Neuroprotective effects of Ilexonin A following transient focal cerebral ischemia in rats

AI-LING XU1,2, GUAN-YI ZHENG1, ZHI-JIAN WANG1,3, XIAO-DONG CHEN4 and QIONG JIANG4

1Department of Traditional Chinese Medicine, The Affiliated Union Hospital of Fujian Medical University, Fuzhou, Fujian 350001; 2Neonatal Department, The People’s Hospital Affiliated to Fujian University of Traditional Chinese Medicine, Fuzhou, Fujian 350004; 3Department of Neurology, Fuzhou Neuro-Psychiatric Hospital, Fuzhou, Fujian 350000; 4Burns Institute of the Affiliated Union Hospital of Fujian Medical University, Fuzhou, Fujian 350001, P.R. China

Received March 24, 2015; Accepted December 30, 2015

DOI: 10.3892/mmr.2016.4921

Abstract. Ilexonin A is a compound isolated from the root of a plant commonly used in traditional Chinese medicine. The aim of the present study was to investigate the possible protective mechanism of Ilexonin A in rats subjected to occlusion of the middle cerebral artery (MCAO). Transient focal cerebral ischemia was induced by 2 h of MCAO, followed by reperfusion. Ilexonin A at doses of 20, 40 and 80 mg/kg were administered via intraperitoneal injection immediately following ischemia/reperfusion. The expression levels of glial fibrillary acidic protein (GFAP), ionized calcium-binding adapter molecule-1 (Iba-1), vascular endothelial growth factor (VEGF), fetal liver kinase-1 (Flk-1) and Nestin were examined using immunostaining and Western blot analysis of the peri-infarct region following ischemia/reperfusion. Ilexonin A significantly decreased the infarct volume and improved neurological deficits in a dose-dependent manner. The expression levels of VEGF, Flk-1 and Nestin were significantly increased in the rats treated with Ilexonin A, compared with the rats administered with saline. Following treatment with Ilexonin A, a higher number of GFAP-positive astrocytes were found in the Ilexonin A-treated rats at 1, 3 and 7 days, compared with the rats exposed to ischemia only, however, there were fewer astrocytes at 14 days, compared with the ischemia group. Ilexonin A significantly decreased the protein expression of Iba-1. The results of the present study suggested that the protective effects of Ilexonin A were associated with revascularization, neuronal regeneration, and the regulation of astrocyte and microglia cell activation.

Introduction

Although stroke is one of the leading contributors to mortality and long-term disability rates among adults around the world, innovative neuroprotective treatments for therapy following a stroke require further investigation. An area of investigation, which is gaining increasing attention is the use of herbal medicines or their extracts for treating stroke. Ilexonin A is a compound isolated from the root of the plant Ilex pubescens, which shows good therapeutic effects when used for the treatment of coronary artery disease, angina and vasculitis (1,3). It has been confirmed that the therapeutic effects of Ilexonin A are achieved through improving blood circulation via its anti-thrombotic and anti-inflammatory activities (4). As early as 1985, Luo et al found that dysfunction induced by cerebral ischemia was ameliorated following treatment with Ilexonin A. Evidence obtained in subsequent studies has suggested that Ilexonin A, which can reduce infarct volume and improve neurological deficit, has a neuroprotective potential following cerebral ischemia/reperfusion injury, the effect of which is partly attributed to enhancement of the secretion of neurotrophic factors, mitigating cerebral edema and promoting neural regeneration (5,6).

Astrocytes and microglia cells reside in the central nervous system (CNS) as ubiquitously distributed quiescent cell populations, which are able to in change morphology, number and function when activated by ischemic conditions (7,8). It has been suggested that reactive astrocytes and microglia are multifunctional cells, which, depending on the microenvironment, can be either beneficial or detrimental following ischemia/reperfusion (9,10). Previous reports have suggested that reactive astrocytes and microglia can promote tissue integrity, seal off injured tissue and restrict neuronal death through cell proliferation (11), phagocytosis of cellular debris and the secretion of neurotrophic factors (11,12). However, they can also mediate brain edema and inflammation by producing neurotoxic substances and pro-inflammatory cytokines following ischemic injury (13,14). The present study aimed to investigate the characteristics of activated astrocytes and microglia following the post-ischemia administration of Ilexonin A.

