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Immune reactions and nerve repair in mice with sciatic nerve injury 14 days after intraperitoneal injection of Brazil☆

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Abstract

BALB/c mice were intraperitoneally injected with 10, 5 or 2.5 mg/kg Brazil for 14 days after sciatic nerve injury. Results demonstrate that the spleen T/B lymphocyte stimulation index and serum circulating immune complex concentration were significantly reduced, and the morphology of the soleus muscle was restored in mice with sciatic nerve injury. These effects of Brazil were dose-dependent. Our experimental findings indicate that Brazil can regulate immune responses after nerve injury and promote sciatic nerve repair.

Key Words: Brazil; peripheral nerve injury; repair; neural regeneration; immune

Abbreviations: CIC, circulating immune complex; SME, *Su Mu* ethanol extract; ELISA, enzyme-linked immunosorbent assay

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INTRODUCTION

The immune response evoked by peripheral nerve injury may aggravate Wallerian degeneration, leading to secondary injury[1], and inhibit the repair and regeneration of peripheral nerve[2-8]. Therefore, the use of immunosuppressants to inhibit the inflammatory response after peripheral nerve injury can help reduce scar formation and promote the repair and regeneration of peripheral nerve[9-10].

Caesalpinia sappan L, a member of the Fabaceae-Caesalpinioideae family, is mainly distributed in India, Myanmar and Vietnam. The dried heartwood of this tree, also known as logwood, is widely used in oriental medicine, particularly for treating inflammation. The main components include brazilein, Brazilin, stearic acid and stigmasterol, among other compounds[11]. Among these, brazilein has been shown to function as an effective immunosuppressant. *Su Mu* ethanol extract (SME) can promote the repair of peripheral nerve after injury[12-13]. SME significantly inhibits the proliferation of lymphocytes in rats[14]. In screenings of natural drug extracts for immune activity[15-17], Brazil was found to possess good immunosuppressive action[18].

In this study, we examined spleen T/B lymphocyte proliferation and transformation, as well as the expression of serum circulating immune complex (CIC), in a broader attempt to elucidate the effects of Brazil on immune function in BALB/c mice with sciatic nerve injury.

RESULTS

Experimental grouping and analysis of animals

A total of 129 BALB/c mice were included in this study and nine were excluded because of accidental death during anesthesia. After the sciatic nerve was unilaterally resected, experimental mice were randomly divided into the saline (control), high-dose, medium-dose and low-dose groups, which were treated, respectively, with 1 mL saline, or 10, 5 or 2.5 mg/kg Brazil *via* intraperitoneal injection. In total, 120 mice were involved in the final analysis, and five mice were selected from each group at 3, 5, 7, 14, 28 days and 12 weeks post-surgery, to examine T/B lymphocyte proliferation and serum CIC concentration. The body mass index and morphological changes of the soleus muscle were also measured in five mice from each group at 12 weeks post-surgery.

Brazil improves blood T/B lymphocyte proliferation and transformation in mice with sciatic nerve injury

Colorimetric analysis of the stimulation index showed that, except for the low-dose group 3 days after sciatic nerve injury, the T/B lymphocyte transformation stimulation

indices in the Brazil groups receiving high, medium and low doses were significantly lower than those in the saline group ($P < 0.05$ or $P < 0.01$). Lymphocyte proliferation increased with higher doses of Brazil (Tables 1, 2).

Table 1 Blood T lymphocyte transformation and stimulation index in each group of mice with sciatic nerve injury

Group	Time after surgery (day)	
	3	7
Brazil		
High-dose	0.621±0.029[b]	0.553±0.021[b]
Medium-dose	0.699±0.031[b]	0.608±0.039[b]
Low-dose	1.044±0.119[a]	0.869±0.013[b]
Saline	1.201±0.199	1.277±0.131

Group	Time after surgery (day)	
	14	28
Brazil		
High-dose	0.533±0.032[b]	0.462±0.066[b]
Medium-dose	0.561±0.019[b]	0.499±0.052[b]
Low-dose	0.817±0.079[b]	0.801±0.039[b]
Saline	1.346±0.179	1.521±0.045

Stimulation index = absorbance value of drug stimulation well/absorbance value of saline group. Data are expressed as mean ± SD of five mice at each time point. Paired *t* test was used to compare differences between groups. [a]$P < 0.05$, [b]$P < 0.01$, *vs.* saline group.

