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Effect of Ascorbic Acid, α-Tocopherol and Its Combination on *Human Umbilical Vein Endothelial Cells* Permeability Exposed to High Concentration of Glucose

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Abstract: *Aim* - To evaluate effect of ascorbic acid, α-tocopherol and its combination on the permeability of human umbilical vein endothelial cells (HUVEC'S) culture exposed to high concentration glucose. *Material and Methods* - The study was performed using HUVEC'S culture on transwell polyester filter where ascorbic acid, α-tocopherol and its combination at a dose of 25-200 µM were given concomitantly with glucose 22 mM on culture media. After seven days of treatment, endothelial permeability was observed by measuring the concentration of tryphan blue-labelled albumin in a luminal compartment with spectrophotometer λ= 592 nm. The morphology observed under an inverted microscope. The data was analyzed using ANOVA test continued with Dunnet test ($p<0,05$). *Results* - HUVEC'S culture exposed to high concentration glucose increased endothelial permeability compared to those exposed to normal concentration glucose ($p<0.05$). Administration of ascorbic acid 200 µM, α-tocopherol 100 µM and 200 µM were able to inhibit increasing of HUVEC'S permeability exposed to high concentration glucose compared to control group ($p<0.05$). Meanwhile two combinations of ascorbic acid and α-tocopherol at a dose of 50 and 100 µM could prevent the increase permeability of HUVEC'S culture compare to control group ($p<0.05$). *Conclusion* - Ascorbic acid, α-tocopherol and its combination could inhibit the increase of endothelial permeability exposed to high concentration of glucose.

Keywords: ascorbic acid, α-tocopherol, permeability, endothelial cells, high glucose

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Introduction

Diabetes Mellitus (DM) is a metabolic syndrome characterized by chronic hyperglycemia due to a disorder in insulin secretion or insulin sensitivity or both [1]. Hyperglycemia results in DM complications through a mechanism that increases the production of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). These are categorized as free radical compounds, and over production of it without a sufficient antioxidant defense would cause oxidative stress conditions [2]. This condition causes vascular endothelial cell damage and results in macroangiopathy and microangiopathy complications on DM [3].

Vascular endothelial cells act as a selective barrier to prevent the transfer of some substances on the vascular system. The structure of endothelial cells is composed of actin-based cytoskeleton which relates to its function as barrier permeability [4]. Endothelial barrier consists of an actin filament, stress fibers, junction-associated actin filament system and adhesion molecule which maintain the cells permeability against solutes, solvents, hormones and macromolecules [5]. Permeability of endothelial cells are also controlled by signaling mechanism which regulate cellular activity such as calcium (Ca) ions, protein kinases and phosphatases [6].

ROS produced in a hyperglycemic condition could destroy the structure and function of endothelial cells through oxidative stress [6]. Accumulation of ROS results intercellular gaps, actin filaments disorganization and the change of cell shape and therefore increasing paracellular permeability [7]. ROS decreases trans-endothelial electric resistance and increase trans-endothelial permeability against plasma proteins such as albumin. Oxidative stress steps up Ca cytosol level which increases endothelial permeability through cell contraction, gap junction formation and actin filament disorganization [8].

Antioxidant works to neutralize free radicals and reduce oxidative stress damage. Ascorbic acid and α-tochopherol are anti-oxidants that have different characteristics. α-tochopherol is a lipid soluble antioxidant protecting cells membranes against free radical, whereas ascorbic acid is water soluble which works extracellularly [9,10]. When antioxidant scavenge ROS, they result in radical substances and therefore increase oxidative stress condition [11]. The use of antioxidant combination is more recommended compared to a single antioxidant as it reduces more oxidative stress damage. Antioxidant combination is expected to increase the efficacy and reduce the toxicity or adverse reaction [12]. As antioxidants, ascorbic acid and α-tochopherol has not been evaluated on endothelial permeability, especially when combined.

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Aim of study: to explore the effect of ascorbic acid, α-tochopherol and its combination on HUVEC'S culture exposed to high glucose concentration.

Material and Methods

Chemical Uses

Ascorbic acid, α-tochopherol, collagenase type II, glucose, Hank's Balance Salt Solution (HBSS), New Calf Serum (NCS), serum free medium, trypsin EDTA and albumin labeled tryphan blue. All chemicals were purchased from Sigma aldrich.

HUVEC's culture

This research protocol was also approved by the institutional research ethical committee from the Faculty of Medicine Brawijaya University, Malang Indonesia. Human umbilical vein endothelial cells (HUVECs) were obtained from umbilical cord of neonates that fulfill inclusion criteria in some private hospital, Malang, after obtaining informed consent. Procedure of HUVEC's culture based on Jones 1996 with slightly modifications. Isolation of endothelial cells by collagenase type II in serum free media and incubated for 7 minutes. Next, the media solution was centrifuged, and pellet was obtained containing endothelial cells to culture in flasks containing M199 media and was put in an incubator at 37°C and with 5% CO2. After the cell grew a monolayer, a passage or subculture step was performed using trypsin solution and the cells were transferred on to transwell polyester filter. Treatment of both glucose (5 mM : 20 mM) and antioxidants were given concomitantly in culture media for seven days after the cells grew a monolayer. Single composition of both ascorbic acid and α–tocopherol were given each of them in three dose, they are 50 µM, 100 µM, 200 µM meanwhile in combination used composition I (25 µM : 25 µM), II (50 µM : 50 µM) and III (100 µM : 100 µM)

Barrier endothelial assay

HUVEC's barrier assay was based on Lum and Roebuck, 2001 with slight modification. Endothelial permeability to albumin was tested on two compartments separated by a filter membrane. Both of the compartments contain thyrode's solution with slight modification by adding NCS 10 %. Luminal compartment contained endothelial cell on transwell polyester filter and 0.2 ml albumin labeled with tryphan blue 31,44 ppm was added in this compartment. Whereas albuminal compartment contained tyrode's solution and was contiunously stirred with a magnetic stirrer. After 30 minutes, solution in albuminal compartment was taken and measured for albumin labeled tryphan blue level by spectrophotometer with λ= 592 nm.

Histopathology of endothelial cells

Endothelial cells (HUVEC's) culture was observed and noted for shape, size, and number at each view under a light microscope with 400x magnification.

Statistical Analysis

All data are expressed as mean ± SD. The statistical analysis of the data was performed by One Way ANOVA test and then followed by Dunnet test with significant value ($p < 0.05$) using SPSS.

Results

Effect of antioxidant ascorbic acid, α-tochopherol and its combination on permeability of HUVEC's culture

Barrier permeability of HUVEC's culture exposed by normal or high concentration glucose and antioxidant are shown in figure 1.

[Figure 1 here]

Ascorbic acid 200 µM, α-tochopherol 100 µM and 200 µM decreased endothelial permeability to 50 %, 50 % and 40 % respectively compared to control group ($p<0.05$). Meanwhile, combination of ascorbic acid and α-tochopherol 1 (50 µM : 50 µM) and α-tochopherol II (100 µM : 100 µM) reduced barrier permeability of endothels to 60% and 50% respectively compared to the control group ($p<0.05$). Administration of the second combination of antioxidant decreased endothelial permeability until it is not different compared to normal group ($p>0.05$). Whereas, HUVEC's culture exposed to high concentration glucose increased its permeability three times fold compared to normal concentration glucose ($p<0.05$).

Effect of ascorbic acid, α-tochopherol and its combinations on structure of HUVEC's culture exposed high glucose concentration

Morphology of HUVEC's culture exposed normal or high concentration glucose and antioxidant are shown in figure 2.

[Figure 2 here]

HUVEC's culture exposed to glucose at normal concentrations showed monolayer cells having a cobblestone shape and a smoothing of cells membran. Whereas, on high concentration glucose, cell apoptosis was observed, characterized by cell shrinkage and nucleus condensation. Meanwhile, administration of ascorbic acid, α-tochopherol and its combination had more potential to protect cells against oxidative damage on endothelial cells exposed high concentration glucose.

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Discussion

Effect of high concentration glucose expose on permeability and morphology of HUVEC's culture

Exposure of high concentration glucose on HUVEC's culture increased cell permeability. Glucose in high concentrations is oxidizied into reactive dicarbonyl sugar which could bind to amino-lipids and proteins of endothelial cell, such as VE-cadherin [8]. Glycation of protein can destroy VE-cadherin structure, therefore causing it to lose their elasticity and function as adhesion molecule. It results in barrier function impairment and detachment of endothelial cells [13]. Based on figure 2, high concentration of glucose on HUVEC's culture increases cell death and cell apoptosis, characterized by shrinkage and nucleus condensation, causing an increase in endothelial permeability. Exposure of glucose at high concentration increases hydrogen peroxide radicals and Ca cytosol level, the highest level of both occurs at the seventh days [14,15]. The increase of cytosolic Ca levels activates phospholipase-C and results in Myosin Light Chain Kinase (MLCK) phosphorylation which initiates cytoskeleton contraction and increases endothelial permeability [6]. On the seventh days of treatment, hydrogen peroxide radical disrupts actin filament which is signaled by an increase of actin depolymeration (G-actin) controlling endothelial barrier function [16,17]. Based on the previous study, permeability assay was performed on the seventh day [6].

HUVEC's culture exposed to high concentration of glucose showed an increase of cell permeability compared to normal concentration. The morphology of endothelial cells exposed to glucose at normal concentration is monolayer, polygonal (cobblestone) shape and smooth of cell membrane. Staining with anti-actin antibodies showed actin filament to be well arranged, whereas, exposure to high concentration of glucose on HUVEC's culture caused a disorganization of actin filament [16]. This structure correlated with the barrier function, in which HUVEC's permeability exposed to glucose at normal concentration was lower than high concentration of glucose. Hiperglycemia condition is analog with high concentration glucose exposure which increases anion superoxide and Protein Kinase C (PKC) activation [9,17]. Increase of PKC induces Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase to produce superoxide radical and stimulate expression of endothelial adhesion molecule resulting in the dysfunction of endothelial barrier [17,18]

Effect of antioxidant ascorbic acid on permeability and morphology of HUVECS culture

Ascorbic acid 200 µM reduces endothelial cells permeability when exposed to high concentration glucose. Ascorbic acid is a general antioxidant scavenging ROS and RNS at liquid phase, especially in cytosol, and therefore protects cell damage caused by free radicals [12]. These include hydroxyl radicals, peroxyl radicals, superoxide anions, nitrogen dioxides and also non-radical species, such as oxygen singlets and nitroxide peroxynitrics [2,12]. Ascorbic acid prevents nitrosation process due to free radicals on target molecules. It can neutralize ROS effect and therefore avoiding the damage of actin filament structure controlling barrier function [16]. Ascorbic acid can also protect VE-cadherin structure regulating endothelial permeability. As VE-cadherin produces complex compound with α-catenin and is connected by cytoskeleton, disconnection of junction with endothelial cells can be prevented [4,20]. In physiologic concentrations, ascorbic acid can inhibit oxidized-Low Density Lipoprotein (LDL) on vascular cells and neutrophil [2,6]. Ascorbic acid can also inhibit modification of LDL oxidative through the scavenging of free radical and others reactive species [11]

Ascorbic acid is entering the cell cells via two transporter system, which are Na dependent Transporter and Glucose Transorter-1 (GLUT-1). Disorders of transporter system results in the decrease of antioxidant defense in intracellularly. The structure of Dehydroascorbic (DHA) is similar to glucose, therefore competes to be transported by GLUT-1 into cytoplasma [21]. Differences in physical and chemical properties of ascorbic acid can affect their potency as antioxidants in decreasing endothelial permeability when exposed to high concentration glucose [12]. Increasing the dosage of ascorbic acid up to 200 µM results in antioxidant entered into intracellularly after competing with glucose at 22 mM, and therefore this dose shows a real effect in increasing HUVEC's permeability exposed to high concentration glucose. The morphology of cells showed cells membrane to be smooth and no damage to cells membrane was found.

Ascorbic acid 50 µM and 100 µM did not significantly decrease endothelial permeability. Low doses of ascorbic acid cannot protect cells against oxidative damage and therefore resulting in the disorder of endothelial permeability. This was signaled by the passage number of albumins which was high in albuminal compartment. On the other hand, low dose ascorbic acid cannot be transported to intracellularly effectively due to it failure to compete with high concentration glucose [12]. Interaction between ROS and ascorbic acid results in DHA through ascorbil radical intermediates. Ascorbic acid is an effective antioxidant scavenger, since, first, both of ascorbic acid and ascorbic radical have a low reduction potential therefore they could react with all of radical and biology oxidant. Secondly, ascorbic radical has a low reactivity due to resonancy stability on free electron [11]. Besides it, ascorbic acid could be regenerated from ascorbic radical or DHA through the reaction of both of dependent and independent enzymatic. Radical ascorbic is reducted by NADPH dependent semi dehydroascorbate reductase and NADPH dependent selenoenzyme thioredoxin reductase [2,11]. Whereas, DHA is changed into ascorbic by non-enzymatic by Glutathione (GSH) and lipoic acid. On the other hand, interaction between ascorbic acid and with metal transition results in a negative effect since it produces hydroxyl radical and alchoxy lipid trough metal ion reaction with hydrogen peroxydes [11,21].

Effect of α-tochopherol on permeability and morphology of HUVECS culture exposed high concentration of glucose.

α-tochopherol 100 µM and 200 µM decreases endhothelial permeability exposed to high concentration glucose. Antioxidant α-tochopherol works by inhibiting lipid peroxidation and lipoprotein damage on cells membrane especially at the lipid phase. α-tochopherol is a lipid soluble antioxidant working in the lipid bilayer of cells membrane, and functions by stop chain reactions, starting by scavenging peroxyl and inhibiting free radical production such as oxygen singlet on cell membrane, and therefore lipoproteins can be protected against oxidation [2,10]. α-tochopherol as an extra cellular antioxidant can neutralize the effects of ROS and protects cell membrane against radical, moreover damage of actin filament and VE-cadherin controlling endothelial

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barrier function could be avoided [19]. α-tochopherol prevents lipid peroxidation on Poly Unsaturated Fatty Acid (PUFA) of cells membranes and minimize LDL oxidation. Besides that, α-tochopherol regulates membrane fluidity, stabilizes membrane and inhibits 5-lypoksigenase activity [Redana, 19]. Lipid peroxidation is initiated trough hydrogen bisalilic on lipid by peroxyl radical resulting lipid and peroxide radical. Oxygen results in peroxyl lipid radical production and lipid peroxidation propagation through chain reaction. α-tochopherol reduces lipid peroxidation through two mechanics, a chain bounding of lipid radical carrier or scavenging of peroxyl radical [2,12].

α-tochopherol at a 100 μM dosage was stronger in inhibiting permeability increase compared 200 μM, based on albumin concentration in the albuminal compartment. This is caused by α-tochopherol 100 μM having a higher activity in protecting the stability of cell membrane lipoproteins from damage due to ROS. Morphology of cells on the figure 2 showed that cells membrane was smooth and no damage to the cell was found. Increasing the dose of α-tochopherol to 200 μM precisely increases endothelial permeability. When acts as antioxidant, α-tochopherol will be oxidized into tochopheryl and this radical form is physiologically converted into tochopherol [10]. Administration of α-tochopherol 200 μM possibly produces tochopheryl radical in a huge concentration which cannot be regenerate by physiologic mechanism. Tochopheryl radical will destroy actin filaments and VE- cadherin structure which moreover results in the dysfunction of the endothelial barrier. Combination with ascorbic acid is suggested in α-tochopherol usage especially in high doses [12,19].

