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Basic Study

Moxibustion therapy improving delayed memory deficits via promoting neurogenesis and angiogenesis of hippocampus in a vascular dementia rat model

灸法通过促进血管性痴呆大鼠海马神经血管新生改善延迟回忆

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Abstract
Objective: To investigate the alteration of delayed memory and its relationship with neurogenesis and angiogenesis in vascular dementia (VD) rats after moxibustion therapy.
Methods: Two hundred adult male SPF Wistar rats were chosen for the experiment. Thirty-six rats were randomly selected as the sham operation group. Except for rats in the sham operation group (*n*=36), the others were made into VD models by bilateral common carotid arteries occlusion (BCCAo). After modeling, the 108 survived rats were randomly divided into 3 groups: a model group, a neural stem cells (NSCs) plus endothelial progenitor cells (EPCs) moxibustion group and a NSCs moxibustion group. Co-transplanted implant was transplanted into the rats in the NSCs plus EPCs moxibustion group, and the rats in the NSCs moxibustion group were transplanted by NSCs only. The NSCs plus EPCs moxibustion group and the NSCs moxibustion group received suspended moxibustion therapy at Baihui (GV 20), Dazhui (GV 14) and Shenting (GV 24), (each group was divided into 3 subgroups by the treatment course as 1, 2 and 3 courses). Every group was measured by Morris water maze to evaluate its delayed memory after 3 treatment courses and the rat's brain was taken out after perfusion of 4% paraformaldehyde one day after 1, 2 and 3 treatment courses, respectively. Marker protein expression was detected by laser confocal microscope to analyze the effect on neurogenesis and angiogenesis.
Results: VD rats showed delayed memory in Morris water maze test 3 d after ischemic injury. After 3 courses of moxibustion therapy, VD-induced delayed memory deficits were improved in the NSCs plus EPCs moxibustion group and the NSCs moxibustion group. The expressions of nestin, doublecortin (DCX) and CD34 increased significantly in the two moxibusiton groups after every treatment course (all $P<0.05$), which might contribute to the neurogenesis and angiogenesis in hippocampus. In addition, compared with the rats in the NSCs moxibustion group, the expressions of nestin, DCX and CD34 increased significantly in the NSCs plus EPCs moxibustion group ($P<0.05$).
Conclusion: Moxibustion can reverse VD-induced delayed memory deficits, which may be related to the promotion of neurogenesis and angiogenesis.
Keywords: Moxibustion Therapy; Point, Baihui (GV 20); Point, Shenting (GV 24); Point, Dazhui (GV 14); Governor Vessel; Dementia, Vascular; Rats

【摘要】目的：观察灸法治疗后血管性痴呆(VD)大鼠延迟记忆的改变及其与神经血管新生的关系。**方法：**共200只SPF级Wistar大鼠进行实验。随机选择36只作为假手术组。除假手术组外的大鼠均通过双侧颈总动脉闭塞(BCCAo)法制作VD模型。造模后，108只存活大鼠被随机分为模型组、神经干细胞(NSCs)加内皮祖细胞(EPCs)艾灸组和NSCs艾灸组，每组36只。将共植体移植入NSCs加EPCs艾灸组，NSCs组仅移植NSCs。NSCs加EPCs艾灸组和NSCs艾灸组使用悬灸百会、大椎和神庭治疗(每组根据疗程分为3个亚组，分别治疗1、2和3个疗程)。各组在治疗3个疗程结束后用Morris水迷宫测量评估延迟记忆；分别在治疗1、2和3个疗程1 d后心脏灌注4%多聚甲醛，然后取脑。通过激光共聚焦显微镜检测标记蛋白表达，分析神经发生和血管生成与艾灸的关系。**结果：**Morris水迷宫试验显示缺血损伤后3 d，VD大鼠延迟回忆障碍。NSCs加EPCs艾灸组和NSCs艾灸组大鼠在3个疗程艾灸治疗后，VD诱导的

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延迟回忆缺陷得到改善。两个治疗组大鼠的巢蛋白(Nestin), 双皮质素(DCX)和CD34的表达在1、2和3个疗程治疗后均显著增加(均$P<0.05$), 这可能有助于海马的神经新生和血管生成。此外, 与移植NSCs的大鼠相比, 艾灸显著增加NSCs加EPCs艾灸组的Nestin、DCX和CD34表达(均$P<0.05$)。**结论：**灸法可以减轻VD诱导的延迟记忆障碍, 这可能与促进神经新生和血管生成有关。

【关键词】灸法; 穴, 百会; 穴, 神庭; 穴, 大椎; 督脉; 痴呆, 血管性; 大鼠

【中图分类号】R2-03　　**【文献标志码】**A

Vascular dementia (VD) is a progressive disease caused by reduced blood flow to the brain, which can affect cognitive abilities. VD patients may suffer from slow thinking, forgetfulness, depression and anxiety, disorientation, and loss of executive functions such as problem solving, working memory, thinking, reasoning, judgment, planning and execution of tasks, with performance declining accompanied by increasing task complexity. VD accounts for about 17%-20% of all dementia patients, making it the second leading form of dementia after Alzheimer's disease (AD). It is prevalent among the older population[1]. Memory dysfunction especially immediate and delayed memory occurs in the early phase of VD.

Recently, some studies have demonstrated that neural stem cells (NSCs), which have a proliferation capacity of self-renewal and generation of both neurons and glia[2], play a key role in endogenous restoration in the mammalian central nervous system (CNS)[3]. Although neural regeneration in the brain declines with age[4], NSCs survive throughout life in a few distinct neurogenic zones, such as the subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone (SVZ) of the lateral ventricles[5]. Endogenous NSCs in these regions are activated after injury[6] and subsequently develop to mature neurons through a complex sequence of developmental steps, including self-renewal, differentiation, migration, targeting, and synaptic integration[5,7]. However, the quantity of activated endogenous NSCs is not sufficient to completely repair nerve injury[3].