Table 2 Blood B lymphocyte transformation and stimulation index in each group of mice with sciatic nerve injury

Group	Time after surgery (day)	
	3	7
Brazil		
High-dose	0.819±0.109[a]	0.633±0.019[b]
Medium-dose	0.982±0.109[a]	0.749±0.033[b]
Low-dose	1.109±0.026	1.039±0.018[a]
Saline	1.168±0.102	1.299±0.329

Group	Time after surgery (day)	
	14	28
Brazil		
High-dose	0.579±0.039[b]	0.411±0.016[b]
Medium-dose	0.608±0.041[b]	0.512±0.007[b]
Low-dose	0.899±0.019[a]	0.749±0.071[a]
Saline	1.439±0.233	1.688±0.015

Stimulation index = absorbance value of drug stimulation well/absorbance value of saline group. Data are expressed as mean ± SD of five mice at each time point. Paired *t* test was used to compare differences between groups. [a]$P < 0.05$, [b]$P < 0.01$, *vs.* saline group.

Brazil reduces serum CIC levels in mice with sciatic nerve injury

Enzyme-linked immunosorbent assay (ELISA) analysis showed that serum CIC levels gradually increased 5–14 days after sciatic nerve injury, while at 28 days, they declined to the initial levels observed at 3 days. The serum CIC levels in the high-, medium- and low-dose groups were significantly lower than in the saline group (Table 3 and Figure 1).

Table 3 Serum circulating immune complex levels (mU/L) in mice with sciatic nerve injury

Time after surgery (day)	High-dose group	Medium-dose group	Low-dose group	Saline group
3	481±56	529±46	556±33	631±29
5	739±56[b]	819±29[a]	933±35[a]	1 081±75
7	1 211±40[b]	1 631±56[b]	1 981±38[a]	2 241±47
14	1 589±83[b]	2 001±73[b]	2 449±42[b]	3 891±73
28	510±36[a]	561±62[a]	659±46	716±64

Data are expressed as mean ± SD of five mice at each time point. Paired *t* test was used to compare differences between groups. [a]$P < 0.05$, [b]$P < 0.01$, *vs.* saline group.

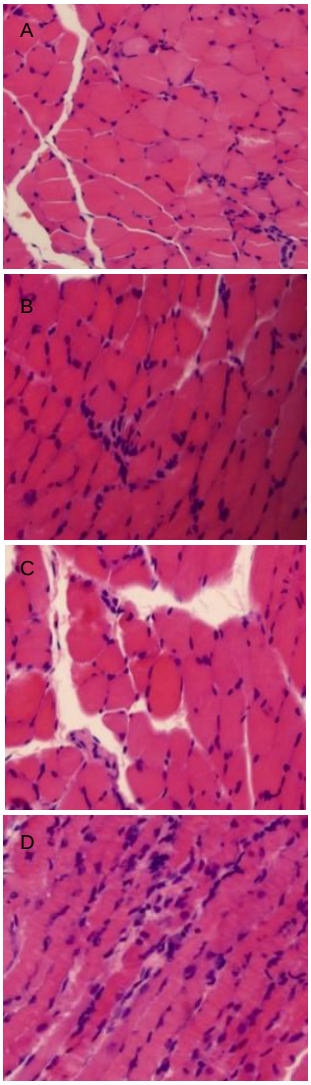


Figure 1 Morphology of soleus muscle cells at 12 weeks post-surgery (hematoxylin-eosin staining, × 20).

The recovery of the soleus muscle in the high-dose (A) and medium-dose (B) groups was significantly better than in the low-dose (C) and saline (D) groups.

Brazil improves soleus muscle structure in mice

At 12 weeks post-surgery, the myocytes of the soleus muscle were tightly packed, and the muscle fiber cross-sectional area was large with only small gaps in all three Brazil dosage groups. In contrast, in the saline (control) group, atrophy of muscle fibers was present, and a small muscle fiber cross-sectional area and wide gaps were observed. The recovery of the soleus muscle in the high- and medium-dose groups was significantly better than in the low-dose and saline groups (Figure 1). At 12 weeks after surgery, the soleus muscle body mass indices in the high- and medium-dose groups were significantly higher than in the low-dose and saline groups (2.29 ± 0.03 and 2.18 ± 0.02 *vs*. 2.01 ± 0.04 and 1.91 ± 0.05, $P < 0.05$ or $P < 0.01$).

DISCUSSION

Before the 1980s, nerves were considered immune-privileged due to the presence of the blood brain barrier. However, an increasing body of research has shown that nerve injury can lead to humoral and cellular immunological changes. In 1985, de Medinaceli *et al* [19] observed an immune reaction at the site of damage after nerve injury, and found that response intensity correlated with the degree of injury. Subsequent studies also pointed out the presence of nerve-specific antibodies at sites of nerve lesion after peripheral nerve injury. Ansselin *et al* [20] demonstrated that the T lymphocyte concentration was high in injured nerves. Pei *et al* [21] detected the accumulation of IgG in the injured nerve by immunohistochemical analysis, and they found that the accumulation was negatively correlated with the repair and regeneration of nerve fibers, as well as the recovery of nerve function. Thus, we speculate that the immune response inhibits neural regeneration.