Single administration of α-tochopherol and ascorbic acid at the same dose (100 μM) showed a different effect on endothelial permeability. α-tochopherol was more potent in protecting the barrier function of endothelial cells exposed high concentration glucose compared to ascorbic acid. α-tochopherol works through dual action both of as a direct and indirect antioxidant. Direct antioxidant mechanism through inhibition of free radical and production of Advance Glycate End Product (AGE), stop chains reaction production whereas indirect antioxidant work by the inhibition of Protein Kinase C (PKC) activation [10]. α-tochopherol was more effective in inhibiting the increase of endothelial permeability exposed to high concentration glucose compared to ascorbic acid. This is caused by α-tochopherol works through dual action whereas ascorbic acid as general antioxidant works scavenge free radical only [12].

Effect combination of ascorbic acid and α-tochopherol on HUVECS culture exposed high glucose concentration

Combination of ascorbic acid and α–tochopherol at dosage 50 μM and 100 μM reduced the permeability of endothelial cells exposed high concentration glucose. The use of ascorbic acid and α–tochopherol combination results in a pharmacodynamic interaction and a synergistic effect to protect cells against free radicals. Aside from that, the combination of antioxidants aims to reduce the doses of both of them and therefore adverse reaction can be minimized [23,24]. α–tochopherol is a lipid soluble antioxidant working on lipid bilayer of cells membranes to inhibit both of chain reaction and free radical production [10,19]. Whereas, ascorbic acid as a general antioxidant works on liquid phase of cytosol to protect cells trough ROS scavenger mechanism [11,12]. They reduce radical reactivity and therefore actin filament structure and VE cadherin moreover endothelial barrier function could be retained. Cell morphology after given antioxidant combination showed cells membrane to be smooth and there are not damage cells. How α–tochopherol and ascorbic acid work as antioxidants have been explained on previously section. The combination of α–tochopherol and ascorbic acid have a beneficial effect, in which tochopheryl radical can be regenerated into tochopherol by ascorbic acid and therefore reduces stress oxidative [12,22].

Based on this study, antioxidant combination needs a lower dose compared to single antioxidant to retain of endothelial barrier function. Ascorbic acid 50 μM and α–tochopherol 50 μM in single composition have not showed inhibition of permeability increase of HUVECS culture. Whereas, in combination of ascorbic acid 50 μM and α–tochopherol 50 μM could reduce the increase of endothelial permeability until it was not different compare to normal group.

The combination of ascorbic acid 100 μM and α–tochopherol 100 μM could prevented permeability increase of HUVECS culture, however it was lower compare to at dose each of 50 μM. The increase of dosage possibly changes it into a pro-oxidant effect due to the production increase of ascorbil and tochopheryl radical [11,12]. Disorder of transporter system of ascorbic acid into cytoplasma have a potency to cause lipoprotein damage of cells membrane [6,11]. They have a function as endothelial barrier therefore the HUVEC's permeability will increase. Metal transition also contribute on the increasing of ROS production beside the expose of high concentration of glucose [2].

Ascorbic acid 25 μM and α–tochopherol 25 μM in combination did not significantly decreases endothelial permeability. This was caused by the low doses of its combination which cannot protect cells against oxidative damage therefore increase endothelial permeability [24]. This was signaled by the passage number of albumins which was high in albuminal compartment.

Our studies showed that the exposure of high concentration glucose on HUVEC's culture increased the endothelial permeability, while ascorbic acid, α-tocopherol and its combination potentially have protective properties by inhibit the increase of endothelial cells permeability exposed high concentration of glucose.

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Conflict of interest

We declare that we have no conflict of interest

Funding

No Funding

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Ethical Concern

"This article does not contain any studies with animals performed by any of the authors."

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Legends

Figure 1. Potency ascorbic acid, α-tochopherol and its combination on permeability of HUVECS culture exposed high concentration glucose ; T1:Glucose 5 mM, T2: Glucose 22 mM, T3: Glucose 22 mM+ Vit C 50 µM, T4: Glucose 22 mM+ Vit C 100 µM, T5: Glucose 22 mM+ Vit C 200 µM, T6: Glucose 22 mM+ Vit E 50 µM, T7: Glucose 22 mM+ Vit C 100 µM, T8: Glucose 22 mM+ Vit C 200 µM, T9: Glucose 22 mM+ Vit C 25 µM + Vit E 25 µM, T10: Glucose 22 mM+ Vit C 50 µM + Vit E 50 µM, T11: Glucose 22 mM+ Vit C 100 µM + Vit E 100 µM

Figure 2. Potency ascorbic acid, α-tochopherol and its combination on structure of HUVEC's culture exposed high concentration glucose. (M=200x); (A). 5 mM glucose: Polygonal / cobblestone-shaped cells and fine-looking cell membranes / smoothing. (B). Glucose 22 mM: Cells undergoing apoptosis characterized by shrinkage cells and core condensation. (C). G22 + VIT C.50 + VIT. 50 µM: Cells and cell membranes do not show structural damage. (D). G22 + VITE.100 µM; The cell membrane is fine and the polygonal cells do not appear to be structural damage. (E). G22 + VIT C.100 µM: Cell membrane is not smooth due to membrane lipoprotein damage (F). G22 + VITC.25 + VIT E. 25 µM: The cells undergo apoptosis and the nucleus is condensed.

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Figures

Figure 1

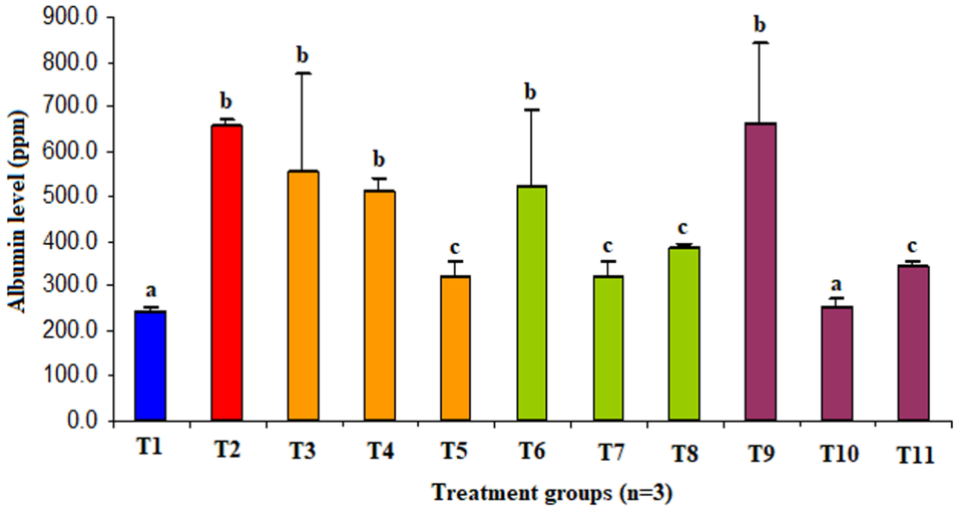
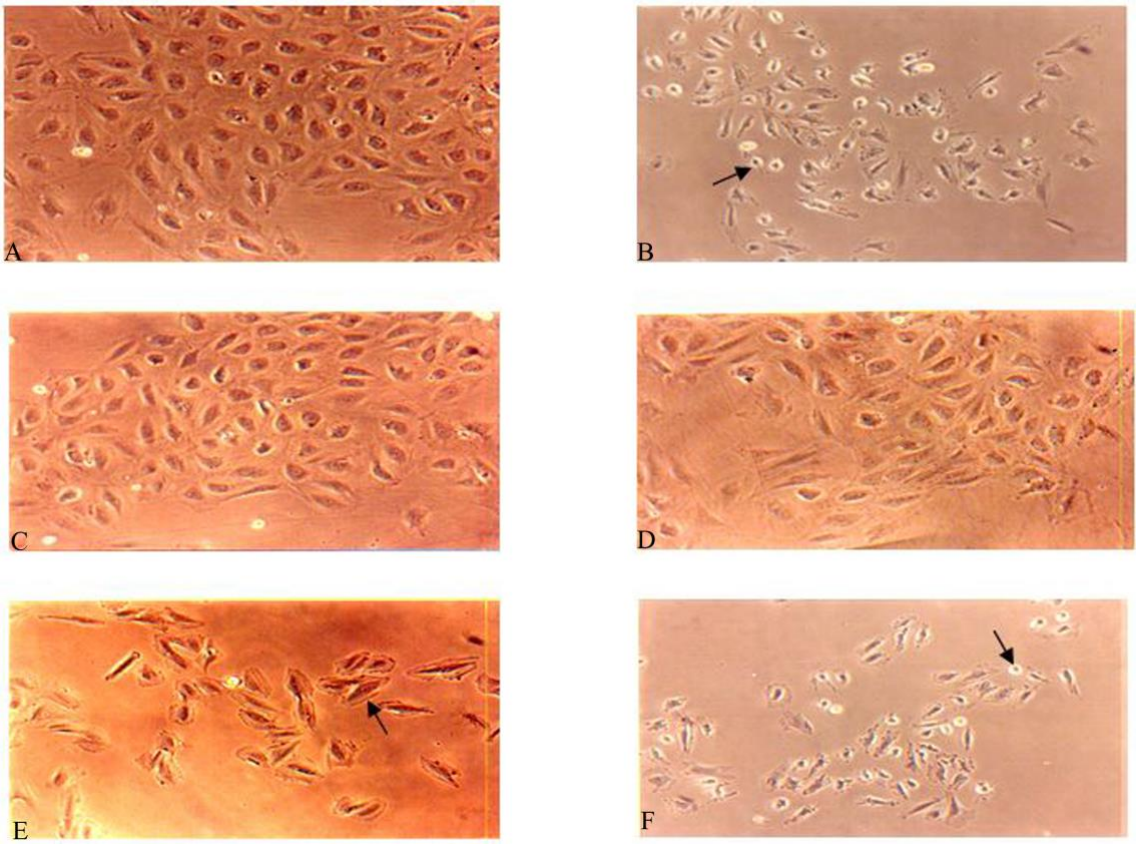


Figure 2



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M. Aris Widodo – Prof. dr. MS.SpFK, Ph.D Head of Doctoral Post Graduate Study in Medicine at Department of Pharmacology, School of Medicine, Brawijaya University, Jl. Veteran, Ketawanggede, Lowokwaru, Ketawanggede, Lowokwaru, Malang, East Java 65145.

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Point-to-point responses

Reviewer 1

No	Issues	Responses
1	All abbreviations should be deciphered at the first mention in the text	Page 2 Line 68 It has been revised Page 3 Line 125 It has been revised
2	How much material was included in the analysis? This needs to be understood in order to evaluate statistical analysis, as the authors use data dispersion measures.	Page 2 Line 71-89 They have been revised
3	Quantitative variables were assessed for compliance with the normal distribution? Was this taken into account when analyzing the data?	Page 2 Line 96-97 The data normal and homogen threfore they are analysed by one way anova
4	For all devices and materials, you must specify the manufacturer and country of origin	Page 2 Line 67-93 They have been revised.
5	Why is HUVEC'S culture not discussed in the Introduction? It appears immediately in the goal.	Page 1 Introduction It has been added.
6	The article has so few results and so much discussion. It is necessary to concentrate on comparing your own results and other studies. The scientific novelty of the research should be evident from the Discussion section. Some paragraphs in the current version are better suited for the review article.	Page 3 Discussion It has been revised
7	Where is the section Conclusion?	Page 4 It has been revised

Assistant Editor

No	Issues	Responses
1	The imprecision in figures as regards quality, resolution and indistinctness do not support their publication in the present form. You are therefore requested to send better quality figures in TIFF or JPG versions. And, the figures must be prepared according to the following instructions: Width ≥8.5 inches; Height ≥11 inches; Pixels/ inches ≥600 DPI. Please have these figures improved either, yourself	Done

	or by professional graphic designers that may be in your organization/ country.	
2	Statement of human rights. When reporting studies that involve human participants, authors should include a statement that the studies have been approved by the appropriate institutional and/or national research ethics committee and have been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. If doubt exists whether the research was conducted in accordance with the 1964 Helsinki Declaration or comparable standards, the authors must explain the reasons for their approach, and demonstrate that the independent ethics committee or institutional review board explicitly approved the doubtful aspects of the study. The following statements should be included in the text before the References section: "Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards." For retrospective studies, please add the following sentence: "For this type of study formal consent is not required."	Ethical approval already added
3	Statement on the welfare of animals. The welfare of animals used for research must be respected. When reporting experiments on animals, authors should indicate whether the international, national, and/or institutional guidelines for the care and use of animals have been followed, and that the studies have been approved by a research ethics committee at the institution or practice at which the studies were conducted (where such a committee exists). For studies with animals, the following statement should be included in in the text before the References section: "Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed." If applicable (where such a committee exists): "All	DONE Page 5 Line 246-247 This study did not involve any animals but parts of human body (umbilical cord) did involved. All of the methods were approved by the institutional research ethical committee from the Faculty of Medicine Brawijaya University, Malang Indonesia. Therefore, the following statement was added in the text before reference section: "This article does not contain any studies with animals performed by any of the authors."

	procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted." If articles do not contain studies with human participants or animals by any of the authors, please select one of the following statements: "This article does not contain any studies with human participants performed by any of the authors." "This article does not contain any studies with animals performed by any of the authors." "This article does not contain any studies with human participants or animals performed by any of the authors."	
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Article type Original Research

Effect of Ascorbic Acid, α-Tocopherol and Its Combination on *Human Umbilical Vein Endothelial Cells* Permeability Exposed to High Concentration of Glucose

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Abstract: *Aim* - To evaluate effect of ascorbic acid, α-tocopherol and its combination on the permeability of human umbilical vein endothelial cells (HUVEC'S) culture exposed to high concentration glucose. *Material and Methods* - The study was performed using HUVEC'S culture on transwell polyester filter where ascorbic acid, α-tocopherol and its combination at a dose of 25-200 µM were given concomitantly with glucose 22 mM on culture media. After seven days of treatment, endothelial permeability was observed by measuring the concentration of tryphan blue-labelled albumin in a luminal compartment with spectrophotometer λ= 592 nm. The morphology observed under an inverted microscope. The data was analyzed using ANOVA test continued with Dunnet test ($p<0,05$). *Results* - HUVEC'S culture exposed to high concentration glucose increased endothelial permeability compared to those exposed to normal concentration glucose ($p<0.05$). Administration of ascorbic acid 200 µM, α-tocopherol 100 µM and 200 µM were able to inhibit increasing of HUVEC'S permeability exposed to high concentration glucose compared to control group ($p<0.05$). Meanwhile two combinations of ascorbic acid and α-tocopherol at a dose of 50 and 100 µM could prevent the increase permeability of HUVEC'S culture compare to control group ($p<0.05$). *Conclusion* - Ascorbic acid, α-tocopherol and its combination could inhibit the increase of endothelial permeability exposed to high concentration of glucose.

Keywords: ascorbic acid, α-tocopherol, permeability, endothelial cells, high glucose

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Introduction

Diabetes Mellitus (DM) is a metabolic syndrome characterized by chronic hyperglycemia due to a disorder in insulin secretion or insulin sensitivity or both [1]. Hyperglycemia results in DM complications through a mechanism that increases the production of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). These are categorized as free radical compounds, and over production of it without a sufficient antioxidant defense would cause oxidative stress conditions [2]. This condition causes vascular endothelial cell damage and results in macro~~angiopathy~~ and micro angiopathy complications on DM [3].

Vascular endothelial cells act as a selective barrier to prevent the transfer of some substances on the vascular system. The structure of endothelial cells is composed of actin-based cytoskeleton which relates to its function as barrier permeability [4]. Endothelial barrier consists of an actin filament, stress fibers, junction-associated actin filament system and adhesion molecule which maintain the cells permeability against solutes, solvents, hormones and macromolecules [5]. Permeability of endothelial cells are also controlled by signaling mechanism which regulate cellular activity such as calcium (Ca) ions, protein kinases and phosphatases [6]. Human umbilical vein endothelial cells (HUVEC'S) culture was used in this study due to it has several advantages. The culture is an *in vitro* model of vascular endothelial that can be used to evaluate both of its structure and function. The use of this culture more represent the condition in human, effective both in time and cost, reliable and reproducible [4].

