NEURAL REGENERATION RESEARCH
Volume 7, Issue 3, January 2012
www.nrronline.org

Cite this article as: Neural Regen Res. 2012;7(3):165-170.

Anchanling reduces pathology in a lactacystin-induced Parkinson's disease model☆

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Abstract

A rat model of Parkinson's disease was induced by injecting lactacystin stereotaxically into the left mesencephalic ventral tegmental area and substantia nigra pars compacta. After rats were intragastrically perfused with *Anchanling*, a Chinese medicine, mainly composed of magnolol, for 5 weeks, when compared with Parkinson's disease model rats, tyrosine hydroxylase expression was increased, α-synuclein and ubiquitin expression was decreased, substantia nigra cell apoptosis was reduced, and apomorphine-induced rotational behavior was improved. Results suggested that *Anchanling* can ameliorate Parkinson's disease pathology possibly by enhancing degradation activity of the ubiquitin-proteasome system.

Key Words: Parkinson's disease; *Anchanling*; ubiquitin-proteasome system; α-synuclein; tyrosine hydroxylase; cell apoptosis

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Received: 2011-06-02
Accepted: 2011-12-20
(N20101004002/YJ)

Li YH, Wu ZZ, Gao XW, Zhu QW, Jin Y, Wu AM, Huang CJA. Anchanling reduces pathology in a lactacystin-induced Parkinson's disease model. Neural Regen Res. 2012;7(3):165-170.

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doi:10.3969/j.issn.1673-5374.2012.03.001

INTRODUCTION

Loss of substantia nigra dopaminergic neurons and intracytoplasmic Lewy body formation are the main pathological changes in Parkinson's disease (PD)[1-4]. Lewy body formation is highly correlated with functional impairment of the ubiquitin-proteasome system (UPS)[5-9]. The UPS is an important pathway involving protein degradation *in vivo*, responsible for clearing mutated, damaged and misfolded proteins, and comprises ubiquitin, ubiquitin-related enzymes and the proteasome. Abnormal changes in ubiquitin or ubiquitin-related enzymes E1, E2, E3 or protease may influence UPS function[10-12]. Recent evidence indicates that intracellular α-synuclein protein is misfolded or overexpressed in response to α-synuclein gene mutations, which inhibits proteasome degradation and induces abnormal α-synuclein accumulation, resulting in neurotoxicity[13]. Overexpression of α-synuclein in nerve cells can induce ubiquitin-dependent proteasome dysfunction and cell apoptosis[14]. Furthermore, proteasome dysfunction can result in abnormal protein aggregation. Stereotaxic injection of the proteasome inhibitor lactacystin into the substantia nigra is toxic to dopaminergic neurons, and induces protein aggregation and inclusion body formation[15-16]. An *in vitro* experiment further demonstrated that lactacystin can induce ubiquitinated protein aggregation and promote intracytoplasmic ubiquitin-positive inclusion body formation[17].

Substitution therapy with levodopa has been commonly used to treat PD. It ameliorates PD symptoms, but induces severe toxicity and side effects and does not control PD progression. Recent studies demonstrated that Chinese medicines can delay PD progression, relieve adverse effects due to Western medicine, and control symptoms of non-motor disturbance in PD[5-18]. However, studies mainly focused on oxidative stress, cell apoptosis, excitatory amino acid toxicity and immune reactions to investigate the mechanism of Chinese medicines[19-35]. *Anchanling* (ACL), a Chinese medicine, is effective in treating early stage PD, and exerts positive effects in PD patients with advanced stage disease, or poor response to dopamine therapy. However, the pharmacodynamic mechanism remains poorly understood. In the present study, a rat model of PD was established using lactacystin, and the effects of ACL on cell apoptosis and UPS function were observed by immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) techniques.

RESULTS

Quantitative analysis of experimental animals

Following one-week adaptation, 60 of 80

Sprague-Dawley rats were selected according to their food intake, behavior and coat[36-37]. Lactacystin was stereotaxically injected in the left mesencephalic ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) of 50 rats to establish a PD model. A total of 49 rats survived after lesion, and 27 were selected following screening of apomorphine-induced behavior. Ten were used as the vehicle-treated (PD) group and 10 as the ACL group, respectively treated with distilled water and ACL by intragastric perfusion. Another 10 of 60 rats were used as the control group. Therefore, 30 rats were included in the final analysis.

