Původní sdělení | Original research article

The Effect of Lisinopril on Angiogenesis of Endothelial Progenitor Cells (EPCs) in Stable Coronary Heart Disease Post COVID-19

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SOUHRN

Kontext: Infekční onemocnění covid-19 je spojeno s endotelovou dysfunkcí. Ověřuje se představa možnosti opravy endotelové buňky pomocí endotelových progenitorových buněk (endothelial progenitor cell, EPC) s použitím inhibitorů angiotenzin konvertujícího enzymu (ACEI); podle této představy by se tak mohla zlepšit angiogeneze EPC.
Metody: Jedná se o skutečně experimentální *in vitro* studii s uspořádáním s kontrolní skupinou vytvořenou až po testu. Mononukleární buňky byly izolovány z periferní žilní krve pacienta s chronickým koronárním syndromem, který měl v anamnéze infekční onemocnění covid-19. Buňky byly kultivovány ve speciálním médiu po dobu sedmi dní a buňky EPC byly identifikovány imunocytochemicky pomocí protilátek proti buňkám CD34 radioaktivně značeným fluoresceinizothiocyanátem (FITC) s vyšetřením pod fluorescenčním mikroskopem. Buňky byly rozděleny do dvou skupin, kontrolní a do skupiny s aplikací lisinoprilu v dávce 50 μM. Morfologie a spojení tubulů byly zkoumány pomocí analyzátoru obrázků výrobce Wimasis. Koncentrace von Willebrandova faktoru (vWF) a CD31 byly měřeny metodou ELISA, následovanou statistickým srovnáním obou skupin, přičemž za statisticky významnou byla považována hodnota $p < 0,05$.
Výsledky: Po 144hodinové kultivaci byly buňky EPC identifikovány pomocí světelné a fluorescenční mikroskopie. Parametry tvorby tubulů včetně pokryté plochy (29,6 ± 15,68 vs. 61,8 ± 25,41; p 0,13), celkového počtu tubulů 387 ± 101,55 vs. 382,67 ± 158,53; p 0,97), větvení (163 ± 72,52 vs. 179,66 ± 53,5; p 0,543) a celkového počtu kliček (40,66 ± 30,73 vs. 52,66 ± 5,77; p 0,543) nevykazovaly mezi kontrolní skupinou a skupinou s aplikací lisinoprilu žádný rozdíl. Pokud se týče sérologických biomarkerů, hodnoty CD31 se mezi kontrolami a skupinou s aplikací lisinoprilu statisticky významně nelišily (2 903,58 ± 578,08 vs. 3 361,89 ± 391,24; p 0,319), nicméně hodnoty vWF byly statisticky významně vyšší ve skupině s aplikací lisinoprilu (98,670 ± 3,240 vs. 91,181 ± 2,443; p 0,033).
Závěr: U pacientů se stabilní ischemickou chorobou srdeční po prodělaném infekčním onemocnění covid-19 může lisinopril ovlivňovat angiogenezi buněk EPC, jak dokazuje zvyšování hodnot parametrů tvorby tubulů a statisticky významný nárůst koncentrace von Willebrandova faktoru.

Keywords:
Angiogenesis
Coronary heart disease
COVID-19
Endothelial progenitor cells
Lisinopril
Tube formation

ABSTRACT

Background: COVID-19 infection is associated with endothelial dysfunction. The concept of endothelial cell repair utilizing endothelial progenitor cells (EPCs) with the use of angiotensin-converting enzyme inhibitors (ACEIs) has been developing which is known for its potential for EPC's angiogenesis improvement.
Methods: This is a true experimental in vitro study with post-test only control group design. Mononuclear cells were isolated from peripheral venous blood of patient with chronic coronary syndrome and history of COVID-19. The cells then cultured on special media for 7 days and EPC was identified using immunocytochemical examination with labelled anti-CD34 cells FITC-under fluorescence microscope examination. The cells were divided into two groups consisted of control group and 50 μM lisinopril-treated group. The morphology and tube connections were analyzed using Wimasis image analyzer. The von Willebrand factor (vWF) and CD31 concentrations were also measured by ELISA. Statistical comparison between both groups was performed and p-value < 0.05 was considered significant.