ROS produced in a hyperglycemic condition could destroy the structure and function of endothelial cells through oxidative stress [6]. Accumulation of ROS results intercellular gaps, actin filaments disorganization and the change of cell shape and therefore increasing paracellular permeability [7]. ROS decreases trans-endothelial electric resistance and increase trans-endothelial permeability against plasma proteins such as albumin. Oxidative stress also steps up Ca cytosol level which increases endothelial permeability through cell contraction, gap junction formation and actin filament disorganization [8].

Antioxidant works to neutralize free radicals and reduce oxidative stress damage. Ascorbic acid and α-tochopherol are anti-oxidants that have different characteristics. α-tochopherol is a lipid soluble antioxidant protecting cells membranes against free radical, whereas ascorbic acid is water soluble which works extracellularly [9,10]. When antioxidant scavenge ROS, they result in radical substances and therefore increase oxidative stress condition [11]. The use of antioxidant combination is more recommended compared to a single antioxidant as it reduces more oxidative stress damage. Antioxidant combination is

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expected to increase the efficacy and reduce the toxicity or adverse reaction [12]. As antioxidants, ascorbic acid and α-tochopherol has not been evaluated on endothelial permeability, especially when combined.

Aim of study: to explore the effect of ascorbic acid, α-tochopherol and its combination on permeability of HUVEC'S culture that was exposed to high glucose concentration.

Material and Methods

Chemical Uses

Ascorbic acid, α-tochopherol, collagenase type II, glucose, Hank's Balance Salt Solution (HBSS), New Calf Serum (NCS), serum free medium, trypsin Ethylendiaminetetraacetic acid (EDTA), phosphat buffer saline (PBS), and albumin labeled tryphan blue. All chemicals were purchased from Sigma aldrich from United States of America (USA).

HUVEC's culture

This research protocol was also approved by the institutional research ethical committee from the Faculty of Medicine Brawijaya University, Malang Indonesia. Human umbilical vein endothelial cells (HUVECs) were obtained from umbilical cord of neonates that fulfill inclusion criteria in some private hospital, Malang, after obtaining informed consent. Procedure of HUVEC's culture based on Jones 1996 with slightly modifications. Isolation of endothelial cells by collagenase type II in 10 ml serum free media and incubated for 7 minutes. Next, the media solution was centrifuged 1000 rpm for 8 minutes. The pellet was obtained containing endothelial cells to culture in flasks coated by gelatin 0.2 % and containing 4 ml M199 media. Then, Flask was put in an incubator at 37°C and with 5% CO2. After the cell grew a monolayer, a passage or subculture step was performed using trypsin solution and the cells were transferred on to transwell polyester filter. Treatment of both glucose (5 mM and 20 mM) as well as ascorbic acid and α–tocopherol were given concomitantly in culture media for seven days after the cells grew a monolayer. Single composition of both ascorbic acid and α–tocopherol were given each of them in three dose, they are 50 µM, 100 µM, 200 µM meanwhile in combination used composition I (25 µM : 25 µM), II (50 µM : 50 µM) and III (100 µM : 100 µM)

Barrier endothelial assay

HUVEC's barrier assay was based on Lum and Roebuck, 2001 with slight modification. Endothelial permeability to albumin was tested on two compartments separated by a filter membrane. Both of the compartments contain thyrode's solution with slight modification by adding NCS 10 %. Luminal compartment contained endothelial cell on transwell polyester filter and 2.5 ml thyrode's solution, 0.2 ml albumin labeled with tryphan blue 31,44 ppm was added in this compartment. Whereas albuminal compartment contained 10 ml thyrode's solution and was contiunously stirred with a magnetic stirrer. After 30 minutes, solution in albuminal compartment was taken and measured for albumin labeled tryphan blue level by spectrophotometer with λ= 592 nm.

Histopathology of endothelial cells

Endothelial cells (HUVEC's) culture was observed and noted for shape, size, and number at each view under a light microscope with 400x magnification.

Statistical Analysis

All data are expressed as mean ± SD. After the data was tested both of the homogenity and normality, therefore the advanced statistical test was selected. The statistical analysis of the data was performed by One Way ANOVA test if the data is normal and homogeneous. Then followed by Dunnet test with significant value ($p < 0.05$) using SPSS.

Results

Effect of antioxidant ascorbic acid, α-tochopherol and its combination on permeability of HUVEC's culture

Barrier permeability of HUVEC's culture exposed by normal or high concentration glucose and antioxidant are shown in figure 1.

[Figure 1 here]

Ascorbic acid 200 µM, α-tochopherol 100 µM and 200 µM decreased endothelial permeability to 50 %, 50 % and 40 % respectively compared to control group ($p<0.05$). Meanwhile, combination of ascorbic acid and α-tochopherol 1 (50 µM : 50 µM) and α-tochopherol II (100 µM : 100 µM) reduced barrier permeability of endothels to 60% and 50% respectively compared to the control group ($p<0.05$). Administration of the second combination of antioxidant decreased endothelial permeability until it is not different compared to normal group ($p>0.05$). Whereas, HUVEC's culture exposed to high concentration glucose increased its permeability three times fold compared to normal concentration glucose ($p<0.05$).

Effect of ascorbic acid, α-tochopherol and its combinations on structure of HUVEC's culture exposed high glucose concentration

Morphology of HUVEC's culture exposed normal or high concentration glucose and antioxidant are shown in figure 2.

[Figure 2 here]

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HUVEC's culture exposed to glucose at normal concentrations showed monolayer cells having a cobblestone shape and a smoothing of cells membran. Whereas, on high concentration glucose, cell apoptosis was observed, characterized by cell shrinkage and nucleus condensation. Meanwhile, administration of ascorbic acid, α-tochopherol and its combination had more potential to protect cells against oxidative damage on endothelial cells exposed high concentration glucose.

Discussion

Effect of high concentration glucose expose on permeability and morphology of HUVEC's culture

Exposure of high concentration glucose on HUVEC's culture increased cell permeability. Glucose in high concentrations is oxidizied into reactive dicarbonyl sugar which could bind to amino-lipids and proteins of endothelial cell, such as VE-cadherin [8]. Glycation of protein can destroy Vascular endothel (VE)-cadherin structure, therefore causing it to lose their elasticity and function as adhesion molecule. It results in barrier function impairment and detachment of endothelial cells [13]. Based on figure 2, high concentration of glucose on HUVEC's culture increases cell death and cell apoptosis, characterized by shrinkage and nucleus condensation, causing an increase in endothelial permeability. Exposure of glucose at high concentration increases hydrogen peroxide radicals and Ca cytosol level, the highest level of both occurs at the seventh days [14,15]. The increase of cytosolic Ca levels activates phospholipase-C and results in Myosin Light Chain Kinase (MLCK) phosphorylation which initiates cytoskeleton contraction and increases endothelial permeability [6]. On the seventh days of treatment, hydrogen peroxide radical disrupts actin filament which is signaled by an increase of actin depolymeration (G-actin) controlling endothelial barrier function [16,17]. Based on the previous study, permeability assay was performed on the seventh day [6].

HUVEC's culture exposed to high concentration of glucose showed an increase of cell permeability compared to normal concentration. The morphology of endothelial cells exposed to glucose at normal concentration is monolayer, polygonal (cobblestone) shape and smooth of cell membrane. Staining with anti-actin antibodies showed actin filament to be well arranged, whereas, exposure to high concentration of glucose on HUVEC's culture caused a disorganization of actin filament [16]. This structure correlated with the barrier function, in which HUVEC's permeability exposed to glucose at normal concentration was lower than high concentration of glucose. Hiperglycemia condition is analog with high concentration glucose exposure which increases anion superoxide and Protein Kinase C (PKC) activation [9,17]. Increase of PKC induces Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase to produce superoxide radical and stimulate expression of endothelial adhesion molecule resulting in the dysfunction of endothelial barrier [17,18]

Effect of antioxidant ascorbic acid on permeability and morphology of HUVECS culture

Ascorbic acid 200 μM reduces endothelial cells permeability when exposed to high concentration glucose. Ascorbic acid is a general antioxidant scavenging ROS and RNS at liquid phase, especially in cytosol, and therefore protects cell damage caused by free radicals [12]. These include hydroxyl radicals, peroxyl radicals, superoxide anions, nitrogen dioxides and also non-radical species, such as oxygen singlets and nitroxide peroxynitrics [2,12]. Ascorbic acid prevents nitrosation process due to free radicals on target molecules. It can neutralize ROS effect and therefore avoiding the damage of actin filament structure controlling barrier function [16]. Ascorbic acid can also protect VE-cadherin structure regulating endothelial permeability. As VE-cadherin produces complex compound with α-catenin and is connected by cytoskeleton, disconnection of junction with endothelial cells can be prevented [4,20]. ~~In physiologic concentrations, ascorbic acid can inhibit oxidized-Low Density Lipoprotein (LDL) on vascular cells and neutrophil [2,6]. Ascorbic acid can also inhibit modification of LDL oxidative through the scavenging of free radical and others reactive species [11].~~ Ascorbic acid is an effective antioxidant scavenger, since, first, both of ascorbic acid and ascorbic radical have a low reduction potential therefore they could react with all of radical and biology oxidant. Secondly, ascorbic radical has a low reactivity due to resonancy stability on free electron [11].

Ascorbic acid is entering the cell cells via two transporter system, which are Na dependent Transporter and Glucose Transorter-1 (GLUT-1). Disorders of transporter system results in the decrease of antioxidant defense in intracellularly. The structure of Dehydroascorbic (DHA) is similar to glucose, therefore competes to be transported by GLUT-1 into cytoplasma [21]. Differences in physical and chemical properties of ascorbic acid can affect their potency as antioxidants in decreasing endothelial permeability when exposed to high concentration glucose [12]. Increasing the dosage of ascorbic acid up to 200 μM results in antioxidant entered into intracellularly after competing with glucose at 22 mM, and therefore this dose shows a real effect in ~~increasing~~ decreasing HUVEC's permeability exposed to high concentration glucose. The morphology of cells showed cells membrane to be smooth and no damage to cells membrane was found.

Ascorbic acid 50 μM and 100 μM did not significantly decrease endothelial permeability. Low doses of ascorbic acid cannot protect cells against oxidative damage and therefore resulting in the disorder of endothelial permeability. This was signaled by the passage number of albumins which was high in albuminal compartment. On the other hand, low dose ascorbic acid cannot be transported to intracellularly effectively due to it failure to compete with high concentration glucose [12]. ~~Interaction between ROS and ascorbic acid results in DHA through ascorbil radical intermediates. Ascorbic acid is an effective antioxidant scavenger, since, first, both of ascorbic acid and ascorbic radical have a low reduction potential therefore they could react with all of radical and biology oxidant. Secondly, ascorbic radical has a low reactivity due to resonancy stability on free electron [11]. Besides it, ascorbic acid could be regenerated from ascorbic radical or DHA through the reaction of both of dependent and independent enzymatic. Radical ascorbic is reducted by NADPH dependent semi dehydroascorbate reductase and NADPH dependent selenoenzyme thioredoxin reductase [2,11]. Whereas, DHA is changed into ascorbic by non-enzymatic by Glutathione (GSH) and lipoic acid. On the other hand, interaction between ascorbic acid and with metal transition results in a negative effect since it produces hydroxyl radical and alchoxy lipid through metal ion reaction with hydrogen peroxydes [11,21].~~

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Effect of α-tochopherol on permeability and morphology of HUVECS culture exposed high concentration of glucose.

α-tochopherol 100 µM and 200 µM decreases endhothelial permeability exposed to high concentration glucose. Antioxidant α-tochopherol works by inhibiting lipid peroxidation and lipoprotein damage on cells membrane especially at the lipid phase. α-tochopherol is a lipid soluble antioxidant working in the lipid bilayer of cells membrane, and functions by stop chain reactions, starting by scavenging peroxyl and inhibiting free radical production such as oxygen singlet on cell membrane, and therefore lipoproteins can be protected against oxidation [2,10]. α-tochopherol as an extra cellular antioxidant can neutralize the effects of ROS and protects cell membrane against radical, moreover damage of actin filament and VE-cadherin controlling endothelial barrier function could be avoided [19]. α-tochopherol prevents lipid peroxidation on Poly Unsaturated Fatty Acid (PUFA) of cells membranes and minimize LDL oxidation. Besides that, α-tochopherol regulates membrane fluidity, stabilizes membrane and inhibits 5-lypoksigenase activity [Redana, 19]. Lipid peroxidation is initiated trough hydrogen bisalilic on lipid by peroxyl radical resulting lipid and peroxide radical. Oxygen results in peroxyl lipid radical production and lipid peroxidation propagation through chain reaction. α-tochopherol reduces lipid peroxidation through two mechanics, a chain bounding of lipid radical carrier or scavenging of peroxyl radical [2,12].

α-tochopherol at a 100 µM dosage was stronger in inhibiting permeability increase compared 200 µM, based on albumin concentration in the albuminal compartment. This is caused by α-tochopherol 100 µM having a higher activity in protecting the stability of cell membrane lipoproteins from damage due to ROS. Morphology of cells on the figure 2 showed that cells membrane was smooth and no damage to the cell was found. Increasing the dose of α-tochopherol to 200 µM precisely increases endothelial permeability. When acts as antioxidant, α-tochopherol will be oxidized into tochopheryl and this radical form is physiologically converted into tochopherol [10]. Administration of α-tochopherol 200 µM possibly produces tochopheryl radical in a huge concentration which cannot be regenerate by physiologic mechanism. Tochopheryl radical will destroy actin filaments and VE- cadherin structure which moreover results in the dysfunction of the endothelial barrier. Combination with ascorbic acid is suggested in α-tochopherol usage especially in high doses [12,19].

Single administration of α-tochopherol and ascorbic acid at the same dose (100 µM) showed a different effect on endothelial permeability. α-tochopherol was more potent in protecting the barrier function of endothelial cells exposed high concentration glucose compared to ascorbic acid. α-tochopherol works through dual action both of as a direct and indirect antioxidant. Direct antioxidant mechanism through inhibition of free radical and production of Advance Glycate End Product (AGE), stop chains reaction production whereas indirect antioxidant work by the inhibition of Protein Kinase C (PKC) activation [10]. α-tochopherol was more effective in inhibiting the increase of endothelial permeability exposed to high concentration glucose compared to ascorbic acid. This is caused by α-tochopherol works through dual action whereas ascorbic acid as general antioxidant works scavenge free radical only [12].

Effect combination of ascorbic acid and α-tochopherol on HUVECS culture exposed high glucose concentration

Combination of ascorbic acid and α–tochopherol at dosage 50 µM and 100 µM reduced the permeability of endothelial cells exposed high concentration glucose. The use of ascorbic acid and α–tochopherol combination results in a pharmacodynamic interaction and a synergistic effect to protect cells against free radicals. Aside from that, the combination of antioxidants aims to reduce the doses of both of them and therefore adverse reaction can be minimized [23,24]. α–tochopherol is a lipid soluble antioxidant working on lipid bilayer of cells membranes to inhibit both of chain reaction and free radical production [10,19]. Whereas, ascorbic acid as a general antioxidant works on liquid phase of cytosol to protect cells trough ROS scavenger mechanism [11,12]. They reduce radical reactivity and therefore actin filament structure and VE cadherin moreover endothelial barrier function could be retained. Cell morphology after given antioxidant combination showed cells membrane to be smooth and there are not damage cells. How α–tochopherol and ascorbic acid work as antioxidants have been explained on previously section. The combination of α–tochopherol and ascorbic acid have a beneficial effect, in which tochopheryl radical can be regenerated into tochopherol by ascorbic acid and therefore reduces stress oxidative [12,22].

