treatment of ischemic stroke should be evaluated in future investigations.

In conclusion, FMT attenuated cerebral ischemic injury in HFD-induced obese rats and the beneficial effects of FMT may be mediated by the attenuation of oxidative stress and the inhibition of apoptosis in the brain. FMT may be a novel approach for the prevention and treatment of stroke.

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Conflict of Interest

The authors declare no conflict of interest.

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