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# Effects of bacterial biofilm on regulation of neurovascular unit functions and neuroinflammation of patients with ischemic cerebral stroke by immunocyte

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**ABSTRACT**

In this experiment, the effects of biofilm on neurovascular unit functions and neuroinflammation of patients with ischemic cerebral stroke were investigated. For this purpose, 20 adult male rats were purchased from Taconic (8 to 10 weeks old, weighing between 20 and 24g) and selected as the research objects. Then, they were randomly divided into an experimental group (10 rats) and a control group (10 rats). Ischemic cerebral stroke rat models were established. Besides, pseudomonas aeruginosa (PAO1) was prepared manually and implanted into the bodies of rats in the experimental group. mNSS scores, cerebral infarction area, and the release of inflammatory cytokines of rats in the two groups were compared. Results showed that mNSS scores for rats in the experimental group at all periods were remarkably higher than those for rats in the control group ($P$<0.05), which demonstrated that the rats in the experimental group suffered much severer neurological impairment than those in the control group. In addition, the release of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, inducible nitric oxide synthase (iNOS), and IL-10 were all higher than those of the control group ($P$<0.05). The cerebral infarction area of the experimental group at all periods was remarkably larger than that of the control group ($P$<0.05). In conclusion, the formation of biofilm led to the aggravation of neurological impairment and inflammatory reactions among patients with ischemic cerebral stroke.

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## Introduction

Cerebral infarction is the global main cause of death and disability among people. In recent years, some progress and development have been made in reperfusion therapy with the continuous development of medical technology. However, only a very small number of patients can benefit from relevant treatment in current clinical practice with the failure of a large number of neuroprotective trials (1). What's more, the mechanism of the occurrence and development of ischemic cerebral stroke is still unclear. In recent years, relevant studies show that inflammation and immunological reaction play a vital role in the occurrence and development of ischemic cerebral stroke (2). Immunological and inflammatory reactions last for the entire course of cerebral stroke (3). According to recent relevant research results, cerebral ischemia causes cell infiltration into the brain. After that, ischemic cerebral injury is further exacerbated through the lysis of ischemic neurons and the enhancement of intracerebral inflammation in the acute phase of stroke (4). Neurovascular units mainly consist of vascular endothelial cells, astrocytes, pericytes, neurons, and capillaries. At present, it is generally believed that vascular neural units play a significant role in the occurrence and development of ischemic cerebral stroke and the reconstruction of peripheral vascular tissues after stroke. After the proposal of the relevant concept of neurovascular units, the cause of the failure of focusing solely on neurons and ignoring vascular injury and other neuroprotective interventions becomes much clearer (5). Neurovascular units have a remarkable effect on the interaction between neurons around cortical capillaries and vascular cells. Besides, neurovascular units are closely related to peripheral vascular smooth muscle cells, arterial endothelial cells, and perivascular neurons (6). Hence, some scholars have proposed that the closely connected system was called the neurovascular network system. The improvement of the functions of this neurovascular network becomes an effective interventional method for ischemic stroke. With the failure of numerous neuroprotection experiments, people gradually realize that the focus of the study on the regulation of ischemic stroke should be the interaction between neurons, astrocytes, and capillaries, which is the concept of neurovascular units. The concept has been widely recognized since it was proposed and it was viewed as the key influencing factor for understanding the pathogenesis of ischemic stroke and developing relevant drugs (7).

Bacterial biofilm (BF) is defined as a large number of bacterial polymer membrane-like materials generated by wrapping bacteria adhering to the surface of the objects in contact with secreted polysaccharide matrix, fibrin, and proteolipid. In brief, biofilm refers to a microbial community generated by wrapping microorganisms with an extracellular polymer matrix (8). Its formation is very beneficial for the survival of bacteria and their adaption to the external environment. Once biofilm comes into being, there are apparent differences between cell pathogenicity, morphology, and sensitivity to the environment and floa-

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ting state. In particular, BF significantly becomes resistant to antibiotics and the immune system, which leads to a series of serious clinical problems and many chronic and refractory infectious recurrent diseases and causes great difficulties and obstacles to the treatment of disease (9). Clinical statistical data show that BF is the main cause of the occurrence of 80% of clinical refractory infectious diseases. During the occurrence and development of cerebral stroke, inflammation and immunological reaction last for the entire process of the occurrence and development of cerebral stroke. Therefore, the appearance of BF among patients with cerebral stroke makes the treatment much more difficult. Hence, it is of great importance to investigate the effect and mechanism of BF in regulating neurovascular unit functions and neuroinflammation of patients with ischemic cerebral stroke by immunocytes. However, there are very few clinically relevant studies at present (10).