Based on this study, antioxidant combination needs a lower dose compared to single antioxidant to retain of endothelial barrier function. Ascorbic acid 50 µM and α–tochopherol 50 µM in single composition have not showed inhibition of permeability increase of HUVECS culture. Whereas, in combination of ascorbic acid 50 µM and α–tochopherol 50 µM could reduce the increase of endothelial permeability until it was not different compare to normal group.

The combination of ascorbic acid 100 µM and α–tochopherol 100 µM could prevented the permeability increase of HUVECS culture, however it was lower compare to at dose each of 50 µM. The increase of dosage possibly changes it into a pro-oxidant effect due to the production increase of ascorbil and tochopheryl radical [11,12]. Disorder of transporter system of ascorbic acid into cytoplasma have a potency to cause lipoprotein damage of cells membrane [6,11]. They have a function as endothelial barrier therefore the HUVEC's permeability will increase. Metal transition also contribute on the increasing of ROS production beside the expose of high concentration of glucose [2].

Ascorbic acid 25 µM and α–tochopherol 25 µM in combination did not significantly decreases endothelial permeability. This was caused by the low doses of its combination which cannot protect cells against oxidative damage therefore increase endothelial permeability [24]. This was signaled by the passage number of albumins which was high in albuminal compartment.

Conclusion

Our studies showed that the exposure of high concentration glucose on HUVEC's culture increased the endothelial permeability, while ascorbic acid 100 µM, α-tocopherol 100 µM and 200 µM, as well as its combination potentially have protective properties by inhibit the increase of endothelial cells permeability exposed high concentration of glucose.

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Conflict of interest

We declare that we have no conflict of interest

Funding

No Funding

Ethical Concern

"This article does not contain any studies with animals performed by any of the authors."

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Legends

Figure 1. Potency ascorbic acid, α-tochopherol and its combination on permeability of HUVECS culture exposed high concentration glucose ; T1:Glucose 5 mM, T2: Glucose 22 mM, T3: Glucose 22 mM+ Vit C 50 µM, T4: Glucose 22 mM+ Vit C 100 µM, T5: Glucose 22 mM+ Vit C 200 µM, T6: Glucose 22 mM+ Vit E 50 µM, T7: Glucose 22 mM+ Vit C 100 µM, T8: Glucose 22 mM+ Vit C 200 µM, T9: Glucose 22 mM+ Vit C 25 µM + Vit E 25 µM, T10: Glucose 22 mM+ Vit C 50 µM + Vit E 50 µM, T11: Glucose 22 mM+ Vit C 100 µM + Vit E 100 µM

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Figure 2. Potency ascorbic acid, α-tochopherol and its combination on structure of HUVEC's culture exposed high concentration glucose. (M=200x); (A). 5 mM glucose: Polygonal / cobblestone-shaped cells and fine-looking cell membranes / smoothing. (B). Glucose 22 mM: Cells undergoing apoptosis characterized by shrinkage cells and core condensation. (C). G22 + VIT C.50 + VIT. 50 μM: Cells and cell membranes do not show structural damage. (D). G22 + VITE.100 μM; The cell membrane is fine and the polygonal cells do not appear to be structural damage. (E). G22 + VIT C.100 μM: Cell membrane is not smooth due to membrane lipoprotein damage (F). G22 + VITC.25 + VIT E. 25 μM: The cells undergo apoptosis and the nucleus is condensed.

Figures

Figure 1

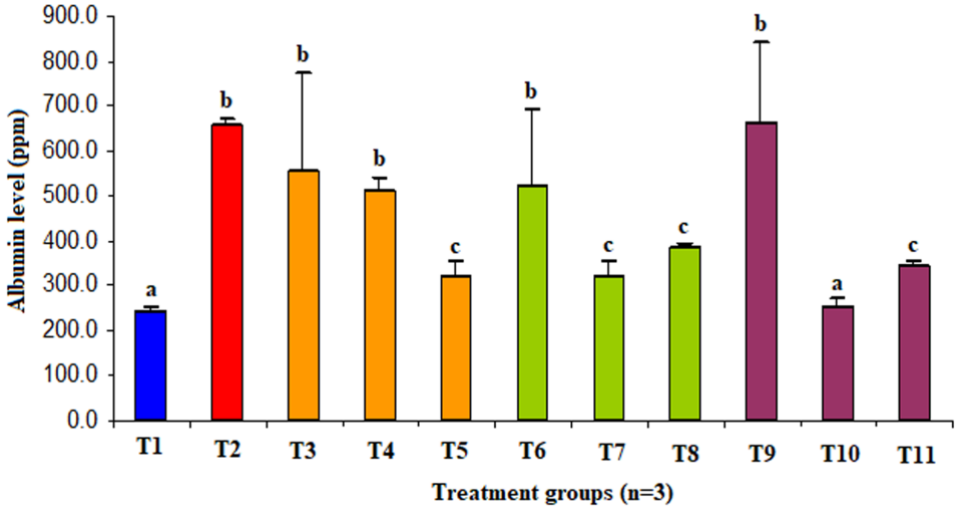
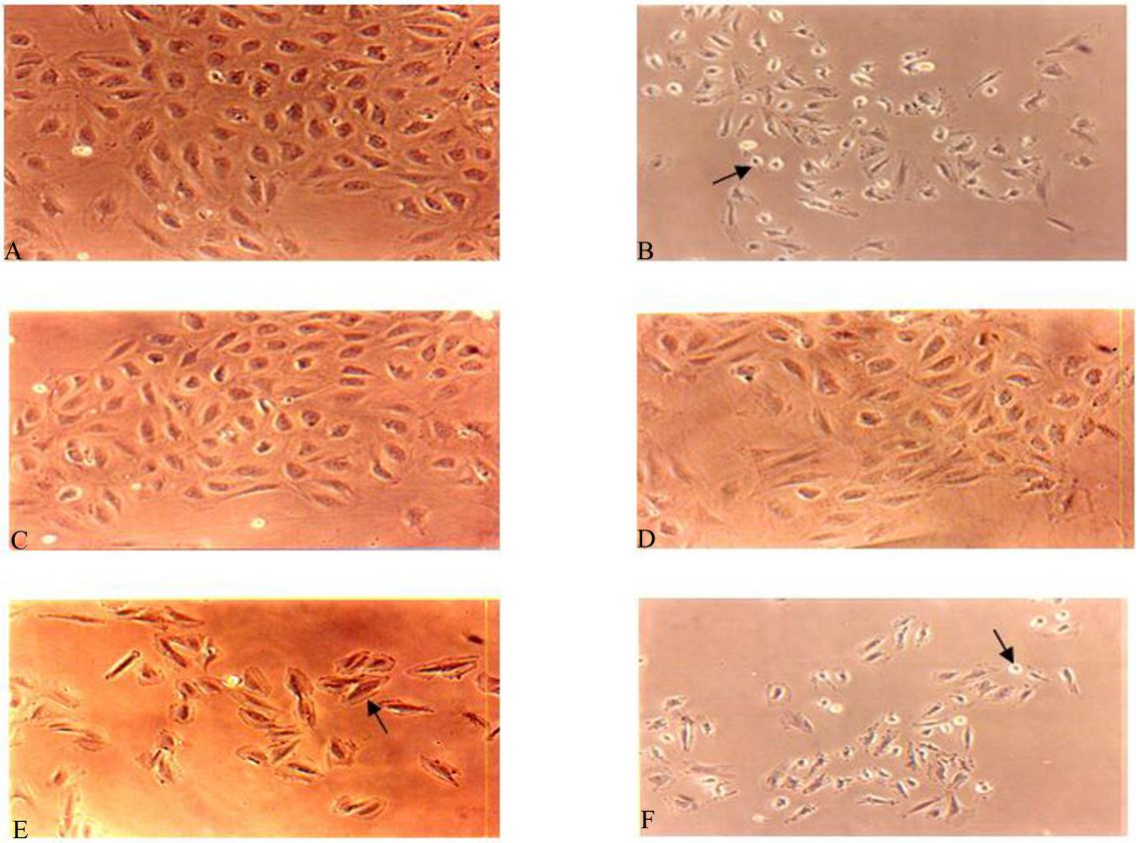


Figure 2



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Effect of Ascorbic Acid, α-Tocopherol and Its Combination on *Human Umbilical Vein Endothelial Cells* Permeability Exposed to High Concentration of Glucose

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Abstract: *Aim* **-** To evaluate the effect of ascorbic acid, α-tocopherol and its combination on the permeability of human umbilical vein endothelial cells (HUVEC'S) culture exposed to high concentration glucose. *Material and Methods* - The study was performed using HUVEC'S culture on transwell polyester filter where ascorbic acid, α-tocopherol and its combination at a dose of 25-200 µM were given concomitantly with glucose 22 mM on culture media. After seven days of treatment, endothelial permeability was observed by measuring the concentration of trypan blue-labeled albumin in a luminal compartment with spectrophotometer λ= 592 nm. The morphology observed under an inverted microscope. The data was analyzed using ANOVA test continued with the Dunnet test (p<0,05). *Results* - HUVEC'S culture exposed to high concentration glucose increased endothelial permeability compared to those exposed to normal concentration glucose (p<0.05). Administration of ascorbic acid 200 µM, α-tocopherol 100 µM and 200 µM were able to inhibit increasing of HUVEC'S permeability exposed to high concentration glucose compared to control group (p<0.05). Meanwhile, two combinations of ascorbic acid and α-tocopherol at a dose of 50 and 100 µM could prevent the increased permeability of HUVEC'S culture compare to control group (p<0.05). *Conclusion* - Ascorbic acid, α-tocopherol, and its combination could inhibit the increase of endothelial permeability exposed to a high concentration of glucose.

Keywords: ascorbic acid, α-tocopherol, permeability, endothelial cells, high glucose

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Introduction

Diabetes Mellitus (DM) is a metabolic syndrome characterized by chronic hyperglycemia due to a disorder in insulin secretion or insulin sensitivity or both [1]. Hyperglycemia results in DM complications through a mechanism that increases the production of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). These are categorized as free radical compounds, and overproduction of it without a sufficient antioxidant defense would cause oxidative stress conditions [2]. This condition causes vascular endothelial cell damage and results in macro and microangiopathy complications on DM [3].

Vascular endothelial cells act as a selective barrier to prevent the transfer of some substances on the vascular system. The structure of endothelial cells is composed of actin-based cytoskeleton which relates to its function as barrier permeability [4]. The endothelial barrier consists of an actin filament, stress fibers, junction-associated actin filament system and adhesion molecule which maintain the permeability of the cells against solutes, solvents, hormones and macromolecules [5]. Permeability of endothelial cells is also controlled by a signaling mechanism which regulates cellular activity such as calcium (Ca) ions, protein kinases, and phosphatases [6]. Human umbilical vein endothelial cells (HUVEC'S) culture was used in this study due to several advantages. The culture is an *in vitro* model of vascular endothelium that can be used to evaluate both of its structure and function. The use of this culture is better in representing the human condition, while effective both in time and cost, reliable and reproducible [4].

ROS produced in a hyperglycemic condition could destroy the structure and function of endothelial cells through oxidative stress [6]. Accumulation of ROS results in intercellular gaps, actin filaments disorganization and the change of cell shape and therefore increasing paracellular permeability [7]. ROS decreases trans-endothelial electric resistance and increases trans-endothelial permeability against plasma proteins such as albumin. Oxidative stress steps up Ca cytosol level which increases endothelial permeability through cell contraction, gap junction formation, and actin filament disorganization [8].

Antioxidant works to neutralize free radicals and reduce oxidative stress damage. Ascorbic acid and α-tocopherol are anti-oxidants that have different characteristics. α-tocopherol is a lipid-soluble antioxidant protecting cells membranes against free radical, whereas ascorbic acid is water soluble which works extracellularly [9,10]. When antioxidant scavenges ROS, they result in radical substances and therefore increase oxidative stress condition [11]. The use of antioxidant combination is more recommended compared to a single antioxidant as it reduces more oxidative stress damage. The antioxidant combination is

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expected to increase the efficacy and reduce the toxicity or adverse reaction [12]. As antioxidants, ascorbic acid and α-tocopherol have not been evaluated on endothelial permeability, especially when combined.

Aim of the study: to explore the effect of ascorbic acid, α-tocopherol and its combination on the permeability of HUVEC'S culture that was exposed to high glucose concentration.

Material and Methods

Chemical Uses

Ascorbic acid, α-tocopherol, collagenase type II, glucose, Hank's Balanced Salt Solution (HBSS), New Calf Serum (NCS), serum-free medium, trypsin Ethylenediaminetetraacetic acid (EDTA), phosphate buffer saline (PBS), and albumin labeled trypan blue. All chemicals were purchased from Sigma Aldrich (USA).

HUVEC's culture

This research protocol was also approved by the institutional research ethical committee from the Faculty of Medicine Brawijaya University (ethical certificate No. 217/EC/KEPK-S3/03/2014), Malang Indonesia. Human umbilical vein endothelial cells (HUVECs) were obtained from the umbilical cord of neonates that fulfill inclusion criteria in some private hospital, Malang, after obtaining informed consent. The procedure of HUVEC's culture based on Jones 1996 with slight modifications. Isolation of endothelial cells by collagenase type II in 10 ml of serum-free media and incubated for 7 minutes. Next, the media solution was centrifuged in 1000rpm for 8 minutes. The pellet, containing endothelial cells, was obtained for culture in 0.2% gelatin-coated flasks that contain 4ml of M199 media. Then, the flask was put in an incubator at 37°C and with 5% CO2. After the cell grew a monolayer, a passage or subculture step was performed using trypsin solution and the cells were transferred on to transwell polyester filter. Treatment of both glucose (5 and 20 mM), ascorbic acid, and α–tocopherol was given concomitantly in culture media for seven days after the cells grew a monolayer. Single composition of both ascorbic acid and α–tocopherol was given each of them in three doses, they are 50 µM, 100 µM, 200 µM meanwhile in combination used composition I (25 µM: 25 µM), II (50 µM: 50 µM) and III (100 µM: 100 µM)

Barrier endothelial assay

HUVEC's barrier assay was based on Lum and Roebuck, 2001 with slight modification. Endothelial permeability to albumin was tested on two compartments separated by a filter membrane. Both of the compartments contain tyrode's solution with slight modification by adding NCS 10 %. The luminal compartment contained endothelial cell on transwell polyester filter and 2.5 ml of tyrode's solution, 0.2 ml albumin labeled with trypan blue 31,44 ppm was added in this compartment. Whereas the albuminal compartment contained 10 ml of tyrode's solution and was continuously stirred with a magnetic stirrer. After 30 minutes, the solution in albuminal compartment was taken and measured for albumin labeled trypan blue level by spectrophotometer with λ= 592 nm. Based on the previous study, the permeability assay was performed on the seventh day [6].

Histopathology of endothelial cells

Endothelial cells (HUVEC's) culture was observed and noted for shape, size, and number at each view under a light microscope with 400x magnification.

Statistical Analysis

All data are expressed as mean ± SD. After the data was tested for homogeneity and normality, the advanced statistical test was performed. The statistical analysis of the data was performed by One Way ANOVA test if the data is normal and homogeneous. Then followed by a Dunnet test with significant value ($p < 0.05$) using SPSS.

Results

Effect of antioxidant ascorbic acid, α-tocopherol and its combination on permeability of HUVEC's culture

Barrier permeability of HUVEC's culture exposed by normal or high concentration glucose and antioxidant are shown in figure 1.

[Figure 1 here]

Ascorbic acid 200 µM, α-tocopherol 100 µM, and 200 µM decreased endothelial permeability to 50%, 50%, and 40% respectively compared to the control group ($p<0.05$). Meanwhile, the combination of ascorbic acid and α-tocopherol 1 (50 µM: 50 µM) and α-tocopherol II (100 µM: 100 µM) reduced barrier permeability of endothel to 60% and 50% respectively compared to the control group ($p<0.05$). Administration of the second combination of antioxidant decreased endothelial permeability until it is not different compared to the normal group ($p>0.05$). Whereas, HUVEC's culture exposed to high concentration glucose increased its permeability three times fold compared to normal concentration glucose ($p<0.05$).

