

Original Article

Comparison of endothelial cell- and endothelial progenitor cell-derived exosomes in promoting vascular endothelial cell repair

Hui Hu, Chunyu Jiang, Ruiting Li, Jungong Zhao

Institute of Diagnostic and Interventional Radiology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China

Received March 13, 2019; Accepted May 24, 2019; Epub July 1, 2019; Published July 15, 2019

Abstract: Background: Timely endothelial repair after intervention-associated vascular injury is critical to prevent restenosis and thrombosis. Compared to living cell therapies, exosomes may be a better alternative. Thus, we aimed to compare the role of exosomes derived from human umbilical vein endothelial cells (HUVECs) versus those derived from endothelial progenitor cells (EPCs) in vascular endothelial repair. Material/methods: Exosomes secreted from HUVECs and EPCs were isolated and characterized respectively. In vitro, the effects of the two types of exosomes on migration and proliferation of endothelial cells were studied. In vivo, rats were systemically treated with the two exosome groups respectively after carotid artery endothelial injury induced by a balloon. The efficacy in promoting re-endothelialization was measured by Evans blue dye and histological examination. Results: Both types of exosomes, sized from 30 nm to 100 nm, had sphere-shaped or cupped morphology, and expressed CD63, CD9, and CD81; but the total yield of exosomes (particles/mL) based on number of cells, was 4.2 times higher from EPCs than HUVECs. Compared with control treatment, both exosome treated groups manifested significant enhancement of migration and proliferation in vitro and vascular recovery at an early stage in vivo. The two exosome-treated groups, however, did not statistically significantly differ. Conclusion: In conclusion, our results indicated that exosomes derived from HUVECs and EPCs had similar morphology, size distributions and characteristics, but those derived from EPCs were more abundant with comparable biologic activity. Therefore, EPCs may be a robust source of exosomes to promote vascular repair.

Keywords: Exosomes, endothelial cells, re-endothelialization, HUVECs, endothelial progenitor cells

Introduction

Endovascular therapies are widely used techniques to treat patients with cardiovascular disease [1-3]. However, such procedures are associated with an approximately 30% risk of lumen restenosis during the first 3 months after intervention, even with drug-eluting balloons [4]. Intervention-related vascular endothelial cell damage is a key factor in the initiation and progression of restenosis [5]. Vascular endothelial injury may bring about potential changes such as inflammation, thrombosis, and smooth muscle cell proliferation, which cause neointimal hyperplasia, adverse arterial remodeling, and restenosis [5, 6]. Thus, timely promotion of endothelial cell (ECs) repair is crucial to prevent progression of restenosis.

Recent studies have revealed that paracrine-mediated migration of ECs from the adjacent healthy endothelium may be important in endothelial regeneration after damage [5, 7, 8]. Exosomes, an important component of cellular paracrine secretion, are eliciting interest for biotherapeutics in endothelial cell repair and angiogenesis [5, 9-11]. Exosomes originate from multivesicular bodies (MVBs), with small bilipid membranes and 40-150 nanometers size [12]. They play critical roles in mediating intercellular communication by shuttling mRNAs and proteins and altering gene expression and cellular behavior of the recipient cells. Compared with classic cell-based therapies, administration of exosomes reduces the risk of thrombosis, unwanted immune responses, or tumorigenesis [13, 14].

Identification of ideal cell types for exosome isolation is important in developing exosome-based therapy for vascular endothelial cell injury. Recent studies show that exosomes secreted from human coronary artery endothelial cells (HCAECs) and human microvascular endothelial cell line (HMEC-1) containing miRNAs could be internalized by recipient ECs to regulate gene expression and stimulate ECs proliferation and migration [5, 9]. However, HCAECs and HMEC-1 are hard to obtain, since harvesting of coronary artery and microvascular tissue may be invasive [15]. In addition, unwanted cell types often contaminate ECs obtained from fat [16].

In contrast, EPCs can be obtained in a non-invasive way [15]. After harvest of peripheral blood and cord blood, mononuclear cells have been isolated and induced to EC-like cells *ex vivo* [15, 17]. Moreover, EPCs are better at cell proliferation and sensitivity to angiogenic factors compared to adult mature vascular endothelial cells [18].