Endothelial progenitor cells (EPCs) are the precursor cells of vascular endothelial cells, which contribute to new vessel formation in postnatal angiogenesis. They are mobilized by physiological and pathological stresses, such as exercise, trauma, tumor, and inflammation[8-10]. Increases in circulating EPCs are reported to improve clinical outcomes of stroke and myocardial infarction[11-12]. We have previously demonstrated that increasing EPCs could promote the functional recovery of brain trauma in a rat model but the effect was not good enough[13-14].

This study used retroviral vector carrying enhanced green fluorescent protein (EGFP) to label rat fatal NSCs that cultivated *in vitro*, then constructed the combinative implants which consisted of NSCs, EPCs and appropriate extracellular matrix (ECM), and transplanted the combinative implants or NSCs into the lateral ventricle of the VD rats respectively in order to simulate the micro-environment of neurogenesis and angiogenesis. The purpose was to finally explore the mechanism of moxibustion therapy on post-plantation VD rats by observing the variation of delayed memory, nestin, doublecortin (DCX) and CD34.

1 Materials and Methods

1.1 Animals

The study was carried out at the Animal Care Facility of Anhui University of Chinese Medicine and the experimental procedure followed the *Guidelines for the Care and Use of Laboratory Animals* published in 2006 by the Ministry of Science and Technology of the People's Republic of China. Two hundred adult male SPF Wistar rats weighing 250-300 g (at the start of the experiment) were purchased from the Animal Experimental Corporation of Beijing Weitonglihua [certification No. SCXK (Jing) 2012-0001]. All animals were housed in a controlled environment [12 h light/dark cycles, (25±1) ℃].

One hundred and ninety-five rats were chosen for the experiment by Morris water maze (MWM). Thirty-six rats were separated into the sham operation group, while the other rats were operated on two-vessel occlusion (2-VO) to make the VD models. VD models were identified 3 d after operation by MWM. The qualified models were randomly divided into a model group, a NSCs plus EPCs moxibustion group and a NSCs moxibustion group. A total of 108 rats survived at the end of the experiment except for the sham operation group: the model group (n=36), the NSCs plus EPCs moxibustion group (n=36) and the NSCs moxibustion group (n=36).

1.2 Vascular dementia model

Bilateral common carotid arteries occlusion (BCCAo) was performed to make the VD model. Rats were forbidden from eating 12 h before the surgery. Briefly, rats were anesthetized with the mixture of 10% chloride hydrate (Guangfu, China) and 25% urethane (Solarbio, China) [5.0 mL/(kg·bw), intraperitoneal injection] and then fixed. After disinfected routinely, the rats were incised in the middle of the neck and the bilateral common carotid arteries were separated and occluded. The wounds were sutured and injected with gentamycin to prevent from infection. The rats in the sham operation group were treated the same as the VD group except for BCCAo. All rats were kept in different cages respectively after the surgery.

1.3 Model identification

Rats in every group were identified with MWM 3 d after modeling. The rat whose mean latency was significantly different from that of the sham operation group was identified as the VD model.

1.4 Cell culture

The NSCs (Weikai Biotechnology Corporation, China) were cultivated in special medium for rats' NSCs. The EPCs (MRC Biotechnology Corporation, China) were cultivated in special medium for rats' EPCs. Cells were cultured at 37 ℃ with 5% CO_2 in an incubator (Thermo Scientific, USA). The NSCs and EPCs medium was changed in 2-3 d and 2 types of cells were passaged at 1:3 and 1:4 respectively in 5 d. NSCs and EPCs that had been passaged 3-5 times were used for the experiment, which strongly maintained their proliferation and differentiation abilities.

1.5 EGFP labeling of NSCs

Retroviral vector carrying EGFP (Hongshan Biotechnology Corporation, China) was used to infect rat fatal NSCs [multiplicity of infection (MOI) = 50]. NSCs were observed under IX51 fluorescence microscopy (OLYMPUS, Japan) with 450-490 nm laser and the green fluorescence could be seen even in the middle of NSCs.

1.6 Combinative implants construction

Digested the passaged EPCs with parenzyme (Solarbio, China) until most cells began to change in form and used EPCs special medium to stop cells digesting. The digested EPCs were mixed with special medium together and cultivated in 6-well plates (Corning, USA), and then grew to monolayer after 24 h.

Primarily got rid of the medium and cultured suspended NSCs in the 6-well plates added with NSCs special medium after coating laminin (Sigma, America). Then got rid of the medium and cultured EPCs in the 6-well plates added with the mixture of NSCs and EPCs special medium after coating laminin 24 h later. Two types of cells grew to multilayers after 24-48 h.

1.7 Transplantation of combinative implants and NSCs

Three days after VD model identification, the rats in the NSCs plus EPCs moxibustion group and the NSCs moxibustion group were anesthetized and received stereotaxic (RWD-68505, Shenzhen, China) transplantation. Combinative implants or NSCs suspension with 1×10^6 cells in 10 μL PBS were injected into the lateral ventricle of the right hemisphere in rats by micro-syringe pump respectively, with the following coordinates: Bregma, −2.4 mm; M-L, −3.6 mm; D-V, −3.5 mm. The injection was delivered at 1 μL/min and the needle was kept *in situ* for 5 min after injection before withdrawn slowly. The wound was then sutured and the animals were returned to the cages separately for follow-up experiments.

1.8 Treatment methods

In the sham operation group and the model group, rats did not undergo any treatment except for fixation.