Effect of ascorbic acid, α-tocopherol and its combinations on the structure of HUVEC's culture exposed high glucose concentration

Morphology of HUVEC's culture exposed normal or high concentration glucose and antioxidant are shown in figure 2.

[Figure 2 here]

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HUVEC's culture exposed to glucose at normal concentrations showed monolayer cells having a cobblestone shape and smoothing of cells membrane. Whereas, on high concentration glucose, cell apoptosis was observed, characterized by cell shrinkage and nucleus condensation. Meanwhile, the administration of ascorbic acid, α-tocopherol and its combination had more potential to protect cells against oxidative damage on endothelial cells exposed high concentration glucose.

Discussion

Effect of high concentration glucose expose on permeability and morphology of HUVEC's culture

Exposure of high concentration glucose on HUVEC's culture increased cell permeability. Glucose in high concentrations is oxidized into reactive dicarbonyl sugar which could bind to amino-lipids and proteins of endothelial cells, such as Vascular endothelial (VE)-cadherin [8]. Glycation of protein can destroy VE-cadherin structure, therefore causing it to lose their elasticity and function as an adhesion molecule. It results in barrier function impairment and detachment of endothelial cells [13]. Based on figure 2, a high concentration of glucose on HUVEC's culture increases cell death and cell apoptosis, characterized by shrinkage and nucleus condensation, causing an increase in endothelial permeability. Exposure of glucose at high concentration increases hydrogen peroxide radicals and Ca cytosol level, the highest level of both occurs at the seventh days [14,15,25]. The increase of cytosolic Ca levels activates phospholipase-C and results in Myosin Light Chain Kinase (MLCK) phosphorylation which initiates cytoskeleton contraction and increases endothelial permeability [6]. On the seventh days of treatment, hydrogen peroxide radical disrupts actin filament which is signaled by an increase of actin depolymerization (G-actin) controlling endothelial barrier function [16,17,26].

HUVEC's culture exposed to a high concentration of glucose showed an increase in cell permeability compared to a normal concentration. The morphology of endothelial cells exposed to glucose at normal concentration is a monolayer, polygonal (cobblestone) shape and smooth of the cell membrane. Staining with anti-actin antibodies showed actin filament to be well arranged, whereas, exposure to a high concentration of glucose on HUVEC's culture caused disorganization of actin filament [16,26]. This structure correlated with the barrier function, in which HUVEC's permeability exposed to glucose at normal concentration was lower than the high concentration of glucose. Hyperglycemia condition is analog with high concentration glucose exposure which increases anion superoxide and Protein Kinase C (PKC) activation [9,17]. Increase of PKC induces Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase to produce superoxide radical and stimulate expression of endothelial adhesion molecule resulting in the dysfunction of endothelial barrier [17,18]

Effect of antioxidant ascorbic acid on permeability and morphology of HUVECS culture

Ascorbic acid 200 μM reduces endothelial cells permeability when exposed to high concentration glucose. Ascorbic acid is a general antioxidant scavenging ROS and RNS at liquid phase, especially in the cytosol, and therefore protects cell damage caused by free radicals [12]. These include hydroxyl radicals, peroxyl radicals, superoxide anions, nitrogen dioxides, and also non-radical species, such as oxygen singlets, nitroxide, and peroxynitrite [2,12]. Ascorbic acid prevents nitrosation process due to free radicals on target molecules. It can neutralize ROS effect and therefore avoiding the damage of actin filament structure controlling barrier function [16]. Ascorbic acid can also protect the VE-cadherin structure regulating endothelial permeability. As VE-cadherin produces a complex compound with α-catenin and is connected by the cytoskeleton, disconnection of the junction with endothelial cells can be prevented [4,20]. The previous study showed that ascorbic acid inhibited Malondialdehyde (MDA) production and retained endothelial-derived relaxing factor (EDRF) especially the level of nitrite oxide [27]. Ascorbic acid is an effective antioxidant scavenger. First, both of ascorbic acid and ascorbic radical have a low reduction potential, so they could react with all of radical and biology oxidant. Secondly, ascorbic radical has a low reactivity due to resonance stability on a free electron [11].

Ascorbic acid is entering the cell cells via two transporter system, which are Na dependent Transporter and Glucose Transporter-1 (GLUT-1). Disorders of the transporter system result in a decrease of antioxidant defense in intracellularly. The structure of Dehydroascorbic (DHA) is similar to glucose, therefore competes to be transported by GLUT-1 into the cytoplasm [21]. Differences in physical and chemical properties of ascorbic acid can affect their potency as antioxidants in decreasing endothelial permeability when exposed to high concentration glucose [12]. Increasing the dosage of ascorbic acid up to 200 μM results in antioxidant entered into intracellularly after competing with glucose at 22 mM, and therefore this dose shows a real effect in decreasing HUVEC's permeability exposed to high concentration glucose. The morphology of cells showed cells membrane to be smooth and no damage to cells membrane was found.

Ascorbic acid 50 μM and 100 μM did not significantly decrease endothelial permeability. Low doses of ascorbic acid cannot protect cells against oxidative damage and therefore resulting in the disorder of endothelial permeability. This was signaled by the passage number of albumins which was high in the albuminal compartment. On the other hand, low dose ascorbic acid cannot be transported to intracellularly effectively due to its failure to compete with high concentration glucose [12].

Effect of α-tocopherol on permeability and morphology of HUVECS culture exposed a high concentration of glucose.

α-tocopherol 100 μM and 200 μM decreases endothelial permeability exposed to high concentration glucose. Antioxidant α-tocopherol works by inhibiting lipid peroxidation and lipoprotein damage on cells membrane especially at the lipid phase. α-tocopherol is a lipid-soluble antioxidant working in the lipid bilayer of cells membrane, and functions by stop chain reactions, starting by scavenging peroxyl and inhibiting free radical production such as oxygen singlet on the cell membrane, and therefore lipoproteins can be protected against oxidation [2,10]. α-tocopherol as an extracellular antioxidant can neutralize the effects of ROS and protects cell membrane against radical, moreover, damage of actin filament and VE-cadherin controlling endothelial barrier function could be avoided [19]. α-tocopherol prevents lipid peroxidation on Poly Unsaturated Fatty Acid (PUFA) of cells

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membranes and minimizes LDL oxidation. The administration of α-tocopherol on the HUVECS culture, which was exposed to a high level of glucose, showed the decrease of MDA level and maintenance of nitrite oxide level [27]. Besides that, α-tocopherol regulates membrane fluidity, stabilizes membrane and inhibits 5-lypoksigenase activity [Redana, 19]. Lipid peroxidation is initiated through hydrogen bisalilic on lipid by peroxyl radical resulting lipid and peroxide radical. Oxygen results in peroxyl lipid radical production and lipid peroxidation propagation through a chain reaction. α-tocopherol reduces lipid peroxidation through two mechanics, a chain bounding of lipid radical carrier or scavenging of peroxyl radical [2,12].

α-tocopherol at a 100 μM dosage was stronger in inhibiting permeability increase compared to 200 μM, based on albumin concentration in the albuminal compartment. This is caused by α-tocopherol 100 μM having a higher activity in protecting the stability of cell membrane lipoproteins from damage due to ROS. Morphology of cells on figure 2 showed that cells membrane was smooth and no damage to the cell was found. Increasing the dose of α-tocopherol to 200 μM precisely increases endothelial permeability. When acts as an antioxidant, α-tocopherol will be oxidized into tocopheryl and this radical form is physiologically converted into tocopherol [10]. Administration of α-tocopherol 200 μM possibly produces tocopheryl radical in a huge concentration which cannot be regenerated by the physiologic mechanism. Tocopheryl radical will destroy actin filaments and VE-cadherin structure which moreover results in the dysfunction of the endothelial barrier. Combination with ascorbic acid is suggested in α-tocopherol usage especially in high doses [12,19].

A single administration of α-tocopherol and ascorbic acid at the same dose (100 μM) showed a different effect on endothelial permeability. α-tocopherol was more potent in protecting the barrier function of endothelial cells exposed high concentration glucose compared to ascorbic acid. α-tocopherol can reach the lipid membrane; therefore, it can protect the membrane from lipid peroxidation. In accordance to the previous study, α-tocopherol prevented MDA production as a result of lipid membrane peroxidation on HUVEC culture exposed to high glucose level [27]. α-tocopherol works through dual action both of as a direct and indirect antioxidant. The direct antioxidant mechanism through inhibition of free radical and production of Advance Glycate End Product (AGE), stop chains reaction production whereas indirect antioxidant work by the inhibition of Protein Kinase C (PKC) activation [10]. α-tocopherol was more effective in inhibiting the increase of endothelial permeability exposed to high concentration glucose compared to ascorbic acid. This is caused by α-tocopherol works through dual action whereas ascorbic acid as general antioxidant works scavenge free radical only [12].

Effect combination of ascorbic acid and α-tocopherol on HUVECS culture exposed high glucose concentration

Combination of ascorbic acid and α–tocopherol at dosage 50 μM and 100 μM reduced the permeability of endothelial cells exposed high concentration glucose. The use of ascorbic acid and α–tocopherol combination results in a pharmacodynamic interaction and a synergistic effect to protect cells against free radicals. Aside from that, the combination of antioxidants aims to reduce the doses of both of them and therefore adverse reaction can be minimized [23,24]. The antioxidant combination reduces radical reactivity and therefore actin filament structure and VE-cadherin moreover endothelial barrier function could be retained. Cell morphology after given antioxidant combination showed cells membrane to be smooth and there are not damage cells. How α–tocopherol and ascorbic acid work as antioxidants have been explained in the previous section. The combination of α–tocopherol and ascorbic acid have a beneficial effect, in which tocopheryl radical can be regenerated into tocopherol by ascorbic acid and therefore reduces stress oxidative [12,22].

Based on this study, antioxidant combination needs a lower dose compared to single antioxidant to retain of endothelial barrier function. Ascorbic acid 50 μM and α–tocopherol 50 μM in single composition has not shown inhibition of permeability increase of HUVECS culture. Whereas, in the combination of ascorbic acid 50 μM and α–tocopherol 50 μM could reduce the increase of endothelial permeability until it was not different compared to the normal group. This combination also strongly decreased the endothelial permeability of the HUVEC culture.

The combination of ascorbic acid 100 μM and α–tochopherol 100 μM could prevented the permeability increase of HUVECS culture, however, it was lower compared to at dose each of 50 μM. The increase of dosage possibly changes it into a pro-oxidant effect due to the production increase of ascorbic and tocopheryl radical [11,12]. Disorder of the transporter system of ascorbic acid into cytoplasm has a potency to cause lipoprotein damage of cells membrane [6,11]. They have a function as an endothelial barrier, therefore, the HUVEC's permeability will increase. The metal transition also contributes to the increase of ROS production besides the expose of high concentration of glucose [2]. Ascorbic acid 25 μM and α–tocopherol 25 μM in combination did not significantly decrease endothelial permeability. This was caused by the low doses of its combination which cannot protect cells against oxidative damage, therefore, increase endothelial permeability [24]. This was signaled by the passage number of albumins which was high in the albuminal compartment.

Conclusion

Our studies showed that the exposure of high glucose concentration on HUVEC's culture increases the endothelial permeability. Meanwhile, ascorbic acid 100 μM, α-tocopherol 100 μM and 200 μM, as well as its combination potentialy have protective properties by inhibiting the increase of endothelial cells permeability exposed high concentration of glucose.

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Conflict of interest

We declare that we have no conflict of interest

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Funding

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Ethical Concern

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors. For this type of study formal consent is not required.

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Legends

Figure 1. Potency ascorbic acid, α-tocopherol and its combination on permeability of HUVECS culture exposed high concentration glucose ; T1:Glucose 5 mM, T2: Glucose 22 mM, T3: Glucose 22 mM+ Vit C 50 µM, T4: Glucose 22 mM+ Vit C 100 µM, T5: Glucose 22 mM+ Vit C 200 µM, T6: Glucose 22 mM+ Vit E 50 µM, T7: Glucose 22 mM+ Vit C 100 µM, T8: Glucose 22 mM+ Vit C 200 µM, T9: Glucose 22 mM+ Vit C 25 µM + Vit E 25 µM, T10: Glucose 22 mM+ Vit C 50 µM + Vit E 50 µM, T11: Glucose 22 mM+ Vit C 100 µM + Vit E 100 µM

Figure 2. Potency ascorbic acid, α-tocopherol and its combination on structure of HUVEC's culture exposed high concentration glucose. (M=200x); (A). 5 mM glucose: Polygonal / cobblestone-shaped cells and fine-looking cell membranes / smoothing. (B). Glucose 22 mM: Cells undergoing apoptosis characterized by shrinkage cells and core condensation. (C). G22 + VIT C.50 + VIT. 50 µM: Cells and cell membranes do not show structural damage. (D). G22 + VITE.100 µM; The cell membrane is fine and the polygonal cells do not appear to be structural damage. (E). G22 + VIT C.100 µM: Cell membrane is not smooth due to membrane lipoprotein damage (F). G22 + VITC.25 + VIT E. 25 µM: The cells undergo apoptosis and the nucleus is condensed.

Figures

Figure 1

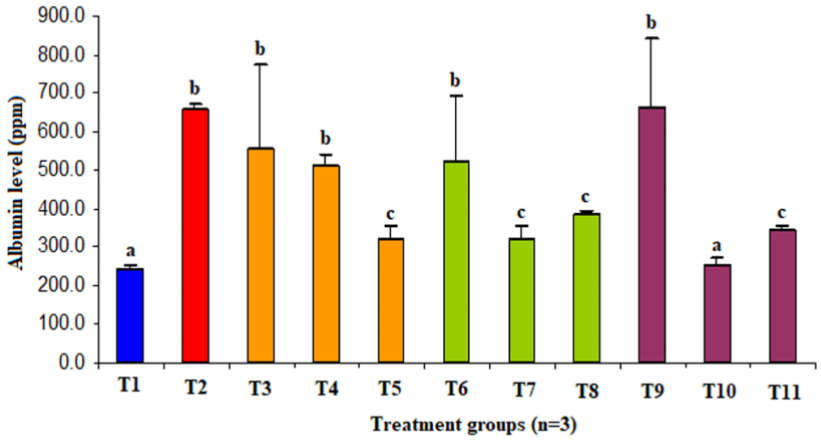
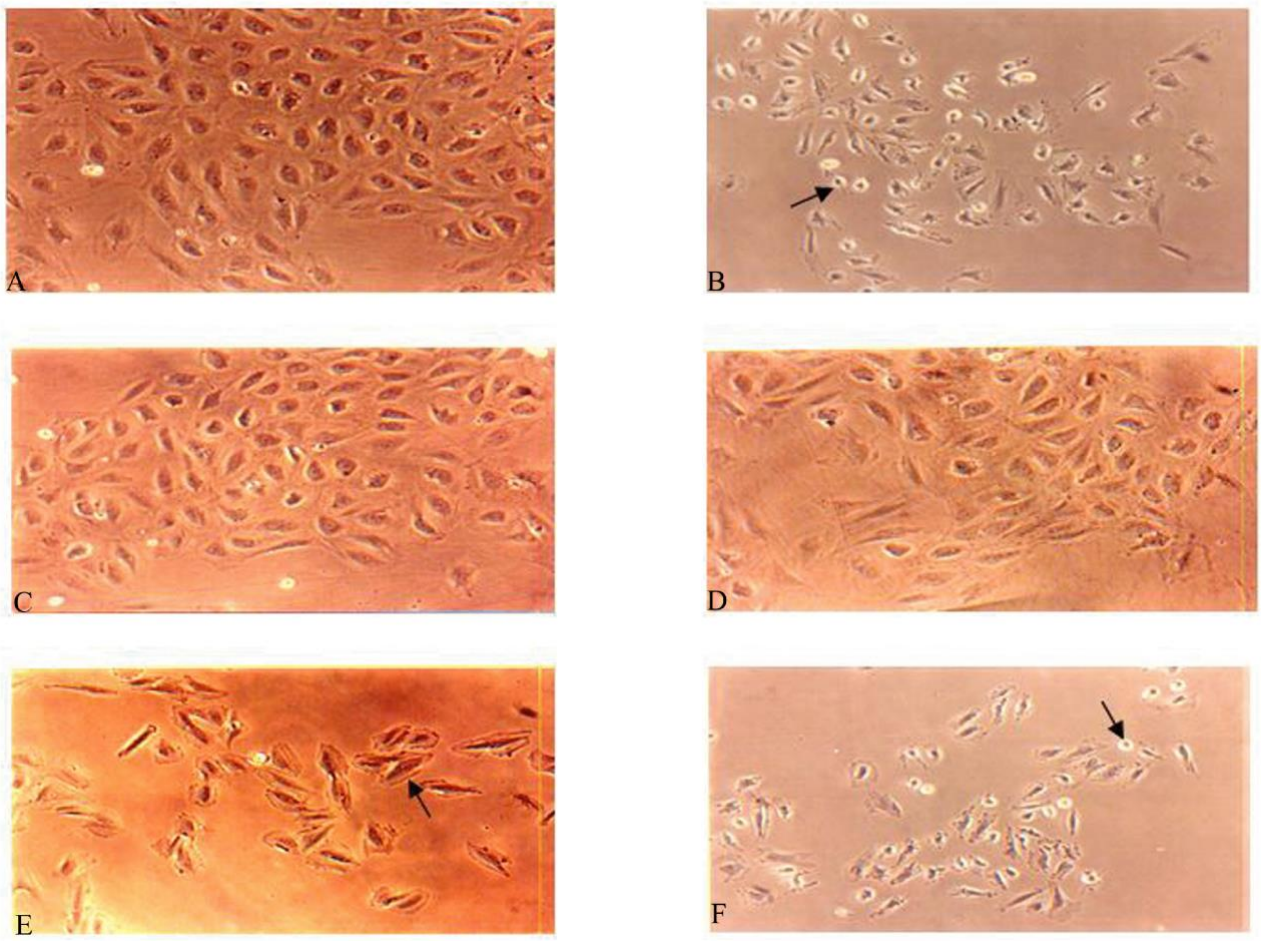