To date, there is no direct comparison of exosomes derived from ECs and EPCs in terms of their features, as well as their therapeutic effects. In this study, we set out to compare exosomes derived from HUVECs and EPCs *in vivo* and *in vitro* to identify a robust source of exosomes to promote vascular repair.

Materials and methods

Exosome isolation

Exosomes from HUVECs and EPCs were collected as described [9]. When grown up to approximately 70% to 80% confluence, HUVECs and EPCs (Lonza) were cultured in serum-free EBM-2 medium (Lonza) for 24-40 hours. The conditioned media of both cell lines were collected and the number of both cell lines was counted. Collected culture medium was first centrifuged at $2,000 \times g$ (Beckman Coulter) for 20 minutes to remove cellular debris, then collected by differential centrifugation (Hitachi), $10,000 \times g$ for 30 minutes, and $100,000 \times g$ for 120 minutes. The pelleted exosomes were resuspended with 15 ml PBS and filtered with a $0.22 \mu\text{m}$ filter (Millipore), then centrifuged at $4,000 \times g$ to $200 \mu\text{L}$.

Exosome identification

Exosome size and concentration were measured by NTA (Nanosight NS300, Malvern, UK). Sample that were 1000-fold diluted were pipetted with a 1 mL syringe and slowly pushed into the sample chamber. The average of the three movie recordings captured with $20 \times$ magnification, 30 frames per second, was taken to measure the size and concentration of the exosomes. The morphologies of both exosomes were observed by transmission electron microscopy (TEM) (Tecnai G2, Spirit Biotwin, USA) as previously described [19]. Western blot was used to analyze the characteristic surface markers of exosomes including CD63, CD9, and CD81. Briefly, exosome samples (total protein of $30 \mu\text{g}/\text{lane}$) were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then transferred to polyvinylidene fluoride membranes (Millipore, USA). The membranes were blocked with 5% BSA in TBST (TBS with 0.2% Tween-20) for 2 h, following by incubation with primary antibodies against CD63, CD9, and CD81 (Abcam, UK) at 4°C overnight. Then they were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. The immunoreactive protein bands were visualized using an ECL detection kit (Thermo Fisher Scientific) and imaged with the ChemiDoc XRS Plus Luminous Image Analyser (Bio-Rad).

Measurement of uptake of both exosomes by HUVECs

To observe exosome uptake by HUVECs, we stained the exosomes from HUVECs and EPCs with Vybrant DiO dye (Molecular Probes) following the manufacturer's protocol, then added them (1×10^{11} particles/mL) to the medium of HUVECs and incubated for 2 hours at 37°C [19]. The cells were then washed with PBS and fixed in 4% paraformaldehyde for 20 minutes, then stained with DAPI for 5 minutes at room temperature. After washing, cells were observed using a laser confocal fluorescence microscope (Leica Microsystems, Wetzlar, Germany) [10].

Cell proliferation assay

HUVECs at an initial density of 1.5×10^3 cells/well were seeded into 96-well plates and cul-

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tured in serum-free EGM media with 1×10^{11} particles/mL of exosomes from HUVECs, EPCs, or PBS, respectively. At days 0, 1, 2, and 3, 20 μ l MTT reagent (0.5 mg/ml) was added to each well media. Then cells were cultured for another 4 h at 37°C, and 150 μ L dimethyl sulfoxide was added to each well. The absorbance representing the proliferation of HUVECs, was measured at 490 nm.

Migration assay

HUVEC migration was evaluated by scratching the confluent HUVEC cell layer (6×10^4 cells/well) in a 12-well plate with a 200 μ L pipette tip. The detached cells were washed with PBS, and cultured in 500 mL of serum-free medium with exosomes (1×10^{11} particles/mL) from HUVECs or EPCs, or without exosomes for 24 hours. Cell migration was assessed by light microscopy at magnification $\times 100$ (Olympus Corporation, Tokyo, Japan) and Image-Pro Plus software.

