ORIGINAL ARTICLES

Effects of benner pury on the function of endothelial progenitor cells in vitro from patients with hypertension in a time-dependent manner

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Abstract

Objective: To investigate the effect of Benner Pury on Endothelial progenitor cells (EPCs) proliferation, migration, adhesion, apoptosis and oxidative stress in vitro from patients with hypertension and its time dependence.
Methods: The patients who were diagnosed as hypertension grade 1 according to the standard "Guidelines for Prevention and Treatment of Hypertension" were enrolled. Mononuclear cells were isolated by density gradient centrifugation and stained with fluorescence chemical acetylated low density lipoprotein marked with DiI (acLDL-DiI) and fluorescein isothiocyanate (FITC)-lectin. Double staining positive cells were considered as the differentiation of EPCs. The control group included healthy subjects matched with study group in age, gender. EPCs cultivated for 5 days were used for study. Cells harvested at different set times under the stimulation of Benner Pury, and the EPCs proliferation, migration, adhesion ability, apoptosis and oxidative stress indicators were detected respectively.
Results: (1) Compared with the control group, peripheral blood EPCs proliferation, migration and adhesion ability were obviously decreased in the hypertension group ($p < .01$), EPCs apoptosis rate and oxidative stress response were significantly increased ($p < .01$). (2) Benner Pury significantly increased the EPCs proliferation, migration, adhesion ability and improved apoptosis and oxidative stress reaction in a time-dependent manner.
Conclusions: Benner Pury can improve the EPCs proliferation, migration, adhesion, apoptosis and oxidative stress in patients with hypertension in a time-dependent manner.

Key Words: Hypertension, Endothelial progenitor cells, Proliferation, Migration, Adhesion, Apoptosis, Oxidative stress

Endothelial progenitor cells (EPCs), which are precursor cells of vascular endothelial cells, can be involved in angiogenesis, vasculogenesis and the repair of endothelial injury, and are closely associated with hypertension and target organ damage caused by hypertension.[1] Angiotensin II plays an important role in the occurrence and the development of hypertension. Angiotensin converting enzyme inhibitors (ACEIs) quell the activity of Angiotensin II by inhibiting angiotensin converting enzymes, and then lower blood pressure. This study used EPCs in vitro from patients with hypertension under the intervention of Benner Pury classified as ACEI drugs, on one hand, to observe the changes in EPCs proliferation, migration, adhesion, apoptosis and oxidative stress at different set times, on the other hand, to investigate the effect of ACEI anti-hypertensive drugs on the function of peripheral circulating EPCs in patients with hypertension and further explore whether therapeutical effects of ACEI on patients with hypertension were related to the improve-

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ment of EPC function.

1 Objects and methods

1.1 Objects

15 cases (9 males and 6 females) of patients who were diagnosed as hypertension grade 1 (the hypertension group) were chosen according to the standards of "2010 Chinese Guidelines for the Management of Hypertension". The objects were with an average age of (47 ± 12). Exclusion criteria: patients who had taken any anti-hypertension drugs before; patients with dyslipidemia/dysglycemia; smokers and alcoholics; patients with infectious diseases (e.g. severe upper respiratory tract infection, pulmonary infection, hepatobiliary infections etc.); patients with hyperpyrexia; patients who used inflammation-inhibiting drugs (e.g. non-steroidal anti-inflammatory drugs [NSAIDs], steroid drugs and opioid drugs). The control group was comprised of 15 cases (10 males and 5 females) of healthy subjects who were equivalent in age and gender to those in the hypertension group; the average age was (46 ± 11).

1.2 Reagents and materials

EGM-2-MV medium was produced by Lonza, acetylated low density lipoprotein marked with DiI (acLDL-DiI) and fluorescein isothiocyanate (FITC)-lectin were produced by BTI, MTT was manufactured by Sigma, human fibronectins were products of Roche and CCK-8 was a product of Sigma. Besides, CD34, CD133, superoxide dismutase (SOD) kits and malondialdehyde (MDA) kits were procured from Beijing Biosynthesis Biotechnology Co., Ltd.