Three days after the cells injection, rats in the NSCs plus EPCs moxibustion group and the NSCs moxibustion group received suspended moxibustion at Dazhui (GV 14), Baihui (GV 20) and Shenting (GV 24) with homemade moxa sticks (120 mm in length and 5 mm in diameter), 20 min for each acupoint, once a day. This treatment continued for 7 consecutive days as one treatment course, there was a 24-hour interval between two courses. Rats in each group were subdivided into three subgroups considering of the course of treatment, 12 rats in each subgroup, they were treated for 1, 2 or 3 courses, respectively. After treatment, all rats were returned to their cages respectively. The rats would be tested by MWM 24 h after treatment and then sacrificed. The brains of rats were made into frozen sections for immunofluorescence test.

1.9 MWM test

Delayed memory was assessed by MWM (JLBehv-M, Shanghai Jiliang, China). Briefly, a rat-used tank (180 cm in diameter and 40 cm in height) was separated into four quarters and filled with water [25 cm in depth, at (26±2) ℃]. A target platform (10 cm in diameter) was hidden 2 cm below the water surface in the first quarter halfway between the center and the wall of the maze. Rats were allowed to adapt to the maze on the platform for 30 s before the visible platform experiment per day for 3 d. Afterwards, the rats were forced to swim in water and try to locate the submerged escape platform. Rats were ordered to swim to find the platform directly without adaption on the fourth day of the test. A computerized tracking system was used to record latency. Four times from four random start positions in four different quarters were tested daily for consecutive 4 d. Rats which failed to find the platform within 2 min were recorded for a maximum latency score of 120 s.

1.10 Immunofluorescent staining

Rats in the model group, the NSCs plus EPCs moxibustion group and the NSCs moxibustion group were deeply anesthetized and heart perfused with 0.9% NaCl followed by 4% paraformaldehyde dissolved in 0.1 mol/L phosphate buffer (PFA). Next, the brains were quickly separated, postfixed in 4% PFA for 24-48 h, and immersed in 30% sucrose solution for storage at 4 ℃ prior to sectioning and frozen serial coronal brain sections (10 μm) were prepared on a cryostat (Leica-CM 1900, Leica, Germany). The sections located in SGZ and SVZ were blocked with 0.1% triton X-100 and 10% normal blocking serum which had original species the same as the secondary antibody in PBS at room temperature for 2 h to avoid unspecific staining. All sections were incubated with mouse primary antibody for anti-EGFP (1:100, MRC Biotechnology Corporation, China) firstly. Then rabbit primary antibody for and anti-nestin (1:100, Abcam, UK), anti-DCX (1:100, Abcam, UK) or anti-CD34 (1:100, Abcam, UK) were added

respectively for different target detection. Briefly, the sections were incubated with both primary antibodies overnight at 4 ℃, followed by anti-mouse FITC (1:200, Santa Cruz, USA) and anti-rabbit TRITC (1∶500, Sigma, USA) for 2 h at room temperature without light. Then the sections were counterstained with DAPI for visualization of nuclei for 10 min, followed by 3 washes of 15 min each in PBST (0.1% triton in PBS) and coverslipped for microscopic observation.

1.11 Quantification and imaging

All the quantification was done using the Image J program. Positive nestin, DCX and CD34 cells were mainly observed in SGZ and SVZ. At least 3 sections per rat were used for nestin and DCX quantification by calculating mean optical density (MOD) of the target protein. Quantification of CD34 needed at least 3 sections every rat. Six fields in the view were chosen per section and new-born microvessel density (MVD) was quantified by counting CD34-positive cells. Microscopic imaging was done using OLYMPUS LSM (FV 1000, OLYMPUS, Japan) confocal microscope. Images were acquired as tile scans with objectives lens: 40×, sampling speed: 8.0 μs/pixel, image size: 2 048×1 536, integration type: Frame Kalman, and analyzed using the OLYMPUS FV 1000 image-analysis software. Images for different experimental interventions were acquired under the same laser and microscopic parameters for the purpose of consistency.

1.12 Statistical analysis

Statistical analysis was conducted using statistical package of social science SPSS version 22.0 software. The measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$) and were subjected to test the normality. The data accorded with normal distribution were analyzed using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test or Dunnett T3 test for multiple group comparisons according to the homogeneity of variance. $P<0.05$ was considered to indicate a statistical difference. The data accorded with skewed distribution were analyzed using Kruskal-Wallis H rank-sum test. $P<0.05$ was considered to indicate a statistically significant difference. Mann-Whitney U test was used for the following multiple-group comparison and the value of $P<\alpha'$ was considered statistically significant. $\alpha'=2\alpha/[k(k-1)]$, k was represented for the groups would be compared.

2 Results

2.1 Model identification

It was showed that the latency of the model group, the NSCs plus EPCs moxibustion group and the NSCs moxibustion group was longer than that of the sham operation group on the 4th day of visible platform experiment by MWM in model identification test after modeling ($P<\alpha'=0.008$), whereas no statistically significant difference was showed among the model group, the NSCs plus EPCs moxibustion group and the NSCs moxibustion group ($P>\alpha'=0.008$). The VD models could be considered successful (Figure 1).

2.2 EGFP labeling of NSCs

Neurospheres began to form 48 h after retroviral vector labeling EGFP infecting NSCs. Cells grew in suspension and green fluorescence could be seen in the neurospheres under fluorescence microscope (Figure 2).

2.3 Combinative implants construction

The combinative implants were irregular under microscope. NSCs had characteristic of reflective rays and formed into big neurosphers. EPCs grew in the shape of rhombus or fusiform and had bigger nucleuses than NSCs. NSCs were encircled by EPCs or cohered each other (Figure 3).

2.4 Improvement of delayed memory after NSCs plus EPCs combinative implant

As expected, latency was significantly shortened on the fourth day of visible platform experiment by MWM after 3 treatment courses compared with that before treatment ($P<0.05$) except the model group, suggesting that the delayed memory had developed in all rats except those in the model group (Table 1). The improvement of rats in the sham operation group might be caused by the continuous training before formal test. However, the escape latency of all rats was influenced by grouping. The rats subjected to NSCs plus EPCs combinative implant transplantation and moxibustion had shorter latency than those in the model group ($P<0.008$).