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Results: The EPCs were identified after 144 hours culture period using light and fluorescence microscope. Tube formation parameters including covered area (29.6 ± 15.68 vs 61.8 ± 25.41; *p* 0.13), total tube 387 ± 101.55 vs 382.67 ± 158.53; *p* 0.97), branching (163 ± 72.52 vs 179.66 ± 53.5; *p* 0.543) and total loops (40.66 ± 30.73 vs 52.66 ± 5.77; *p* 0.543) showed no differences between control and lisinopril-treated group. In the term of serologic biomarker, CD31 concentration was not significantly different between control and treatment group (2903.58 ± 578.08 vs 3361.89 ± 391.24; *p* 0.319), however, vWF was significantly higher (98,670 ± 3,240 vs 91,181 ± 2,443; *p* 0.033) in lisinopril-treated group.
Conclusion: Lisinopril may affect the angiogenesis of EPCs in patients with stable coronary heart disease post COVID-19 reflected by descriptive increase in tube formation assays and significant increase of the concentration of von Willebrand factor.

Introduction

SARS-CoV-2 virus as the agent causing COVID-19 pandemic has a serious global problem. To date, more than 750 million cases and 6.8 million deaths have been recorded worldwide. The SARS-CoV-2 virus is known to infect lung cells primarily, but evidence suggests it can also attack the extrapulmonary vascular system.[1,2]

Coronary heart disease (CHD) is a chronic cardiovascular disease that is often present in patients with COVID-19. In general, patients with coronary heart disease have a higher risk of complications if infected with the SARS-CoV-2 virus. This is because the expression of ACE2 in the cardiovascular system is higher than in the lungs and causing chronic systemic inflammation through several mechanisms, increasing oxidative stress, platelet activation, and thrombosis, disrupting blood pressure regulation via the ACE2 receptor, and even acute myocarditis can cause cardiac ventricular dysfunction which risks increasing complications of heart attacks, heart failure, arrhythmias, and even death in patients with coronary heart disease after COVID-19 infection.[3–6]

COVID-19 infection causing endothelial dysfunction due to direct or indirect mechanism which causes a cytokine storm. The concept of repairing damaged endothelial cells with endothelial progenitor cells (EPC) has been widely studied, where one agent that has been proven clinically and in vivo to affect the function of EPC are angiotensin-converting enzyme inhibitors (ACEI).[7] Evidence from previous studies also shows that CHD patients experience a reduction in number and migratory function of EPC in the circulation, which results in impaired neovascularization of ischemic tissue.[8]

Methods

This type of research is a true experimental in vitro study with post-test only control group design. This laboratory research was carried out in the Tissue and Cell Bank Installation Laboratory, Diagnostic Center Building, Hospital. Dr. Soetomo Surabaya. The research was carried out in the period from July to August 2023. The research samples were mononuclear cells isolated from peripheral venous blood of patients with stable coronary heart disease aged 30–65 years who met the inclusion criteria which are male gender, aged 40–59 years, diagnosed with stable CHD. Confirmed by the results of coronary angiography which shows a lesion with stenosis ≥ 50% in the left main coronary artery or ≥ 70% in one or more other major coronary arteries based on angiography, has been exposed to COVID-19 infection and is willing to participate in the research procedures and has the informed consent signed. Exclusion criteria were acute myocardial infarction, acute limb ischemia, and a history of coronary artery bypass graft surgery (CABG). Descriptive analysis was carried out by displaying the mean and standard deviation for each group. Tube formation assay was carried out using the Wim Tube software to obtain data on covered area, total tube length, total branching points, total loops, and total nets, while data on VWF and CD31 concentrations were obtained through ELISA examination.