The research was conducted to investigate the effects of BF on neurovascular unit functions and neuroinflammation of patients with ischemic cerebral stroke. Rats were used for the construction of ischemic cerebral stroke models and pseudomonas aeruginosa (PAO1) BF was prepared and implanted into the bodies of rats. Besides, the calculation of cerebral infarction area, mNSS score, and real-time quantitative polymerase chain reaction (PCR) were adopted to explore the differences in neurological functions and the occurrence of inflammation among rats between two groups to provide reference and basis for the clinical treatment of related diseases.

## Materials and Methods

### Research objects

20 adult male rats were purchased from Taconic (8 to 10 weeks old, weighing between 20 and 24g) and selected as the research objects. Then, they were randomly divided into an experimental group (10 rats) and a control group (10 rats) and kept in a clean animal room with constant temperature and humidity and the alternation of light and dark for 12 hours. All rats could take food freely and they conformed to relevant national regulations and standards. Researchers should strictly follow animal experimental standards to minimize pain during surgery and feeding.

### Preparation of cerebral ischemia-reperfusion model

Firstly, anesthesia induction was carried out. After the rats were completely anesthetized, they were fixed in the supine position. During the surgery, 1% to 2% isoflurane and oxygen supply needed to be maintained. Besides, rats' body temperature should be measured to ensure they remained within the normal range. After that, an opening was made in the middle of the rats' necks and then subcutaneous tissues were separated to expose the carotid sheath. The surgery should be performed very carefully to avoid injury to the vagus nerve. Next, two silk sutures were threaded underneath blood vessels for later use. Then, the subcutaneous tissues were separated upward to the bifurcation of internal and external carotid arteries. After that, some connective tissues were removed. Next, two silk sutures were threaded through the neck and external carotid artery and then the silk suture at the external carotid artery was knotted. Besides, the silk suture at the internal carotid artery was pulled with an arteriolar clamp. After that, the silk suture at the proximal end of the common carotid artery was knotted and that at the distal end was prepared. Then, an oblique 45° incision was made below the silk suture at the distal end and above the knotted site of the common carotid artery with microsurgical scissors. Next, a cotton swab was used to remove part of the blood and then cord No. 1418 was inserted into the common carotid artery with forceps. After that, the silk suture pulling on the internal carotid artery was released to allow all cords to pass through. If the insertion was not smooth, the cord should be tilted to the rats' nasal side. The decrease of the base value of blood flow by 10% was set as the time to start occlusion. The cord was removed after 60 days after the beginning of the experiment. At that time, blood flow recovered to more than 50% (except for no obvious recovery).

### Implantation of BF

PAO1 stored at -80° was taken out and resuscitated. After that, it was inoculated into Langmuir-Blodgett (LB) tablet and cultured at 37°C for 18 hours. Then, a colony was selected and implanted into a 40 mL LB culture solution. After constant incubation on a shaking table for 18h to 20h, the bacteria solution was centrifuged. After that, the suspension was adjusted to OD600=0.5 with 0.9% NaCl. Then, emulsion tube carriers sterilized with ethylene oxide were rinsed with aseptic distilled water for later use. Next, a 100 mL shake flask was added with 40mL experimental strain solutions and cultured at constant temperature and 110rpm for 20 hours for bacterial adhesion. Then, iodophor was employed to disinfect the left inferior abdomen of rats and 3mL of 3% phenobarbital sodium was injected into the abdomen for anesthesia. After that, 1cm incision was made in the left inguen of rats and then the skin and subcutaneous tissues were exposed layer by layer. Finally, the abdominal cavity was exposed and PA BF carriers. Next, the incision was sutured with silk suture. In addition, 2 mL of sterile physiological saline was injected into the neck to avoid dehydration. BF was only implanted in the rats in the experimental group, while it was not implanted in rats in the control group. BF was represented and observed by scanning electron microscope (SEM) and pathological tissue sectioning.