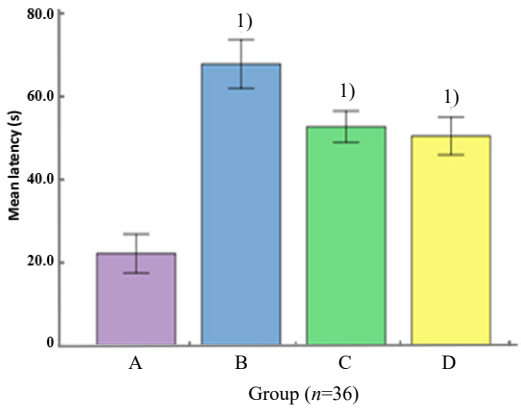
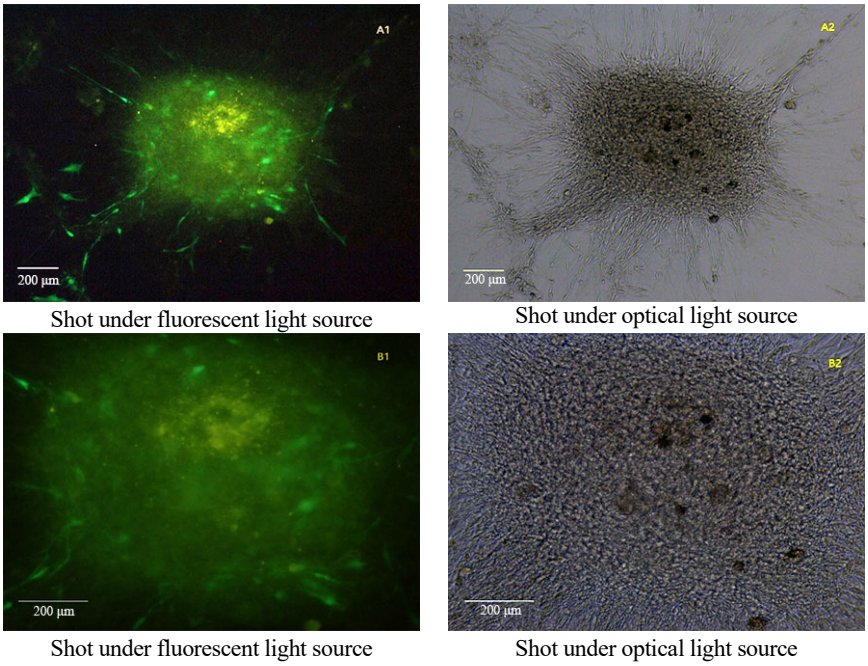


Figure 1. VD model identification with MWM

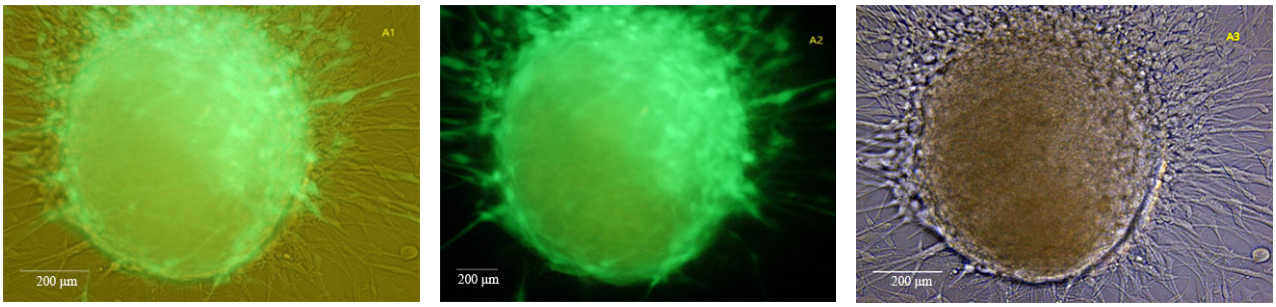
Note: A=Sham operation group; B=Model group; C=NSCs plus EPCs moxibustion group; D=NSCs moxibustion group; compared with the sham operation group, 1) $P<0.05$



Shot under fluorescent light source　Shot under optical light source

Shot under fluorescent light source　Shot under optical light source

Figure 2. EGFP labeling of NSCs (×200)



Shot under fluorescent and optical light sources　Shot under fluorescent light source　Shot under optical light source

Figure 3. NSCs plus EPCs combinative implants（×200）

Table 1. Comparisons of delayed memory among groups ($\bar{x}$ ±s, s)

Group	*n*	Before treatment	After treatment
Sham operation	36	22.21±32.50	7.69±8.21[2)]
Model	36	67.80±43.91[1)]	46.08±38.37
NSCs plus EPCs	36	52.71±33.68[1)]	22.73±21.54[2)3)]
NSCs	36	50.49±38.45[1)]	28.98±29.54[2)]

Note: Compared with the sham operation group before treatment, 1) $P<0.05$; compared with the same group before treatment, 2) $P<0.05$; compared with the model group after treatment, 3) $P<0.008$

2.5 Increased neurogenesis and angiogenesis in the right lateral SVZ after treatment

The sham operation group and the model group were not compared since no positive cells could be found under microscope.

The level of neurogenesis was assessed by quantifying nestin and DCX-positive cells, a specific marker of NSCs proliferation and differentiation respectively. The level of angiogenesis was assessed by counting CD34-positive cells, a specific marker of new-born micro-vessel. Our results proved that neurogenesis and angiogenesis increased in the lateral SVZ after moxibustion but the changes were different in different groups and subgroups. The positive cells showed yellow cytoplasm with blue cell nucleus in them and the yellow arrows were used to indicate the results.