Correspondence to: Dr Guan-Yi Zheng, Department of Traditional Chinese Medicine, The Affiliated Union Hospital of Fujian Medical University, 29 Xinquan Road, Gulou, Fuzhou, Fujian 350001, P.R. China
E-mail: guanyi.zheng@yahoo.com

Key words: Ilexonin A, revascularization, transient focal cerebral ischemia, neuroprotection
It has been suggested that angiogenesis and neurogenesis occurring in the peri-infarct region are associated with long-term recovery (15,16); and the two processes appear to be governed by ischemia-induced growth factors. One of the most important neurotrophic and angiogenic factors found during stroke recovery, which is fundamental for adult angiogenesis, is vascular endothelial growth factor (VEGF) (17). A previous study suggested that VEGF mediates neuroprotection and promotes neural regeneration when it is bound by its specific receptor, fetal liver kinase 1 (Flk-1) (18). Endogenous neurogenesis and neovascularization are substantially activated and occur in close proximity to the ipsilateral neocortex of the ischemic brain (15).

The present study was performed in order to explore the underlying protective mechanisms of Ilexonin A in rats, and to provide a reliable scientific basis for treating ischemic cerebrovascular disease with Ilexonin A. The current study investigated the effects of Ilexonin A on the expression levels of VEGF, Flk-1 and Nestin in the peri-infarct region following transient focal cerebral ischemia.

Materials and methods

Reagents. All reagents were purchased from Beijing Zhongshan Biotechnology Co., Ltd. (Beijing, China), unless otherwise stated. Ilexonin A was supplied by Guangdong Boro Pioneer Pharmaceutical Group Co., Ltd. (Guangdong, China; approval no. Z44023366).

Animal treatment and administration. A total of 108 male Sprague-Dawley rats (Grade II) weighing 250±10 g, aged 6-8 weeks, were supplied by Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China; certificate no. 2007-0005). The rats were housed in temperature (22‑25˚C) and humidity (40-70%) controlled conditions with a 12 h light/dark cycle and ad libitum access to food and water. The study was approved by the ethics committee of The Affiliated Union Hospital of Fujian Medican University (Fuzhou, China). The rats were divided into six groups for investigation: Normal group; Sham group; Ischemia group, and Ilexonin A groups receiving 20, 40, and 80 mg/kg, respectively. Each group was divided into four subgroups (n=6), based on the onset of reperfusion, set at 1, 3, 7 or 14 days following ischemia. The normal control rats were anesthetized and underwent identical surgery, with the exception that the nylon suture was not inserted.

Inclusion criteria of MCAO and neurological severity scoring of animals. Following recovery of the rats from anesthesia, neurological severity was evaluated for the determination of MCAO using a five point score, according to Longa's method (19): 0, no deficit; 1, failure to extend right forelimb fully; 2, circling to the right; 3, falling down to the right; 4 no spontaneous walking with a depressed level of consciousness. Achievement of MCAO was confirmed using a score of 1-3 points. The rats were re-evaluated using the same method prior to sacrifice by anaesthetic overdose using 10% chloral hydrate.

Tetrazolium chloride staining. The rats were anesthetized and received cardiac perfusion with 100 ml saline, followed by 200 ml 4% paraformaldehyde (Beijing Donglin Changsheng Biotechnology Co., Ltd.) in 0.1 M phosphate buffer (pH 7.4). The brains were carefully removed when the liver and extremities turned white in color. The brains were frozen at -20˚C for 20 min, and then cut from the anterior pole into five coronal slices of 2 mm thickness. The slices were stained with 2% 3, 5-triphenyltetrazolium chloride solution (Sigma-Aldrich, St. Louis, MO, USA) in the dark at 37˚C in an incubator for 30 min, and turned over every 5 min. A 10% buffered-formalin solution (Beijing Donglin Changsheng Biotechnology Co., Ltd.) was used for fixation (24 h) prior to imaging with a digital camera (VPC-SHI; Sanyo Electric Co., Ltd., Osaka, Japan). The normal brain tissue was stained red, whereas the ischemic area remained unstained.

