

Simvastatin attenuates TNF- α -induced apoptosis in endothelial progenitor cells via the upregulation of SIRT1

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Received February 6, 2014; Accepted April 1, 2014

DOI: 10.3892/ijmm.2014.1740

Abstract. Endothelial progenitor cells (EPCs) originate from the bone marrow and can be classified as either early or late EPCs. The focus of this study was on late EPCs, as they play an important role in angiogenesis and vascular proliferation. Evidence suggests that inflammatory and oxidative changes can increase EPC apoptosis. Of note, tumor necrosis factor- α (TNF- α) is a contributing risk factor to the development of atherosclerosis and plays a key role as both an inflammatory mediator and an inducer of apoptosis in endothelial cells. Additionally, a member of the sirtuin family, silent information regulator type-1 (SIRT1), promotes cell survival by repressing p53- and non-p53-dependent apoptosis in response to DNA damage and oxidative stress. Statins have also been shown to play a key role in the prevention of endothelial apoptosis and senescence via their lipid-lowering and anti-inflammatory actions. However, there is little evidence that statins themselves attenuate EPC apoptosis induced by TNF- α . The aim of this study was to demonstrate the effectiveness of one of the most commonly used statins, simvastatin, on decreasing TNF- α -induced apoptosis in EPCs. The results indicated that SIRT1 protein expression was decreased by TNF- α in a time- and dose-dependent manner and that while TNF- α caused a marked increase in the percentage of apoptotic EPCs, application of simvastatin decreased this percentage. A high concentration of simvastatin promoted the expression of SIRT1 and increased the proliferation of EPCs. In conclusion, findings of this study showed that simvastatin is crucial in

counteracting the TNF- α -induced apoptosis of EPCs and that this protection may involve the actions of SIRT1.

Introduction

Endothelial progenitor cells (EPCs) originate in the bone marrow. They are recruited into peripheral circulation in response to tissue ischemia or back to their site of origin to areas of injured endothelium. In this manner, they participate in the repair of damaged tissues, thereby improving blood flow and attenuating the progression of atherosclerosis (1). Since both animal models and human studies have shown that EPCs can contribute to neovascularization and re-endothelialization, EPCs have been examined as potential treatments for various types of ischemic disease, including stroke (2), ischemic myocardium (3,4), kidney injury (5), and ischemic vascular disease (6). Moreover, it has been reported that a low EPC level is an independent risk factor for future cardiovascular events such as unstable angina and myocardial infarction (MI) (7,8). MI (9) and limb ischemia (10,11) themselves have been associated with an increase in circulating EPC numbers, and vascular traumas such as acute MI with ST elevation (12) and percutaneous coronary intervention (13) have been suggested to induce the rapid mobilization of EPCs.

Evidence from *in vitro* and clinical studies suggested that inflammatory and oxidative changes can influence EPC apoptosis (14,15). Additionally, tumor necrosis factor- α (TNF- α) is a contributing risk factor in atherosclerosis and common metabolic disturbances including insulin resistance and dyslipidemia (16). More specifically, TNF- α plays a key role as both an inflammatory mediator and inducer of apoptosis in endothelial cells (17,18). Evidence of these roles can be found in the increased plasma levels of TNF- α in diabetic patients, which have been shown to impair the function of endothelial cells, as well as to enhance their aging and apoptosis (19-21). A previous *in vitro* study has shown that incubation of EPCs with TNF- α increases p38-phosphorylation, resulting in a reduction of total EPCs (22). Clinically, statins are widely used in the treatment of dyslipidemia and associated vascular abnormalities. In fact, clinical trials with statins have shown considerable benefits in patients with ischemic heart and peripheral disease, irrespective of their cholesterol

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Key words: endothelial progenitor cells, tumor necrosis factor- α , silent information regulator type-1, simvastatin, apoptosis

concentration (23). Evidence from *in vitro* and *in vivo* data have demonstrated that statins exert pleiotropic actions beyond their lipid-lowering effects, including immune regulation, anti-inflammatory effects, maintenance of plaque stability, and anti-platelet effects, in addition to anti-fibrotic and anti-oxidant properties (24-27).

