

Combined effects of shear stress and glucose on the morphology, actin filaments, and VE-cadherin of endothelial cells in vitro



Erna Sulistyowati^{a,*}, Nur Permatasari^b, M. Aris Widodo^b

^a Department of Pathology, Faculty of Medicine, Malang Islamic University, Malang, East Java, Indonesia

^b Department of Pharmacology, Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia

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ABSTRACT

Objective: The purpose of the present study was to analyze the effects of glucose and shear stress on the morphology and density of endothelial cells, actin filamen, and the expression of VE-cadherin.

Methods: After confluency of endothelial cells (3–4 days), 22 mM of D-glucose was administered for 7 days. Endothelial cells were exposed to shear stress of 10 dyne/cm² for varied durations of 5, 8, 12, and 15 min. Morphology of ECs was observed using an inverted microscope before and after shear stress exposure. VE-cadherin and actin filament were analyzed immunohistochemically.

Results: Exposure to high glucose induces more shrinkage and the cell density decreased at 15 min. High glucose reduced actin filaments and the more globular ones, especially around the nuclei. There was a decline in VE-cadherin scores with significant differences between treatments with 5 mM and 22 mM of glucose.

Conclusion: Combination exposure of shear stress and high glucose changes morphology, reduces actin filament and VE-cadherin, of endothelial cells.

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1. Introduction

Until recently, atherosclerosis remains a problem in many parts of the world. High-density lipoprotein (HDL) and low-density lipoprotein (LDL) have opposite roles in the pathomechanisms of atherosclerosis [1, 2]. In most cases, atherosclerosis occurs in the bifurcation areas, bends, or stenosis of the artery. At these sites, blood flows are impaired in which the vortical flows and zones are separated. These events predispose to atherosclerosis. In addition to impaired blood flows, the slowing down of the blood flow may occur. This slowing down flow causes interaction between the low-density lipoprotein and blood vessel walls and then there is penetration into the vascular walls [3].

Vascular endothelial cells (ECs) are constantly exposed to the shear stress of blood flows. Endothelial cells are highly responsive to shear stress characterized by conversion of mechanic stimulus into biological-relevant signals (mechanotransduction). Early stages of this response include cell-anchoring integrins and activation of membrane receptors, such as vascular endothelial growth factor receptor-2 (VEGFR-2) and G-protein coupled receptors [4–7]. Subsequent to the initial activation of the molecules, the signals are transmitted into cells through the activation of various signal transduction pathways, such as mitogen-activated protein kinase (MAPK) pathway, protein kinase B (PKB/Akt) pathway, and endothelial nitric oxide synthase (eNOS)

signals. The processes lead to functional changes of apoptosis and cell proliferation, inflammatory sensitivity, and cytoskeletal remodeling [8–10].

A variety of vascular complications such as micro and macro-angiopathies often are found in individuals with type 1 diabetes mellitus with impaired blood sugar control. Incidence of vascular complications increases morbidity and mortality of patients [11]. Hyperglycemia represents one of the complication-accelerating factors, interacting with various factors of blood flow such as shear stress. To the best of our knowledge, no study has evaluated the combined effects of hyperglycemia and shear stress on ECs so far. The purpose of the present study was to analyze the effects of hyperglycemia and shear stress on the morphology and density of ECs. In addition, it will evaluate the effects of these factors on the expression of VE-cadherin.

2. Material and methods

2.1. Endothelial cells culture

This study was of experimental in vitro. Umbilical veins that have been cleaned with phosphate-buffered saline A (PBS-A) solutions were isolated enzymatically by the use of type II collagenase dissolved in a serum free medium consisting of M199 (Gibco), 100 µ/ml of penicillin and 100 µ/ml of streptomycin (Sigma), 21 mM/ml solution of sodium bicarbonate-phenol red, 2 mM glutamine. Cells were incubated for 7 min and rinsed with 10 ml of PBS-A. Solutions containing ECs were

* Corresponding author.

E-mail address: dr_erna@unisma.ac.id (E. Sulistyowati).

centrifuged at 1000 rpm for 8 min in order to obtain pellets of ECs. ECs were grown on glass objects in 6-well culture plates. Upon formation of monolayers and confluence of endothelial cells (3–4 days), 22 mM of D-glucose was administered for 7 days. Media were replaced every 2 days.

2.2. Shear stress

Cone and plate were used for treatment with shear stress, as shown in Fig. 1. ECs were exposed to shear stress of 10 dyne/cm² for varied durations of 5, 8, 12, and 15 min. The variation of shear-stress duration was made between 5 and 15 min to determine their effects on changes in the morphology, density, the structure of VE-cadherin, and actin filaments of endothelial cells.

2.3. Endothelial morphology and density

Morphology of ECs was observed using an inverted microscope before and after shear stress. Cell density was counted manually under an inverted microscope on day 7 of treatment with 22 mM of glucose.

