Effects of leukemia inhibitory factor and basic fibroblast growth factor on free radicals and endogenous stem cell proliferation in a mouse model of cerebral infarction☆

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Abstract

The present study established a mouse model of cerebral infarction by middle cerebral artery occlusion, and monitored the effect of 25 µg/kg leukemia inhibitory factor and (or) basic fibroblast growth factor administration 2 hours after model establishment. Results showed that following administration, the number of endogenous neural stem cells in the infarct area significantly increased, malondialdehyde content in brain tissue homogenates significantly decreased, nitric oxide content, glutathione peroxidase and superoxide dismutase activity significantly elevated, and mouse motor function significantly improved as confirmed by the rotarod and bar grab tests. In particular, the effect of leukemia inhibitory factor in combination with basic fibroblast growth factor was the most significant. Results indicate that leukemia inhibitory factor and basic fibroblast growth factor can improve the microenvironment after cerebral infarction by altering free radical levels, improving the quantity of endogenous neural stem cells, and promoting neurological function of mice with cerebral infarction.

Key Words

leukemia inhibitory factor; basic fibroblast growth factor; endogenous neural stem cells; free radical; malondialdehyde; nitric oxide; glutathione peroxidase; superoxide dismutase; neuroprotection

Research Highlights

Leukemia inhibitory factor and basic fibroblast growth factor can promote endogenous neural stem cell proliferation and recovery of neurological function by altering free radical levels and improving the microenvironment after cerebral infarction.

Abbreviations

MCAO, middle cerebral artery occlusion; MDA, malondialdehyde; GSH-Px, glutathione peroxidase; NO, nitric oxide; SOD, superoxide dismutase

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INTRODUCTION

Studies have shown that endogenous neural stem cells are activated after cerebral infarction, and play an important role in repair following nerve injury[1-2]. However, the quantity of activated endogenous neural stem cells is not sufficient to completely repair nerve injury. Thus, how to increase the number of endogenous neural stem cells has become a major research focus. Leukemia inhibitory factor is involved in multiple neuroprotective mechanisms. It can promote neurotrophin-3 expression, improve neuronal differentiation, repair injured tissue and enhance endogenous neural stem cell proliferation[3]. Basic fibroblast growth factor is a multifunctional signaling protein that exhibits

neuroprotective effects and promotes endogenous neural stem cell differentiation[4]. However, its mechanism of action remains poorly understood.

Internal environmental changes can affect cell survival. Free radical changes can influence the microenvironment of the brain; aggravate nerve injury, and play an important role in cell apoptosis and survival. However, little data is available regarding the influence of free radicals on endogenous neural stem cell survival and proliferation.

The present study investigated the neuroprotective mechanism of leukemia inhibitory factor and basic fibroblast growth factor by assessing motor function in mice, endogenous neural stem cell proliferation, and malondialdehyde (MDA), glutathione peroxidase (GSH-Px), nitric oxide (NO) and superoxide dismutase (SOD) levels.

RESULTS

Quantitative analysis of experimental animals

Mice (n = 144) were randomly assigned to six groups (n = 24): normal, sham-surgery, model (middle cerebral artery occlusion (MCAO) + normal saline), leukemia inhibitory factor (MCAO + cerebrospinal fluid containing leukemia inhibitory factor), basic fibroblast growth factor (MCAO + cerebrospinal fluid containing basic fibroblast growth factor), and leukemia inhibitory factor + basic fibroblast growth factor (MCAO + cerebrospinal fluid containing leukemia inhibitory factor and basic fibroblast growth factor) groups. Eight mice from each of the six groups were selected at 1, 2 and 3 weeks following model establishment. All 144 mice were included in the final analysis.

Leukemia inhibitory factor and basic fibroblast growth factor increased the number of endogenous neural stem cells in brain tissue from the infarcted hemisphere

Anti-nestin immunofluorescence staining showed no nestin-positive endogenous neural stem cells in the normal or sham-surgery group, but a large number of endogenous neural stem cells was observed in the model, leukemia inhibitory factor, basic fibroblast growth factor, and leukemia inhibitory factor + basic fibroblast growth factor groups (Figure 1).

