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Comparison of angiogenic and proliferative effects of three commonly used agents for pulmonary artery hypertension (sildenafil, iloprost, bosentan): is angiogenesis always beneficial?

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Abstract. – OBJECTIVE: Pulmonary artery hypertension (PAH) is devastating disease that has very serious outcomes. Dysregulated angiogenesis is one of the main responsible courses in pathophysiology of disease. Our experimental research intends to find out and compare the angiogenic effects of medications used sildenafil, iloprost, and bosentan in the treatment of PAH.

MATERIALS AND METHODS: This study was performed in Department of Biochemistry and Cancer and Stem Cell Research Laboratory of our institutes between August and October 2014. Angiogenic activity of sildenafil, iloprost, and bosentan were examined *in vivo* in chick chorioallantoic membrane (CAM) model and *in vitro* tube formation assay of human umbilical vein endothelial cells (HUVECs). Proliferative activity of these three agents was also determined through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on HUVECs.

RESULTS: In CAM assay, when compared to the control and drug groups, treatment with sildenafil solutions resulted in a significant dose-dependent increase (budding, sprouting, extravasation) on CAM vessel growth. While there was no significant proliferative effect with iloprost and bosentan, presence of sildenafil caused a statistically significant proliferation on HUVECs following 24 and 48 h incubation ($p < 0.05$) compared to the control group. Comparing the tube length/area ratio values, there was statistically significant increase in sildenafil group with respect to the other 2 groups ($p < 0.05$). Iloprost and bosentan did not show a significant effect.

CONCLUSIONS: The results provide evidence that sildenafil but not iloprost and bosentan induces angiogenesis *in vitro* and *in vivo*. Dysregulated angiogenesis, as an important pathophysiological part in the progression of PAH, may be triggered by the chronic ingestion of sildenafil in the long treatment period and may cause negative effects.

Key Words:
Angiogenesis, Sildenafil, Iloprost, Bosentan, HUVEC, Endothelial cell tube formation, CAM assay.

Introduction

Pulmonary arterial hypertension (PAH) whether primary or secondary is a disorder characterized by an elevation in pulmonary artery pressure that can lead to right ventricular failure and death[1-3]. Contemporary one-, three-, five-, and seven-year survival rates from time of diagnostic right-sided heart catheterization are 85%, 68%, 57%, and 49%, respectively[4]. PAH is a multifactorial disease involving abnormal vascular tone, endothelial dysfunction, inflammation, dysregulated angiogenesis, and enhanced thrombosis[5,6].

Our understanding of angiogenesis has evolved substantially in the past two decades. To a very near time, pathophysiology of PAH has been thought to result from vasoconstriction of the pulmonary vascular resistance vessels or from elevated shear stress due to increased shunt blood flow. Now, the new concepts related to angiogenesis have caught up with a need to understand, at a molecular level. Angiogenesis can

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partly help to understand several aspects related to the pathological presentation of PAH, that can not solely explained by vasoconstriction[7].

There are three commonly used classes of medications that have shown efficacy in the treatment of PAH: phosphodiesterase-5 inhibitors, prostanoids, endothelin receptor antagonists. These medications have different pathway targets, mechanisms of action, indications, delivery routes, and side-effect profiles[8].

Despite dysregulated angiogenesis is one of the main responsible course in the pathophysiology of PAH, we don't know how these agents affect on angiogenesis. Our experimental research intends to find out and compare the angiogenic effects of medications used in the treatment of PAH.

Materials and Methods

Sildenafil, Iloprost and Bosentan Solutions

Various sildenafil solutions were prepared with serial dilutions from a stock solution of 1,5 mM, which was prepared by dissolving 5 mg sildenafil (Sigma-Aldrich, I, Seelze, Germany) in a final volume of 5 mL solution. Stock solutions of 50 μM iloprost (Ilomedin, Bayer Schering Pharma AG, Bergkamen, Germany) and 10 mM bosentan (Tracleer, Actelion Pharmaceuticals Ltd, Allschwil, Switzerland) were used for the preparations of required concentrations. The required concentrations for each assay were determined according to the recommended dose per kg, on patients with PAH.