Immunohistochemical examination. The rats were anesthetized and perfused in the same manner as that described above. The brains were removed and post-fixed for 24 h in the same fixative solution, and dehydrated in a sucrose gradient (15, 20 and 30%) in 0.1 M phosphate buffer until they sank to the bottom. Subsequently, 8 µm sections were cut using a cryomicrotome (Microm HM525; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for use in immunohistochemical probing.
Immunohistochemistry (IHC) was performed in strict accordance with the datasheet of the Elivision kit (Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China), with rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:5,000; ab7260; Abcam, Cambridge, UK) to identify astrocytes, goat polyclonal anti-ionized calcium-binding adapter molecule-1 (Iba-1) antibody (1:100; ab5076; Abcam) to identify microglia, and mouse monoclonal anti-Nestin antibody (1:100; sc-33677; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) to identify neural progenitor cells. In the negative controls, 0.01 M PBS was substituted for the primary antibody. The results were visualized using a 3,3′-diaminobenzidine kit (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). A microscope (CX40; Olympus Corporation, Tokyo, Japan) was used for image acquisition. The positive cells in each section were evaluated using Image Pro Plus 5.0 (Media Cybernetics, Inc., Rockville, MD, USA) in five high power microscopic fields (CX40; Olympus Corporation, Tokyo, Japan; magnification, x400).

**Western blot analysis.** The rats were infused with 100-200 ml saline following anesthesia and the brains were removed. The peri-infarct region was disassociated and lysed in 10 µl/µg radiolmmunoprecipitation assay lysis buffer with 0.01 M phenylmethanesulfonylfluoride (both from Beyotime Institute of Biotechnology, Haimen, China). The samples were homogenized by sonication and centrifuged at 14,000 x g at 4˚C for five min, and 20 µg was loaded onto SDS-PAGE gels (12% for Nestin and Flk-1). Following electrophoresis, the proteins were electrically transferred onto a nitrocellulose membrane (pore size, 0.45 µm; EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat milk in Tris-buffered saline with tween (TBST) containing Tris-Hcl (0.1 M; pH 7.5.) 0.9% NaCl and 0.05% Tween-20, at room temperature for 1 h on a shaking table. The membranes were subsequently incubated with the following primary antibodies in TBST at 4˚C overnight: Rabbit polyclonal anti-GFAP, (1:20,000; ab7260), goat polyclonal anti-Iba-1 (1:200; ab5076; both purchased from Abcam), mouse monoclonal anti-Nestin (1:300), mouse monoclonal anti-VEGF (1:200; sc-7269), mouse monoclonal anti-Flk-1 (1:200; sc-6251) and mouse monoclonal anti-β-actin (1:2,000; sc-47778) (all obtained from Santa Cruz Biotechnology, Inc.).

Following being washed with TBST, the membranes were incubated with secondary antibodies (1:6,000; Beijing Zhongshan Golden Bridge Biotechnology, Co., Ltd.) at room temperature for 2 h and washed with TBST. The immunoblots were visualized using enhanced chemiluminescence detection reagents (KPL, Gaithersburg, MD, USA). Densitometry was performed using ImageMaster® VDS gel imaging and analysis system (AlphaImager 2200; Alpha Innotech Corporation, San Jose, CA, USA), with β-actin as the loading control.

**Statistical analysis.** All data are expressed as the mean ± standard deviation and were analyzed using one-way analysis of variance using the SPSS 17.0 Software package (SPSS, Inc., Chicago, IL, USA). The differences between the groups were analyzed using the least significant difference test for homogeneity of variance and the Games-Howell test for heterogeneity of variance. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Tetrazolium chloride staining.** The induction of ischemic infarction by MCAO involves the striatum and cortex in shorter time spans and other regions at later time points (20). In the present study, brain atrophy induced by tissue degeneration and necrosis was observed in the ischemic core. Following treatment with Ilexonin A, the infarct volume was decreased, particularly at 7 days, in the Ilexonin A 40 mg/kg group (Fig. 2).

**Effect of Ilexonin A on neurological deficits.** Neurological symptoms, including unconsciousness, ptosis and tonic seizures, were exhibited on day 1 in the rats subjected to MCAO, with or without administration of Ilexonin A. With prolongation of reperfusion, the neurological deficits gradually disappeared, and rats treated with Ilexonin A recovered at a faster rate, compared with those in the ischemia group (Table I).