Quantification of nestin-positive cells showed significant decrease after the 2nd and 3rd treatment courses compared with that after the 1st treatment course in the NSCs plus EPCs moxibustion group ($P<0.05$). Similar variation trend was found in the NSCs moxibustion group that quantification decreased after the 3rd treatment course compared with that after the 1st treatment course ($P<0.05$). And the quantification in the NSCs moxibustion group was significantly lower than that in the NSCs plus EPCs moxibustion group after the 1st treatment course and the 3rd treatment course ($P<0.05$), (Figure 4 and Figure 5).

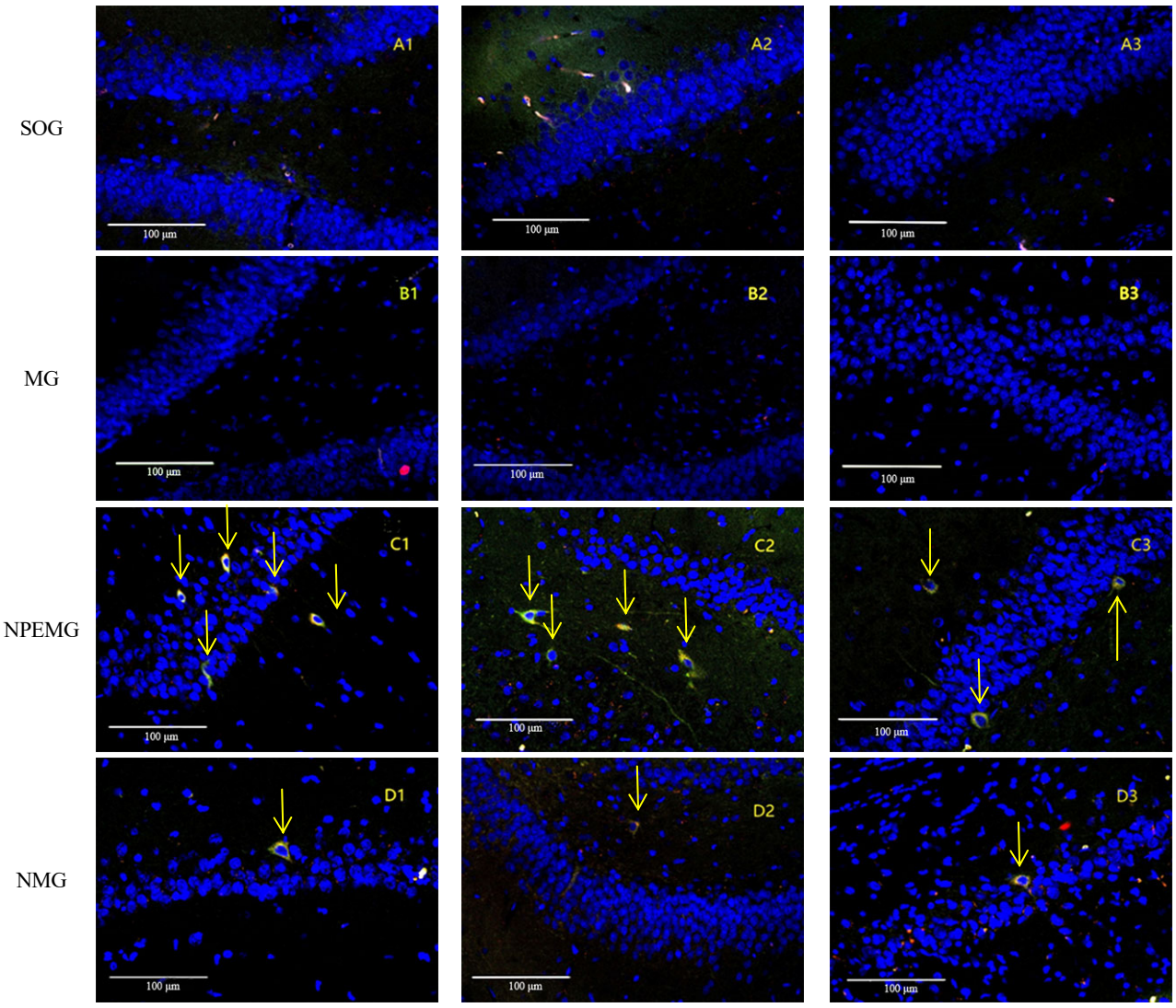


Figure 4. Immunofluorescent expression of nestin in the right hippocampus of rats [*n*=36, immunofluorescent staining for DAPI (blue), EGFP (green) and nestin (red), EGFP/nestin double labeled (yellow), ×400]

Note: SOG=Sham operation group; MG=Model group; NPEMG=NSCs plus EPCs moxibustion group; NMG=NSCs moxibustion group

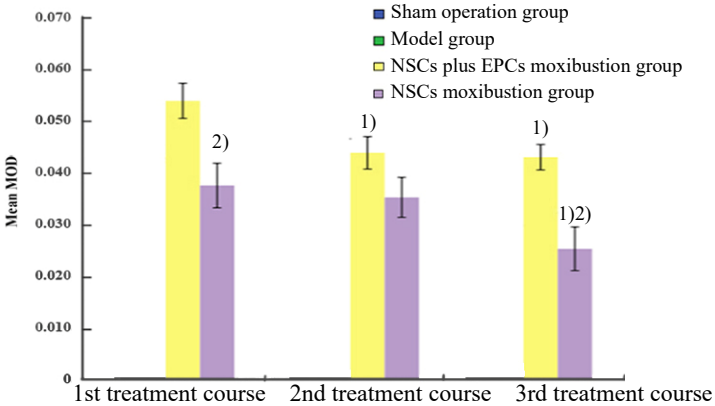


Figure 5. Mean MOD of nestin in different groups with different treatment courses

Note: Compared with the result after the 1st treatment course in the same group, 1) $P<0.05$; compared with the NSCs plus EPCs moxibustion group at the same time point, 2) $P<0.05$

Quantification of DCX-positive cells indicated significant increase after the 2nd treatment course compared with that after the 1st treatment course and the 3rd treatment course in the NSCs plus EPCs moxibustion group (P<0.05) and the quantification in the NSCs plus EPCs moxibustion group was significantly higher than that in the NSCs moxibustion group after the 2nd treatment course (P<0.05), (Figure 6 and Figure 7).