Normality data testing was analyzed with Shapiro–Wilk test. For normally distributed data, statistical test analysis is carried with unpaired numerical comparative test or Mann–Whitney test if it is not normally distributed. It is considered statistically significant if *p* value

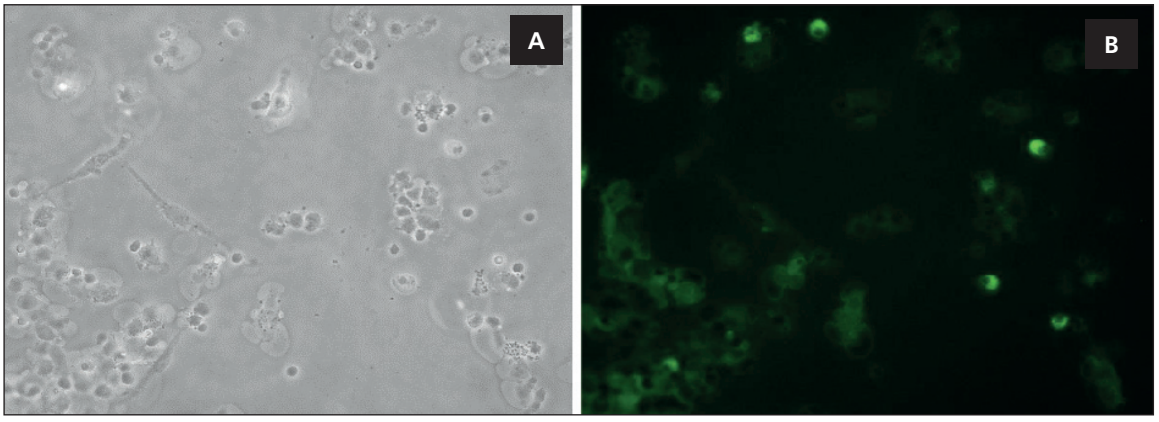

Fig. 1 – (A) Identification of EPCs using a microscope shows cells shaped like spindles. (B) Identification of EPCs using the immunocytochemical staining method.

Table 1 – Demographic data and clinical characteristics of patients

Clinical examination	
Type sex	Man
Age (year)	43
Tall body (cm)	164
Heavy body (kg)	79
Body mass index (kg/m^2)	29.3
Pressure blood (mmHg)	109/71
Rate heart (beats/min) Electrocardiography	65 Sinus rhythm, 62 bpm, normal frontal axis, horizontal axis CCWR
Laboratory results	
Cholesterol – total (mg/dL)	285
Triglycerides (mg/dL)	178
Cholesterol LDL (mg/dL)	142
Cholesterol HDL (mg/dL)	55
Hemoglobin (mg/dL)	14.2
Sugar – blood random (mg/dL)	140
Echocardiography	
Left ventricle ejection fraction (%)	60 (biplane)
Left ventricle internal diameter (cm)	3.8
Wall motion	Normokinetics
Valve	No obtained abnormality
Coronary angiography	
Left playing coronary arteries (LMCA)	Normal
Left anterior descending artery (LAD)	Normal
Left coronary circumflex arteries (LCX)	Critical stenosis 99% in distal LCx
Right coronary arteries (RCA)	Stenosis significant 70% proximal RCA; critical stenosis 99% mid RCA, significant stenosis of the distal 90% of the RCA

is < 0.05. This study already had been by the health research ethics committee of Soetomo General Academic Hospital, Surabaya City, Indonesia (no. 039/Komitlitkes/2022).

Results

The research sample was taken from a 43-year old gentleman with a history of CHD > 1 year, with complaints of stable angina pectoris with grading Canadian Cardiovascular Society (CCS) angina grade scale of II–III already on optimal medical therapy. Clinical subject's characteristical data are shown in **Table 1**.