Influence of ACL on substantia nigra tyrosine hydroxylase (TH) expression in lesioned rats

Immunofluorescent labeling showed that TH was expressed in cells of the *substantia nigra* of control group rats and the number of TH-positive cells was 293.8 ± 13.0 per field of view (× 200) (n = 6). After 5 weeks on vehicle treatment, the number of TH-positive cells was significantly reduced in the lesioned group (53.50 ± 14.05 per field of view (× 200); n = 6) compared with control group (P < 0.05). 5 weeks of ACL increased TH-positive cells in the *substantia nigra* of rats (130.33 ± 11.91 per field of view (× 200); n = 6) compared with the vehicle-treated group (P < 0.05), but this remained lower than the control group (P < 0.05; Figure 1).

Influence of ACL on substantia nigra α-synuclein and ubiquitin expression in lesioned rats

Immunofluorescence and thioflavin S (a chromogenic marker of amyloid substance) labeling were used to examine protein aggregation. The rate of thioflavin S and α-synuclein double labeling, as well as thioflavin S and ubiquitin double labeling was significantly increased at 5 weeks in the vehicle-treated group compared with the control group. ACL significantly reduced thioflavin S and α-synuclein double labeling, as well as thioflavin S and ubiquitin double labeling (Figures 2, 3).

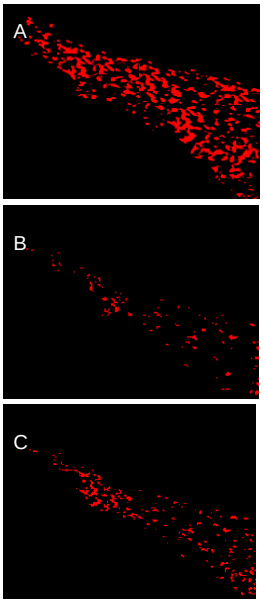

Figure 1 Substantia nigra tyrosine hydroxylase (TH) expression in rats (immunofluorescent staining, × 200).

TH-positive reaction was represented by red fluorescence. A large number of TH-positive cells were observed in the control group (A). Compared with the control group, the number of TH-positive cells was significantly decreased in the vehicle-treated (model) group (B). *Anchanling* (C) increased TH-positive cells in rats compared with the vehicle-treated group.

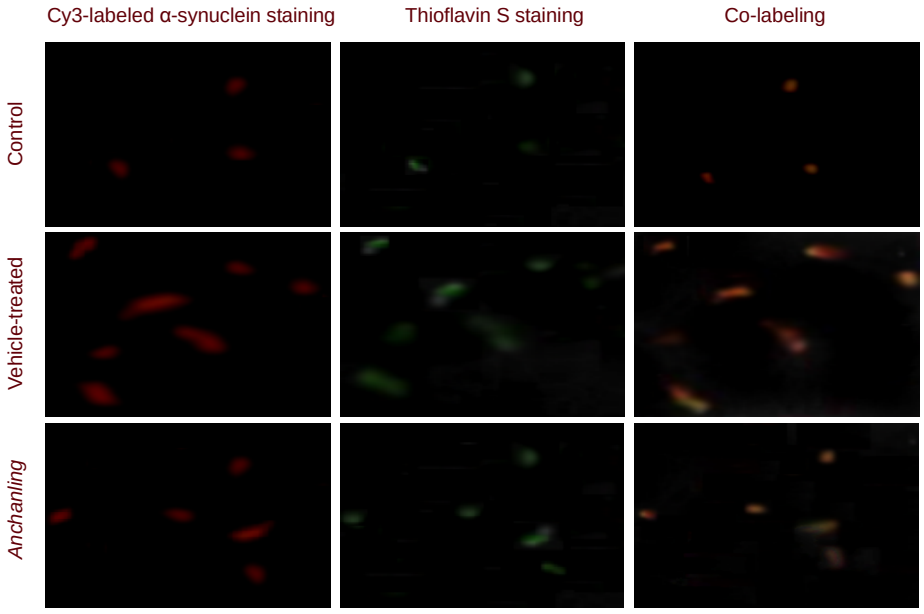

Figure 2 Alpha-synuclein protein expression in rat substantia nigra (immunofluorescence double-labeling staining, × 200).