**Effects of Ilexonin A on astrocyte activation.** In the IHC rat brain sections, GFAP-positive cells were observed sporadically, primarily in the cortex, in the normal and sham groups, whereas...
their numbers were markedly increased in the peri-infarct region following ischemia/reperfusion (Fig. 3A). Over time, the GFAP-positive cells in the ischemia group became markedly activated, with a marked change in morphology, which included swelling of the soma, darkening of the cytoplasm and growth and thickening of cell processes, which even interlaced with each other, like a network, at day 14. Compared with the ischemia group, an increase in GFAP-immunoreactivity was observed from day 1 following ischemia/reperfusion in the Ilexonin A groups, persisting until day 7 (Fig. 3B). Of note, the GFAP-immunoreactivity decreased per contra 14 days following ischemia/reperfusion in all Ilexonin A groups, and the morphology was altered less, particularly in the 40 mg/kg dose Ilexonin A group.
Following reperfusion, the expression level of GFAP in the peri-infarct region was markedly increased, and reached a peak at day 7 in the ischemia and Ilexonin A group, as demonstrated by Western blot analysis (Fig. 3C). Compared with the ischemia group, the levels of GFAP in the Ilexonin A group were higher on days 1, 3 and 7, however, there were fewer GFAP-positive cells on day 14, compared with the ischemia rats (Fig. 3D).

**Effect of Ilexonin A on the activation of microglia.** The IHC results revealed no significant difference in the numbers of Iba-1-positive cells between the normal and sham groups, with only quiescent microglia in the peri-infarct region. In the ischemia group, the number of Iba-1-positive cells was increased between days 1 and 14, peaking 7 days post-ischemia/reperfusion (Fig. 4A and B). The quiescent microglia, which had elliptic and small soma, and more cell processes, gradually changed into rod-like cells, which had enlarged soma and thinner, shorter processes. These finally changed to amoeba-like macrophages, with large rounded soma and fewer or no cell processes. The numbers of Iba-1 positive cells, particularly the round macrophage-like cells, in the Ilexonin A groups were significantly reduced at each time point, compared with the ischemia group. The change was most marked at 7 days post-ischemia/reperfusion in the 40 mg/kg Ilexonin A group.

As shown in Fig. 4, compared with the normal and sham groups at time points >1 day, cerebral ischemia in the rats subjected to MCAO and Ilexonin A treatment significantly induced the expression of Iba-1, as detected using Western blot analysis (Fig. 4C and D). The expression of Iba-1 peaked 7 days following reperfusion in the ischemia and Ilexonin A
groups. Compared with the ischemia group, Ilexonin A treatment significantly decreased the expression of Iba-1, compared with the group exposed to reperfusion alone, particularly in the 40 mg/kg Ilexonin A group.

**Effect of Ilexonin A on the proliferation of neural stem cells.** In the normal and sham groups, a number of small vessels and micro-vascular endothelial cells were weakly positive for Nestin. Following ischemia/reperfusion, the numbers of Nestin-positive cells around the ischemia core increased significantly on days 3, 7 and 14, with a peak at 7 days, in the ischemia and Ilexonin A groups (Fig. 5A and B). In the rats subjected to MCAO treatment, a significant induction of Nestin was observed in the peri-infarct region. A further increase was observed in all Ilexonin A groups (Fig. 5C and D).

**Effect of Ilexonin A on the expression of VEGF.** Compared with the normal and sham group, the expression of VEGF was increased at day 1 post-ischemia/reperfusion, and was highest at 7 days. The expression of VEGF was further increased in the rats receiving Ilexonin A at each time point, particularly at 7 days in the 80 mg/kg group (Fig. 6).

**Effect of Ilexonin A on the expression of Flk-1.** The expression levels of Flk-1 were increased 3 days following ischemia/reperfusion, and reached a peak at 7 days in the ischemia group, compared with the normal and sham groups. Following treatment with Ilexonin A, the expression levels of Flk-1 were further increased at each time point, particularly at 7 days in the 80 mg/kg Ilexonin A group (Fig. 7).