Cell Viability Assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method was used to evaluate the cell viability assay with the same procedures according to our previously published study (9). Shortly, the cells (10-20.000/well) were incubated in a 96-well plate in the presence of various concentrations of sildenafil (0.5 μM, 1 μM, 2.5 μM and 5 μM), iloprost (0.25, 0.5, 1 and 2 μM), and bosentan (0.1, 0.25, 0.5 and 1 μM) in a final volume of 0.2 mL for 24-48 hours to determine the effect on endothelial cell proliferation.

In vitro Endothelial Cell Tube Formation Assay

1 μM of these drug solutions were used to determine their effect on tube formation. This assay was performed following the steps as described previously[9].

Chick Chorioallantoic Membrane (CAM) Assay

CAM assay was used for *in vivo* evaluation of angiogenesis. Different concentrations of sildenafil (50, 100 and 150 μM), iloprost (0.5, 2.5 and 5 μM), and bosentan (100, 250 and 500 μM) were prepared, and 50 μL of each solution was used to determine the effect of each solution on angiogenesis *in vivo*. A standardized procedure including the same steps with our published study was followed to perform CAM assay, and the effect on CAM vascular area was scored[9].

Statistical Analysis

Chi-square test was used for the non-parametric tests. In addition, Yates correction analysis was performed for statistically significant difference. Statistical analysis was carried out using MedCalc for Windows, version 12.5 (MedCalc Software, Ostend, Belgium). We used in the same data situations as a Pearson's correlation. The statistical analysis was carried out using Statistical Package for the Social Sciences software for Windows, Version 15.00 (SPSS Inc., Chicago, IL, USA). The value of $p \leq 0.05$ was considered statistically significant.

Results

Cell Viability Assays of Sildenafil, Iloprost and Bosentan

While there was no significant proliferative effect with iloprost and bosentan, presence of sildenafil caused a statistically significant proliferation on human umbelical vein endothelial cells (HUVECs) following 24 and 48 h incubation ($p < 0.05$) compared to the control group (Figure 1).

Effects of sildenafil, Iloprost and Bosentan on Tube Formation

In the tube formation assay, on the 18th hour of incubation, the results were evaluated. Comparing the tube length/area ratio values, there was statistically significant increase in sildenafil group with respect to the other 2 groups ($p < 0.05$). So, it was found that sildenafil had proangiogenic effect (Figures 2 and 3). Iloprost and bosentan did not show a significant effect.

Sildenafil Induces Angiogenesis on CAM

The vessel formation on each chorioallantoic membrane (CAM) area was macroscopically

