The Potential Efficacy of Endothelial Progenitor Cells With and Without Nitric Oxide Inducer as Cell Therapy for Right Ventricular Impairments Caused by Pulmonary Hypertension in Rats

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Abstract

Background: A number of studies now suggest that endothelial progenitor cells (EPCs) may induce neovascularisation and could be a promising approach for cell based therapy for pulmonary artery hypertension (PAH).

Purpose: We investigated whether or not intravenous injection of bone marrow-derived endothelial progenitor cells (EPCs), alone or combined with NO inducer restore pulmonary hemodynamics and increase microvascular perfusion in the rat monocrotaline (MCT) model of pulmonary artery hypertension so improve right ventricle performance.

Material and Methods: Rats were divided into control, rats with PAH, PAH rats receiving EPCs, PAH rats receiving NO inducer (L-Arginine) and PAH rats receiving EPCs plus NO inducer, after 1 month, serum creatine phosphokinase enzyme, VEGF level in heart and lung were assessed. Histopathological analysis of both heart and lung tissues was performed.

Results: The level of VEGF was increased in all treated groups. Immunohistochemical staining showed perivascular, intravascular and peribronchial CD34 positive cell aggregates in the groups received EPCs whether alone or combined with NO inducer.

Conclusion: The present study proved that, administration of BM-EPCs alone, NO alone or EPCs plus NO, produce therapeutic effect in restoring pulmonary hemodynamics and so improving right ventricle performance in PAH. This effect may be through angiogenic action of EPCs and NO.

Key Words: Pulmonary artery hypertension – EPCs – NO and angiogenesis.

Introduction

PAH is defined as an increase in arterial pressure above 25mmHg [1]. There are cellular and structural changes in the walls of pulmonary arteries, which result in endothelial dysfunction and vascular remodeling [2]. In turn vasoactive mediator imbalance occurs resulting in vasoconstriction. Structural remodeling in pulmonary vessels occurs as a result of excessive pulmonary arterial smooth muscle cell proliferation and impaired apoptosis [3]. Ultimately pulmonary arteries endothelial cell injury is central to the subsequent development of lumen-obliterative lung vascular lesions [4].

Endothelial progenitor cells are bone marrow-derived mononuclear cells that circulate in the blood and are believed to repair blood vessel damage by migrating to sites of vascular injury and differentiating into endothelial cells [5]. EPCs therapy is now emerging as a potential regenerative approach to PAH [6]. So far, established therapy is aimed at restoring the balance of vasoactive substances but does not target repair of the endothelium, creating need for treatments such as EPC therapy [7].

Nitric oxide (NO) has been proposed as the major physiologic regulator of blood vessel tone. It acts on smooth muscle cells producing vasodilatation and inhibiting its proliferation [8]. The bioavailability of NO is reduced in PAH, most likely as a consequence of post-translational alterations of the enzyme producing nitric oxide, eNOS and/or enhanced NO degradation [9]. Some data suggest that an adequate NO regulation is also needed for the angiogenic properties of EPCs. eNOS inhibition by the compound L-NAME significantly reduced microtubule formation by EPCs in a culture model of angiogenesis [10].

The aim of the work was to investigate whether or not intravenous injection of bone marrow-derived endothelial progenitor cells (EPCs), alone or combined with NO inducer can restore pulmonary
hemodynamics and increase microvascular perfusion in the rat monocrotaline (MCT) model of PAH so improve right ventricle performance.

**Material and Methods**

This study was performed at the Unit of Biochemistry and Molecular Biology at The Medical Biochemistry Department, Faculty of Medicine, Cairo University, Egypt during the period from February 2013 to March 2014.

**Preparation of the animal model:**

This study included fifty healthy, male adult white albino rats (200-250g body weight). Animals were inbred in the Experimental Animal Unit, Faculty of Medicine, Cairo University. Rats were bred and maintained in an air conditioned animal house with specific pathogen free conditions and were subjected to a 12: 12-h daylight/darkness. Animals were fed a semi-purified diet that contained (gm/kg): 200 casein, 555 sucrose, 100 cellulose, 100 fat blends, 35 vitamin mix and 35 mineral mix. All animal experiments received approval from the Institutional Animal Care Committee.