A number of studies[22-26] have employed immunosuppressive agents to promote peripheral nerve regeneration, and have clearly shown that FK506 can inhibit the expression of immune response, thereby promoting neural regeneration. As a natural mushroom extract, FK506 has some side effects[27]. Results of our present study demonstrate that lymphocyte proliferation in the Brazil-treated groups was significantly lower than in the saline group, and that the amount of proliferation was dose-dependent. The serum CIC concentrations in the Brazil groups were significantly lower than in the saline group. The recovery of the soleus muscle in the high- and medium-dose groups was significantly better than in the low-dose and saline groups.

In summary, Brazil inhibits immunological overreaction in mice with sciatic nerve injury, in a clearly dose-dependent fashion.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment.

Time and setting

Experiments were performed between July and September in 2011 at the Immunology Laboratory, Jilin University, China.

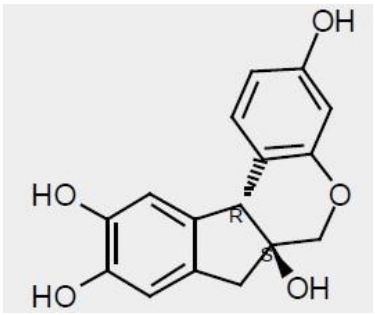
Materials

Animals

A total of 129 clean, adult, male BALB/c mice, aged 8 weeks, weighing 25 ± 2 g, were provided by the Experimental Animal Center of Jilin University School of Basic Medicine, China (license number: SCXK (Ji) 2007-0001). BALB/c mice were housed in cages in a room at 20°C, with free access to complete pellet feed (Experimental Animal Center of Jilin University, Changchun, Jilin Province, China) and water. Some of the bedding material was changed every other day. All experimental manipulations of the animals were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[28].

Drugs

Brazil was extracted from the SME (Brazil 5%; Shaanxi Province Changyue Phytotech Co., Ltd., Xi'an, Shaanxi Province, China). Its chemical structure is shown below:



SME was concentrated under vacuum at 50°C, separated and extracted with petroleum ether, and condensed at normal atmospheric pressure. The purified extracts (95% purity) were identified by high-performance liquid chromatography analysis[29]. Powders were weighed with an analytical balance (Keda Testing Instrument Factory, Shanghai, China) and dissolved in dimethyl sulfoxide to a final concentration of 100 μg/mL. The dimethyl sulfoxide final concentration was 0.05%. Brazil solution was sterilized using a membrane filter (0.02 μm micropore), stored at −20°C, and used within one month.

Methods

Establishment of sciatic nerve injury models

Mice were intraperitoneally anesthetized with 1% thiopental sodium, 100 mg/kg, and the sciatic nerve was exposed (Figure 2). The nerve trunk was completely resected 0.5 cm below the ischial tuberosity and sutured using a 11/0 atraumatic needle under a 12 × surgical microscope (Zhenjiang Microscopic Surgical Instrument Factory, Jiangsu Province, China).

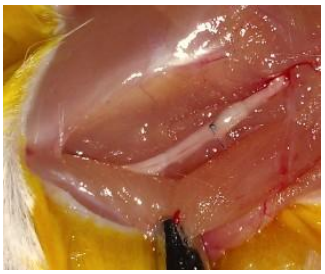


Figure 2 Sciatic nerve injury model.

Medication and dosing

The dose for intraperitoneal administration into mice was based on the clinical dose of hematoxylin crude drug[30], considered a medium dose. Next, high and low doses were determined. High-, medium- and low-dose of Brazil was 10, 5 and 2.5 mg/kg per day, respectively. The compound was administered from 1 day before to 14 days after surgery.