1.3 Separation, culture and identification of human EPCs

Fresh anticoagulated peripheral blood (20 ml) was taken respectively from subjects in the hypertension group and the control group. Mononuclear cells were isolated by a conventional method called density gradient centrifugation. With a seeding density of 1×10^5 cells/well, the mononuclear cells were inoculated in 6-well cell culture plates precoated with human fibronectins. Added EGM-2-MV medium (hEGF, Hydrocortisone, VEGF, FG-FB, R3-IGF-1) containing 5% patients' serums from cells and placed them into the CO_2 incubator (5% CO_2) for constant temperature incubation. After 4-day incubation, non-adherent cells were rinsed out by use of PBS. In order to maintain the original cell living environment, added EGM-2-MV medium containing 20% patients' serums from cells to make the fifth-day incubation for various examinations and analyses. Adherent cells were stained with fluorescence chemical acLDL-DiI and FITC-lectin, and observed with the aid of a laser scanning confocal microscope. Double staining positive cells were considered as the differentiation of EPCs. 15 fields of microscope ($\times$ 200) were chosen from each well randomly for EPCs counting. The counting was averaged and converted to the number of cells per square millimeter for data-recording.

1.4 Experiment grouping and detection indicators

EPCs cultivated for 5 days were used for study. The experiment was grouped into the control group, the hypertension group and the Benner Pury (10 μmol/L) group. According to different set times under the stimulation of Benner Pury, the Benner Pury group was subdivided into three groups: Benner Pury group (12 h), Benner Pury group (24 h) and Benner Pury group (48 h). In the next day after enrollment, fasting blood was required of all subjects. EPCs were isolated from peripheral blood for cultivation to detect EPCs proliferation, migration, adhesion ability, apoptosis and oxidative stress indicators in the hypertension group and the control group; EPCs in the hypertension group were harvested at different set times (12 h, 24 h and 48 h) under the stimulation of Benner Pury (10 μmol/L), and the changes in EPCs proliferation, migration, adhesion ability, apoptosis and oxidative stress indicators were detected respectively.

1.5 Detection methods

1.5.1 Detection of EPCs proliferation by MTT

With a seeding density of 5×10^3 cells/well, EPCs were inoculated in 96-well cell culture plates. Being adherent after 24-hour cultivation, cells were rinsed twice by use of PBS and cultured for another 12 hours with the addition of EGM-2-MV medium containing 1% patients' serums from cells for experimental use. The experiment was started by another 4-hour cultivation with the medium containing 20% patients' serums from cells, MTT was added to each well until the final MTT concentration reached up to 500 μg/ml, and then the cultivation was continued for another 24 hours. Abandoned the culture medium and added 150 μl of DMSO. After oscillation and crystal dissolution, the value of A490 nm was measured by the enzyme mark instrument.

1.5.2 Detection of EPCs migration

EPCs were cultured for 12 hours in EGM-2-MV medium containing 1% patients' serums from cells for the experimental use. Injected 30 μl of 20% patients' serum culture medium into the lower compartment of the modified Boyden chamber, and 50 μl of culture medium containing 3×10^4 EPCs into the upper compartment of the modified Boy-

den chamber. Scraped cells unmoved off the membrane and the remaining cells were immobilized by use of formaldehyde and stained with Giemsa. It was required to randomly choose cells migrated to the lower in 5 fields of microscope ($\times$ 200).

1.5.3 Detection of EPCs adhesion

96-well plates were precoated with PBS (50 μl/well) containing 20 mg/L human fibronectins, and reserved at 4°C overnight. The experiment was started after 12-hour continuous cultivation with EGM-2-MV medium containing 1% patients' serums from cells. The cell concentration was adjusted to 1×10^8 cells/L by 24-hour cultivation with 20% patients' serum culture medium, and 100 μl/well of cells were added to the coated 96-well plates. The cells kept adherent for 24 minutes under the condition of 37°C and 5% CO_2. Then, rinsed out the cells unattached with PBS. Added 10 μl of CCK-8 solution to each well, and compared optical density (OD) values of the hypertension group to those of other groups.

1.5.4 Effects of Benner Pury on EPCs apoptosis

The experiment was started after 12-hour continuous cultivation with EGM-2-MV medium containing 1% patients' serums from cells. The cells were cultivated for 24 hours with 20% patients' serum culture medium and stained with fluorescence FITC-Annexin-V/PI as instructed by Annexin-V-FLUOS staining kit, and then observed under the fluorescence microscope ($\times$ 400). Besides, the flow cytometer was applied to the counting of apoptosis cells in proportion to the whole cells. Each group was made in triplicate, and the experiment was made three times. $FITC^+$ PI^- indicated the cells were the early apoptosis cells, and $FITC^+$ PI^+ suggested the cells were the late apoptosis cells.

1.5.5 Detection of EPCs oxidative stress under the stimulation of Benner Pury

The experiment was begun after 12-hour continuous cultivation with EGM-2-MV medium containing 1% patients' serums from cells. The cells were cultivated for 24 hours with 20% patients' serum culture medium. The xanthine oxidase assay kit was applied to the measurement of the activity of SOD in the supernatant, and the thiobarbituric acid test was conducted to measure the MDA level. Values of OD in each group were measured by the spectrometer. Followed the instructions of kits and then defined the activity of SOD and MDA level.