Thioflavin S was used as a chromogenic marker of amyloid substance. Red fluorescence represents α-synuclein staining, and green represents thioflavin S staining. In the vehicle-treated (model) group, co-labeling of α-synuclein and thioflavin S was evident. In the *Anchanling* group, the co-labeling was reduced compared with the model group. Only weak red and green fluorescence was observed in the control group.

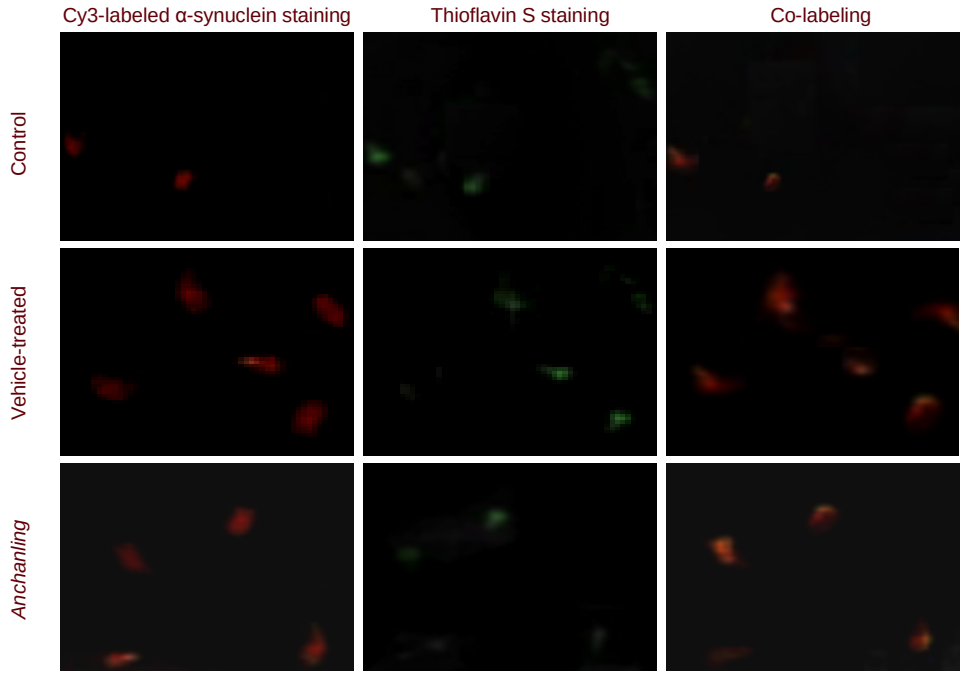

Figure 3 Ubiquitin protein expression in rat substantia nigra (immunofluorescence double-labeling staining, × 200).

Thioflavin S was used as chromogenic marker of amyloid substance. Red fluorescence represents ubiquitin staining, and green represents thioflavin S staining. In the vehicle-treated (model) group, co-labeling of ubiquitin and thioflavin S was evident. In the *Anchanling* group, the co-labeling was reduced compared with the model group. Only weak red and green fluorescence was observed in the control group.

Influence of ACL on nigral apoptosis in lesioned rats

TUNEL showed that cell apoptosis was significantly increased in the substantia nigra of the vehicle treated group compared with the control group ($P < 0.05$). However, it was significantly decreased in the ACL group compared with the vehicle treated group ($P < 0.05$; Table 1).

Table 1 Cell apoptosis in substantia nigra of rats

Group	Number of apoptotic cells (cells/ 4 mm × 4 mm field of view)	Apoptotic index (%)
Control	2.94±1.76	4.00±1.19
Vehicle-treated	42.22±11.84[a]	74.30±9.41[a]
Anchanling	21.28±7.31[b]	37.04±8.40[b]

[a]$P < 0.05$, *vs*. control group; [b]$P < 0.05$, *vs*. vehicle-treated (model) group. Data were expressed as mean ± SD of six rats in each group. Apoptotic index (%) = number of apoptotic cells/total number of cells in the field of view × 100%. Intergroup differences were compared using Tamhane test in heterogeneity of variance.