### Evaluation of neurological impairment

mNSS scoring method was employed to evaluate the neurological impairment among the included rats. It was a comprehensive evaluation scale for the sensorimotor balance of rodents. In addition, the double-blind method was adopted for manual evaluation. The total score was 18 points. A higher score indicated more significant neurological impairment. The specific scoring standards and contents were as follows (Table 1).

### Calculation method for cerebral infarction area

An animal dedicated 7T magnetic resonance imaging (MRI) was employed to calculate the volume of cerebral infarction among rats. During the imaging, a reflector coil 72mm in length and rat surface receiver coil were used for MRI. Besides, 3.5% isoflurane was adopted for anesthesia induction and 1.0-2.0% isoflurane mixed with 70% nitrogen and 30% oxygen was used for nasal supply to maintain anesthesia during MRI. The scanning sequence was as follows. Time of repetition (TR)=4000ms, effec-

**Table 1.** mNSS detection scale of the sensorimotor balance of rodent.

| Items | | | Scores |
|---|---|---|---|
| Motor detection | Tail suspension test | Fore limb buckling | 1 |
| | | Posterior limb buckling | 1 |
| | | Positive tail suspension test | 1 |
| | Placement of rats on the floor | Walled normally | 0 |
| | | Unable to walk in a straight line | 1 |
| | | Turned in circles to the paralyzed side | 2 |
| | | Tilted to the paralyzed side | 3 |
| | Sensory test | Visual and tactile tests in a quiet environment | 1 |
| | | Proprioceptive sensation test | 1 |
| | Balance beam test | Normal | 0 |
| | | Grasped the edge of the balance beam | 1 |
| | | Held the balance beam tightly with the limbs on one side dropped | 2 |
| | | Held the balance beam tightly with the limbs on both sides dropped or rotated on it for over 60s | 3 |
| | | Tried to keep the balance on the balance beam and fell off (>40s) | 4 |
| | | Tried to keep the balance on the balance beam and fell off (>20s) | 5 |
| | | Fell off and didn't try to keep the balance on the balance beam (<20s) | 6 |
| | Reflection function | Auricle reflection | 1 |
| | | Cornea reflection | 1 |
| | | Panic reflection | 1 |
| | | Epilepsy, clonus, and dystonia | 1 |
| Total score | | | 18 |

tive time of echo (TE)=60ms, number of average=4, field of view (FOV)=19.2mm×19.2 mm, matrix size=192×192, slice thickness=0.5 mm, and time advance (TA)=6m 24s. T2WI hyperintensity (white) was used to represent the infarction area. Besides, different slice thickness was set. The cerebral infarction volume of each slice ($mm^2$) could be calculated by the scale. After that, it was multiplied by slice thickness to obtain the approximate value of cerebral infarction volume.

**Detection of intracerebral inflammatory cytokine expression by real-time quantitative PCR**

In the research, real-time quantitative PCR was used to detect the expression of intracerebral inflammatory cytokines. The specific procedures of real-time quantitative PCR detection were as follows.

A. Rats were deeply anesthetized and fixed in the supine position. Then, a median thoracic incision was made to expose the heart. After that, the pre-cooled phosphate buffer solution (PBS) was perfused into the incision. After the successful perfusion, the cervical vertebra was severed with scissors. After the brain tissues were separated carefully, they should be quickly placed into a freezing tube and then stored in liquid nitrogen.

B. After brain tissues were completely frozen, they were ground in an ice mortar until they were completely powdered. Next, 1mL Trizol was added into the mortar for lysis. After they were completely lysed, 200ul trichloromethane was added. After that, lysis liquid was transferred to a 1.5 mL centrifuge tube using a pipette and then put into a high-speed centrifuge pre-cooled to 4°C to be centrifuged at 12,000rpm for 15 minutes.

C. The upper liquid was aspirated into a 1.5mL centrifuge and added with 500μL isopropyl alcohol. After it was mixed evenly, the mixed solution was placed at room temperature for 10 minutes. Next, it was put into the pre-cooled centrifuge and centrifuged at 12,000rpm for 15 minutes.

D. After it was taken out again, the upper liquid was sucked off. After that, 1.5mL isopropyl alcohol was added and then centrifuged at 12,000rpm for 10 minutes. Next, the supernatant was removed and then 75% ethyl alcohol solution configured with 1mL diethyl pyrocarbonate (DEPC) water was added. After the solution was mixed evenly, it was put into the pre-cooled centrifuge (7,500g) and centrifuged for 5 minutes.