Quantification results showed that the number of endogenous neural stem cells significantly increased in the infarcted hemisphere of the leukemia inhibitory factor, basic fibroblast growth factor, and leukemia inhibitory factor + basic fibroblast growth factor groups compared with the model group at 1, 2 and 3 weeks after model establishment (P < 0.05). In particular, the number of

endogenous neural stem cells was greatest in the leukemia inhibitory factor + basic fibroblast growth factor group, which was significantly greater than the leukemia inhibitory factor group and basic fibroblast growth factor group (P < 0.05; Table 1).
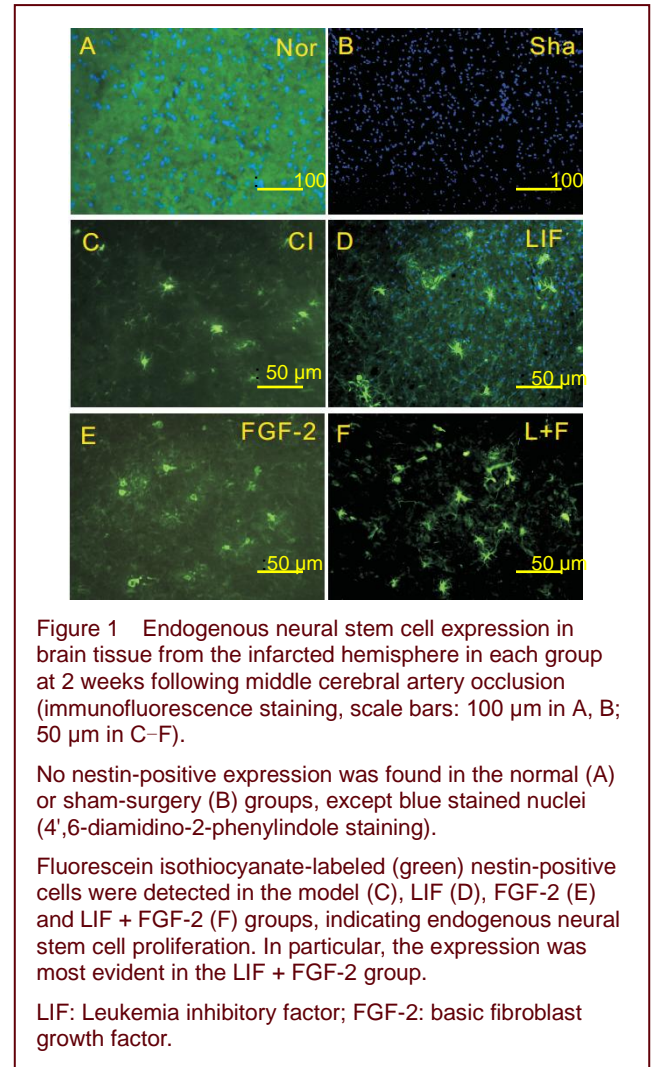

Figure 1 Endogenous neural stem cell expression in brain tissue from the infarcted hemisphere in each group at 2 weeks following middle cerebral artery occlusion (immunofluorescence staining, scale bars: 100 μm in A, B; 50 μm in C−F).

No nestin-positive expression was found in the normal (A) or sham-surgery (B) groups, except blue stained nuclei (4',6-diamidino-2-phenylindole staining).

Fluorescein isothiocyanate-labeled (green) nestin-positive cells were detected in the model (C), LIF (D), FGF-2 (E) and LIF + FGF-2 (F) groups, indicating endogenous neural stem cell proliferation. In particular, the expression was most evident in the LIF + FGF-2 group.

LIF: Leukemia inhibitory factor; FGF-2: basic fibroblast growth factor.

Table 1 Quantitative results of endogenous neural stem cells (n/μm^2) in brain tissue from the infarcted hemisphere of each group

Group	Time after model establishment (week)		
	1	2	3
Normal	2.1±3.3	1.8±3.1	4.7±3.2
Sham	2.8±2.4	2.6±3.2	2.0±2.1
Model	983.6±532.0	1 394.6±693.4	1 732.9±858.5
LIF	3 245.7±456.8[a]	3 732.4±345.6[a]	4 656.6±657.3[a]
FGF-2	2 721.7±548.7[a]	3 452.5±573.8[a]	3 053.6±495.7[a]
LIF+FGF-2	3 068.5±901.3[a]	5 646.9±1 503.3[abc]	5 182.3±1 398.3[ab]

Data were expressed as mean ± SEM of four mice in each group at each time point. [a]P < 0.05, *vs.* model group; [b]P < 0.05, *vs.* FGF-2 group; [c]P < 0.05, *vs.* LIF group (one-way analysis of variance). LIF: Leukemia inhibitory factor; FGF-2: basic fibroblast growth factor; Sham: sham-surgery.