1.6 Statistical treatment

SPSS11.0 was applied to statistical analysis of data, and the measurement data were represented by $\bar{x} \pm s$. One-way ANOVA was used in statistics, and the difference ($p < .05$) was statistically significant.

2 Results

2.1 Identification of EPCs

Under the inverted microscope, it was observed on the fifth day that the cells were short-spindled on a single layer. After being stained with acLDL-DiI and ulex europaeus agglutinin I marked with FITC (FITC-UEA-I), the cells should be observed under the fluorescence microscope: cells with red fluorescence were acLDL-DiI positive cells; cells with green fluorescence were UEA-I positive cells; and double staining positive cells were considered as EPCs in the process of differentiation. According to the analysis made by the flow cytometer, the positive rates of CD34 and CD133 were (72.55 $\pm$ 5.17)% and (71.31 $\pm$ 4.39)% respectively (see Figure 1).

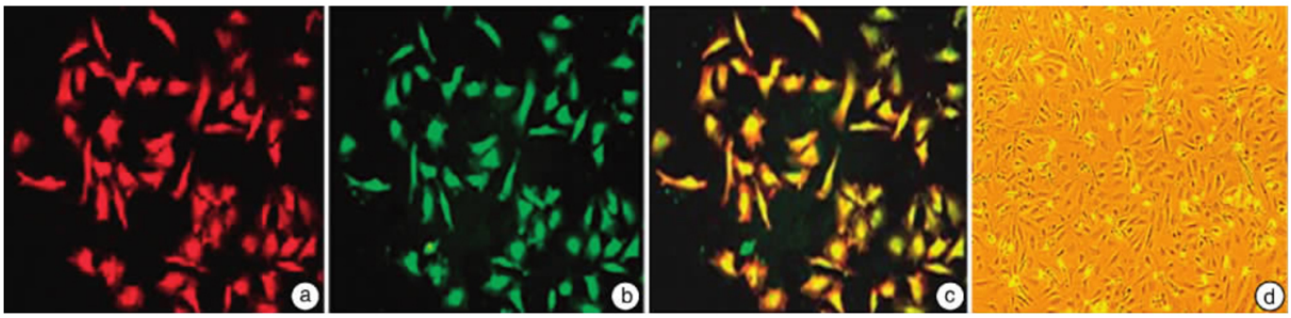

Figure 1: a: cells absorbing acLDL-DiI showed red fluorescence; b: cells absorbing FITC-lectin showed green fluorescence; c: cells absorbing both fluorescent dyes showed yellow fluorescence; d: endothelioid cells, of which adherent cells turned spindled after 5 days

2.2 Effects of Benner Pury on EPCs proliferation, migration, adhesion ability at different times

Compared with the control group, EPCs proliferation, migration and adhesion ability were obviously decreased in the hypertension group ($p < .01$). The changes in EPCs proliferation, migration and adhesion ability of the hypertension were detected at different culture times (12 h, 24 h and 48 h) under the stimulation of Benner Pury (10 μmol/L). The results showed that, with the time going by, EPCs proliferation, migration and adhesion ability were increased under the stimulation of Benner Pury in a time-dependent manner ($p < .05$) (see Table 1 and Figure 2).

Table 1: Effects of Benner Pury on EPCs proliferation, migration and adhesion of endothelial progenitor cells at different times ($\bar{x} \pm s$)

Group	Proliferation	Migration	Adhesion
Control Group	0.241 ±0.021	31.2 ±2.9	36.4 ±5.1
Hypertension Group			
• Benner Pury Group (0 h)	0.126 ±0.054 *	18.2 ±3.1 *	24.5 ±3.9 *
• Benner Pury Group (12 h)	0.149 ±0.051 **	21.4 ±2.8 **	28.9 ±2.8 **
• Benner Pury Group (24 h)	0.191 ±0.039 **	24.9 ±1.8 **	31.4 ±3.9 **
• Benner Pury Group (48 h)	0.228 ±0.033 **	28.1 ±4.2 **	34.9 ±4.2

Note. In comparison with the control group, * $p < .01$; In comparison with Benner Pury Group (0 h), ** $p < .05$