Silent information regulator type-1 (SIRT1) is a member of the sirtuin family of nicotinamide adenine dinucleotide (NAD)-dependent class III histone deacetylases (HDACs). Previous studies have shown that SIRT1 promotes cell survival by repressing p53-dependent apoptosis in response to DNA damage and oxidative stress. This repression occurs via physical interaction with both p53 and the forkhead transcription factor (FOXO) family of proteins (28-30). Additionally, evidence suggests that SIRT1 may be involved in pathways of telomere maintenance, giving them a putative role in aging and survival (31). It has been reported that statins can activate SIRT1, which raises the possibility of their epigenetic regulation of inflammatory responses. Moreover, SIRT1 plays a key role in the prevention of endothelial apoptosis and senescence through its direct effect on endothelial nitric oxide synthase (eNOS) activation (32,33). However, there is little evidence that statins can attenuate TNF- α -induced apoptosis in EPCs. Furthermore, the possible anti-apoptotic effects of SIRT1 are currently unclear.

Thus, the present study aimed to clarify the roles of statin application and activation of SIRT1 on EPC apoptosis. The results showed that simvastatin, one of the most commonly used statins, is capable of reducing TNF- α -induced apoptosis in EPCs, and that SIRT1 may play a critical role in the prevention of EPC apoptosis.

Materials and methods

Materials. TNF- α was obtained from PeproTech, Inc. (Rocky Hill, NJ, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). SIRT1 antibody was obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). The FITC Annexin V Apoptosis Detection kit was purchased from BD Pharmingen (San Diego, CA, USA). EGM-2MV was obtained from Clonetics (San Diego, CA, USA). Lymphoprep™ was purchased from Axis-shield (Oslo, Norway), and Matrigel from BD Biosciences (San Diego, CA, USA).

Cell culture. EPCs derived from human umbilical cord blood were obtained by Lymphoprep™ density gradient centrifugation at 800 x g, for 20 min at a temperature of 4°C. Following centrifugation, the mononuclear cells (MNCs) layer was harvested and washed twice in 0.9% saline. The MNCs were cultivated in endothelial differentiation medium (EGM-2MV), which contained 5% fetal bovine serum, VEGF, fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor-1 and ascorbic acid. Cell cultures were maintained at 37°C with 5% CO₂ and 21% O₂ in a humidified atmosphere. Three days after plating, the non-adherent cells were discarded and fresh medium was applied. To maintain optimal culture conditions, the medium was changed on alternate days. The cells were selected for *in vitro* study after three total passages.

Western blot analysis. EPCs were homogenized in phosphate-buffered saline containing a protease inhibitor cocktail (50 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 10% glycerol). The samples were incubated overnight at 4°C with rabbit anti-SIRT1 (1:500) (Cell Signaling Technology, Inc.). Antibody signal was detected using a Chemiluminescent Detection kit according to the manufacturer's protocol (Beyotime, Haimen, China). The results were normalized to GAPDH. The relative band intensities of the blots were determined with Adobe Photoshop software.

Apoptosis analysis. After treatment for 48 h, EPCs were harvested, washed in ice-cold PBS, resuspended in 500 μ l of binding buffer, and incubated in the dark with 5 μ l of propidium iodide (PI) and 5 μ l of Annexin V-fluorescein isothiocyanate for 15 min. The samples were washed and resuspended in 500 μ l PBS, before analyzing with flow cytometry.

MTT growth curve. EPCs were detached and seeded into 96-well plates (4,000 cells per well). At the indicated time-points (24, 48 and 72 h after culture), the cells were stained with MTT (5 mg/ml) in PBS for 3 h, and then dissolved with 50% N,N-dimethylformamide and 10% SDS for 3 h at 37°C. The optical density at 570 nm was then determined. Each point was determined in triplicate and an average was obtained for analysis.

Tube formation assay. To demonstrate capillary tube formation, 250 μ l of Matrigel was added to 24-well plates and solidified for 30 min at 37°C. EPCs were then seeded (5×10^4 cells/well) on the solidified Matrigel and suspended in 300 μ l of EGM2-MV medium. After 12- and 24-h incubations, the cells were incubated at 37°C with 5% CO₂ for 5-24 h.