Rat model of balloon-induced vascular injury and treatment

All procedures were approved by the Animal Research Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. Female SD rats (200-250 g) were intraperitoneally injected with 50 mg/kg pentobarbital for anesthesia and 100 U/kg heparin sodium for heparinization. After exposing the left common carotid artery, the left internal carotid artery, and external carotid artery, we ligated the distal external carotid artery and temporarily occluded the common carotid artery and internal carotid artery using artery clips. For endothelial injury, a 2F Fogarty arterial embolectomy balloon catheter (Edwards Lifesciences) was inserted through the internal carotid artery to the common carotid artery. The balloon was slowly distended with saline and was pulled back with rotation three times. The total length of denudation was about 5-6 mm from the bifurcation of the carotid arteries. Following the wire removal, the external carotid artery near the bifurcation was ligated permanently, and the artery clamps in the internal carotid artery and common carotid artery were taken away to restore antegrade blood flow. After injury for 2 h and 24 h, the two experimental groups were injected with 2×10^{12} exosome particles from HUVECs or EPCs dissolved in 200 μ L of PBS

respectively, and the control group was injected with an equal volume of PBS through the tail vein.

Evans blue dye analysis of rat carotid artery re-endothelialization

On day 7, the rats were anesthetized according to the method described above. After intravenous injection of the Evans blue dye (3%), dye was allowed to circulate for 10 minutes. Then 2 mL of 0.9% NaCl was administered to wash away the unbound Evans blue dye. The left common carotid artery was harvested after rats were euthanized by anesthesia. The area stained blue represents nonendothelialization, while the area not stained represents re-endothelialization. Images were observed using a stereo microscope (Leica M205 FA). Re-endothelialization is expressed as the ratio of the remaining area to the total area.

Histologic analysis

On day 7, the left common carotid artery of both the exosome-treated groups and the control group were harvested. Then the samples were fixed in 10% formalin for 10 minutes, followed by dehydration and embedding in paraffin. Five transverse sections of 5 μ m thickness of each sample were collected, and stained with hematoxylin and eosin according to standard protocols. The area of intima and media, and the ratio of intimal and medial area (I/M) were observed and measured with a Nikon E200 microscope and Image-Pro Plus 6 software.

Statistical analysis

SPSS 18 was used for statistical analysis. All experiments were carried out with at least three replicates per group, and in vitro experiments were repeated three times. Data are expressed as means \pm standard deviation (SD). Differences were compared by one-way analysis of variance (ANOVA). $P < 0.05$ was considered significant.

Results

Characterization of exosomes

Characterization of exosomes derived from HUVECs and EPCs was determined by NTA, TEM, and western blot. NTA measurement