Colorimetric analysis of lymphocyte proliferation and transformation

The optimal final concentration of Concanavalin A was determined to be 5 μg/mL, and that of lipopolysaccharide was found to be 40 μg/mL, according to preliminary experiments. Five rats from each group were selected at 3, 5, 7, 14 and 28 days after injury, and anesthetized using 1% thiopental sodium (100 mg/kg) *via* intraperitoneal injection. Mice were decapitated after 0.5 mL blood samples were removed from the eyeballs. The spleen was taken out and placed in a petri dish containing RPMI1640 (Gibco, Carlsbad, CA, USA), triturated uniformly and filtered using a 200-mesh steel mesh to obtain a cellular suspension. Cells were incubated with RPMI1640 culture medium at 1×10^7 cells/mL in a micro-well culture plate, 0.1 mL in each well. Using 20% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, Zhejiang Province, China; fetal bovine serum: RPMI 1640 = 1:4), the concentration of Concanavalin A was adjusted to 10 μg/mL and that of lipopolysaccharide to 80 μg/mL. Each sample was aliquoted into three wells and each well was treated with Concanavalin A/ lipopolysaccharide (Sigma, St. Louis, MO, USA) 0.1 mL, while three control wells were treated with 20% fetal bovine serum 0.1 mL. Cells were incubated in a 37°C CO_2 incubator for 48 hours and treated with methylthiazolyldiphenyl-tetrazolium bromide (Sigma; 5 mg/mL), 20 μL/well, for an additional 4 hours. After the supernatant was removed, DMSO stock solution was added, 100 μL/well, and the plate was agitated for 10 minutes. The absorbance was read using a microplate reader (USA Bio-Rad, Chicago, IL, USA) at 570 nm[12]. Stimulation index = mean absorbance of drug stimulation well/mean absorbance of blank well.

ELISA determination of serum CIC levels

The eyeball blood samples were placed in a sterile EP tube for 2 hours at room temperature and stored overnight in a 4°C refrigerator. Tissues were centrifuged at 5 000 r/min for 3 minutes the next day and the supernatant was stored at −20°C for further use. All procedures were in strict accordance with the instructions of the CIC ELISA kit (Rapidbio Lab, Calabasas, CA, USA). Before the test, the kit was equilibrated to room temperature, the required strips were taken out, and the CIC sample was diluted. The CIC concentration in the well of the ELISA coated plate was 900, 600, 300, 150 or 75 mU/L. The experimental well received 40 μL of sample and 10 μL of serum. The membrane was sealed and the plate was incubated at 37°C for 30 minutes. Then the membrane was removed and the culture liquid was discarded, and each well was rinsed with phosphate buffered saline five times, for 30 seconds each. Each well was treated with horseradish peroxidase 50 μL, followed by cell incubation and washing. Each well was treated with H_2O_2 50 μL, and then with 3,3',5,5'-tetramethylbenzidine 50 μL. Cells were subject to the color reaction in the dark at 37°C for 15 minutes. The reaction was terminated by adding 50 μL of 2 M H_2SO_4 into each well. The blank well was adjusted to zero and the absorbance value at 450 nm was measured within 15 minutes after termination. The standard curve was plotted with the absorbance value of the CIC as the vertical axis and the CIC concentration as the abscissa[13], to calculate the serum CIC concentration. CIC standard curve formula: $Y = (14.12 + 2.16 X^{0.66})/(235.44 + X^{0.66})$; Y: CIC absorbance value; X: CIC concentration (supplementary Figure 1 online).

Determination of soleus muscle body mass index and histological observation

Five mice were selected from each group at 12 weeks after injury. Muscles were bluntly dissected after anesthesia. The triceps muscle of the calf ipsilateral to the injury was exposed and bluntly dissected along with the soleus muscle. The soleus muscle on the injury side was cut and weighed on an electronic analytical balance (Wuxi Keda Test Instrument Factory, Wuxi, Jiangsu Province, China) after the connective tissue was removed and surface blood was cleaned with filter paper. According to the methods of Cuadros[31], soleus muscle body mass index = soleus muscle weight/body weight × 100.

The soleus muscle was fixed with 10% neutral formalin for more than 72 hours, ethanol dehydrated and embedded in paraffin. The widest segment was cut into sections for hematoxylin-eosin staining. The morphology of muscle fibers was observed under light microscopy (Olympus, Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± SD. Paired *t* tests between groups were performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). A level of $P < 0.05$ was considered a statistically significant difference.

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Author contributions: Jian Cao had full access to data acquisition, integration and analysis, as well as study concept and design, and he wrote the manuscript. Lisen Li and Yongan Wang integrated data. Yiwen Jiang was responsible for data analysis. Zhongpin Niu and Weitian Yin validated papers. Haoyu Liu was responsible for statistical analysis. Lisen Li was in charge of funds. Binfeng Wang provided technical and information support. Weitian Yin instructed the experiments.
Conflicts of interest: None declared.
Ethical approval: The pilot project was given approval by Jilin University Animal Ethics Committee, China.
Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org, and entering Vol. 7, No. 9, 2012 item after selecting the “NRR Current Issue” button on the page.

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