E. After the centrifugation, the supernatant was removed and then an appropriate amount of DEPC water was added into the centrifuge tube to be mixed thoroughly with dried ribonucleic acid (RNA). After that, RNA concentration was measured.

F. The mixture was reversely transcribed to complementary deoxyribonucleic acid (cDNA) using a reverse transcription kit called PrimeScriptTM RT reagent kit. After DNA was purified, it was stored at -80°C in a refrigerator.

G. After all, samples were collected, SYBR (Roche, Indianapolis, IN, USA) was used for amplification.

**Statistical analysis**

SPSS 18.0 statistical software was adopted in the research. Quantitative data were expressed as mean±standard deviation and the distribution of indicators in all groups was described. Qualitative data were described by rate. *t*-test was adopted for the comparison of the means between the two groups. Pearson X2 test was employed for the comparison of the sample rate between qualitative data. $P<0.05$ indicated that the differences revealed statistical significance.

## Results

### Observation on BF pathological tissue sections

The observation results of BF pathological tissue sections were shown in Figure 1 below. According to Figure 1, local cell infiltration occurred among the rats in both groups with an increase in the duration of infection. Besides, they suffered from severer and severer inflammation. However, rats implanted with BF suffered from more serious inflammation and cell infiltration.

### Observation on BF with SEM

The results of the observation on BF with SEM were displayed in Figure 2 below. It was demonstrated that plenty of colonies in the experimental group adhered to the surface versus those in the control group. Besides, a three-dimensional structure was gradually formed and original short rod-like colonies gradually became round over time. In contrast, the number of bacteria in the control group gradually increased without the formation of BF over time.

### Comparison of mNSS scores for rats in two groups

The comparison of mNSS scores for rats in two groups was presented in Figure 3 below. mNSS scores for experimental and control groups on the 3rd, 5th, and 7th day after BF implantation amounted to 6±1.22 vs 4±1.08, 12±2.21 vs 8±3.11, and 17±3.03 vs 13±2.88, respectively. MNSS scores for the experimental group at all periods were higher than those for the control group ($P$<0.05). The above finding suggested that the rats in the experimental group suffered from much more serious neurological impairment than those in the control group.

### Comparison of cerebral infarction volume of rats between two groups

The results of the comparison of cerebral infarction volume among rats between the two groups were presented in Figure 4 below. Cerebral infarction areas of rats in the experimental and control group on the 3rd, 5th, and 7th day after BF implantation amounted to 22.8±11.3 vs 16.7±13.6, 35.1±12.07 vs 26.03±10.08, 43.3±22.1 vs 38.4±14.33, respectively. The cerebral infarction areas of rats in the experimental group at all periods were larger than those of rats in the control group ($P$<0.05).

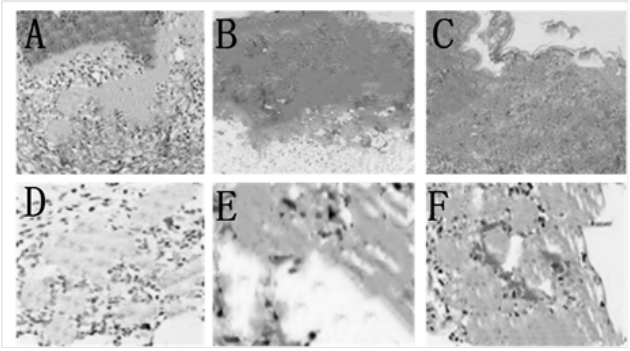


**Figure 1.** Pathological tissue sections of rats in two groups at different periods. Note: A, B, and C showed the pathological tissue sections in the experimental group on the 3rd, 5th, and 7th day, respectively. D, E, and F displayed the pathological tissue sections in the control group on the 3rd, 5th, and 7th days, respectively.