Statistical analysis. Data are expressed as mean \pm SD. An unpaired Student's t-test was used to evaluate statistical differences between the groups. Differences were considered significant at a value of P<0.05.

Results

TNF- α induces apoptosis and decreases SIRT1 expression of EPCs. To investigate whether SIRT1 expression could be regulated by TNF- α in EPCs, EPCs were treated with TNF- α (10, 20 and 40 ng/ml) for 24 h and the expression of SIRT1 was determined by western blot analysis. We found that TNF- α reduced SIRT1 protein expression in EPCs in a concentration-dependent manner (Fig. 1A). We also studied the time course of SIRT1 expression in EPCs by treating EPCs with TNF- α (20 ng/ml) for 12, 24 and 36 h. Our results showed that SIRT1 protein expression decreased in a time-dependent manner when compared with the control (Fig. 1B).

Simvastatin inhibits TNF- α -induced-apoptosis of EPCs. EPCs were treated with TNF- α (20 ng/ml) for 24 h and two groups of EPCs were then selected and treated with two concentrations of simvastatin (10^{-7} or 10^{-8} mol/l). Cell apoptosis was then evaluated via flow cytometry. The results showed that TNF- α

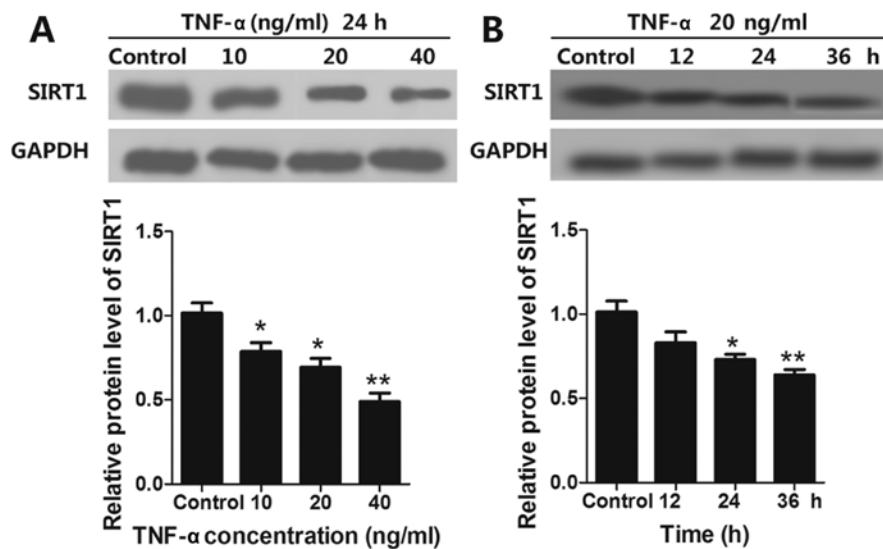


Figure 1. (A) Effects of tumor necrosis factor- α (TNF- α) on silent information regulator type-1 (SIRT1) levels in endothelial progenitor cells (EPCs) is concentration- and time-dependent. EPCs were treated with TNF- α at concentrations of 10, 20, and 40 ng/ml for 24 h and SIRT1 expression was determined by western blot analysis. (B) EPCs were treated with TNF- α (20 ng/ml) for the indicated time-points and SIRT1 expression was determined by western blot analysis. * $P < 0.05$ and ** $P < 0.01$ compared to the control group, $n = 3$ for each group for western blot analysis.

caused a marked increase in the percentage of apoptotic EPCs. However, application of simvastatin decreased the number of apoptotic cells when compared with cells treated solely with TNF- α . Moreover, we found that a higher concentration of simvastatin had a greater reduction in apoptotic cell number ($P < 0.05$) (Fig. 2A and C).

Simvastatin increases TNF- α -induced decreases in SIRT1 levels. To investigate whether the TNF- α -induced decrease of SIRT1 expression could be raised by simvastatin application, EPCs were treated with TNF- α and a high or low concentration simvastatin (10^{-7} or 10^{-8} mol/l). Expression of SIRT1 was then determined by western blot analysis. We found that a high concentration of simvastatin resulted in a marked increase in SIRT1 expression levels when compared to the TNF- α -only group ($P < 0.05$) (Fig. 2A and B). By contrast, we found that a low concentration of simvastatin resulted in a less obvious increase.