Leukemia inhibitory factor and basic fibroblast

growth factor influenced free radical expression in brain tissues from the infarcted hemisphere

MDA content in brain tissue homogenates significantly reduced, and NO content, and GSH-Px and SOD activity were significantly elevated in the leukemia inhibitory factor, basic fibroblast growth factor, and leukemia inhibitory factor + basic fibroblast growth factor groups compared with the model group at 1, 2 and 3 weeks after model establishment ($P < 0.05$). MDA content significantly declined, and NO content, and GSH-Px and SOD activity were significantly elevated in the leukemia inhibitory factor + basic fibroblast growth factor group compared with the basic fibroblast growth factor group ($P < 0.05$). GSH-Px and SOD activities were significantly elevated in the leukemia inhibitory factor + basic fibroblast growth factor group compared with the leukemia inhibitory factor group ($P < 0.05$; Table 2).

Leukemia inhibitory factor and basic fibroblast growth factor improved the motor function of mice with cerebral infarction (Tables 3, 4)

Table 2 Malondialdehyde (nmol/mg), glutathione peroxidase (U/mL), nitric oxide (µM) and superoxide dismutase (U/mL) levels in brain tissue homogenates from each group

Group	Time after model establishment (week)	Malondialdehyde	Nitric oxide	Glutathione peroxidase	Superoxide dismutase
Normal	1	7.66±0.37	2.41±0.06	348.54±6.99	440.67±5.81
	2	7.88±0.14	2.47±0.02	346.18±2.76	438.34±7.97
	3	7.96±0.37	2.42±0.03	343.29±3.00	434.35±2.53
Sham-surgery	1	9.23±0.04	2.20±0.05	332.68±3.42	420.73±3.66
	2	8.90±0.37	2.31±0.07	333.78±4.06	414.48±6.29
	3	8.76±0.24	2.19±0.09	338.52±1.56	423.42±6.63
Model	1	15.24±0.26	1.33±0.03	225.64±0.36	320.51±1.62
	2	14.23±0.15	1.29±0.04	232.21±3.54	325.15±3.65
	3	13.95±0.34	1.33±0.03	231.14±1.26	320.88±2.95
LIF	1	10.95±0.38[a]	1.74±0.03[a]	259.29±1.15[a]	358.03±3.63[a]
	2	10.19±0.04[a]	1.69±0.02[a]	259.35±1.29[a]	353.54±5.86[a]
	3	9.72±0.27[a]	1.71±0.04[a]	260.63±4.99[a]	353.81±8.05[a]
FGF-2	1	11.46±0.04[a]	1.62±0.06[a]	288.45±3.51[a]	360.88±2.73[a]
	2	11.03±0.23[a]	1.55±0.04[a]	283.99±2.08[a]	367.71±2.80[a]
	3	10.22±0.08[a]	1.61±0.05[a]	277.55±7.09[a]	369.94±1.44[a]
LIF+FGF-2	1	9.11±0.10[ab]	1.91±0.04[ab]	322.03±4.29[abc]	392.52±2.47[abc]
	2	8.86±0.31[ab]	1.88±0.03[ab]	332.25±6.06[abc]	395.24±6.26[abc]
	3	8.51±0.32[ab]	1.85±0.03[ab]	333.15±2.59[abc]	397.57±6.33[abc]

Data were expressed as mean ± SEM of four mice in each group at each time point. [a]$P < 0.05$, *vs.* model group; [b]$P < 0.05$, *vs.* FGF-2 group; [c]$P < 0.05$, *vs.* LIF group (one-way analysis of variance). LIF: Leukemia inhibitory factor; FGF-2: basic fibroblast growth factor.