### Comparison of the release of intracerebral inflammatory cytokines among rats between two groups

The results of the comparison of the release of intracerebral inflammatory cytokines among rats in two groups were displayed in Figure 5 below. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, inducible nitric oxide synthase (iNOS), and IL-10 of rats in experimental and control groups 7 days after BF implantation amounted to 2.21±0.38 vs 1.12±0.66, 2.88±0.99 vs 0.99±0.13,

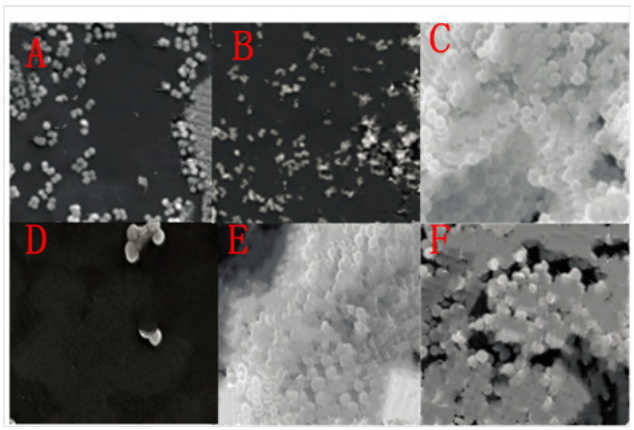


**Figure 2.** SEM images of BF. Note: A, B, and C showed SEM images in the experimental group on the 3rd, 5th, and 7th days. D, E, and F displayed SEM images in the control group on the 3rd, 5th, and 7th days.

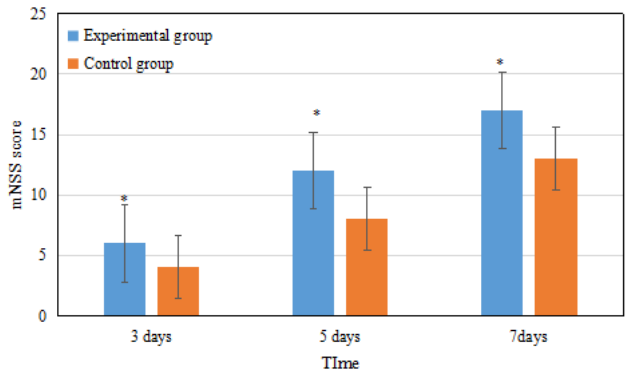


**Figure 3.** Comparison of mNSS scores for rats in two groups. Note: The differences in mNSS scores between the two groups revealed *$P$<0.05.

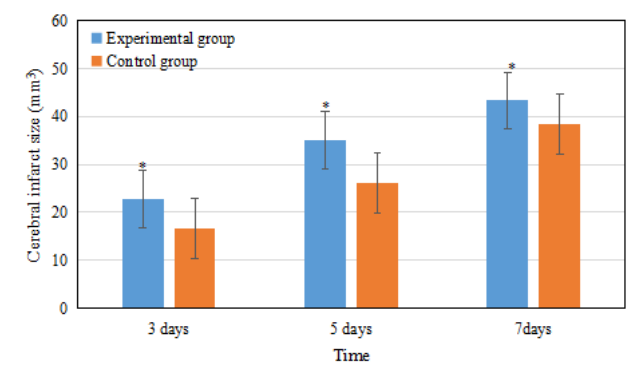


**Figure 4.** Comparison of cerebral infarction areas among rats between two groups. Note: The difference in cerebral infarction areas between the two groups revealed *$P$<0.05.

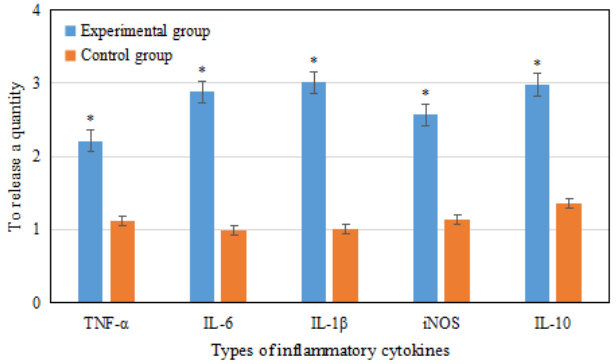


**Figure 5.** The comparison of the release of intracerebral inflammatory cytokines among rats between two groups. Note: The comparison with the release of intracerebral inflammatory cytokines in the control group revealed *$P$<0.05.

3.01±0.73 vs 1.01±0.73, 2.57±0.67 vs 1.13±0.52, and 2.98±1.01 vs 1.36±0.77, respectively. The differences between groups indicated statistical significance ($P$<0.05).