Identification of EPCs was initially cultured for 7 days and then using the immunocytochemical method using the CD34 marker to identify EPC, where CD34 is one of the positive markers for EPC and found in young and mature EPC. Observations carried out using a fluorescence microscope showed that cells expressing CD34 gave off a green glow (**Fig. 1**).

EPC in the 50 μM lisinopril group (P1) that had been cultured were measured and compared with several assessment criteria against the control group (P0). In the P1 treatment group, there was an increase in covered area more than twice than the control. Total tube, tube length, branching, total loop were also increase with and analyzed by the Wimasis Image program, Also an accumulating concentration of VWF and CD31 were also found with the ELISA technique. The data results obtained for each assessment component are shown in **Table 2**.

Discussion

The role of EPC in revascularizing damaged tissue/ischemia has been proven in animal and human tests which show that endothelial dysfunction will trigger EPC response to the damaged vascular areas to provide endothelial cells for new vessels and stimulate growth factors to activate surrounding cells. Asahara et al. first discovered EPC in bone marrow and peripheral blood isolated based on the CD34 marker. These cells then differentiate into mature endothelial cells on a platform of fibronectin and angiogenic factors. EPCs were obtained through isolation of cultured peripheral blood cells, identified based on CD34 binding.[9,10]

One stage of the physiological process known as angiogenesis, the emergence of new vasculature originating from pre-existing cells within the vasculature, is the tube formation. At this stage, contact occurs between endothelial cells, which causes the formation of a lumen structure that resembles a tube, which is an organized network of blood vessels.[11] Methods for assessing tube formation in in vitro studies include the use of *inverted microscopes* for phase contrast and the use of software

Table 2 – Statistical test results for EPC sample variables

	Covered area	Total tube	Tube length	Branching	Total loops	Concentration VWF	Concentration CD31
P0	29.6 ± 15.68	387 ± 101.55	30921 ± 9398.77	163 ± 72.52	40.66 ± 30.73	91,181 ± 2,443	2903.58 ± 578.08
P1	61.8 ± 25.41	382.67 ± 158.53	33371.67 ± 5837.19	179.66 ± 53.5	52.66 ± 5.77	98,670 ± 3240	3361.89 ± 391.24
p	0.130	0.970	0.313	0.765	0.543	0.033*	0.319

P0 – control group; P1 – EPC + lisinopril 50 μM group.
* $p < 0.05$: statistically significant.

that can count tube numbers, lengths, loops, and branching points. The WimTube application was used in this study to collect quantitative data from pipes formed between groups.

In this study, both the control group and the llisinopril group showed tube formation. This suggests that EPCs isolated from peripheral blood play a role in tube formation. In addition, Lisinopril administration had no impact on EPC culture tube formation in comparison with the control group, though lacking statistical significance. Although not statistically significant, the lisinopril group showed an increase in several tube formation parameters, such as covered area, number of tubular structures, overall length of tubes, total branching points, and total loops.

Once cell culture was initiated, Corning Matrigel basement membrane matrix was administered, according to the Embedded 3D (Corning Matrigel Basement Membrane Matrix) culture protocol. Pictures of tube formation in both groups were taken on the fourteenth day after administration of basement membrane matrix extract (BME). This differs from the protocol used by DeCicco-Skinner, where BME administration was performed on the fourteenth day of cell culture. Results are recorded for 2, 4, 6, and 24 hours. Studies show that tube formation begins within two to four hours after BME administration, and peak tube formation may occur within three to twelve hours, depending on the concentration of angiogenic factors in the medium. The life of this tube is limited to 18 hours and begins to break down due to the death of endothelial cells.[12]