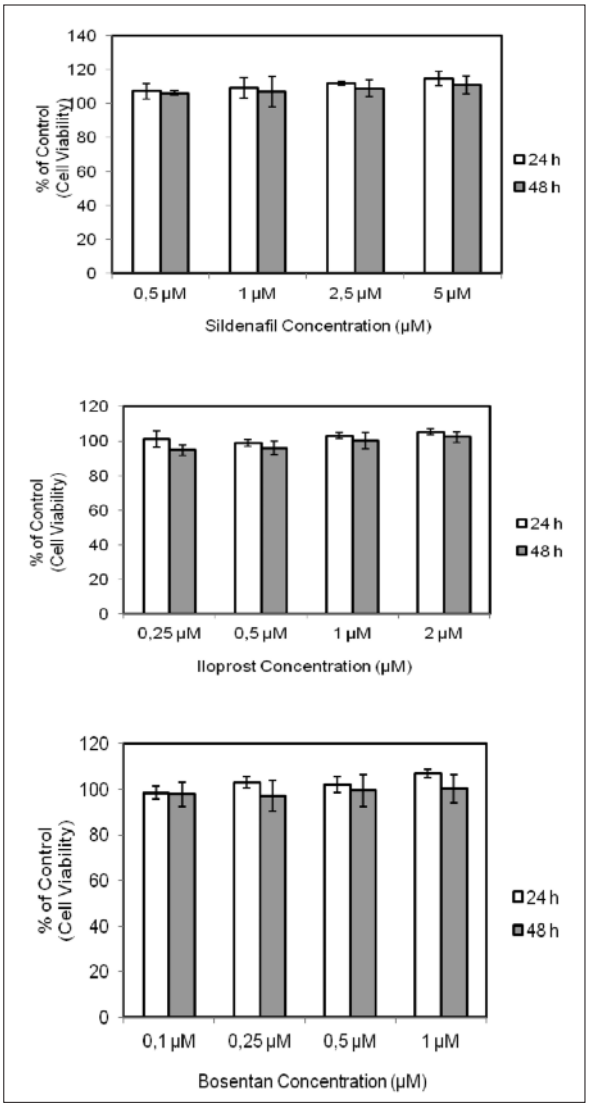

Figure 1. Cell proliferation of HUVECs under sildenafil, iloprost and bosentan incubation (cell viability was indicated as percentage of control). HUVECs: human umbilical vein endothelial cells.

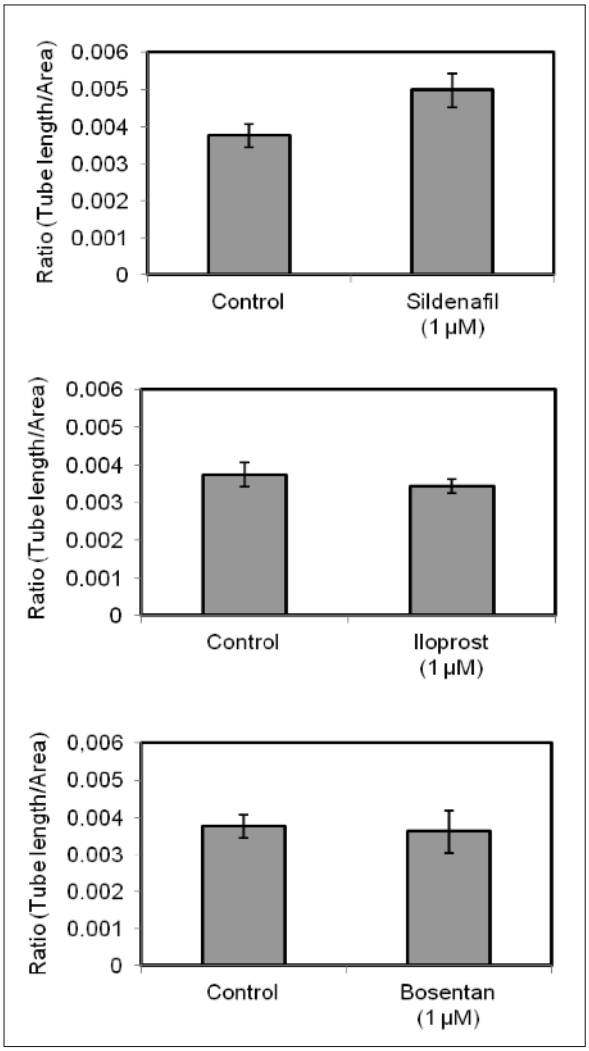

Figure 2. Ratio of tube length to area under sildenafil, iloprost and bosentan incubation and control.