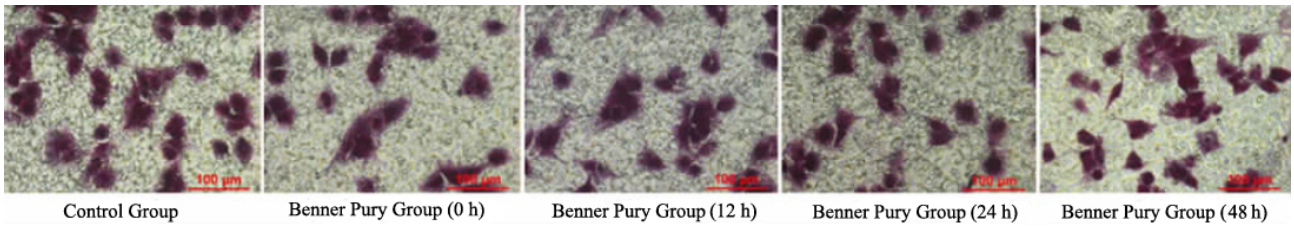

Figure 2: Effects of Benner Pury on EPCs migration at different times (× 200)

2.3 Effects of Benner Pury on EPCs apoptosis rate at different times

Compared with the control group, EPCs apoptosis rate was significantly increased in the hypertension group ($p < .01$). The change in EPCs apoptosis rate was detected at different culture times (12 h, 24 h and 48 h) under the stimulation of Benner Pury (10 μmol/L). The results showed that, with the time going by, the early and the late apoptosis rates of EPCs were decreased gradually under the stimulation of Benner Pury in a time-dependent manner ($p < .05$) (see Table 2 and Figure 3).

Table 2: Effects of Benner Pury on EPCs apoptosis rate at different times ($\bar{x} \pm s$)

Group	Early Apoptosis	Late Apoptosis
Control Group	3.2 ±2.1	4.7 ±2.9
Hypertension Group		
• Benner Pury Group (0 h)	9.2 ±3.1 *	8.6 ±3.2 *
• Benner Pury Group (12 h)	7.6 ±2.5 **	6.4 ±1.9 **
• Benner Pury Group (24 h)	5.3 ±2.4 **	4.6 ±3.1 **
• Benner Pury Group (48 h)	3.4 ±1.8 **	2.7 ±1.8 **

Note. In comparison with the control group, * $p < .01$; In comparison with Benner Pury Group (0 h), ** $p < .05$

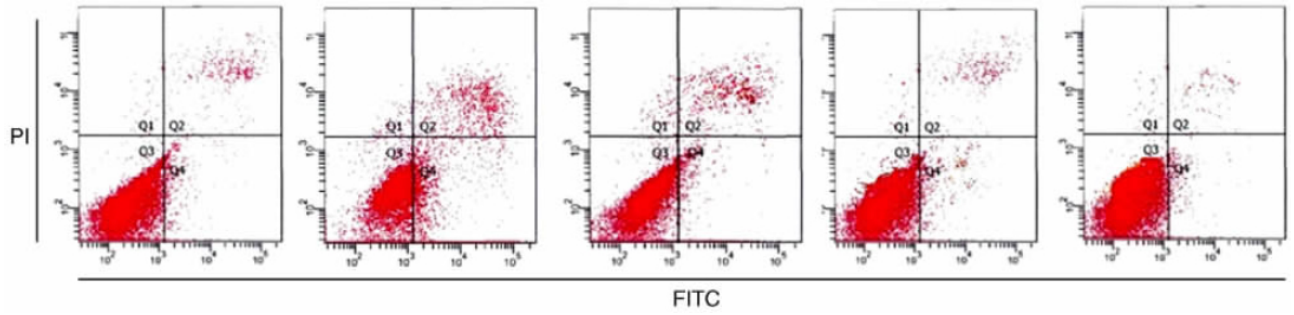

Figure 3: Effects of Benner Pury on EPCs apoptosis rate at different times

2.4 Effects of Benner Pury on EPCs oxidative stress

In comparison with the control group, the activity of SOD was obviously decreased and the MDA level was apparently increased in the hypertension group ($p < .01$). The changes in EPCs oxidative stress in the Benner Pury group (10 μmol/L) were detected at different culture times (12 h, 24 h and 48 h). The results showed that, with the time going by, the activity of SOD was gradually increased and the MDA level was decreased under the stimulation of Benner Pury in a time-dependent manner ($p < .05$) (see Table 3).