The study conducted by Zhao et al. researched the effect of Naringin, an active ingredient derived from the Chinese herbal plant *Drynaria fortunei* on endothelial progenitor cells. Results indicate that naringin promotes the proliferation of EPCs and the formation of tubes facilitated by the CXCL12/CXCR4 pathway axis, suggesting its potential as a therapeutic agent for ischemic diseases.[13] Tube formation was recorded at 12 hours of observation after administration of the basement membrane matrix. ImageJ software was used for quantification of pipe formation images. The results showed that administration of naringin increased pipe length and area significantly ($p < 0.05$). Images of pipe formations were documented with an inverted microscope. However, as shown in this study, the pipe formation does not have a co-relation between naringin and the control group. This shows that several factors influence optimal pipe formation and formation assay. Some of these are the concentration of angiogenic factors in the preconditioning medium, variations in the optimal duration for tube formation, and inhomogeneous protocols.

A number of growth factors contribute to each stage of angiogenesis, such as VEFG and FGF are growth factors that play a role in the tube formation process. The study by Soro et al. which shows that peptides derived from vascular endothelial growth factor receptor-1 (VEGFR-1) can induce tube formation in vitro. VEGFR-1 is also a tyrosine kinase receptor for growth factors of the VEFG family.[14] The in vivo study by Ebrahimian et al. conducted in animal trials on ischemic limbs using ACEI (perindopril; candesartan), angiotensin 1 receptor blocker (ARB) showed an increase in VEFG protein levels by 1.4-fold. Nevertheless, this proangiogenic effect of ACEI was not seen in subjects with B2R deficiency, indicating that bradykinin pathway may have proangiogenic effects of ACEI.[15]

The glycoprotein Von Willebrand factor (VWF) is essential for hemostasis and is a specific marker of mature endothelial cells. VWF expression is related to the number of functional endothelial cells and plays a role in angiogenesis. A number of adhesion molecules, including integrins and VWF, influence growth factor signaling pathways. VWF is a protein in the blood that helps platelets aggregate to the walls of blood vessels, surface, and also functions for circulating factor VIII in the circulation.[16] In this study, the VWF concentration of the lisinopril group was more elevated ($p < 0.05$) indicating that there were probably more functional mature endothelial cells affected by lisinopril.

The immunoglobulin gene consists of a transmembrane glycoprotein called CD31 which is widely expressed on endothelial cells and is also expressed in different levels on hematopoietic cells such as platelets, granulocytes and monocytes. CD31 expression is related to the angiogenic potential of cells.[17] In this study, the CD31 concentration of lisinopril group exhibited higher levels compared to the control group with statistically significant value ($p > 0.05$). This indicates that cultured EPC cells have angiogenic properties, both with and without lisinopril.

Limitations of the study

This study has limitations related to the standard protocol for assaying tube formation, especially regarding the time of observation of tube formation, limited resources, and limitations to continue measuring proangiogenic factor markers, so it cannot identify the angiogenic factor signaling pathways involved in tube formation.

Conclusions

There was an increase in VWF concentration in EPCs cultures given 50 μM lisinopril, while an increase in tube formation and CD31 concentration also occurred but did not show statistical significance. The results provide data to supports the possible influence of ACEI (lisinopril) on EPCs in patients with stable coronary heart disease post COVID-19.

Highlights

This study showed the effect of Lisinopril increase in VWF concentration in EPCs cultures, thus making it possible to increase angiogenesis factor in patients with stable coronary heart disease post COVID-19.

An increase in tube formation did occur, suggesting the possibility of llisinopril influence on EPCs in patients with stable coronary heart disease post COVID-19.

An increase of CD31 concentration in treated group suggesting the possibility of lisinopril influence on EPCs in patients with stable coronary heart disease post COVID-19.

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Competing interests
The authors declare that they have no competing interests.

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Ethical statement
This research has been approved by the Health Research Ethics Committee of the Dr. Soetomo General Academic Hospital, Surabaya, Indonesia. The ethic number is No. 039/Komitlitkes/2022.

Availability of data and materials
All data are available in the manuscript

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