Figure 2



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Original article

Effect of ascorbic acid, α-tocopherol and its combination on human umbilical vein endothelial cells permeability exposed to high concentration of glucose

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Abstract: *Aim* — To evaluate the effect of ascorbic acid, α-tocopherol, and its combination on the permeability of human umbilical vein endothelial cells (HUVEC'S) culture after high glucose concentration exposure.
Material and Methods — The study was performed using HUVEC'S culture on transwell polyester filter. The ascorbic acid, α-tocopherol, and its combination (25-200 µM) were concomitantly given with glucose 22 µM to the culture media. After seven days of treatment, endothelial permeability was observed by measuring the concentration of trypan blue-labeled albumin in a luminal compartment with a spectrophotometer (λ=592 nm). The morphology of the cells was observed under an inverted microscope. The data was analyzed using ANOVA test followed by Dunnet test ($p<0.05$).
Results — HUVEC'S culture exposed to high concentration glucose increased endothelial permeability better than those which exposed to normal concentration ($p<0.05$). The treatment of ascorbic acid 200 µM, α-tocopherol 100 µM and 200 µM were capable to inhibit the increase HUVEC'S permeability exposed to high concentration glucose better than control group ($p<0.05$). Moreover, two combinations of ascorbic acid and α-tocopherol, respectively 50 and 100 µM, could prevent the increased permeability of HUVEC'S culture better than control ($p<0.05$).
Conclusion — Ascorbic acid, α-tocopherol, and its combination could inhibit the increase of endothelial permeability exposed to a high concentration of glucose.

Keywords: ascorbic acid, α-tocopherol, permeability, endothelial cells, high glucose.

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Introduction

Diabetes mellitus (DM) is a metabolic syndrome characterized by chronic hyperglycemia due to the disorder of insulin secretion or insulin sensitivity or both at the same time [1]. Hyperglycemia could lead to DM through a mechanism which increases the production of radical compounds, reactive oxygen species (ROS) and reactive nitrogen species (RNS). They overproduct without a sufficient antioxidant defense would cause oxidative stress conditions [2]. This condition causes vascular endothelial cell damage and results in macro and microangiopathy complications on DM [3].

Vascular endothelial cells act as a selective barrier to prevent the transfer of some substances on the vascular system. The structure of endothelial cells is composed of actin-based cytoskeleton which relates to its function as barrier permeability [4]. The endothelial barrier consists of an actin filament, stress fibers, junction-associated actin filament system, and adhesion molecule which maintain the permeability of the cells against solutes, solvents, hormones, and macromolecules [5]. Permeability of endothelial cells is also controlled by a signaling mechanism which regulates cellular activity such as calcium (Ca) ions, protein kinases, and phosphatases [6]. Human umbilical vein endothelial cells (HUVEC'S) culture was used in this study due to several advantages. The culture is an *in vitro* model of vascular endothelium that can be used to evaluate its structure and function. The use of this culture is better in representing the actual human condition, while reliable, reproducible, and effective both in time and cost [4].

ROS, produced in a hyperglycemic condition, could damage the structure and function of endothelial cells through oxidative stress [6]. ROS accumulation causes intercellular gaps, actin filaments disorganization, and the change of cell shape, therefore increasing the paracellular permeability [7]. ROS decreases trans-endothelial electric resistance and increases trans-endothelial permeability against plasma proteins such as albumin. Oxidative stress steps up the Ca level in the cytosol, which increases endothelial permeability through cell contraction, gap junction formation, and actin filament disorganization [8].

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Antioxidant works to neutralize free radicals and reduce oxidative stress damage. Ascorbic acid and α-tocopherol are anti-oxidants that have different characteristics. α-tocopherol is a lipid-soluble antioxidant protecting cells membranes against free radical, whereas ascorbic acid is water soluble which works extracellularly [9, 10]. When antioxidant scavenges ROS, they result in radical substances and therefore increase oxidative stress condition [11]. The application of antioxidant combination is more recommended compared to a single antioxidant as it reduces more oxidative stress damage. It is expected to increase the efficacy and reduce the toxicity or adverse reaction [12]. As antioxidants, ascorbic acid and α-tocopherol have not been evaluated on endothelial permeability, especially when combined.

Aim of the study: To explore the effect of ascorbic acid, α-tocopherol and its combination on the permeability of HUVEC'S culture that was exposed to high glucose concentration.

Material and Methods

Chemical uses

Ascorbic acid, α-tocopherol, collagenase type II, glucose, Hank's Balanced Salt Solution (HBSS), New Calf Serum (NCS), serum-free medium, trypsin Ethylenediaminetetraacetic acid (EDTA), phosphate buffer saline (PBS), and albumin labeled trypan blue. All chemicals were purchased from Sigma Aldrich (USA).

HUVEC's culture

This research protocol was also approved by the institutional research ethical committee from the Faculty of Medicine Brawijaya University (ethical certificate No. 217/EC/KEPK-S3/03/2014), Malang Indonesia. Human umbilical vein endothelial cells (HUVECs) were obtained from the umbilical cord of neonates. Samples were collected based on inclusion criteria in some private hospital, Malang, after obtaining the informed consent. The procedure of HUVEC's culture based on Jones 1996 with slight modifications. Endothelial cells were isolated by collagenase type II in 10 ml of serum-free media and incubated for 7 minutes. Next, the media solution was centrifuged in 1000rpm for 8 minutes. The pellet, containing endothelial cells, was collected for further culture isolation in 0.2% gelatin-coated flasks, which contain 4ml of M199 media. Then, the flask was stored in an incubator at 37°C and with 5% CO_2. After the cell grew as monolayer, a passage or subculture step was performed using trypsin solution and the cells were transferred on to transwell polyester filter. Glucose (5 and 20 mM), ascorbic acid, and α–tocopherol was administrated concomitantly in culture media for seven days after the cells grew as monolayer. Single composition of both ascorbic acid and α–tocopherol was administrated each of them in three doses, 50 µM, 100 µM, 200 µM, respectively. To compare the effectivity, the antioxidant also combined under these following doses: I (25 µM: 25 µM), II (50 µM: 50 µM) and III (100 µM: 100 µM)

Barrier endothelial assay

HUVEC's barrier assay was based on Lum and Roebuck, 2001 with slight modification. Endothelial permeability to albumin was assayed on two compartments separated by a filter membrane. Both of the compartments contain tyrode's solution with slight modification by adding NCS 10 %. The luminal compartment contained endothelial cell on transwell polyester filter and 2.5 ml of tyrode's solution, 0.2 ml albumin labeled with trypan blue 31,44 ppm was added in this compartment. Whereas the albuminal compartment contained 10 ml of tyrode's solution and was continuously stirred with a magnetic stirrer. After 30 minutes, the solution in albuminal compartment was taken and measured for albumin labeled trypan blue level by spectrophotometer with λ=592 nm. Based on the previous study, the permeability assay was performed on the seventh day [6].

Histopathology of endothelial cells

Endothelial cells (HUVEC's) culture was observed to characterize its morphology (such as: shape and size) and countable cells under a light microscope (M=400x).

Statistical analysis

All data are expressed as mean ± SD (standard deviation). Data was statistically tested using homogeneity and normality test before the sequel statistical test was performed. If the data is normal and homogeneous, the statistical analysis of the data was performed by One Way ANOVA test. Statistical test followed by a Dunnet test ($p < 0.05$) using SPSS (IBM).

Results

Effect of antioxidant ascorbic acid, α-tocopherol and its combination on the permeability of HUVEC's culture

Barrier permeability of HUVEC's culture exposed by normal or high concentration glucose and antioxidant are shown in *Figure* 1.

Ascorbic acid 200 µM, α-tocopherol 100 µM, and 200 µM decreased endothelial permeability to 50%, 50%, and 40% respectively better than control group ($p<0.05$). Meanwhile, the combination of ascorbic acid and α-tocopherol 1 (50 µM: 50 µM) and α-tocopherol II (100 µM: 100 µM) reduced barrier permeability of endothelial to 60% and 50% respectively better than control group ($p<0.05$). Administration of the second combination of antioxidant decreased endothelial permeability until no difference to the normal group was observed ($p>0.05$). Moreover, HUVEC's culture exposed to high concentration glucose increased its permeability three folds compared to normal concentration ($p<0.05$).

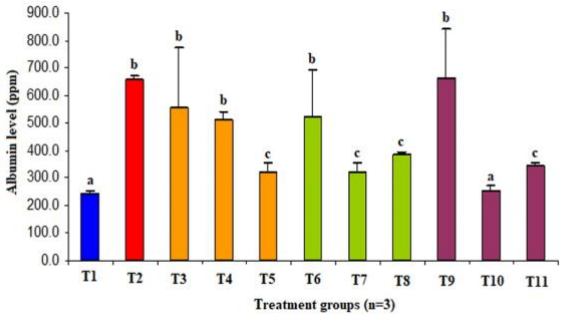

Figure 1. Potency ascorbic acid, α-tocopherol and its combination on permeability of HUVECS culture exposed high concentration glucose. T1, glucose 5 µM; T2, glucose 22 µM; T3, glucose 22 µM + vit C 50 µM; T4, glucose 22 µM + vit C 100 µM; T5, glucose 22 µM + vit C 200 µM; T6, glucose 22 µM + vit E 50 µM; T7, glucose 22 µM + vit C 100 µM; T8, glucose 22 µM + vit C 200 µM; T9, glucose 22 µM + vit C 25 µM + vit E 25 µM; T10, glucose 22 µM + vit C 50 µM + vit E 50 µM; T11, glucose 22 µM + vit C 100 µM + vit E 100 µM.

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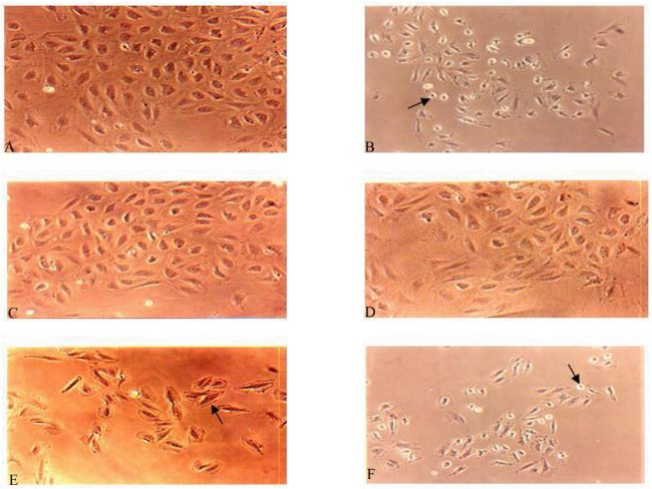

Figure 2. Potency ascorbic acid, α-tocopherol and its combination on structure of HUVEC's culture exposed high concentration glucose (M=200x). (A) 5 µM glucose: polygonal/cobblestone-shaped cells and fine-looking cell membranes/smoothing. (B) glucose 22 µM: cells undergoing apoptosis characterized by shrinkage cells and core condensation. (C) glucose 22 µM + vit C 50 µM + vit 50 µM: cells and cell membranes do not show structural damage. (D) glucose 22 µM + vit E 100 µM; the cell membrane is fine and the polygonal cells do not appear to be structural damage. (E) glucose 22 µM + vit C 100 µM: cell membrane is not smooth due to membrane lipoprotein damage. (F) G22 + VITC.25 + VIT E. 25 µM: the cells undergo apoptosis and the nucleus is condensed.

Effect of ascorbic acid, α-tocopherol and its combinations on the structure of HUVEC's culture exposed high glucose concentration

Morphology of HUVEC's culture exposed normal or high concentration glucose and antioxidant are shown in *Figure* 2.

HUVEC's culture exposed to glucose at normal concentrations showed monolayer cells with a cobblestone shape and smooth cell membrane. Cell apoptosis was observed on high concentration glucose, characterized by cell shrinkage and nucleus condensation. Ascorbic acid, α-tocopherol, and its combination had more potential to protect cells against oxidative damage on endothelial cells exposed high concentration glucose.

Discussion

Effect of high concentration glucose expose on permeability and morphology of HUVEC's culture

The exposure of high concentration glucose on HUVEC's culture increased cell permeability. Glucose in high concentrations is oxidized into reactive dicarbonyl sugar which could bind to amino-lipids and proteins of endothelial cells, such as Vascular endothelial (VE)-cadherin [8]. Glycation of protein can destroy VE-cadherin structures, make they lose their elasticity and function as an adhesion molecule, resulting in barrier function impairment and detachment of endothelial cells [13]. Based on *Figure* 2, a high concentration of glucose could increases cell apoptosis rate, indicated by shrinkage and nucleus condensation, which increase endothelial permeability. Exposure of glucose at high concentration increases hydrogen peroxide radicals and Ca cytosol level, the highest level of both occurs at the seventh days [14, 15, 24]. The increase of cytosolic Ca levels activates phospholipase-C and results in myosin light chain kinase (MLCK) phosphorylation which initiates cytoskeleton contraction and increases endothelial permeability [6]. On the seventh days of treatment, hydrogen peroxide radical disrupts actin filament which is signaled by an increase of actin depolymerization (G-actin) controlling endothelial barrier function [16, 17, 25].