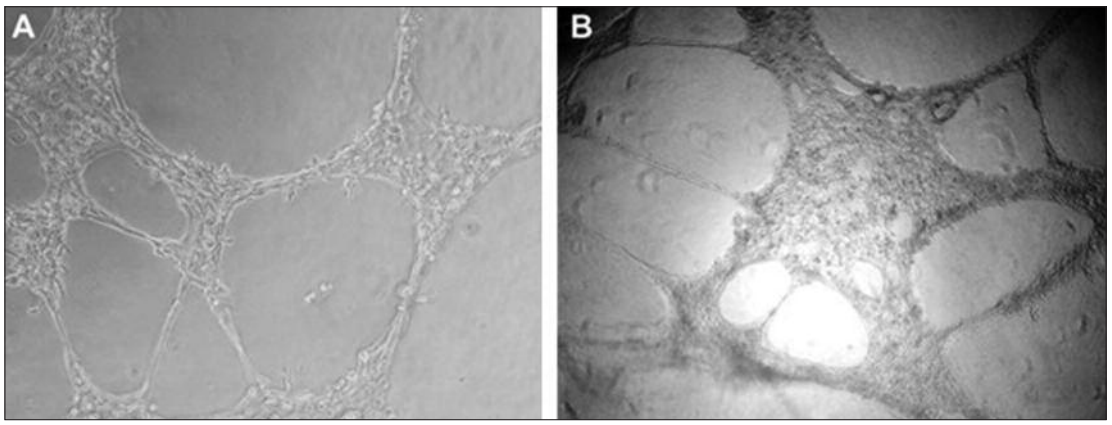

Figure 3. Tube formations of control group ***(A)*** and under sildenafil incubation ***(B)***.

scored as described in previous studies[9] and the results were summarized in Table I. Compared to the control and drug groups, treatment with sildenafil solutions resulted in a significant dose-dependent increase (budding, sprouting, extravasation) on CAM vessel growth. Affected vessels were thicker and had an increased number of branching points. Sprouting of new vessels from existing ones can be clearly seen in Figure 4. The efficacies at different doses of sildenafil were compared using χ^2 test, and there was a statistically significant difference (Yates correction $\chi^2 = 15.191$, $p < 0.05$). The efficacy of increasing doses was evaluated with Spearman's correlation test and a strong correlation was found ($r = 0.804$, $p < 0.001$), indicating the dose dependent effect of sildenafil on angiogenesis *in vivo*. The angiogenic effect was clear especially for the doses of 100 μM and higher.

However, iloprost and bosentan showed no significant effect. The efficacies at different doses of iloprost were compared using χ^2 test, and there was not statistically significant difference (Yates correction $\chi^2 = 4.996$), similarly with bosentan (Yates correction $\chi^2 = 4.557$).

Discussion

The past two decades have seen a marked evolution in our approach to the diagnosis and management of PAH. Multiple randomized controlled trials have been performed in PAH resulting in the regulatory FDA approval of three pharmacological classes: phosphodiesterase type-5 inhibitors, prostanoids, and endothelin-receptor antagonists[10]. Recently, a soluble guanylate cyclase stimulator has been also added to armamentarium of treatment options[11].

PAH mechanisms by which the pulmonary arteries in the pulmonary circulation narrow include pulmonary vasoconstriction, pulmonary vascular remodeling, and thrombosis *in situ*[12,13]. Vascular remodeling involves all 3 layers of the vascular wall. It is complicated by the finding that cellular heterogeneity exists within the compartment of the pulmonary arterial wall[12,14]. Pulmonary vascular remodeling in PAH includes endothelial angiogenesis, smooth muscle cell proliferation and hypertrophy, adventitial fibroblast proliferation, myofibroblast differentiation, and extracellular matrix deposition. Hypoxia is considered as the predominant factor in the pathogenesis of PAH. During the early period of hypoxic exposure, angiogenesis in the mature pulmonary circulation is a potentially beneficial adaptation for gas exchange[15,16]. The lung vascular homeostasis involves maintaining an ideal number of capillaries per unit of lung volume. However, the sustained chronic hypoxia leads to disorder of the process and excess angiogenesis, which would impose more pressure on the proximal pulmonary artery and complicate the course of PAH, suggesting that excess angiogenesis is a crucial player in the pathogenesis of PAH[17].