Table 3 Stay time (second) on rotation apparatus in the rotarod test for each group

Time after model establishment	Normal	Sham-surgery	Model	LIF	FGF-2	LIF+FGF-2
24 hours	180±0.00	180±0.00	2.33±0.33	2.00±0.58	2.33±0.88	2.00±0.58
1 week	180±0.00	180±0.00	17.33±3.84	18.33±3.53	17.67±2.40	24.00±2.89
2 weeks	180±0.00	180±0.00	42.67±2.33	46.33±3.71	49.33±1.45	62.33±5.53[a]
3 weeks	180±0.00	180±0.00	54.33±2.73	89.33±3.18[a]	86.00±4.04[a]	102.00±5.51[ab]

Data were expressed as mean ± SEM of eight mice in each group at each time point. [a]$P < 0.05$, *vs.* model group; [b]$P < 0.05$, *vs.* FGF-2 group (one-way analysis of variance). The rotarod test was performed for 180 seconds. Longer stay times indicate good motor function. LIF: Leukemia inhibitory factor; FGF-2: basic fibroblast growth factor.

Table 4 Bar Grab motor function scores in each group

Time after model establishment	Normal	Sham-surgery	Model	LIF	FGF-2	LIF+FGF-2
24 hours	4.00±0.00	4.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.25±0.25
1 week	4.00±0.00	4.00±0.00	0.50±0.29	1.25±0.25	1.25±0.25	1.50±0.29
2 weeks	4.00±0.00	4.00±0.00	0.75±0.25	1.50±0.29	1.75±0.25	2.75±0.25[a]
3 weeks	4.00±0.00	4.00±0.00	1.00±0.41	2.75±0.25[a]	3.00±0.41[a]	3.50±0.29[a]

Data were expressed as mean ± SEM of eight mice in each group at each time point. [a]$P < 0.05$, *vs.* model group (one-way analysis of variance). High Bar Grab scores indicate good motor function. LIF: Leukemia inhibitory factor; FGF-2: basic fibroblast growth factor.

Stay time in the rotarod test and Bar Grab scores were significantly increased in the leukemia inhibitory factor + basic fibroblast growth factor group when compared to the model group at 2 weeks following model establishment ($P < 0.05$).

The stay time in the rotarod test and Bar Grab scores were significantly higher in the leukemia inhibitory factor, basic fibroblast growth factor, and leukemia inhibitory factor + basic fibroblast growth factor groups when compared with the model group at 3 weeks following model establishment ($P < 0.05$). In particular, the greatest increase was observed in the leukemia inhibitory factor + basic fibroblast growth factor group (Tables 3, 4).

DISCUSSION

Normally, endogenous neural stem cells are in a resting state, but become activated and migrate during disease onset. They can be induced to differentiate into corresponding nerve cells to repair nerve injury[5-6]. However, activated endogenous neural stem cells mainly differentiate into astrocytes, and occasionally into neurons or oligodendrocytes, in the absence of specific intervention. Therefore, they cannot effectively repair neurological dysfunction[7].

leukemia inhibitory factor can promote peripheral neuronal survival and participate in physical stress and metabolic regulation[8]. basic fibroblast growth factor alone or in combination with other neurotrophic factors can promote cortical endogenous neural stem cell differentiation and antagonize glutamate-induced neurotoxicity through FGF receptors, thereby exhibiting a neuroprotective effect[9-10]. Results from the present study showed that leukemia inhibitory factor and basic fibroblast growth factor improved motor function and increased endogenous neural stem cell quantity in mice with cerebral infarction.

Oxidative stress is a major factor for cerebral ischemia and ischemia/reperfusion injury[11-13]. A recent study showed that oxidative stress can regulate the expression of fibroblast growth factor 2, vascular endothelial growth factor and other neurotrophic factors[14]. Therefore, measurement of MDA, NO, SOD and GSH-Px levels can accurately and objectively reflect the degree of oxidative damage in cerebral ischemia/ reperfusion injury, the ability of an organism to clear free radicals, and cell antioxidant capacity[11-13]. Results from the present study showed that after cerebral infarction, MDA content in infarcted brain tissue homogenates increased, but NO content and SOD and GSH-Px activity significantly decreased, indicating ischemia induced oxidative stress, aggravating brain injury.