HUVEC's culture showed increase cell permeability higher than normal cells. The morphology of normal endothelial cells is a monolayer, polygonal (cobblestone) shape and smooth cell membrane. Based on observation after staining process using anti-actin antibodies, it showed actin filament to be well arranged. On the other hand, the HUVEC's culture has disorganization of actin filament [16, 25]. This structure is correlated with the barrier function, in which HUVEC's permeability exposed to glucose at normal concentration was lower than the high concentration of glucose. Hyperglycemia condition is analog with high concentration glucose exposure which increases anion superoxide and protein kinase C (PKC) activation [9, 17]. Increase of PKC induces nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to produce superoxide radicals and stimulate expression of endothelial adhesion molecule resulting in the dysfunction of endothelial barrier [17, 18]

Effect of antioxidant ascorbic acid on permeability and morphology of HUVECS culture

Ascorbic acid 200 µM reduces endothelial cells permeability if exposed to high concentration glucose. Ascorbic acid is a general ROS and RNS scavenging antioxidant at liquid phase, especially in the cytosol. Therefore ascorbic acid protects cells from damage caused by free radicals [12]. These include hydroxyl radicals, peroxyl radicals, superoxide anions, nitrogen dioxides, and also non-radical species, such as oxygen singlets, nitroxide, and peroxynitrite [2, 12]. Ascorbic acid prevents nitrosation process due to free radicals on target molecules. It can neutralize ROS effect and therefore avoiding the damage of actin filament structure which controls the barrier function [16]. Ascorbic acid can also protect the VE-cadherin structure which is regulating endothelial permeability. As VE-cadherin produces a complex compound with α-catenin and is connected by the cytoskeleton, disconnection of the junction with endothelial cells can be prevented [4, 20]. The previous study showed that ascorbic acid inhibited malondialdehyde (MDA) production and retained endothelial-derived relaxing factor (EDRF) and the level of endothelial nitric oxide synthase [26]. Ascorbic acid is an effective antioxidant scavenger. Firstly, it is due to both ascorbic acid and ascorbic radical have a low reduction potential, so they could react with all of radical and biology oxidants. Secondly, ascorbic radical has a low reactivity due to resonance stability on a free electron [11].

Ascorbic acid is entering the cells via two transporter systems, which are Na dependent transporter and glucose transporter-1 (GLUT-1). Disorders of the transporter system result in a decrease of antioxidant defense in intracellularly. The structure of dehydroascorbic (DHA) is similar to glucose, therefore competes to be transported by GLUT-1 into the cytoplasm [21]. Differences in physical and chemical properties of ascorbic acid can affect their potency as antioxidants in decreasing endothelial permeability when exposed to high concentration glucose [12]. Increasing the dosage of ascorbic acid up to 200 µM results in antioxidant entered into intracellularly after competing with glucose at 22 µM, and therefore this dose shows a real effect in decreasing HUVEC's

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permeability exposed to high concentration glucose. The morphology of cells showed cells membrane to be smooth and no damage to cells membrane was found.

Ascorbic acid 50 µM and 100 µM did not significantly decrease endothelial permeability. Low doses of ascorbic acid cannot protect cells against oxidative damage and therefore resulting in the disorder of endothelial permeability. This was signaled by the passage number of albumins which was high in the albuminal compartment. On the other hand, low dose ascorbic acid cannot be transported to intracellularly effectively due to its failure to compete with high concentration glucose [12].

Effect of α-tocopherol on permeability and morphology of HUVECS culture exposed a high concentration of glucose.

α-tocopherol 100 µM and 200 µM decreases endothelial permeability after exposed to high concentration glucose. Antioxidant α-tocopherol works by inhibiting lipid peroxidation and lipoprotein damage on cells membrane especially at the lipid phase. α-tocopherol is a lipid-soluble antioxidant working in the lipid bilayer of cells membrane and functions by stopping chain reactions by scavenging peroxyl and inhibiting free radical production such as oxygen singlet on the cell membrane. Therefore lipoproteins can be protected against oxidation [2, 10]. α-tocopherol as an extracellular antioxidant can neutralize the effects of ROS and protects cell membrane against free radical. Eventually, the damage of actin filament and VE-cadherin that controlling the endothelial barrier function could be avoided [19]. α-tocopherol prevents lipid peroxidation on Poly Unsaturated Fatty Acid (PUFA) of cells membranes and minimizes LDL oxidation. The administration of α-tocopherol on the HUVECS culture, which was exposed to a high level of glucose, showed the decrease of MDA level and maintenance of nitric oxide level [26]. Besides that, α-tocopherol regulates membrane fluidity, stabilizes membrane and inhibits 5-lypoksigenase activity [19]. Lipid peroxidation is initiated through hydrogen bisalilic on lipid by peroxyl radical resulting in lipid and peroxide radicals. Oxygen is the result in peroxyl lipid radical production and lipid peroxidation propagation through a chain reaction. α-tocopherol reduces lipid peroxidation through two mechanics, a chain bounding of lipid radical carrier or scavenging of peroxyl radical [2, 12].

α-tocopherol at a 100 µM dosage was stronger in inhibiting permeability increase compared to 200 µM, based on albumin concentration in the albuminal compartment. This is caused by α-tocopherol 100 µM having a higher activity in protecting the stability of cell membrane lipoproteins from damage due to ROS. Cell morphology showed that the cell's membrane was smooth and no damaged to the cell was found (*Figure* 2). Increasing the dose of α-tocopherol to 200 µM precisely increases the endothelial permeability. When α-tocopherol acts as an antioxidant, it will be oxidized into tocopheryl and this radical form is physiologically converted into tocopherol [10]. Administration of α-tocopherol 200 µM possibly produces tocopheryl radical in a huge concentration which cannot be regenerated by the physiological mechanism. Tocopheryl radical can disrupt actin filaments and VE-cadherin structure which results in the dysfunction of the endothelial barrier. Combination with ascorbic acid is suggested in α-tocopherol usage, especially in high doses [12, 19].

A single administration of α-tocopherol and ascorbic acid at the same dose (100 µM) obtained a different effect on endothelial permeability. α-tocopherol was more potent in protecting the barrier function of endothelial cells exposed high concentration glucose compared to ascorbic acid. α-tocopherol can reach the lipid membrane; therefore, it can protect the membrane from lipid peroxidation. Following the previous study, α-tocopherol prevented MDA production as a result of lipid membrane peroxidation on HUVEC culture exposed to high glucose concentration [26]. α-tocopherol works through dual-action both of as a direct and indirect antioxidant. The direct antioxidant mechanism through inhibition of free radical and production of Advance Glycate End Product (AGE), stop chains reaction production whereas indirect antioxidant work by the inhibition of Protein Kinase C (PKC) activation [10]. α-tocopherol was more effective in inhibiting the increase of endothelial permeability compared to ascorbic acid. This is caused by α-tocopherol works through dual-action whereas ascorbic acid works by scavenging free radical only [12].

Effect of combination of ascorbic acid and α-tocopherol on HUVECS culture exposed high glucose concentration

Combination of ascorbic acid and α–tocopherol at dosage 50 µM and 100 µM reduced the permeability of endothelial cells exposed high concentration glucose. The use of ascorbic acid and α–tocopherol combination results in a pharmacodynamic interaction and a synergistic effect to protect cells against free radicals. Moreover, the combination of antioxidants aims to reduce the doses of both of them and therefore adverse reaction can be minimized [23, 24]. The antioxidant combination reduces radical reactivity and therefore actin filament structure and VE-cadherin, as well as its barrier function, could be maintained. Cell morphology after given antioxidant combination showed cells membrane to be smooth and there are no damage cells. The combination of α–tocopherol and ascorbic acid has a beneficial effect, in which tocopheryl radical can be regenerated into tocopherol by ascorbic acid and therefore reduces stress oxidative [12, 22].

Based on this study, antioxidant combination needs a lower dose compared to single antioxidant to retain of endothelial barrier function. Ascorbic acid 50 µM and α–tocopherol 50 µM in single composition has not shown inhibition of permeability increase of HUVECS culture. Whereas, in the combination of ascorbic acid 50 µM and α–tocopherol 50 µM could reduce the increase of endothelial permeability until it was not different compared to the normal group. This combination also strongly decreased the endothelial permeability of the HUVEC culture.

The combination of ascorbic acid 100 µM and α–tocopherol 100 µM could prevent the permeability increase of HUVECS culture. However, it was lower compared to a dose each of 50 µM. The increase of dosage possibly changes it into a pro-oxidant effect due to the production increase of ascorbic and tocopheryl radical [11, 12]. Disorder of the transporter system of ascorbic acid has a potency to cause lipoprotein damage of cells membrane [6, 11]. They have a function as an endothelial barrier, therefore, the HUVEC's permeability will increase. The metal transition also contributes to the increase of ROS production besides the expose of high concentration of glucose [2]. Ascorbic acid 25 µM and α–tocopherol 25 µM in combination did not significantly decrease endothelial permeability. This was caused by the low doses of its combination which cannot protect cells against oxidative damage, therefore, increase endothelial permeability [23]. This was

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signaled by the passage number of albumins which was high in the albuminal compartment.

Conclusion

Our studies showed that the exposure to high glucose concentration on HUVEC's culture increases the endothelial permeability. Meanwhile, ascorbic acid 100 µM, α-tocopherol 100 µM, and 200 µM, as well as its combination potentially have protective properties by inhibiting the increase of endothelial cells permeability exposed a high concentration of glucose.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Conflict of interest

We declare that we have no conflict of interest

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Original article

Effect of ascorbic acid, α-tocopherol and its combination on human umbilical vein endothelial cells permeability exposed to high concentration of glucose

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Abstract: *Aim* — To evaluate the effect of ascorbic acid, α-tocopherol, and its combination on the permeability of human umbilical vein endothelial cells (HUVEC'S) culture after high glucose concentration exposure.
Material and Methods — The study was performed using HUVEC'S culture on transwell polyester filter. The ascorbic acid, α-tocopherol, and its combination (25-200 μM) were concomitantly given with glucose 22 μM to the culture media. After seven days of treatment, endothelial permeability was observed by measuring the concentration of trypan blue-labeled albumin in a luminal compartment with a spectrophotometer (λ=592 nm). The morphology of the cells was observed under an inverted microscope. The data was analyzed using ANOVA test followed by Dunnet test ($p<0.05$).
Results — HUVEC'S culture exposed to high concentration glucose increased endothelial permeability better than those which exposed to normal concentration ($p<0.05$). The treatment of ascorbic acid 200 μM, α-tocopherol 100 μM and 200 μM were capable to inhibit the increase HUVEC'S permeability exposed to high concentration glucose better than control group ($p<0.05$). Moreover, two combinations of ascorbic acid and α-tocopherol, respectively 50 and 100 μM, could prevent the increased permeability of HUVEC'S culture better than control ($p<0.05$).
Conclusion — Ascorbic acid, α-tocopherol, and its combination could inhibit the increase of endothelial permeability exposed to a high concentration of glucose.

Keywords: ascorbic acid, α-tocopherol, permeability, endothelial cells, high glucose.

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Introduction

Diabetes mellitus (DM) is a metabolic syndrome characterized by chronic hyperglycemia due to the disorder of insulin secretion or insulin sensitivity or both at the same time [1]. Hyperglycemia could lead to DM through a mechanism which increases the production of radical compounds, reactive oxygen species (ROS) and reactive nitrogen species (RNS). They overproduct without a sufficient antioxidant defense would cause oxidative stress conditions [2]. This condition causes vascular endothelial cell damage and results in macro and microangiopathy complications on DM [3].

Vascular endothelial cells act as a selective barrier to prevent the transfer of some substances on the vascular system. The structure of endothelial cells is composed of actin-based cytoskeleton which relates to its function as barrier permeability [4]. The endothelial barrier consists of an actin filament, stress fibers, junction-associated actin filament system, and adhesion molecule which maintain the permeability of the cells against solutes, solvents, hormones, and macromolecules [5]. Permeability of endothelial cells is also controlled by a signaling mechanism which regulates cellular activity such as calcium (Ca) ions, protein kinases, and phosphatases [6]. Human umbilical vein endothelial cells (HUVEC'S) culture was used in this study due to several advantages. The culture is an *in vitro* model of vascular endothelium that can be used to evaluate its structure and function. The use of this culture is better in representing the actual human condition, while reliable, reproducible, and effective both in time and cost [4].

ROS, produced in a hyperglycemic condition, could damage the structure and function of endothelial cells through oxidative stress [6]. ROS accumulation causes intercellular gaps, actin filaments disorganization, and the change of cell shape, therefore increasing the paracellular permeability [7]. ROS decreases trans-endothelial electric resistance and increases trans-endothelial permeability against plasma proteins such as albumin. Oxidative stress steps up the Ca level in the cytosol, which increases endothelial permeability through cell contraction, gap junction formation, and actin filament disorganization [8].

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Antioxidant works to neutralize free radicals and reduce oxidative stress damage. Ascorbic acid and α-tocopherol are anti-oxidants that have different characteristics. α-tocopherol is a lipid-soluble antioxidant protecting cells membranes against free radical, whereas ascorbic acid is water soluble which works extracellularly [9, 10]. When antioxidant scavenges ROS, they result in radical substances and therefore increase oxidative stress condition [11]. The application of antioxidant combination is more recommended compared to a single antioxidant as it reduces more oxidative stress damage. It is expected to increase the efficacy and reduce the toxicity or adverse reaction [12]. As antioxidants, ascorbic acid and α-tocopherol have not been evaluated on endothelial permeability, especially when combined.

Aim of the study: To explore the effect of ascorbic acid, α-tocopherol and its combination on the permeability of HUVEC'S culture that was exposed to high glucose concentration.

Material and Methods

Chemical uses

Ascorbic acid, α-tocopherol, collagenase type II, glucose, Hank's Balanced Salt Solution (HBSS), New Calf Serum (NCS), serum-free medium, trypsin Ethylenediaminetetraacetic acid (EDTA), phosphate buffer saline (PBS), and albumin labeled trypan blue. All chemicals were purchased from Sigma Aldrich (USA).

HUVEC's culture

This research protocol was also approved by the institutional research ethical committee from the Faculty of Medicine Brawijaya University (ethical certificate No. 217/EC/KEPK-S3/03/2014), Malang Indonesia. Human umbilical vein endothelial cells (HUVECs) were obtained from the umbilical cord of neonates. Samples were collected based on inclusion criteria in some private hospital, Malang, after obtaining the informed consent. The procedure of HUVEC's culture based on Jones 1996 with slight modifications. Endothelial cells were isolated by collagenase type II in 10 ml of serum-free media and incubated for 7 minutes. Next, the media solution was centrifuged in 1000rpm for 8 minutes. The pellet, containing endothelial cells, was collected for further culture isolation in 0.2% gelatin-coated flasks, which contain 4ml of M199 media. Then, the flask was stored in an incubator at 37°C and with 5% CO_2. After the cell grew as monolayer, a passage or subculture step was performed using trypsin solution and the cells were transferred on to transwell polyester filter. Glucose (5 and 20 mM), ascorbic acid, and α–tocopherol was administrated concomitantly in culture media for seven days after the cells grew as monolayer. Single composition of both ascorbic acid and α–tocopherol was administrated each of them in three doses, 50 µM, 100 µM, 200 µM, respectively. To compare the effectivity, the antioxidant also combined under these following doses: I (25 µM: 25 µM), II (50 µM: 50 µM) and III (100 µM: 100 µM)

Barrier endothelial assay

HUVEC's barrier assay was based on Lum and Roebuck, 2001 with slight modification. Endothelial permeability to albumin was assayed on two compartments separated by a filter membrane. Both of the compartments contain tyrode's solution with slight modification by adding NCS 10 %. The luminal compartment contained endothelial cell on transwell polyester filter and 2.5 ml of tyrode's solution, 0.2 ml albumin labeled with trypan blue 31,44 ppm was added in this compartment. Whereas the albuminal compartment contained 10 ml of tyrode's solution and was continuously stirred with a magnetic stirrer. After 30 minutes, the solution in albuminal compartment was taken and measured for albumin labeled trypan blue level by spectrophotometer with λ=592 nm. Based on the previous study, the permeability assay was performed on the seventh day [6].