It was revealed that CD 34 positive cells expressed significantly most after the 2nd treatment course compared with that after the 1st treatment course and the 3rd treatment course in both NSCs plus EPCs moxibustion group and NSCs moxibustion group (P<0.05). The expression after the 3rd treatment course was the least (P<0.05). The count in the NSCs moxibustion group was always less compared with that in the NSCs plus EPCs moxibustion group at different time points (P<0.05), (Figure 8 and Figure 9).

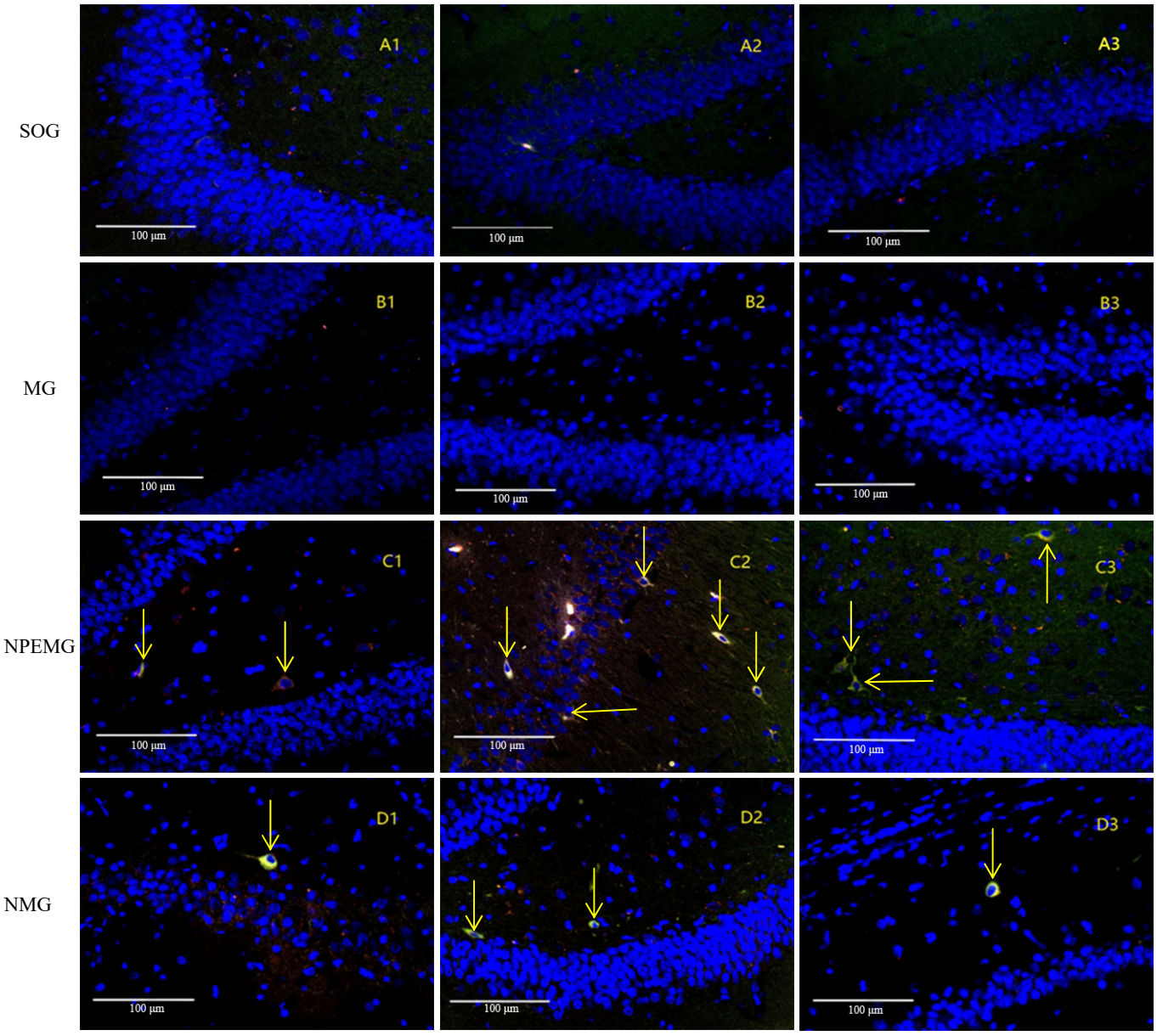


Figure 6. Immunofluorescent expression of DCX in the right hippocampus of rats [n=36, immunofluorescent staining for DAPI (blue), EGFP (green) and DCX (red), EGFP/DCX double labeled (yellow),×400]

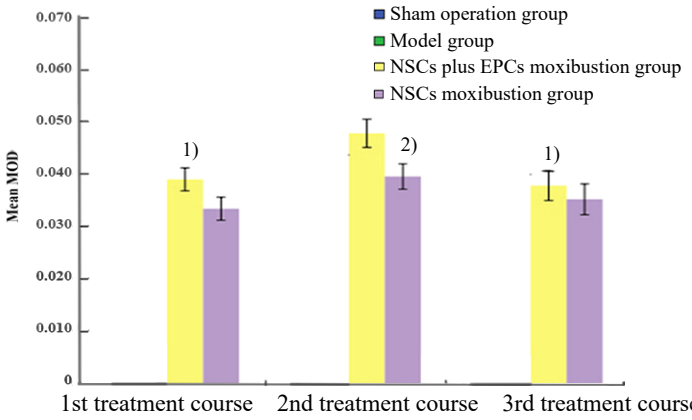


Figure 7. Mean MOD of DCX in different groups with different treatment courses

Note: Compared with the result after the 2nd treatment course in the same group, 1) $P<0.05$; compared with the NSCs plus EPCs moxibustion group at the same time point, 2) $P<0.05$