Cool et al[18] wrote that "The most significant alteration of endothelial cell phenotype in PAH is the abnormal proliferation of endothelial cells in pulmonary arteries; in particular, the growth involving branch points of muscularized pulmonary arteries. Remarkably, the intravascular growth of endothelial cells results in bulging of the vascular wall of the compromised vessel, which imparts a larger vessel diameter than the proximal (feeder) vascular segment". The obligatory lining of pulmonary arteries with a monolay-

Table I. Macroscopic evaluation of the effect of sildenafil treatment on CAM.

Group		Efficacy			
		Ineffective	+1	+2	Total
Control	n	6	0	0	6
	%	100.0	0	0	100
50 μM	n	4	2	0	6
	%	66.67	33.33	0	100
100 μM	n	2	3	1	6
	%	33.33	50.0	16.67	100
150 μM	n	0	2	5	7
	%	0	28.57	71.43	100
Total	n	12	7	6	25
	%	48	28	24	100

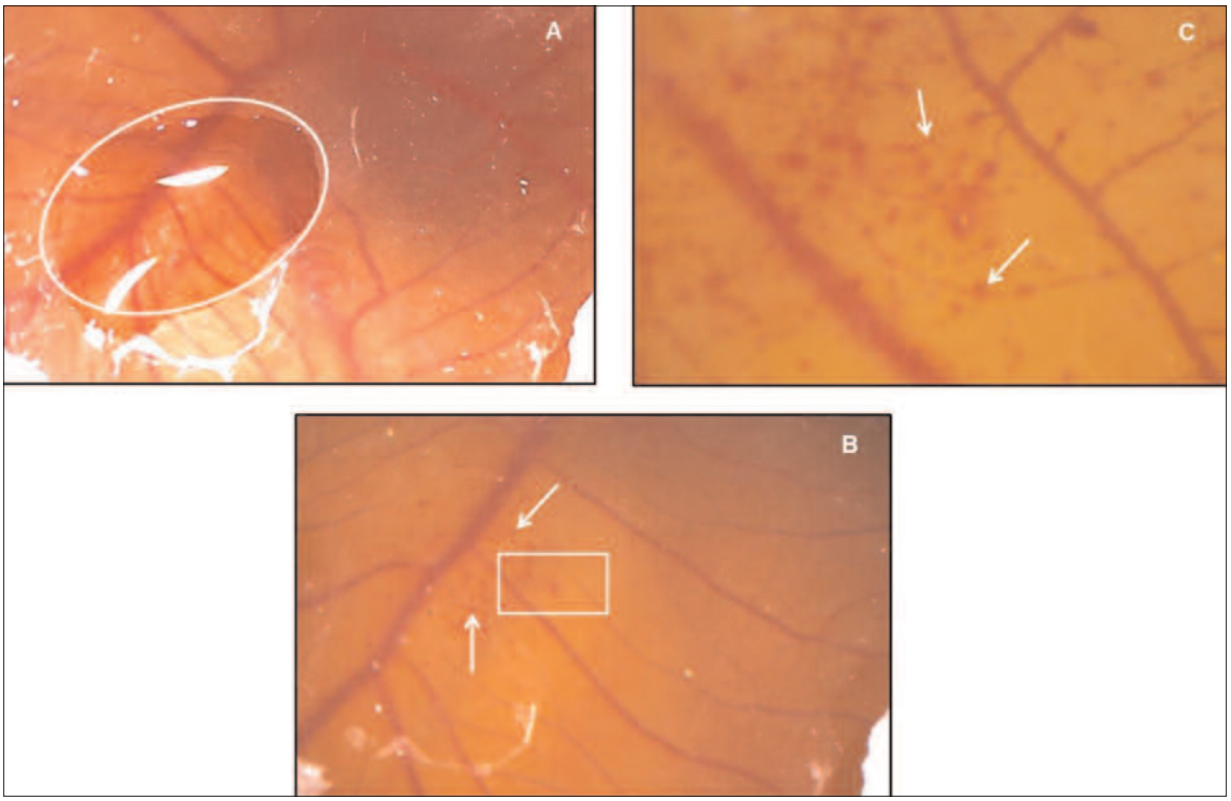

Figure 4. Effect of sildenafil solution on CAM before ***(A)*** and after 24 h ***(B, C)***. ***A,*** and ***B,*** were photographed using ×1.0. ***C,*** was photographed using ×4.0 magnification of the white rectangular area on B. White circle shows the area where sildenafil was placed on first. White arrows show extravasation, budding and sprouting of new vessels from existing vessels. CAM: chorioallantoic membrane.

er of endothelial cells is disrupted in PAH; therefore, the proliferated endothelial cells break the "law of the monolayer"[19]. Dysregulated endothelial cell and myofibroblast proliferation, smooth muscle hypertrophy, intimal fibrosis, and enhanced thrombosis – features evident in obstructive and obliterative arteriolar and multi-channeled "plexiform" lesions of PAH – have been interpreted by some to reflect an underlying process of dysregulated angiogenesis[5].

Recent observations have provided evidence for dysregulated angiogenic activity in PAH. Administration of imatinib, an inhibitor of platelet-derived growth-factor receptor beta (PDGF-Rβ), which regulates smooth muscle proliferation and pericyte recruitment, attenuates PAH in rats, and possibly in humans. In contrast, administering SU-5416, an antiangiogenic inhibitor of vascular endothelial growth-factor receptor and PDGF-Rβ signaling, triggers severe pulmonary vascular remodeling and PAH in rodents. The potent PDGF-Rβ and SRC inhibitor dasatinib attenuates PAH in rats, whereas chronic dasatinib administration has been associated with the development of PAH in man. Angiogenic signaling cascades have important roles in the development and progression of PAH[20].