Influence of ACL on behavior in lesioned rats

Three weeks after lesion locomotion was significantly reduced. Crawling movements and escape responses to external stimuli were delayed. Spontaneous rotational behavior, sniffing and back bending appeared. At five weeks, all rats underwent apomorphine induction. Rotation toward the healthy side was significantly increased in the vehicle-treated group and ACL group ($P < 0.05$), but the frequency of apomorphine-induced rotation in the *Anchanling* group is less than vehicle-treated group ($P < 0.01$). The control group rats did not show aberrant rotational behavior (Table 2).

Table 2 Frequency of apomorphine-induced rotation (rotations/30 minutes) before and after *Anchanling* treatment

Group	Before treatment	After treatment
Control	20.3±2.12	22.6±3.10
Vehicle-treated	285.4±4.12	302.7±7.15[b]
Anchanling	286.7±5.70	247.8±10.37[ab]

[a]$P < 0.01$ ($F = 145.80$), *vs*. model group; [b]$P < 0.05$ ($F = 21.30$), *vs*. before treatment. Data were expressed as mean ± SD of 10 rats in each group. Intergroup differences were compared using Levene's test of homogeneity of variance.

DISCUSSION

In the present study, PD symptoms were induced in rats by injection of lactacystin into the SNc and VTA. As previously published[38], thioflavin S was used as a chromogenic marker of amyloid substance to observe abnormal protein aggregation in cells. Immunofluorescent double labeling showed stronger co-staining of α-synuclein and thioflavin S in the lesioned, vehicle-treated group compared with the control group, suggesting α-synuclein aggregation in the former. In addition, co-staining of ubiquitin and thioflavin S was evident in the vehicle-treated group, indicating ubiquitination of amyloid protein. TUNEL showed that cell apoptosis in the substantia nigra was significantly increased in the vehicle-treated group compared with the control group. Therefore, microinjection of lactacystin into the SNc and VTA can successfully induce

pathological characteristics of PD. This is consistent with a correlation between proteasome dysfunction, α-synuclein aggregation and cell apoptosis in the substantia nigra. That is, functional disturbance of proteasomes can induce α-synuclein aggregation and cell apoptosis in the substantia nigra. Proteasome dysfunction can trigger cell cycle signals in dopaminergic neurons by upregulating cell cycle regulating factor expression, thereby inducing neuronal apoptosis and degeneration[36]. The proteasomal inhibitor lactacystin can activate caspase-3 and induce PC12 apoptosis[37]. Therefore, we conclude that lactacystin inhibits proteasome function in the UPS, thereby blocking α-synuclein degradation, inducing α-synuclein aggregation, cell structural damage, triggering apoptosis, and leading to cell degeneration and death.

In the present study, ACL reduced α-synuclein aggregation and cell apoptosis in the substantia nigra compared with the vehicle-treated group, possibly by improving proteasomal degradation of α-synuclein, reducing protein aggregation and inclusion body formation, and/or activating apoptosis-inhibiting signal pathways to protect nigral cells.

In conclusion, UPS dysfunction can induce α-synuclein and ubiquitin aggregation and inclusion body formation, resulting in neuronal apoptosis in the substantia nigra. ACL may ameliorate PD pathology by enhancing the function of the ubiquitin-proteasome system.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment was used.

Time and setting

The experiment was performed at the SPF Animal Laboratory, Shenzhen Institute for Biochemical Drug Control, and Central Laboratory of First Affiliated Hospital of Shenzhen University, China from June 2007 to February 2009.

Materials

Animals

A total of 80 healthy, adult, male Sprague Dawley rats, weighing 200–250 g, were provided by the Medical Animal Experimental Center of Guangdong Province (No. SCXK (Yue) 2003-0002; 2006A015). The rats were housed in humidity of 40–70% at 20–26°C. The 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 the People's Republic of China[39].

Drugs

ACL was provided by the Shenzhen Institute of Gerontology. The crude drugs were purchased from Shenzhen Accord Pharmaceutical Co., Ltd., China. ACL comprised magnolia bark, fourstamen stephania root and Chinese Magnolivine Fruit and prepared by routine water-ethanol method[40]. Suspension was prepared by mixing with distilled water and administrated intragastrically. The active component was magnolol (5, 5'-Diallyl-2, 2'-biphenyldiol, molecular weight 266.32) and honokiol (3', 5-Diallyl-2-4'-dihydroxybiphenyl, molecular weight 266.33). They were isomeric[41]. Their chemical structural formulas are shown in Figure 4.