## Discussion

In the early phase, ischemic stroke is considered a vascular disease. Hence, it was initially classified as cerebrovascular disease. With the continuous deepening of the understanding of the disease, people gradually paid more and more attention to neurons in the study on ischemic stroke to determine neuroprotective strategy more effectively and clearly (11). Meanwhile, the concept of a neurovascular unit was proposed. At present, it is generally believed that the neurovascular unit consists of vascular endothelial cells, astrocytes, pericytes, neurons, and capillaries in the clinical field (12). According to considerable research outcomes, the neurovascular unit has remarkable effects and impacts on the occurrence and development of stroke and the reconstruction of peripheral vascular tissues. After the concept was proposed, people have a more comprehensive and clearer understanding of the pathogenesis of stroke. However, there are also some defects and shortcomings (13). For instance, only the neuron itself was focused while the cause of the failure of the protection of injured blood vessels by the neuronal intervention was often ignored. Nonetheless, the recovery of injured blood vessels has a significant and prominent effect on the prognosis for patients. In most cases, the focus of the neurovascular unit is the interactions between the neurovascular unit and vascular cells and their impacts (14). The neurovascular unit is closely associated with peripheral vascular smooth muscle cells, arterial endothelial cells, and peripheral vascular neurons. Therefore, many scholars proposed that the closely connected systems were called neurovascular networks. At present, the improvement of the functions of the neurovascular network for the effective intervention of ischemic stroke has become a widely concerned and important topic (15).

Inflammation and immunological reaction play an important role in the occurrence and development of cerebral stroke. According to relevant studies, cerebral ischemia causes cell infiltration to the brain (16). Besides, it further lyses neurons and enhances intracerebral inflammatory reactions, which lead to the continuous aggravation of the ischemic cerebral injury. At present, the main clinical therapeutic drugs for stroke are antioxidant/anti-excitotoxicity neuroprotective drugs (17). However, the therapeutic window of the above drugs mostly stays within several hours after stroke. Most patients with stroke receive treatment in the hospital within 12 hours after onset. Consequently, they missed the treatment time of these drugs when they underwent treatment in the hospital, which led to the failure of numerous clinical trials on neuroprotective drugs (18). In contrast, the immunological inflammatory reaction was involved in secondary cerebral injury after stroke, which usually lasts for a long time. Hence, it can provide a long time window for the treatment of cerebral stroke. At present, the mechanism of inflammatory reaction among patients with ischemic cerebral stroke is still not very clear (19).

Currently, many domestic and foreign scholars agree that BF is the refuge for bacteria under adverse conditions and it often results in continuous chronic infection because of the slow release of bacterioplankton. In the clinical field, BF is defined as a large number of bacterial polymer membrane-like materials generated by wrapping bacteria adhering to the surface of the objects in contact with secreted polysaccharide matrix, fibrin, and proteolipid. In brief, biofilm refers to a microbial community generated by wrapping microorganisms with an extracellular polymer matrix (20). A great number of studies on BF show that the activity of bacteria is very characteristic, such as the adhesion, growth, and proliferation on the surface of carriers. As a result, pathogenic microorganisms become highly resistant to antimicrobial agents. In addition, related microscopic studies demonstrate that organisms are usually formed on the surface of inanimate tissues such as medical devices. They can also be formed on the surface of living matter. In general, PAO1 is the priority for the research field and a diverse bacteria group with ecological significance (21). PAO1 is the commonest bacteria in nosocomial infection and fatal infection among patients with immune deficiency. At present, researchers gradually become clear about the pathogenicity and the formation of BF. Hence, PAO1 was selected to prepare BF in the research. At present, there is no clinical study on the effects of BF on neurovascular unit functions and neuroinflammation of patients with ischemic cerebral stroke (22).

To investigate the effects of BF on neurovascular unit functions and neuroinflammation of patients with ischemic cerebral stroke, rats were used to establish ischemic cerebral stroke models and PAO1 BF was prepared and implanted into the bodies of rats. Besides, the differences in neurological functions and the occurrence of inflammation among rats in the two groups were explored by calculating cerebral infarction area, mNSS score, and real-time quantitative PCR. It was demonstrated that the mNSS score for the rats implanted with BF was higher, the cerebral infarction area was larger, and more inflammatory cytokines were released than those of the rats without implanted BF. The above findings demonstrated that the formation of BF resulted in slow rehabilitation of neurological function and severer inflammation among patients with ischemic cerebral stroke.

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