Simvastatin cannot restore the angiopoietic ability of TNF- α -treated EPCs. The angiopoietic ability of simvastatin was assessed using the tube formation assay. We found that the angiopoietic ability of EPCs was significantly decreased by TNF- α (20 ng/ml, 24 h). We also found that two different concentrations of simvastatin (10^{-7} or 10^{-8} mol/l) were not able to restore the angiopoietic ability of the EPCs.

Simvastatin promotes cell proliferation that was reduced by TNF- α application. The cell viability of EPCs was evaluated by an MTT assay. As shown in Fig. 3, the proliferation of EPCs treated with TNF- α (20 ng/ml) was markedly inhibited when compared to the controls ($P < 0.01$). High concentrations of simvastatin were able to partially enhance cell proliferation when compared to the TNF- α -only group ($P < 0.05$). No differences were observed in cell proliferation between the TNF- α group and low concentrations of simvastatin.

Discussion

EPCs are critical for angiogenesis in ischemic disease and inflammation. Numerous animal studies have suggested that the vaso- and atheroprotective effects of EPCs are associated with their cell replacement and non-cellular differentiation effects. These include trophic support and enhancement of the endogenous repair process (3,34,35). EPCs are heterogeneous and can be classified into early or late EPCs. In *ex vivo* culture systems, early EPCs appear within 4-7 days, while late EPCs develop after 2-3 weeks. It has been hypothesized that early EPCs may primarily provide trophic support, while late EPCs, which express a variety of endothelial markers, differentiate into mature endothelial cells and contribute to vascular repair (36). Considerable evidence indicates that a reduction in the number of EPCs predicts future cardiovascular events. Thus, enhancement of the number of EPCs is of potential therapeutic benefit to individuals with cardiovascular diseases. In this study, late EPCs were selected as our subject due to their role in angiogenesis and proliferation (37).

Statins are beneficial for atherosclerosis via mechanisms independent of their lipid-lowering ability. For instance, increased EPC levels in the early post-infarction phase by statin treatment were associated with improved cardiac function and increased capillary density in the peri-infarct area after a MI (38). A number of mechanisms have been suggested by which statins may attenuate atherosclerosis disease and cellular senescence in EPCs. Several of these mechanisms involve the ability of statin therapy to enhance bone marrow VEGF protein levels, their ability to phosphorylate Akt, eNOS activity, and their ability to decrease oxidative DNA damage and prevent telomere shortening due to oxidative stress in EPCs (38-40). As a powerful factor in inflammatory stimulation, TNF- α has been reported to induce apoptosis and senescence of EPCs both *in vitro* and *in vivo*. However, there are no previous studies focusing on the ability of statins to