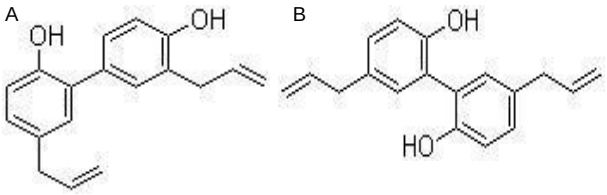

Figure 4 Chemical structures of honokiol (A) and magnolol (B).

Methods

Establishment of PD model

Following behavioral tests, animals without rotational behavior were included for subsequent experiments. The rats were anesthetized by intraperitoneal injection of 2% pentobarbital (45 mg/kg; Sigma, St. Louis, MO, USA), and placed at the stereotaxis instrument (RWD Life Science, Shenzhen, China). The head was routinely sterilized, and the scalp was cut open, and the periosteum was dissected. According to rat stereotaxic atlas (supplementary Figure 1 online), SNc and VTA location was identified[42]: SNc: 5.0 mm posterior to bregma, 1.7 mm left to stereotaxic atlas, 7.6 mm below cranium surface; VTA: 4.6 mm posterior to bregma, 0.9 mm left to stereotaxic atlas, 7.5 mm below cranium surface. According to body mass and cranium size, the coordinates were confirmed and marked using three edged needle. The cranium was drilled using dental drill, and a microsyringe (Hamilton, CH-7402 Bonaduz, GR, Switzerland) was slowly inserted according to coordinates to inject lactacystin (Sigma; 8 μg (4 μL) per point) in vehicle-treated and ACL groups, while the control group was injected with the same volume of normal saline. The injection was inserted to a depth of the center of pinhole slope (zero at cerebral dura mater), 1 μL/min. The needle was maintained for 10 minutes, and slowly removed. The wound was sutured, followed by intraperitoneal injection of penicillin (10×10^4 U/kg/d) for one week to prevent infection. In addition, the wound was disinfected with iodine tincture, once a day. The sutures were removed seven days later.

Evaluation of model

Behaviors were observed at 9: 00 am every seven days from one week postoperatively. The rats were intraperitoneally injected with 0.5 mg/kg apomorphine (Sigma). The rotational behaviors of rats were observed in a plastic tub (40 cm diameter) and rotations within five minutes after injection were recorded for three weeks. Successful lesioning was identified if the rats rotated towards the right within 30 minutes, with mean speed > 7 r/min. Rats with rotations towards the left or rotations towards the right at < 7 r/min or without rotation were excluded.

ACL administration

The ACL group rats were administrated ACL at a dose of 10 times of that for a 60 kg human, dissolved in normal saline, 1.5 mL/100 g[43], 9: 00–10: 00 a.m., once a day. Animals were weighed every week, and the dose was adjusted accordingly. The drugs were administrated for five consecutive weeks. The vehicle-treated and control groups were perfused with the same volume of normal saline. All rats were administrated immediately following model establishment.

Behavioral detection

The rats were induced with apomorphine at 1, 3, 5 weeks after ACL administration to observe rotations and other abnormal behaviors. Changes in rotation frequency before and after administration and among groups were compared.

Immunofluorescent staining for TH expression in the substantia nigra

Five weeks after administration, two rats were randomly selected from each group, anesthetized by intraperitoneal injection with 2% pentobarbital (45 mg/kg), followed by perfusion of 4% paraformaldehyde via left ventricular intubation. Brains were harvested, fixed in postfixation solution containing 20% sucrose at 4°C for 2 hours, followed by postfixation solution containing 30% sucrose at 4°C for 2 hours. Serial coronal sections were prepared originating from the substantia nigra using freezing microtome (Microm HN525, Therm, Walldrorf, Germany), 30 μm thick. Symmetrical sections from the substantia nigra were selected for TH immunohistochemistry. The sections were washed with 0.01 M phosphate buffered saline (PBS) (pH 7.4), blocked in 2% bovine serum albumin at room temperature for 30 minutes, incubated with mouse anti-TH polyclonal antibody (1: 200; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4°C overnight, washed, incubated with Cy3-labeled goat anti-rat secondary antibody dilution (1: 200; Santa Cruz Biotechnology Inc.) at 37°C for 1 hour, shaken, rinsed, mounted with 50% neutral resin, and observed by fluorescence microscope (Leica Inc.). The mean number of TH-positive cells in bilateral substantia nigra sections under 200 × magnification was calculated.