**Discussion**

Ilexonin A is a compound, which is extracted from the herb, *Ilex pubescens*, and has been shown to have antithrombotic effects, anti-inflammatory effects and to improve blood flow.
In the present study, it was observed that Ilexonin A effectively reduced infarct volume and improved neurological deficits, in a dose-dependent manner, which demonstrated that Ilexonin A was a neuroprotectant. Previous studies have suggested that the neuroprotective effect of Ilexonin A may include enhancing the secretion of endogenous neural trophic factors, including FGF and GAP-43, promoting neurogenesis in the neocortical and subependymal zones (SVZs), mitigating cerebral edema, inhibiting free radical production and lipid peroxidation following cerebral ischemia/reperfusion. However, the specific mechanism underlying the neuroprotective effects of Ilexonin A remain to be fully elucidated and requires further investigation.

The astrocyte response to ischemia in adults is complex and remains to be fully elucidated. A previous study found that reactive astrocytes with nuclear membranes increase cell size and number following MCAO (21). It is accepted that the moderate activation and proliferation of astrocytes can have a protective effect following ischemia/reperfusion; the protective mechanism underlying the neuroprotective effects of Ilexonin A remain to be fully elucidated and requires further investigation.

Microglia are the resident immune cells in the CNS. When there is a stimulatory signal in the brain, for example cerebral ischemia, the microglia become activated and can undergo phenotypic and morphological changes (10). Based on the expression of immunocytochemical markers, the microglia become amoeba-like cells following ischemia/reperfusion, which are morphologically indistinguishable from blood-derived macrophages (34). Therefore, Iba-1 positive cells are likely derived from resident microglial and blood-derived macrophages. Novel data, based on the use of antioxidants to promote repair (26-29). If the activation of astrocytes is attenuated, the infarct volume expands 2-3-fold (30), and neuronal death is further enhanced following brain ischemia (25). The axonal growth cones grow only when reactive astrocytes survive, whereas this does not happen if astrocytes are injured in the same area following ischemia injury (31,32). These findings indicate that reactive astrocytes are essential for neuronal survival and regeneration. However, the glial scar, which is formed by the excessive proliferation of reactive astrocytes in the later stage following ischemia/reperfusion, rebuilds the integrity of the CNS, but inhibits axonal regeneration (33). In the present study, Ilexonin A either enhanced the activation of astrocytes in the early stage (1, 3 or 7 days post-MCAO) or inhibited the excessive proliferation of astrocytes in the later stage (14 days post-MCAO) in the peri-infarct region following ischemia/reperfusion. This dual effect indicated that Ilexonin A promotes favorable conditions for neuronal restoration.
of GFP radiation bone marrow-chimeric mice, suggests that blood-derived macrophages are a low percentage of the population of cells in the peri-infarct region at day 2 post-ischemia, peak at day 7 and decrease thereafter (35). By contrast, resident microglial cells are rapidly activated at day 1 post-ischemia. Notably, even at days 4 and 7, the majority of macrophage-like cells remain resident microglia-derived cells (36,37). In addition, previous reports have suggested that microglia expressing different phenotypes and morphologies have different functions when the brain receives different signals from different diseases; and the function (neuroprotective or neurotoxic) may be determined by the steady-state conditions among various microglial factors released into the microenvironment (38-42). A previous study also suggested that resident microglial and newly recruited macrophages assume a ‘healthy’ M2 phenotype in the 7 days following MCAO, which protects neurons against ischemic injury. This is followed by a transition to an ‘unhealthy’ M1 phenotype in the peri-infarct region, which exacerbates neuronal death during ischemic stroke (43). The dual and opposing roles of microglial/macrophages suggest that ischemic stroke therapy requires a focus on adjusting the balance between beneficial and detrimental microglia and macrophage responses, rather than simply suppressing microglia/macrophages.

In conclusion, the present study, found that the number of amoeba-like cells significantly decreased, whereas the number of rod-like cells increased following treatment with Ilexonin A. This indicated that the rod-like microglia, which appeared within the first 3 days of MCAO and were maintained by the administration of Ilexonin A, may have a protective effect against cerebral ischemia/reperfusion injury. The neuroprotective effects of Ilexonin A may be achieved through adjusting the balance of microglia/macrophage responses. In previous studies, the protective effect of microglia/macrophages were reported to occur through reduction in the secretions of neuronal plasticity proteins and neurotrophic factors, including IGF-1 and GDNF (42-45). However, the evidence for this is limited, and further investigation is required.