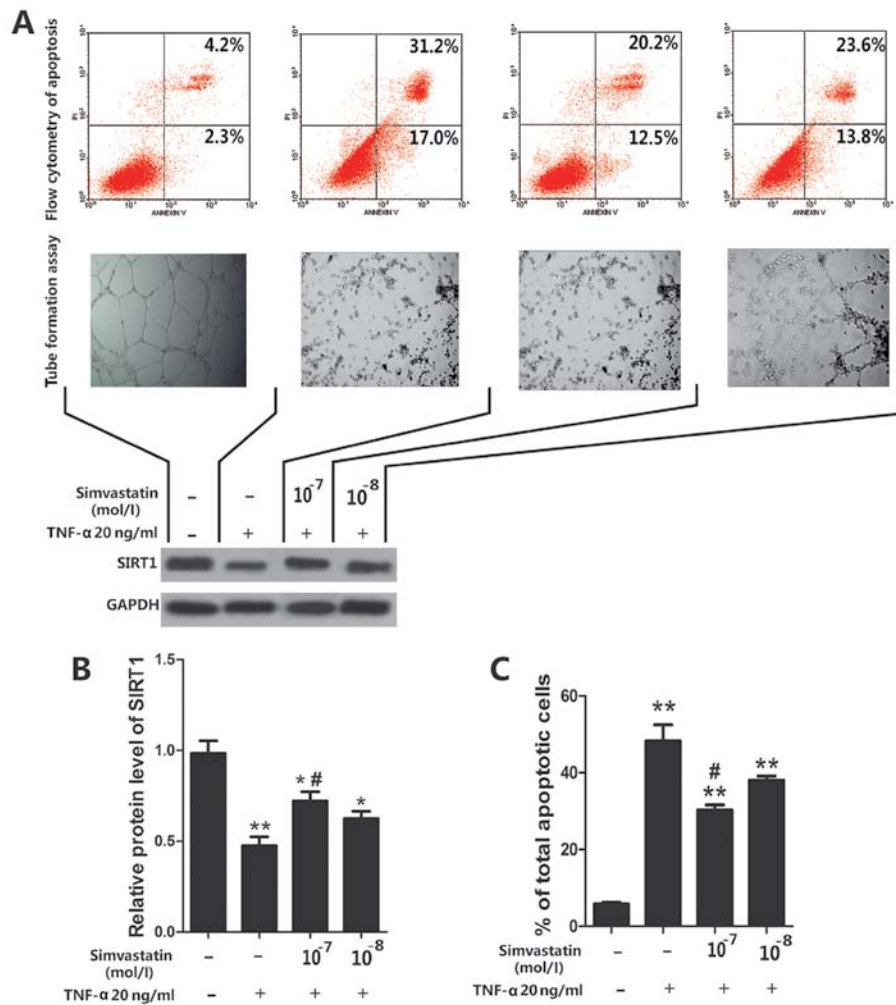


Figure 2. Effect of tumor necrosis factor- α (TNF- α) and simvastatin on apoptosis and angiogenesis in endothelial progenitor cells (EPCs). (A) Cells were stained with both Annexin V/propidium iodide (PI) dual and analyzed by flow cytometry to determine the cell population in early and late apoptosis. Left lower quadrant, viable cells; right lower quadrant, early apoptotic cells; right upper quadrant, late apoptotic cells. (B and C) The total percentage of apoptotic cells within each treatment group is shown in the bar diagram. An *in vitro* study was used to examine tube formation as a function of TNF- α and simvastatin application on EPCs. (A) Controls (without TNF- α and simvastatin) showed a robust angiogenic ability and simvastatin had little effect on TNF- α -induced degeneration. (B) Silent information regulator type-1 (SIRT1) expression of each group was determined by western blot analysis and the TNF- α -induced decrease in SIRT1 expression was significantly increased by high concentrations of simvastatin. *P<0.05 and **P<0.01 compared to the control group, #P<0.05 versus the TNF- α group, n=3 for each group for western blot analysis.

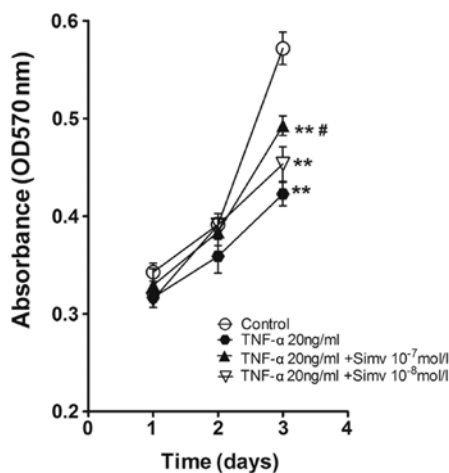


Figure 3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay results showed the cell proliferation in three treatment groups. Data are expressed as mean \pm SD, **P<0.01 compared to the control group, #P<0.01 versus tumor necrosis factor- α (TNF- α) group, n=5 for each group.

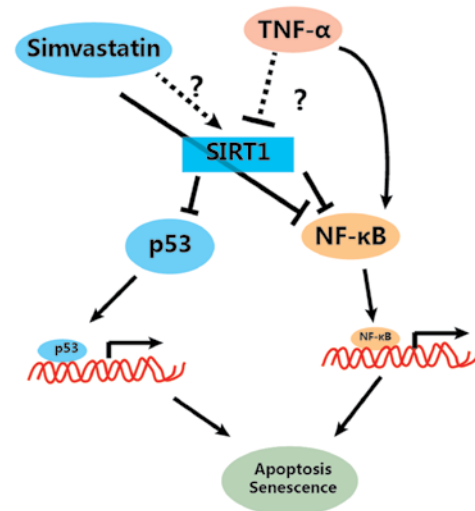


Figure 4. Proposed model for the mechanism of tumor necrosis factor- α (TNF- α), simvastatin, silent information regulator type-1 (SIRT1), p53 and NF- κ B signaling pathway involved in cell apoptosis and senescence.