Histopathology of endothelial cells

Endothelial cells (HUVEC's) culture was observed to characterize its morphology (such as: shape and size) and countable cells under a light microscope (M=400x).

Statistical analysis

All data are expressed as mean ± SD (standard deviation). Data was statistically tested using homogeneity and normality test before the sequel statistical test was performed. If the data is normal and homogeneous, the statistical analysis of the data was performed by One Way ANOVA test. Statistical test followed by a Dunnet test ($p < 0.05$) using SPSS (IBM).

Results

Effect of antioxidant ascorbic acid, α-tocopherol and its combination on the permeability of HUVEC's culture

Barrier permeability of HUVEC's culture exposed by normal or high concentration glucose and antioxidant are shown in *Figure* 1.

Ascorbic acid 200 µM, α-tocopherol 100 µM, and 200 µM decreased endothelial permeability to 50%, 50%, and 40% respectively better than control group ($p<0.05$). Meanwhile, the combination of ascorbic acid and α-tocopherol 1 (50 µM: 50 µM) and α-tocopherol II (100 µM: 100 µM) reduced barrier permeability of endothelial to 60% and 50% respectively better than control group ($p<0.05$). Administration of the second combination of antioxidant decreased endothelial permeability until no difference to the normal group was observed ($p>0.05$). Moreover, HUVEC's culture exposed to high concentration glucose increased its permeability three folds compared to normal concentration ($p<0.05$).

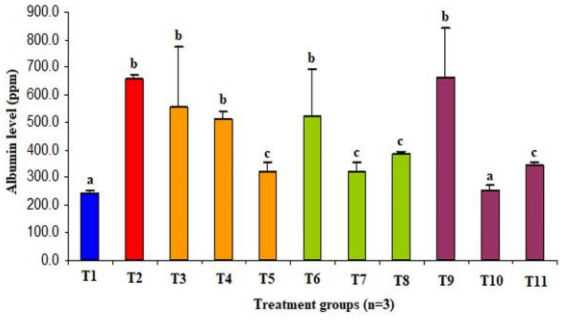

Figure 1. Potency ascorbic acid, α-tocopherol and its combination on permeability of HUVECS culture exposed high concentration glucose. T1, glucose 5 µM; T2, glucose 22 µM; T3, glucose 22 µM + vit C 50 µM; T4, glucose 22 µM + vit C 100 µM; T5, glucose 22 µM + vit C 200 µM; T6, glucose 22 µM + vit E 50 µM; T7, glucose 22 µM + vit C 100 µM; T8, glucose 22 µM + vit C 200 µM; T9, glucose 22 µM + vit C 25 µM + vit E 25 µM; T10, glucose 22 µM + vit C 50 µM + vit E 50 µM; T11, glucose 22 µM + vit C 100 µM + vit E 100 µM.

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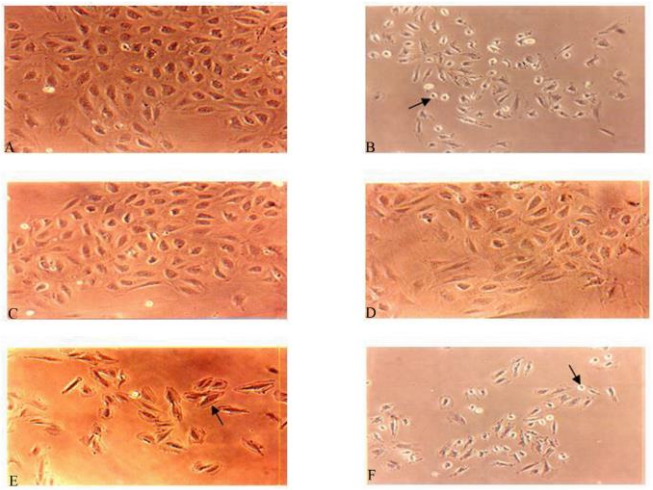

Figure 2. Potency ascorbic acid, α-tocopherol and its combination on structure of HUVEC's culture exposed high concentration glucose (M=200x). (A) 5 µM glucose: polygonal/cobblestone-shaped cells and fine-looking cell membranes/smoothing. (B) glucose 22 µM: cells undergoing apoptosis characterized by shrinkage cells and nucleus condensation. (C) glucose 22 µM + vit C 50 µM + vit 50 µM: cells and cell membranes do not show structural damage. (D) glucose 22 µM + vit E 100 µM; the cell membrane is fine and the polygonal cells do not appear to be structural damage. (E) glucose 22 µM + vit C 100 µM: cell membrane is not smooth due to membrane lipoprotein damage. (F) G22 + VITC.25 + VIT E. 25 µM: the cells undergo apoptosis and the nucleus is condensed.

Effect of ascorbic acid, α-tocopherol and its combinations on the structure of HUVEC's culture exposed high glucose concentration

Morphology of HUVEC's culture exposed normal or high concentration glucose and antioxidant are shown in *Figure* 2.

HUVEC's culture exposed to glucose at normal concentrations showed monolayer cells with a cobblestone shape and smooth cell membrane. Cell apoptosis was observed on high concentration glucose, characterized by cell shrinkage and nucleus condensation. Ascorbic acid, α-tocopherol, and its combination had more potential to protect cells against oxidative damage on endothelial cells exposed high concentration glucose.

Discussion

Effect of high concentration glucose expose on permeability and morphology of HUVEC's culture

The exposure of high concentration glucose on HUVEC's culture increased cell permeability. Glucose in high concentrations is oxidized into reactive dicarbonyl sugar which could bind to amino-lipids and proteins of endothelial cells, such as Vascular endothelial (VE)-cadherin [8]. Glycation of protein can destroy VE-cadherin structures, make they lose their elasticity and function as an adhesion molecule, resulting in barrier function impairment and detachment of endothelial cells [13]. Based on *Figure* 2, a high concentration of glucose could increases cell apoptosis rate, indicated by shrinkage and nucleus condensation, which increase endothelial permeability. Exposure of glucose at high concentration increases hydrogen peroxide radicals and Ca cytosol level, the highest level of both occurs at the seventh days [14, 15, 24]. The increase of cytosolic Ca levels activates phospholipase-C and results in myosin light chain kinase (MLCK) phosphorylation which initiates cytoskeleton contraction and increases endothelial permeability [6]. On the seventh days of treatment, hydrogen peroxide radical disrupts actin filament which is signaled by an increase of actin depolymerization (G-actin) controlling endothelial barrier function [16, 17, 25].

HUVEC's culture showed increase cell permeability higher than normal cells. The morphology of normal endothelial cells is a monolayer, polygonal (cobblestone) shape and smooth cell membrane. Based on observation after staining process using anti-actin antibodies, it showed actin filament to be well arranged. On the other hand, the HUVEC's culture has disorganization of actin filament [16, 25]. This structure is correlated with the barrier function, in which HUVEC's permeability exposed to glucose at normal concentration was lower than the high concentration of glucose. Hyperglycemia condition is analog with high concentration glucose exposure which increases anion superoxide and protein kinase C (PKC) activation [9, 17]. Increase of PKC induces nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to produce superoxide radicals and stimulate expression of endothelial adhesion molecule resulting in the dysfunction of endothelial barrier [17, 18]

Effect of antioxidant ascorbic acid on permeability and morphology of HUVECS culture

Ascorbic acid 200 µM reduces endothelial cells permeability if exposed to high concentration glucose. Ascorbic acid is a general ROS and RNS scavenging antioxidant at liquid phase, especially in the cytosol. Therefore ascorbic acid protects cells from damage caused by free radicals [12]. These include hydroxyl radicals, peroxyl radicals, superoxide anions, nitrogen dioxides, and also non-radical species, such as oxygen singlets, nitroxide, and peroxynitrite [2, 12]. Ascorbic acid prevents nitrosation process due to free radicals on target molecules. It can neutralize ROS effect and therefore avoiding the damage of actin filament structure which controls the barrier function [16]. Ascorbic acid can also protect the VE-cadherin structure which is regulating endothelial permeability. As VE-cadherin produces a complex compound with α-catenin and is connected by the cytoskeleton, disconnection of the junction with endothelial cells can be prevented [4, 20]. The previous study showed that ascorbic acid inhibited malondialdehyde (MDA) production and retained endothelial-derived relaxing factor (EDRF) and the level of endothelial nitric oxide synthase [26]. Ascorbic acid is an effective antioxidant scavenger. Firstly, it is due to both ascorbic acid and ascorbic radical have a low reduction potential, so they could react with all of radical and biology oxidants. Secondly, ascorbic radical has a low reactivity due to resonance stability on a free electron [11].

Ascorbic acid is entering the cells via two transporter systems, which are Na dependent transporter and glucose transporter-1 (GLUT-1). Disorders of the transporter system result in a decrease of antioxidant defense in intracellularly. The structure of dehydroascorbic (DHA) is similar to glucose, therefore competes to be transported by GLUT-1 into the cytoplasm [21]. Differences in physical and chemical properties of ascorbic acid can affect their potency as antioxidants in decreasing endothelial permeability when exposed to high concentration glucose [12]. Increasing the dosage of ascorbic acid up to 200 µM results in antioxidant entered into intracellularly after competing with glucose at 22 µM, and therefore this dose shows a real effect in decreasing HUVEC's

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permeability exposed to high concentration glucose. The morphology of cells showed cells membrane to be smooth and no damage to cells membrane was found.

Ascorbic acid 50 µM and 100 µM did not significantly decrease endothelial permeability. Low doses of ascorbic acid cannot protect cells against oxidative damage and therefore resulting in the disorder of endothelial permeability. This was signaled by the passage number of albumins which was high in the albuminal compartment. On the other hand, low dose ascorbic acid cannot be transported to intracellularly effectively due to its failure to compete with high concentration glucose [12].

Effect of α-tocopherol on permeability and morphology of HUVECS culture exposed a high concentration of glucose.

α-tocopherol 100 µM and 200 µM decreases endothelial permeability after exposed to high concentration glucose. Antioxidant α-tocopherol works by inhibiting lipid peroxidation and lipoprotein damage on cells membrane especially at the lipid phase. α-tocopherol is a lipid-soluble antioxidant working in the lipid bilayer of cells membrane and functions by stopping chain reactions by scavenging peroxyl and inhibiting free radical production such as oxygen singlet on the cell membrane. Therefore lipoproteins can be protected against oxidation [2, 10]. α-tocopherol as an extracellular antioxidant can neutralize the effects of ROS and protects cell membrane against free radical. Eventually, the damage of actin filament and VE-cadherin that controlling the endothelial barrier function could be avoided [19]. α-tocopherol prevents lipid peroxidation on Poly Unsaturated Fatty Acid (PUFA) of cells membranes and minimizes LDL oxidation. The administration of α-tocopherol on the HUVECS culture, which was exposed to a high level of glucose, showed the decrease of MDA level and maintenance of nitric oxide level [26]. Besides that, α-tocopherol regulates membrane fluidity, stabilizes membrane and inhibits 5-lypoksigenase activity [19]. Lipid peroxidation is initiated through hydrogen bisalilic on lipid by peroxyl radical resulting in lipid and peroxide radicals. Oxygen is the result in peroxyl lipid radical production and lipid peroxidation propagation through a chain reaction. α-tocopherol reduces lipid peroxidation through two mechanics, a chain bounding of lipid radical carrier or scavenging of peroxyl radical [2, 12].

α-tocopherol at a 100 µM dosage was stronger in inhibiting permeability increase compared to 200 µM, based on albumin concentration in the albuminal compartment. This is caused by α-tocopherol 100 µM having a higher activity in protecting the stability of cell membrane lipoproteins from damage due to ROS. Cell morphology showed that the cell's membrane was smooth and no damaged to the cell was found (*Figure* 2). Increasing the dose of α-tocopherol to 200 µM precisely increases the endothelial permeability. When α-tocopherol acts as an antioxidant, it will be oxidized into tocopheryl and this radical form is physiologically converted into tocopherol [10]. Administration of α-tocopherol 200 µM possibly produces tocopheryl radical in a huge concentration which cannot be regenerated by the physiological mechanism. Tocopheryl radical can disrupt actin filaments and VE-cadherin structure which results in the dysfunction of the endothelial barrier. Combination with ascorbic acid is suggested in α-tocopherol usage, especially in high doses [12, 19].

A single administration of α-tocopherol and ascorbic acid at the same dose (100 µM) obtained a different effect on endothelial permeability. α-tocopherol was more potent in protecting the barrier function of endothelial cells exposed high concentration glucose compared to ascorbic acid. α-tocopherol can reach the lipid membrane; therefore, it can protect the membrane from lipid peroxidation. Following the previous study, α-tocopherol prevented MDA production as a result of lipid membrane peroxidation on HUVEC culture exposed to high glucose concentration [26]. α-tocopherol works through dual-action both of as a direct and indirect antioxidant. The direct antioxidant mechanism through inhibition of free radical and production of Advance Glycate End Product (AGE), stop chains reaction production whereas indirect antioxidant work by the inhibition of Protein Kinase C (PKC) activation [10]. α-tocopherol was more effective in inhibiting the increase of endothelial permeability compared to ascorbic acid. This is caused by α-tocopherol works through dual-action whereas ascorbic acid works by scavenging free radical only [12].

Effect of combination of ascorbic acid and α-tocopherol on HUVECS culture exposed high glucose concentration

Combination of ascorbic acid and α–tocopherol at dosage 50 µM and 100 µM reduced the permeability of endothelial cells exposed high concentration glucose. The use of ascorbic acid and α–tocopherol combination results in a pharmacodynamic interaction and a synergistic effect to protect cells against free radicals. Moreover, the combination of antioxidants aims to reduce the doses of both of them and therefore adverse reaction can be minimized [23, 24]. The antioxidant combination reduces radical reactivity and therefore actin filament structure and VE-cadherin, as well as its barrier function, could be maintained. Cell morphology after given antioxidant combination showed cells membrane to be smooth and there are no damage cells. The combination of α–tocopherol and ascorbic acid has a beneficial effect, in which tocopheryl radical can be regenerated into tocopherol by ascorbic acid and therefore reduces stress oxidative [12, 22].

Based on this study, antioxidant combination needs a lower dose compared to single antioxidant to retain of endothelial barrier function. Ascorbic acid 50 µM and α–tocopherol 50 µM in single composition has not shown inhibition of permeability increase of HUVECS culture. Whereas, in the combination of ascorbic acid 50 µM and α–tocopherol 50 µM could reduce the increase of endothelial permeability until it was not different compared to the normal group. This combination also strongly decreased the endothelial permeability of the HUVEC culture.

The combination of ascorbic acid 100 µM and α–tocopherol 100 µM could prevent the permeability increase of HUVECS culture. However, it was lower compared to a dose each of 50 µM. The increase of dosage possibly changes it into a pro-oxidant effect due to the production increase of ascorbic and tocopheryl radical [11, 12]. Disorder of the transporter system of ascorbic acid has a potency to cause lipoprotein damage of cells membrane [6, 11]. They have a function as an endothelial barrier, therefore, the HUVEC's permeability will increase. The metal transition also contributes to the increase of ROS production besides the expose of high concentration of glucose [2]. Ascorbic acid 25 µM and α–tocopherol 25 µM in combination did not significantly decrease endothelial permeability. This was caused by the low doses of its combination which cannot protect cells against oxidative damage, therefore, increase endothelial permeability [23]. This was

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signaled by the passage number of albumins which was high in the albuminal compartment.

Conclusion

Our studies showed that the exposure to high glucose concentration on HUVEC's culture increases the endothelial permeability. Meanwhile, ascorbic acid 100 µM, α-tocopherol 100 µM, and 200 µM, as well as its combination potentially have protective properties by inhibiting the increase of endothelial cells permeability exposed a high concentration of glucose.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Conflict of interest

We declare that we have no conflict of interest

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