In the present study, the expression of Nestin was induced on day 1 following MCAO, increased progressively until day 7, when the highest expression was observed, and was sustained for >2 weeks. These results are consistent with those of previous studies (15,46). The astrocyte-like Nestin-positive cells observed around the ischemic area following 3 days of MCAO indicated a trend for neural stem cells to predominantly differentiate into astrocytes. By contrast, a previous study suggested that either endogenous or transplanted neural stem cells at the site of brain injury predominantly differentiated into gliocytes, with the exception of SVZs and subgranular zones (47). In addition, reactive astrocytes express the Nestin protein and assist in neural stem cell proliferation and differentiation (48,49). Although neural stem cells that proliferate into the peri-infarct region predominantly differentiate into gliocytes, a number of newly matured neurons are observed in the striatum and cortex at later time points post-ischemia/reperfusion (50,51). It has been suggested that newly matured neurons in the striatum and cortex may be associated with neuronal migration from the SVZ (52). Our previous study suggested that the number of newly matured neurons were increased in the cortex, and that neurogenesis and neuronal migration from the SVZ were increased following treatment with Ilexonin A (6).

VEGF, one of the most important angiogenic factors, is a double-edged sword in ischemic injury. VEGF is known as a potent mitogen for angiogenesis in the ischemic boundary, and is a neuroprotective factor involved in reducing infarct volume, improving behavioral recovery, and enhancing cortical, striatal and SVZ neurogenesis and neuron migration following cerebral ischemia in rats (53-55). In addition, VEGF acts as an instructive signal and induces responsive astroglial cells toward neuronal differentiation (56). However, VEGF is a potent vascular permeability factor, which may mediate a harmful response through blood-brain barrier leakage, brain edema, hemorrhage, aggravation of inflammatory responses and aberrant systemic hemodynamics (57-59). Therefore, a major challenge is to accelerate cerebral angiogenesis without exacerbating edema in the brain of stroke patients.

Previous studies have suggested that the results of VEGF administration are variable and are associated with the following aspects of treatment: i) Dose-effectiveness, in which there are dose-dependent effects when different doses are administered by different methods, and excess VEGF administration may have a detrimental effect in functional recovery (60-62); ii) Route dependence, in which local VEGF administration consistently enhances neurologic recovery, whereas acute intravenous delivery exacerbates brain infarcts due to enhanced brain edema; suggesting exogenous VEGF can be directly neurotoxic (63); iii) Time-dependence, in which VEGF has a therapeutic time window within the first 3 h or following 24 h of transient MCAO (62-64); iv) Special receptors, in which VEGF is known to be rapidly induced following focal cerebral ischemia and to bind to two tyrosine kinase receptors, fms-like tyrosine kinase-1 (Flt-1) and fetal liver kinase-1 (Flk-1), on the surface of endothelial cells. These receptors are induced following ischemia and show a similar temporal pattern of expression, however, the post-ischemic increase of Flk-1 is higher than that of Flt-1 (65). A previous study suggested that activation of Flt-1, but not Flk-1, is sufficient to induce hyper-permeability by hypoxia and VEGF (66). The results of the present study suggested that VEGF and Flk-1, which were progressively increased following transient focal cerebral ischemia and administration of Ilexonin A, predominantly exerted neuroprotective effects, which may be dose-dependent.

According to the observation of the dose-dependent effects of Ilexonin A, the present study found that the dose of 40 mg/kg produced the optimal protective effects on the brains of rats following transient focal cerebral ischemia. This result is in accordance with previously published data from Zheng and Shi (6).

In conclusion, neurologic recovery is a complex process, which requires the integration of numerous intrinsic and extrinsic cues to establish intact circuits. No single factor alone is sufficient to support long-term neurologic recovery. The results of the present study suggested that Ilexonin A provided a favorable microenvironment for neurological recovery through regulating the activation of gliocytes, and promoting revascularization and neurogenesis.
Acknowledgements
This study was supported by the Program for New Century Excellent Talents in Fujian Province University, China (grant no. NCETFJ-0704) and the Professorial Academic Development Foundation of Fujian Medical University (grant no. JS09014). The authors would like to thank Clarity Manuscript Consultants LLC for their assistance with manuscript editing.

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