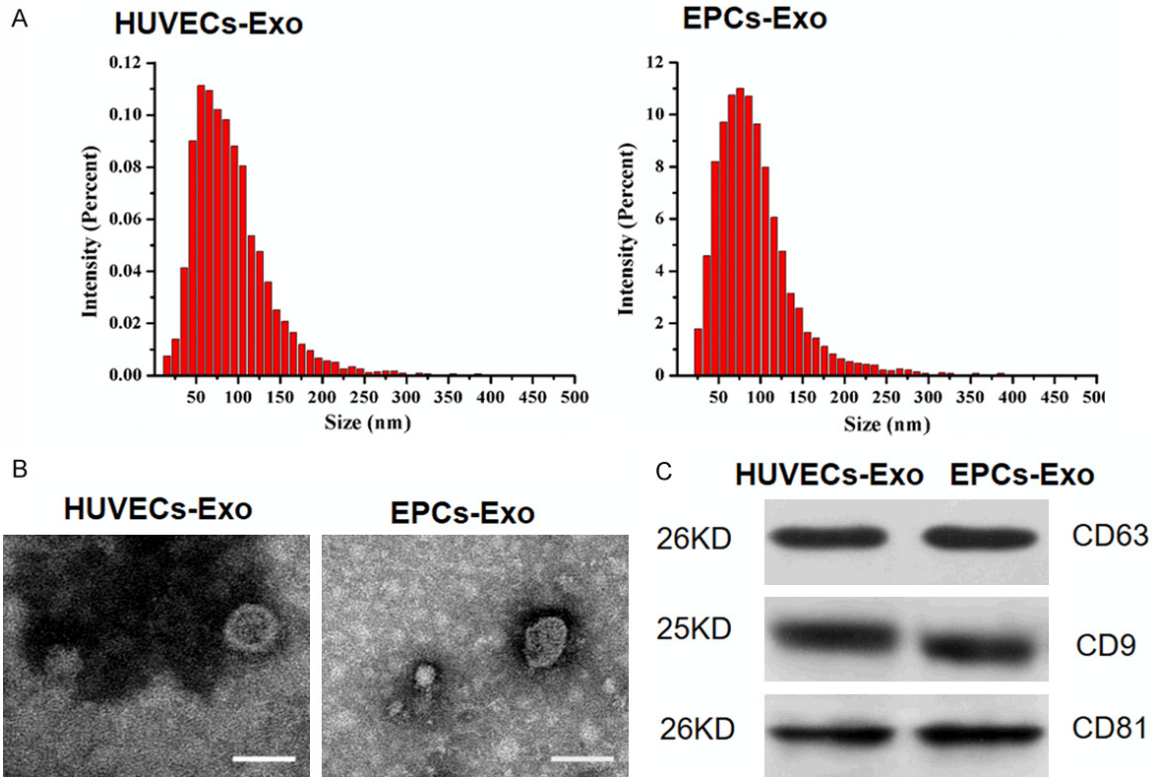


Figure 1. Characterization of HUVEC-derived exosomes (HUVECs-Exo) and EPC-derived exosomes (EPCs-Exo). A. Particle size distribution of HUVECs-Exo and EPCs-Exo was measured by NTA. B. Morphology of HUVECs-Exo and EPCs-Exo was observed with transmission electron microscopy. Scale bar: 50 nm. C. Western blot analysis of exosomal surface markers CD63, CD9, and CD81 in HUVECs-Exo and EPCs-Exo.

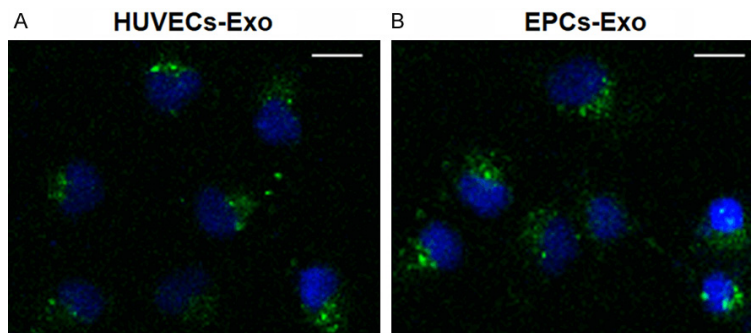


Figure 2. Fluorescence microscopy analysis of Vybrant DiO-labeled HUVECs-Exo and EPCs-Exo internalization by HUVECs. The green-labeled exosomes are visible in the perinuclear region of HUVEC proliferation. * $P < 0.05$. Scale bar: 50 μ m.

showed that the size of both exosome groups ranged from approximately 30 nm to 100 nm (**Figure 1A**). For comparison of exosome yields from HUVECs and EPCs, total cell counts were adjusted. The results of NTA revealed that the particles/mL of exosomes from EPCs was 4.2 times higher than those from HUVECs, independent of exosome size (95% CI 1.58×10^{11} , 8.22

$\times 10^{12}$, $P < 0.001$). TEM showed that both exosomes exhibited a sphere-shaped or cupped morphology (**Figure 1B**). Western blotting indicated the expression of exosomal surface markers including CD63, CD9, and CD81 in both groups (**Figure 1C**).

Endothelial cell internalization of exosomes derived from HUVECs and EPCs

To determine whether exosomes from HUVECs and EPCs could be internalized by ECs,

HUVECs (as an example) were cultured with the DiO-labeled exosomes derived from HUVECs and EPCs for 2 hours. Fluorescence microscopy showed that the labeled exosomes were transferred to the perinuclear region of HUVECs, demonstrating that the EPCs and HUVECs exosomes were incorporated into HUVECs (**Figure 2**).