Immunofluorescent double labeling for α-synuclein and ubiquitin in the substantia nigra

The sections were washed with 0.01 M PBS (pH 7.4), blocked in 2% bovine serum albumin at room temperature for 30 minutes, incubated with mouse anti-α-synuclein (1: 500) and ubiquitin polyclonal antibodies (1: 400; Sigma) at 4°C overnight, washed, incubated with Cy3-labeled goat anti-mouse secondary antibody dilution (1: 200; Santa Cruz Biotechnology Inc.) at 37°C for 1 hour, followed by 0.1% thioflavin S (Sigma) for 10 minutes. The sections were differentiated with 80% ethanol for 5 minutes, shaken, rinsed, mounted with 50% neutral resin, and observed by fluorescence microscope.

TUNEL detection for cell apoptosis in the substantia nigra

After administration, two rats were randomly selected from each group, anesthetized, and the midbrain was harvested, fixed in 4% paraformaldehyde for 24 hours, dehydrated by gradient ethanol, paraffin embedded, followed by coronal sectioning, 5 μm thick. Five sections were selected from each block of brain tissues. TUNEL was performed according to TUNEL kit (R&D Systems, Minneapolis, MN, USA). Briefly, the sections were dewaxed, and washed with PBS, 3 × 5 minutes after antigen was retrieved by microwave, incubated with 20% normal goat serum at room temperature for 30 minutes. TUNEL reaction solution was added to the sections and incubated at 37°C for 90 minutes, washed with PBS, 3 × 5 minutes, followed by 3% H_2O_2 methanol solution at room temperature for 10 minutes and incubation at 37°C for 90 minutes. The sections were treated with POD transforming agent at 37 °C for 30 minutes, washed with PBS, 3 × 5 minutes, visualized with diaminobenzidine/H_2O_2, stained with hematoxylin, dehydrated, cleared, and mounted with neutral resin. Six sections were selected from each group, and three 4 mm × 4 mm fields of view were selected. The number of TUNEL-positive cells and apoptotic index were analyzed using Leica Qwin image analysis system software (Leica Microsystems Imaging Solutions Ltd., Software Version 3.0, Cambridge, UK).

Statistical analysis

Data were analyzed using SPSS version 10.0 (SPSS, Chicago, IL, USA) and are expressed as mean ± SD. In homogeneity of variance, data were analyzed using repeated measurement of analysis of variance, one-way analysis of variance or least significant difference-*t* test; while data were analyzed with Tamhane test in heterogeneity of variance.

Author contributions: Yinghong Li analyzed data, revised manuscript, provided technical and data support. Zhengzhi Wu conceived and designed this study, was in charge of funds, and revised manuscript. Xiaowei Gao provided experimental data and contributed to statistical analysis, and wrote manuscript. Qingwei Zhu conducted the experiments, provided technical and data support. Yu Jin, Anmin Wu and Andrew C. J. Huang provided technical and data support.

Conflicts of interest: None declared.

Funding: This study was supported by the National Natural Science Foundation of China (Influence of Anchanling on endoplasmic reticulum stress and ubiquitin-proteasome system in PD model), No. 30772757.

Ethical approval: This study received permission from the Animal Ethnics Committee of First Affiliated Hospital of Shenzhen University, China.

Acknowledgments: We thank the staff of Animal Laboratory, Shenzhen Institute for Biochemical Drug Control, and Clinical Institute of Chinese and Western Medicine, First Affiliated Hospital of Shenzhen University, China, for technical support.

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. 3, 2012 after selecting the “NRR Current Issue” button on the page.

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(Edited by Zhao Y, Wang Z/Su LL/Wang L)