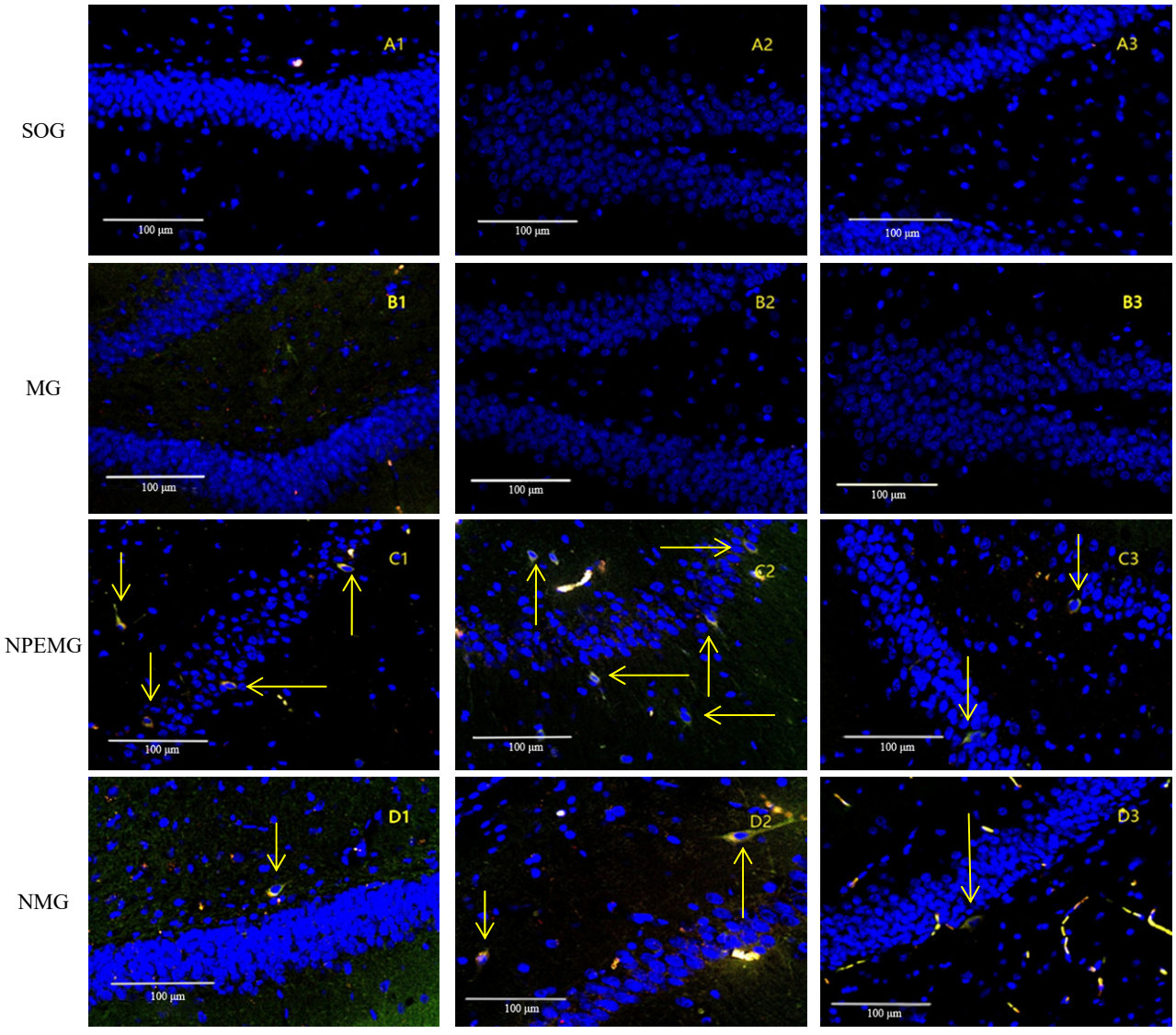


Figure 8. Immunofluorescent expression of CD34 in the right hippocampus of rats [*n*=36, immunofluorescent staining for DAPI (blue), EGFP (green) and CD34 (red), EGFP/CD34 double labeled (yellow), ×400]

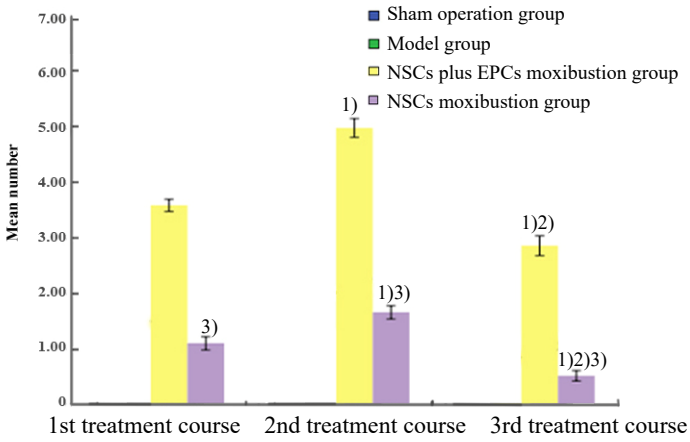


Figure 9. Mean number of CD34 in different groups with different treatment courses

Note: Compared with the result after the 1st treatment course in the same group, 1) $P<0.05$; within-group comparison after the 2nd treatment course, 2) $P<0.05$; compared with the NSCs plus EPCs moxibustion group at the same time point, 3) $P<0.05$

4 Discussion

In Chinese medicine, VD is often associated with abnormal behaviors. Mental activities are regulated by brain and brain marrow is the foundation of mental activities. Consequently, insufficient marrow is mainly responsible for VD: blood stagnation in collaterals also plays a role. In this study, suspended moxibustion was used at Baihui (GV 20), Dazhui (GV 14) and Shenting (GV 24). The treatment can dredge meridians, resolve stagnation, replenish the brain and refresh mind.