Exosomes from HUVECs and EPCs for endothelial cell repair

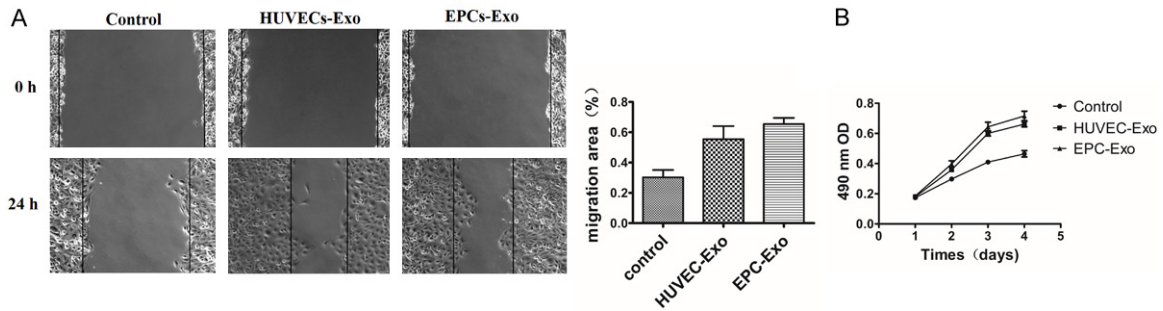


Figure 3. A. A scratch wound assay demonstrated that HUVECs treated with HUVECs-Exo and EPCs-Exo had increased migration compared to the control group. B. MTT analysis of exosomes derived from HUVECs and EPCs in promoting HUVEC proliferation. * $P < 0.05$.

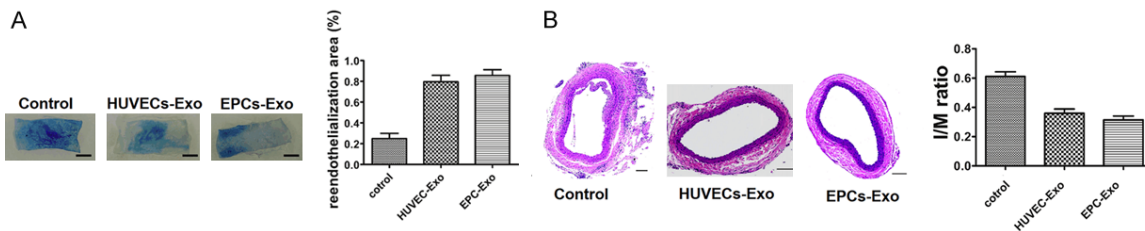


Figure 4. In vivo effect of exosomes on carotid artery injury. A. Evans blue dye was used to calculate the re-endothelialization ratio. Nonendothelialized lesions are represented by blue staining and the re-endothelialized area displays as white. The index of re-endothelialization was calculated as the white area/total area ratio. Data are displayed as mean \pm SD, $P < 0.05$, Scale bar: 1 mm. B. Hematoxylin-eosin staining of carotid artery sections. The intimal to medial area ratio (I/M) was used to evaluate neointimal hyperplasia and is shown as the mean \pm SD, $P < 0.05$, Scale bar: 100 μ m.

Exosomes derived from HUVECs and EPCs promote endothelial target cell proliferation and migration in vitro

The effect of exosomes secreted from HUVECs and EPCs on proliferation of HUVECs was examined by MTT analysis. Exosomes from both groups could significantly promote the proliferation of HUVECs compared to controls ($P < 0.05$), but there was no significant difference between the two exosome treatment groups ($P > 0.05$) (Figure 3B). The role of exosomes derived from HUVECs and EPCs in ECs migration was determined by scratch wound assay. Both exosome treatment groups markedly enhanced the motility and migration of HUVECs compared to controls ($55.46 \pm 8.7\%$, $65.41 \pm 4.1\%$ versus $30.26 \pm 4.8\%$; $P < 0.001$, $P = 0.002$) (Figure 3A), but there was no significant difference between the two exosome-treated groups ($P = 0.096$) (Figure 3A).

EPCs exosomes promote re-endothelialization after vascular injury

The result of endothelial repair in injured arteries on postoperative day 7 was assessed by

Evans blue staining (Figure 4A). The re-endothelialized area appeared white, whereas non-endothelialized lesions were stained blue. The re-endothelialization index was expressed by the ratio of white area to total area. Both exosome treatments significantly accelerated re-endothelialization of the injured arteries compared with the PBS control ($79.57 \pm 6.2\%$, $85.61 \pm 5.6\%$ versus $24.87 \pm 5.1\%$; $n = 4$; $P < 0.001$), but there was no significant difference between the two exosome treatment groups ($P = 0.239$). Quantitative analysis of intimal area/medial area ratios (I/M) in the lumen of injured vessels revealed that neointimal hyperplasia was significantly reduced by treatment with both exosomes compared with the control (0.31 ± 0.03 , 0.36 ± 0.02 versus 0.61 ± 0.03 ; $n = 4$; $P < 0.001$), but there was no significant difference between the two exosome treatment groups ($P = 0.108$) (Figure 4B).