Leukemia inhibitory factor levels significantly increased locally, which can antagonize inflammation, accelerate free radical clearance and restore the internal environment, but basic fibroblast growth factor receptor signaling participates in the inflammatory reaction and free radical metabolism[15-16]. In the present study, leukemia inhibitory factor and basic fibroblast growth factor decreased MDA content and increased NO, SOD and GSH-Px levels in brain tissues. This indicates that leukemia inhibitory factor and fibroblast growth factor can induce free radical clearance and attenuate oxidative injury. Moreover, leukemia inhibitory factor and fibroblast growth factor exhibit a synergistic effect on free radical clearance and oxidative stress inhibition.

In addition, leukemia inhibitory factor and basic fibroblast growth factor have been shown to influence endogenous neural stem cell proliferation[3,10], although the mechanism remains uncertain. In the present study, leukemia inhibitory factor and basic fibroblast growth factor promoted endogenous neural stem cell proliferation and played a role in clearing free radicals and inhibiting cell oxidative injury, demonstrating leukemia inhibitory factor and basic fibroblast growth factor may promote endogenous neural stem cell proliferation by changing free radical levels and improving the endogenous neural stem cell microenvironment.

In summary, the neuroprotection induced by leukemia inhibitory factor and basic fibroblast growth factor may function by altering free radical levels, improving the microenvironment, and enhancing endogenous neural stem cell proliferation. However, the precise mechanism requires further investigation.

MATERIALS AND METHODS

Design
A randomized, controlled, animal experiment.

Time and setting
The experiment was performed in Tianjin Haoyang Biological Manufacture Co., Ltd., China and the Faculty of Medicine, The University of Melbourne, Australia, from 2007 to 2009.

Materials
A total of 144 healthy, male c57BL mice, 8 weeks old, of specific-pathogen free grade, were provided by the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (License No. 0006775). The mice were separately housed for 1 week prior to experimentation for adaptation at 25−30°C in a dried environment with a natural day/night cycle, and allowed free access to food and water. They were deprived for food and water for 8 hours prior to surgery. Animal experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[17].

Methods

Establishment of cerebral infarction model and intervention

Cerebral infarction was established by right middle cerebral artery occlusion[5]. The left middle cerebral artery distal to the heart was ligated, and blood flow was monitored. Blood flow decreased to at least 75%. ALZET osmium drug pump (DURECT, New York, NY, USA) intubation was conducted 2 hours following model establishment[5]. Leukemia inhibitory factor, basic fibroblast growth factor and leukemia inhibitory factor + basic fibroblast growth factor were slowly released into the cerebrospinal fluid through the pump tube. Leukemia inhibitory factor (25 µg/kg; Thermo Fisher Scientific, Boston, MA, USA) and basic fibroblast growth factor (25 µg/kg; Thermo Fisher Scientific), dissolved in normal saline (50 µg in 20 mL), was added to the ALZET pump. The drugs were released over 21 days. The model group was administrated an equal amount of normal saline. The sham-surgery group was subjected to right middle cerebral artery occlusion and ALZET osmium drug pump intubation, but the artery was not ligated and the tube was not placed. The normal group was not treated.

Neurological function evaluation

Rotarod and Bar Grab functional tests were conducted 1 week prior to, 24 hours, 1, 2, and 3 weeks following model establishment. The rotarod test[5] recorded the stay time of a mouse on the rotation apparatus at a constant rotation speed (16 r/min, 3.6 cm diameter; Ugo Basile, Collegeville, PA, USA). The maximum testing time was 180 seconds. The Bar Grab test[5] utilized a 5-mm wood rod to determine claw grip strength: score 0: no action; 1: limb motion, but no griping; 2: griping with no strength; 3: gripping with little strength; 4: powerful gripping.

Immunofluorescence for the number of nestin-positive cells in infarcted brain tissue

The mice (four from each group) were anesthetized by intraperitoneal injection of pentobarbital sodium at 1, 2, and 3 weeks following model establishment, perfused with normal saline at room temperature, followed by 4% (w/v) paraformaldehyde. The brain was harvested, fixed for 24 hours, dehydrated with 30% (w/v) sucrose solution for 24 hours, paraffin embedded, and serially sectioned (8 µm thick). The third section from every 10th section was used for immunohistochemistry. One set was used for nestin immunofluorescence, one as a positive control and a negative control, and the last set was used for other experiments. The sections were washed in mouse tonicity PBS for 20 minutes × 3 times, and mixed with nestin-fluorescein isothiocyanate (license No. MAB353, Chemicon, Santa Cruz, CA, USA; 1:50) overnight at 4°C. The negative control was not incubated with the antibody.