attenuate TNF- α -induced apoptosis in EPCs (41). In this study, proliferation, apoptosis and angiogenesis of EPCs co-cultured with TNF- α and simvastatin were examined. We found that simvastatin exerts a mild anti-apoptotic effect on EPCs treated with TNF- α and that it enhances proliferation of EPCs that were previously inhibited by TNF- α incubation (Fig. 3). We found no effect of simvastatin treatment on the restoration of angiopoiesis (Fig. 2).

SIRT1 was originally identified as a nuclear protein that deacetylates proteins, contributing to cell regulation. Increasing SIRT1 allows for greater cell survival, particularly during periods of stress that usually trigger apoptosis. This occurs through regulation of the activity of several proteins, including FOXO, p53 and Ku70, all of which are involved in apoptosis or the initiation of cell repair (42-44). SIRT1 is located in the nucleus and translocated into the cytoplasm in response to hydrogen peroxide. This translocation results in an increased sensitivity to apoptosis (45). TNF- α is the prototypical member of a family of cytokines that also includes FasL, CD40L, and TRAIL. TNF- α is a potent inducer, which triggers inflammation, apoptosis, differentiation, and cell activation. TNF- α is found in the extracellular matrix, endothelium, and vessel walls of fibro-vascular tissue of proliferative diabetic retinopathy (46). Although SIRT1 levels can alter as a result of TNF- α exposure, its effects may differ depending on cell type, concentration, and time course. For instance, Wang *et al* (47) found that SIRT1 was highly expressed in the nucleus of vascular adventitial fibroblasts (VAFs) and translocated into the cytoplasm in response to TNF- α . Dvir-Ginzberg *et al* (48) also found that SIRT1 expression was reduced in human chondrocytes that had been stimulated with TNF- α (50 mg/ml) for 24 h. Conversely, results of a recent study showed that the expression levels of SIRT1 mRNA and protein were markedly increased in vascular smooth muscle cells (VSMCs) treated with TNF- α at concentrations of 30 and 50 mg/ml for 8 h (49). Similarly, we found that TNF- α reduced SIRT1 protein expression levels in EPCs in a concentration- and time-dependent manner (Fig. 1). Our results have shown that the ability of angiopoiesis and proliferation of EPCs was markedly inhibited by TNF- α . This conclusion was based on our data, which showed that incubation of EPCs with TNF- α significantly increased the percentage of apoptotic EPCs and simultaneously decreased the expression of SIRT1 (Fig. 2). Thus, we conclude that SIRT1 is involved in TNF- α -induced apoptosis in EPCs.

However, the molecular mechanism through which statins exert their anti-apoptotic effect is unclear. Although we found that the expression of SIRT1 can be decreased by TNF- α and increased by simvastatin, additional experiments are required to identify the molecular mechanism between statins, SIRT1, and TNF- α . Based on our results and the available literature, we have proposed a model demonstrating the role of simvastatin in apoptosis of EPCs (Fig. 4). We suggest that simvastatin inhibits cell apoptosis, in part, via the elevation of SIRT1. This elevation consequently inactivates NF- κ B activity by decreasing its acetylation state (NF- κ B-Ac). Additionally, simvastatin may inhibit NF- κ B activity directly, as has been previously reported in the literature (50,51). Moreover, simvastatin-dependent SIRT1 increases could also decrease the activity of p53 by decreasing its acetylation, thus inhibiting the p53-dependent apoptosis.

In summary, we have shown that simvastatin plays an important role in the TNF- α -induced apoptosis of EPCs and that this protection may involve SIRT1. These findings provide new evidence for the anti-inflammatory role of statins, which surpass their known effects on cholesterol metabolism.

Acknowledgements

This study has been supported by the Key Subject Construction of The First Affiliated Hospital of Jinan University, China.

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