Tuder et al[7] stated that "The pulmonary vascular growth uses angiogenic molecules in order to expand and cause PAH in rats, therefore, novel broad-spectrum therapies against angiogenesis have been developed. These therapies include thalidomide, angiostatin, endostatin, and thrombospondin among others. One approach is to use these antiangiogenic approaches to treat PAH. One important development of such an approach will be to validate the causal role of the abnormal proliferation of endothelial cells in pulmonary arteries of patients as the most important contributor to PAH".

Endothelial progenitor cells were investigated as a potential treatment option for their possible role in vascular homeostasis and angiogenesis. This area was complicated by a controversy about identification of circulating progenitors and their contribution to angiogenesis. This con-

troversy *ex vivo*-expanded endothelial-like progenitor cells have shown therapeutic benefit in short-term studies in animal models of PAH and in a randomized controlled trial in humans with idiopathic PAH[21-23].

Pathway, target, mechanisms of action, indications, delivery routes, and side-effect profiles of the sildenafil, bosentan and iloprost are well known, but their angiogenic effects are unknown while pathophysiology of PAH is consisting of dysregulated angiogenesis. Although Pyriochou et al[24] demonstrated that sildenafil stimulates angiogenesis through a mitogen-activated protein kinase pathway; we do not have any information about the effect of bosentan and iloprost on angiogenesis and comparison of their effect on angiogenesis so far. In the current study, we demonstrated that while sildenafil has stimulator effect for angiogenesis and proliferative effect for endothelial cells *in vitro* and *in vivo*, both bosentan and iloprost had no similar effects. From this point of view, it can be speculated that long-term exposure to sildenafil may provoke dysregulated angiogenesis by its angiogenic potential. Besides its beneficial effect, may cause worsening effects by triggering angiogenesis.

Conclusions

The results of the current study provide evidence that sildenafil but not iloprost and bosentan induces angiogenesis *in vitro* and *in vivo*. Dysregulated angiogenesis, as an important pathophysiological part in the progression of PAH, may be triggered by the chronic ingestion of sildenafil in the long treatment period and may cause negative effects. However, this must be confirmed by large randomized clinical trials.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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