**Animals were divided into 5 groups (Table 1):**

- **Group 1**: Healthy control.
- **Group 2**: Positive control (PAH): Received 1ml PBS IV.
- **Group 3**: PAH+EPCs: Received $10^6$ EPCs per rat given by intravenous injection at the rat tail vein [11].
- **Group 4**: PAH+NO: Received NO inducer (L-Arginine 300mg/kg body weight intraperitoneally) [12].
- **Group 5**: EPCs +PAH+NO.

**Table (1): Classification of studied groups.**

<table>
<thead>
<tr>
<th>Groups (10 rats each)</th>
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**Flow Cytometry Analysis (FACS analysis)** for CD34+ of cultured EPCs was done as an identification surface marker of EPCs.

**Detection of CD34+ by immunohistochemistry:**

Unstained positively charged slides were prepared from each paraffin block for immunostaining.
using monoclonal rabbit anti-human antibody (anti-CD34, Lab vision, USA. and ultra-vision detection system) (HRP/DAB, Lab vision, USA). Positive immunoreactivity to CD34 antibody gives a brown staining.

**Serum creatine phosphokinase (CPK) estimation:**

Venous blood was collected from the retro-orbital vein from rats of all groups, left to clot for 30 minutes, and centrifuged at 10,000rpm for 20 minutes. Serum CPK was measured by using EnzyChromTM Creatine Kinase Assay Kit (ECPK-100).

**Estimation of the levels of VEGF in heart and lung tissues:**

VEGF levels in heart and lung tissues were assayed by a commercially available Enzyme-linked immunosorbent assay (ELISA) kit supplied by R and D system USA according to manufacturer instructions.

**Histopathological examination of heart and lung tissues:**

The hearts and lungs were fixed in 10% formaldehyde for 48h and embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin for qualitative histopathological analysis. Other paraffin sections were stained with Trichrome to evaluate fibrosis and collagen deposition.

**Statistical analysis:**

Data were expressed as mean ±SD. Significant differences were determined by using ANOVA and post-hoc tests for multiple comparisons using SPSS 16 computer Software. Results were considered significant at p<0.05.

**Results**

This study was conducted on fifty healthy male adult white albino rats (200-250g body weight).

**Serum CPK level:**

The level of CPK-MB in the serum of the MCT induced pulmonary artery hypertensive (PAH) group shows a significant increase compared to the control healthy group which confirmed the cardiac affection in PAH Fig. (1).

**Estimation of the levels of VEGF in heart and lung tissues by ELISA:**

Level of VEGF was increased in the treated groups in both heart and lung Fig. (6).

**Histopathological and immunohistochemical results:**

Histopathological changes in both the right ventricle and lung were observed by HE and MT staining. There was increased number of small and large vessels in the treated groups. Immunohistochemical staining showed perivascular, intravascular and peribronchial CD34 positive cell aggregates in the groups received EPCs whether alone or combined with NO inducer Figs. (4,5).

EPCs isolation, propagation and identification:

A- EPCs in culture:

EPCs were isolated from rat bone marrow, cultured and propagated for 7 days on fibronectin coated wells using media supplemented with specific growth factors as; VEGF-1 and IGF-1.

B- EPCs- CFU characterization in culture:

EPCs were characterized in culture by formation of CFU Fig. (6).

B- Analysis of EPCs based on cell surface maker expression:

EPCs were characterized by their specific double fluorescent staining with DiLDL-UAE-1 stains Fig. (7I). EPCs stained with DAPI to ensure their viability and survival in vitro Fig. (7II).
Fig. (2): Comparison between the VEGF levels in rat lung tissues of studied groups.

Fig. (3): Comparison between the VEGF levels in rat cardiac tissues of studied groups.