Discussion

In this study, we performed the first comparison of the characteristics of exosomes derived from HUVECs and EPCs and their effect on repair of endothelial damage. Our data showed

that exosomes derived from both HUVECs and EPCs were similar in morphology, size distribution, and presence of surface markers, while the production of exosomes derived from EPCs was 4.2 times higher than those of HUVECs under the same culture conditions. Moreover, the ability of both exosomes derived from HUVECs and EPCs in expediting re-endothelialization at the early stage after endothelial injury in the rat carotid artery and promoting proliferation and migration of endothelial cells *in vitro* showed no significant difference. Our results indicated that abundant exosome-derived EPCs had comparable biologic activity. Therefore, EPCs may be a robust source of exosomes in promoting vascular repair.

Recently, the characterization of human EPC-derived exosomes has been described [10, 11, 19, 20] and the first study of HUVECs-derived exosomes was published in 2017 [21]. It has been reported that exosome content and characteristics may be influenced by different cell culture conditions and isolation methods [22-24]. Therefore, in this study, the classic and widely employed methods were selected for both cell lines. We found that total exosome yield from EPCs according to the numbers of cells adjusted was higher than that of HUVECs. This can be attributed to EPCs exhibiting an inordinately higher self-renewal and expansion rate compared to HUVECs [18].

In this study, exosomes derived from HUVECs and EPCs both improved EC repair after vascular damage in the rat carotid artery *in vitro* and EC proliferation and migration *in vitro*. van Balkom et al. [9] reported that endothelial cell-derived exosomes stimulated migration and proliferation of neighboring target cells through miR-214, which repressed the expression of ataxia telangiectasia mutation, and hence prevented senescence and promoted vascularization. Li et al. [10] demonstrated that EPC-derived exosomes promoted vascular endothelial cell repair by increasing expression of angiogenesis-related molecules. Our result is consistent with previous studies [5, 9-11]. The underlying mechanism of exosome uptake in murine endothelium was recently investigated. Murine ECs took up intravenously injected exosomes of human origin in a developmental endothelial locus-1 dependent manner [5, 25]. The local uptake of exosomes by non-professional phagocytic cells (e.g., ECs) might play a key role in

altering the biologic behavior of adjacent cells, such as inhibition of senescence, stimulation of proliferation, and migration of initiating countermeasures against perceived damage [5, 9].

Contrary to expectations, with the same number of exosomes of the two groups employed *in vivo* and *in vitro*, the results showed no significant difference either in promoting vascular injury or in EC proliferation and migration. We speculate that components such as microRNAs or proteins in exosomes may play a crucial role. Future studies should focus on the components present in exosomes from HUVECs and EPCs that take part in vascular damage repair, and the underlying mechanism.

This study had several limitations. First, the exact mechanisms through which exosomes secreted by HUVECs and EPCs promote endothelial cell recovery remain unclear. Second, the effects of differing concentrations of both exosome sources on *in vivo* endothelial cell recovery at different timepoints need further study. Third, the effects of both exosome sources on smooth muscle cells (SMCs) have not been investigated. All of these represent important and intriguing avenues for future investigation.

Conclusions

In sum, our results indicate that exosomes originating from both ECs and EPCs may promote vascular repair in rat carotid artery injury. However, total exosome yield (particles/mL) was higher from EPCs than HUVECs. Further studies are needed to investigate the exact mechanisms by which both exosome sources promote vascular repair, in order to develop robust exosome-based therapy for vascular diseases.

Acknowledgements

This study was supported by grant from the National Natural Science Foundation of China (81671791).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jungong Zhao, Institute of Diagnostic and Interventional Radiology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, #600, Yishan Rd, Shanghai 200-233, China. E-mail: zhaojungongradio@hotmail.com

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