In VD, chronic hypoperfusion and thromboembolic events can lead to a decrease in cerebral blood flow (CBF), hypoxia, oxidative stress and trigger inflammatory responses[15]. These changes can cause damage to vessel endothelia, glial and neural cells, which will further result in neurovascular uncoupling[16-18], neuro-degeneration, cell death[19], the impairment of neurogenesis, neural progenitor cell proliferation, synaptic plasticity, dendritic spine density, CBF reduction and so on[20]. The periventricular white matter (WM), basal ganglia, and hippocampus are highly susceptible to hypoperfusion. When the hypoperfusion occurs, hippocampus-based learning and memory deficits will ensue[15].

Current studies have demonstrated that acupuncture can improve VD by reducing oxidative stress[21-22], attenuating neuron apoptosis[23-24], increasing the number of pyramidal neuron in the hippocampus CA1 region[25], relieving neuroinflammation, regulating glucose metabolism[26], modulating neurotransmitters[27] and improving synaptic plasticity[28] and blood vessel function[29-31], etc. Neuroscience has found that the cognitive function and memory of VD rats can be improved by inducing neurogenesis in the hippocampus[32]. Neurogenesis is a process that NSCs reside and continuously proliferate and differentiate into new neurons[33]. Nestin is a kind of intermediate filament protein which is recognized as a marker of NSCs located in the brain neurogenic niches[2,34]. Nestin-positive neural precursor cells (NPCs) are reported to have a potential source of newborn brain neurons[35-37]. DCX is a microtubule-associated protein expressed by NPCs, and is associated with neural regeneration[38]. It is associated with the normal brain development processes of neural cell birth and migration[39-41]. It has been demonstrated that ischemia and nerve injury can cause increased nestin and DCX expression but the effect is not enough for functional recovery[33,42]. Acupuncture has been proved to improve ischemia-induced neural damage, mainly by promoting neural regeneration, which is shown by increased nestin and DCX expression[43-44]. EPCs are a population of cells which participate in vessel formation in both physiological and pathological processes, and demonstrate the characteristics of both endothelial and progenitor cells[45]. They have great potential as a source of cells for the repair of vasculogenic injuries[46] and been used to repair ischemic or damaged cardiac tissue in animal models[47] by creating blood vessels in postnatal angiogenesis[48-49]. VEGF is one of the important factors of angiogenesis. There are some reports that moxibustion could increase the VEGF expression in VD rat brain[50]. CD34 has been regarded to express on EPCs[51]. There is a micro-environment called neurovascular niche which is crucial to the neurogenesis and angiogenesis following stroke in adult brains[52]. In this neurovascular niche, angiogenesis can up-regulate NSCs expansion for neurogenesis and in turn NSCs can regulate the development of functional integrity in the neurovascular niche by secreting the factors that induce angiogenesis[53-54]. Accordingly, we

proposed the hypothesis that moxibustion can promote neurogenesis and angiogenesis by inducing the proliferation, differentiation, maturation of NSCs and EPCs respectively.

Using a well-characterized VD model, we examined dysfunction of delayed memory in rats subjected to BCCAo and correlated such changes with no positive nestin, DCX and CD34 cells in SGZ and SVZ.

In this experiment, MWM was used to select experimental rats, identify VD models and test treatment effects. The results illustrated that the successful rate of model replication by 2-VO could reach about 80% and had good survival rate. The 2-VO is a good method to make VD models. Although some rats died during treatment but the mortality rate was not high. Thirty-six rats in every group were included in the experiment and statistically analyzed finally.

We found that rats developed delayed memory defects after VD modeling and those treated with moxibustion after NSCs plus EPCs or NSCs transplantation showed improvement in delayed memory after treatment. The rats treated with NSCs plus EPCs transplantation and moxibustion had higher development compared with those in the model group. In our study, after moxibustion and NSCs plus EPCs or NSCs transplantation, the VD rats significantly showed the trend of increasing level of nestin, DCX and CD34 compared with the rats in the model group. An obvious advantage was also exhibited in the NSCs plus EPCs moxibustion group than in the NSCs moxibustion group. Therefore, apparent reversal of the BCCAo-induced delayed memory deficits and other effects observed in SGZ of the hippocampal dentate gyrus and SVZ of the lateral ventricle could be considered as a consequence of the moxibustion and cells transplantation. Additionally, the different effects between the two moxibustion groups may be caused by the interaction of NSCs and EPCs. Regarding the previous researches, it has been proved that neurogenesis and angiogenesis could be mutually facilitated. Based on these considerations, we confirmed experimentally that moxibustion could promote neurogenesis in brains of VD rats and angiogenesis can regulate neurogenesis actively likewise.

In conclusion, rats treated with moxibustion can improve VD-associated dysfunction of delayed memory. This benefit should be associated with the improvement of neurogenesis and angiogenesis in the injured brain by regulating neurovascular niche. In addition, high proliferation and differentiation levels of NSCs and EPCs are crucial to neurogenesis and angiogenesis.

Conflict of Interest

The authors declared that there was no potential conflict of interest in this article.

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Statement of Human and Animal Rights

The treatment of animals conformed to the ethical criteria.

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