Table 3: Effects of Benner Pury on SOD and MDA of EPCs at different times ($\bar{x} \pm s$)

Group	SOD	MDA
Control Group	78.49 ±8.99	9.41 ±1.61
Hypertension Group		
• Benner Pury Group (0 h)	40.64 ±9.84 *	16.17 ±2.41 *
• Benner Pury Group (12 h)	53.09 ±10.31 **	14.59 ±2.07 **
• Benner Pury Group (24 h)	64.74 ±10.84 **	12.71 ±1.93 **
• Benner Pury Group (48 h)	75.91 ±11.31 **	9.78 ±1.69 **

Note. In comparison with the control group, * $p < .01$; In comparison with Benner Pury Group (0 h), ** $p < .05$

3 Discussions

EPCs are a subtype of precursor cells that have the capacity to specifically home to injured tissues and differentiate into mature vascular endothelial cells.[2] The changes in the number and the function of EPCs play an important role in endothelial maintenance and repair,[3] and are closely associated with the pathogenetic process of hypertension, as well as the occurrence and the development of target organ damage and complications caused by hypertension.[4] Previous researches have shown that, the number of peripheral blood EPCs from bone marrows decreases under the condition of hypertension. Meanwhile, hypertension has an increasingly intensified damage to EPCs with the increase of blood pressure and the development of hypertension pathogenetic process.[5]

This research showed that, in comparison with the control group, the proliferation ability of peripheral blood EPCs was obviously decreased in the hypertension group, which conformed to previous studies ($p < .01$).[5] Furthermore, authors of this article found that, in comparison with the control group, EPCs migration and adhesion ability were also obviously decreased in the hypertension group. The results suggested that, not only the number of peripheral blood EPCs, but also their migration and adhesion ability were degraded under the condition of hypertension.

MDA, a type of main metabolites reflecting lipid peroxidation damage, can cause toxic stress in cells and reflect the degree of cellular oxidative damage; meanwhile, SOD is a main enzyme that can eliminate free radical systems from cells, and the activity of SOD indirectly reflects the capacity of an organism to eliminate oxygen free radicals. The activity of SOD and the MDA level can reflect the degree of peroxidation damage to some extent.[6]

The research indicated that, in comparison with the control group, the early and the late apoptosis rates of EPCs were obviously increased in the hypertension group ($p < .01$), i.e., EPCs apoptosis rate was increased in patients with hypertension, which conformed to previous studies; compared with the control group, the ability of SOD was obviously decreased and the MDA level was apparently increased in the hypertension group, suggesting EPCs oxidative stress response was improved in the hypertension group. In the case of oxidative stress, oxidative stress promotes EPCs apoptosis to affect the function and the number of EPCs by weakening the antioxidant enzyme expression and enhancing the oxidant enzyme expression of EPCs, consequently, the number and the function of peripheral blood EPCs are decreased in patients with hypertension.[7]

Angiotensin II, as an essential active peptide of RAS system, plays a crucial role in a series of pathological reactions caused by the cardiovascular system. It can induce the release of reactive oxygen species from vascular walls, facilitate the excessive aggregation of reactive oxygen species and stimulate the occurrence of oxidative stress to accelerate EPCs aging and result in the damage to EPCs proliferation; furthermore, it can also regulate expression levels of apoptosis proteins or inhibitors of apoptosis proteins, so as to result in EPCs aging or apoptosis.[8]

Benner Pury in ACEI can decrease the activity of angiotensin II by inhibiting angiotensin converting enzymes, and then lower blood pressure. Authors of this article applied 10 μmol/L of Benner Pury to in-vitro culture of EPCs in patients with hypertension, and detected the changes in EPCs proliferation, migration and adhesion ability at different times (12 h, 24 h and 48 h) under the stimulation of Benner Pury. Research data showed that, with time going by, the EPCs proliferation, migration and adhesion ability were increased under the stimulation of Benner Pury in a time-dependent manner ($p < .05$). It is indicated that Benner Pury can improve peripheral blood EPCs proliferation, migration and adhesion ability in patients with hypertension.

The research showed that Benner Pury in ACEI could increase the activities of antioxidant enzymes of EPCs and inhibit the generation of lipid peroxides, in order to improve EPCs dysfunction and extend the lifespan of EPCs.[9, 10] The research found that, by detecting the EPCs oxidative stress response under the stimulation of Benner Pury, which could significantly improve the oxidative stress condition of EPCs. Furthermore, with time going by, the activity of SOD was gradually increased and the MDA level was decreased under the stimulation of Benner Pury in a time-dependent manner ($p < .05$); the early and the late apoptosis rates of EPCs were gradually decreased in a time-dependent manner ($p < .05$). The results showed that Benner Pury could improve apopto-

sis and oxidative stress of peripheral blood EPC in patients with hypertension.

In conclusion, this experiment proves that Benner Pury in ACEI can improve EPCs proliferation, migration, adhesion, apoptosis and oxidative stress in patients with hypertension in a time-dependent manner; and discloses effects of Benner Pury on the function of peripheral blood EPCs in patients with hypertension and its preliminary mechanism.

Conflicts of Interest Disclosure

The authors have no conflicts of interest related to this article.

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