Fig. (4): Histopathological examination. (A) Control. (B) PAH. (C) EPCs. (D) NO. (E) EPCs+NO.

Fig. (5): Immunohistochemical staining. (A) Control. (B) EPCs. (C) EPCs+No.
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Fig. (6): EPCs-CFU characterization in culture. (A): At 24 hours culture. (B): At 7 days of culture.

Fig. (7I): DiLDL-UAE-1 double staining of EPCs in culture.

Fig. (7II): Characterization of viability of cultured EPCs by positive-DAPI blue cytoplasm staining.

Discussion

Endothelial dysfunction plays a prominent role in the pathogenesis of pulmonary arterial hypertension. A variety of evidence suggests that circulating Endothelial Progenitor Cells (EPCs) play an integral role in vascular repair [15,16]. The bioavailability of NO is reduced in PAH, most likely as a consequence of post-translational alterations of the enzyme producing nitric oxide, eNOS and/or enhanced NO degradation [9].

In the present study, we aimed to investigate the effect of intravenous injection of BMEPCs, alone or combined with NO inducer in restoring pulmonary hemodynamics and increasing microvascular perfusion in the rat monocrotaline (MCT) model of PAH so improving right ventricle performance.

There was a significant increase in the level of CPK-MB in the serum of the MCT induced pulmonary artery hypertensive (PAH) (diseased) group when compared to the control healthy group which confirmed the cardiac affection in PAH.

As for vascular endothelial growth factor (VEGF), it is a pivotal regulator of angiogenesis. Some findings suggest a central role of VEGF in PAH which is mediator of angiogenesis and also a factor involved in permeability and inflammatory processes in the vascular endothelium [17]. In the present study there was a significant decrease in the VEGF level in the PAH group compared to the control group. All the groups which received treatment showed significant increase in the VEGF level in heart tissue when compared to PAH group but there was no significant difference in its level between the treated groups. In the lung tissue, there was significant increase in VEGF level in all treated group when compared to the PAH group. This agrees with Lu et al., [18] who found that increase in lung mRNA levels of VEGF in dehydromonocrotaline (DMCT)-induced PAH of rat model compared to control group when intratracheal administration of bone marrow-derived mononuclear cells (BM-MNCs) was carried out.

Histopathological changes in both the right ventricle and lung were observed by HE and MT staining. All the diseased groups showed hypertrophied muscle of the wall of pulmonary vessels of different sizes with luminal narrowing and obliteration in some cases. This agree with Tuder et al., [19] who found that intimal lesions account for most of the reduction of luminal area of small pulmonary arteries and potentially largely influence the overall pulmonary vascular resistance and these intimal lesions consist of eccentric intima thicken-
ing and fibrotic, pleomorphic, concentric and dilation/angiomatoid lesions. Also Suzuki et al., [20] found that the lung vascular morphology of MCT-treated rats revealed significantly larger medial thicknesses of pulmonary arteries.

In the present study, the groups received EPCs either alone or combined with NO inducer showed round cell aggregates in the perivascular and peribronchial areas. There was increased vascularity (increased number of small and mostly large arteries) in all treated groups. This agree with Sun et al., [22] who found that H & E stained lung of MCT model showed remarkable increase in number of muscularized arterioles after induction of pulmonary hypertension being significantly suppressed by either mono-treatment with endothelial progenitor cells (EPCs)/sildenafil (Sil) or a combined regimen.

Immunohistochemical staining of lung tissue with CD34 antibody showed immunohistoreactivity of CD34 positive cells mostly forming perivascular and peribronchial aggregates in the groups received EPCs either alone or combined with NO inducer. CD34 is perhaps the most frequently used marker for isolating EPCs and may be considered the most widely applied method of antigen-based EPC selection [22].

In conclusion, the present study proved that, administration of BM-EPCs alone, NO alone or EPCs plus NO produce therapeutic effect in restoring pulmonary hemodynamics and so improving right ventricle performance in PAH. This effect may be through angiogenic action of EPCs and NO.

References