After three washes in mouse tonicity PBS, the sections were counterstained with 4',6-diamidino-2-phenylindole (1:2 000; Thermo Fisher Scientific) for 5 minutes, washed with mouse tonicity PBS for 20 minutes × 3 times, mounted with fluorescence mediator (Thermo Fisher Scientific), and observed using a red-green-blue fluorescence microscope (Olympus, Tokyo, Japan). Images were processed using Microfire (Life Technologies, Carlsbad, CA, USA) and Imagepro software (Life Technologies). The number of positive cells was quantified using a confocal counting system (Life Technologies) and results are expressed as $n/\mu m^2$.

Preparation of brain tissue homogenates

Brain tissue from the infarcted hemisphere was harvested and homogenized at 1, 2, and 3 weeks following model establishment (four mice from each group) for detection of free radicals. Cells from the brain hemispheres were prepared as follows: brains were collected and placed in ice-cold RPMI 1640 medium. Samples were homogenized with a glass homogenizer and filtered through a 100 mm filter. Samples were centrifuged at 300 × g, incubated with anti-CD16/32 Fc-receptor block for 10 minutes and washed in FACS buffer (2% (w/v) background solution, 0.1% (w/v) NaN$_3$ in PBS) before surface staining[18].

MDA determination in brain tissue homogenates

MDA was determined using thiobarbituric acid spectrophotometric colorimetry[19]. The kit was purchased from Thermo Fisher Scientific. MDA in degraded products of peroxidized lipid condensed with thiobarbituric acid and formed red products, which display a maximum absorption peak at 532 nm. After the reaction, the SP-75 ultraviolet spectrophotometer (Shanghai Spectrum, Shanghai, China) was adjusted to 532 nm, 1 cm light path, zeroed with distilled water, and the absorbance was determined. MDA content was calculated according to the formula:
MDA content (nmol/mg) = [($A_{\text{determined tube}}/A_{\text{standard tube}}$ $-A_{\text{blank tube}}/A_{\text{standard blank tube}}$) ×standard sample concentration (10 nmol/mL)]/protein content (mg/mL)

NO determination in brain tissue homogenates

NO content was determined using the nitrate reductase method[20]. Reagent prepared solution was purchased from the Tianjin No.3 Chemical Reagent Factory (Tianjin, China). Absorbance readings were obtained at 540 nm. After the reaction, the SP-75 ultraviolet spectrophotometer was used to determine the absorbance of the standard solution at different concentrations at 540 nm. Linear regression was performed, and sample concentrations were determined using the standard curve.

GSH-Px determination in brain tissue homogenates

GSH-Px was determined using modified dithio-bis-nitrobenzoic acid direct spectrophotometry[18]. Reagent prepared solution was purchased from the

Tianjin No.3 Chemical Reagent Factory. After the reaction, an SP-75 ultraviolet spectrophotometer was used to determine the absorbance at 422 nm.

SOD determination in brain tissue homogenates

SOD was determined using nitrite spectrophotometry[18]. Reagents were purchased from Thermo Fisher Scientific. The SP-75 ultraviolet spectrophotometer was used to determine the absorbance at 550 nm based on the fact that determination tube absorbance values were less than the control tube during SOD colorimetry using the xanthine and xanthine oxidase reaction system.

Statistical analysis

Data were analyzed using Prism (Graph Pad Software Inc., San Diego, CA, USA) and are expressed as mean ± SEM. Differences were compared using one-way analysis of variance. A value of $P < 0.05$ was considered statistically significant.

Author contributions: Dawei Zang conceived and designed the study, revised the manuscript and was in charge of funds. Weihui Huang integrated and analyzed the experimental data, and drafted the manuscript. Yadan Li, Yufeng Lin and Xue Ye conducted the analyses.
Conflicts of interest: None declared.
Ethical approval: This study received permission from the Animal Ethics Committee of the Faculty of Medicine, The University of Melbourne, Australia.

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