2.4. Immunohistochemistry

ECs grown on cover slips were washed 3 times with sterile PBS of pH 7.4 and fixed with methanol for 5–8 min. Then, each well was washed and incubated with sterile PBS of 2 ml for 5 min. Subsequently, it was washed 3 times with sterile PBS. Incubate with 1 ml of H₂O₂ 3% for 10 min. Wash each well 3 times with 2 ml of sterile PBS for 5 min. Subsequently, each well was incubated with 1 ml of 5% FBS for 1 h. Incubate each well overnight with 1 ml of the primary antibody to VE-cadherin/actin filament mouse monoclonal IgG1 1:100. Wash each well 3 times with 2 ml of sterile PBS for 5 min. Incubate each well with 1 ml of Conjugated Gout Human Anti Mouse IgG adsorbed B Biotin of 1:200 for 1 h. Wash each well 3 times with 2 ml of sterile PBS for 5 min. Incubate each well with 1 ml of 1:500 SA-HRP for 40 min. Wash each well 3 times with 2 ml of sterile PBS for 5 min. Incubate each well with 1 ml of DAB (diamino benzidine) of 1 grain for 10 ml of DAB buffer for 20 min. Wash 3 times with 2 ml of sterile PBS for 5 min. Wash with 2 ml of

sterile distilled water for 5 min. Then, each well was counterstained with 1 ml of Meyer hematoxylin for 10 min. Wash with tap water. Place on glass slides and covered with cover slips.

2.5. Scoring of the VE-cadherin structure

VE-cadherin was observed by the use of a scoring system in view of the utilization of a binocular microscope. Each 100 endothelial cells was subjected to scoring of the structure of VE-cadherin that resembled threads interconnecting ECs in the periphery. Scoring was repeated three times. A score of 2 was given when the observation indicated that 50% or more of the structure of VE-cadherin of ECs were intact. A score of 1 was given when the observation indicated that 50% or more of the structure of VE-cadherin of ECs were broken on the area around the center. Otherwise, a score of 0 was given when the observation indicated that 50% or more of the structure of VE-cadherin of ECs were entirely broken.

3. Results

3.1. Effect of glucose and shear stress on the morphology of endothelial cells

As shown in Fig. 2, exposure to 5 mM of glucose for 7 days prior to treatment with shear stress resulted in ECs having regularly arranged cobble stone-like shape (fusiform). Subsequent to treatment with shear stress for 5 min there were considerable morphological changes in which ECs were irregularly arranged, polygonal, and elongated, and exhibited a decreased ratio of nuclei to cytoplasm. These changes did not occur to the entire ECs, some of them still had fusiform shape. Almost all the entire ECs exhibited changes on minute 8. They were more elongated with nuclei more flattened and the ratio of nuclei to cytoplasm decreased to a greater extent in comparison with that of the treatment with shear stress for 5 min. It appeared that some ECs were already dead, detaching from the base. On minute 12 of the exposure, it was obvious that ECs were even more elongated and lined up according to the direction of shear stress. The most severe damage to the cells was observed on minute 15.

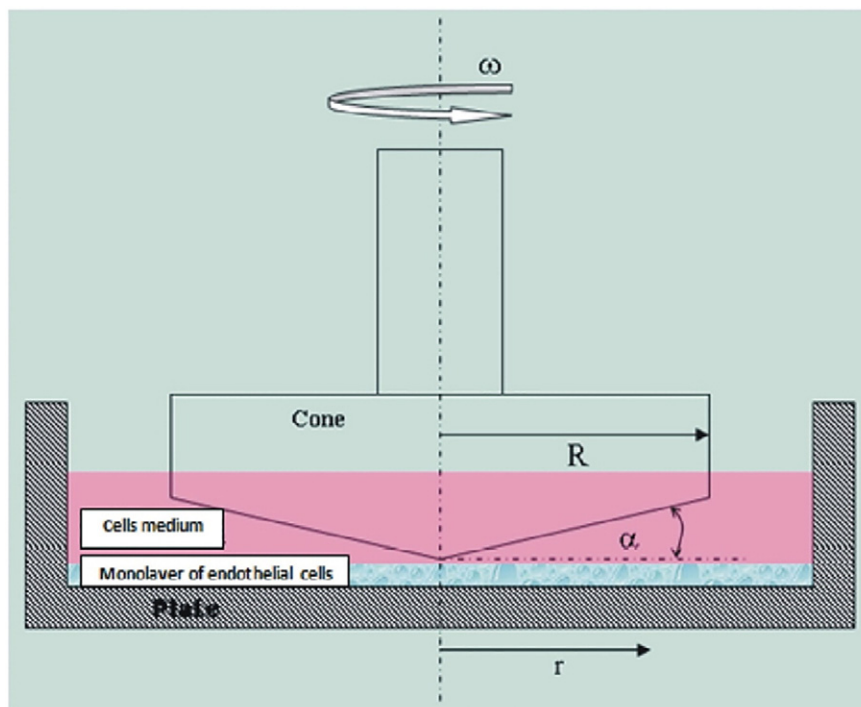


Fig. 1. The design of equipment for inducing shear stress on endothelial cells culture.

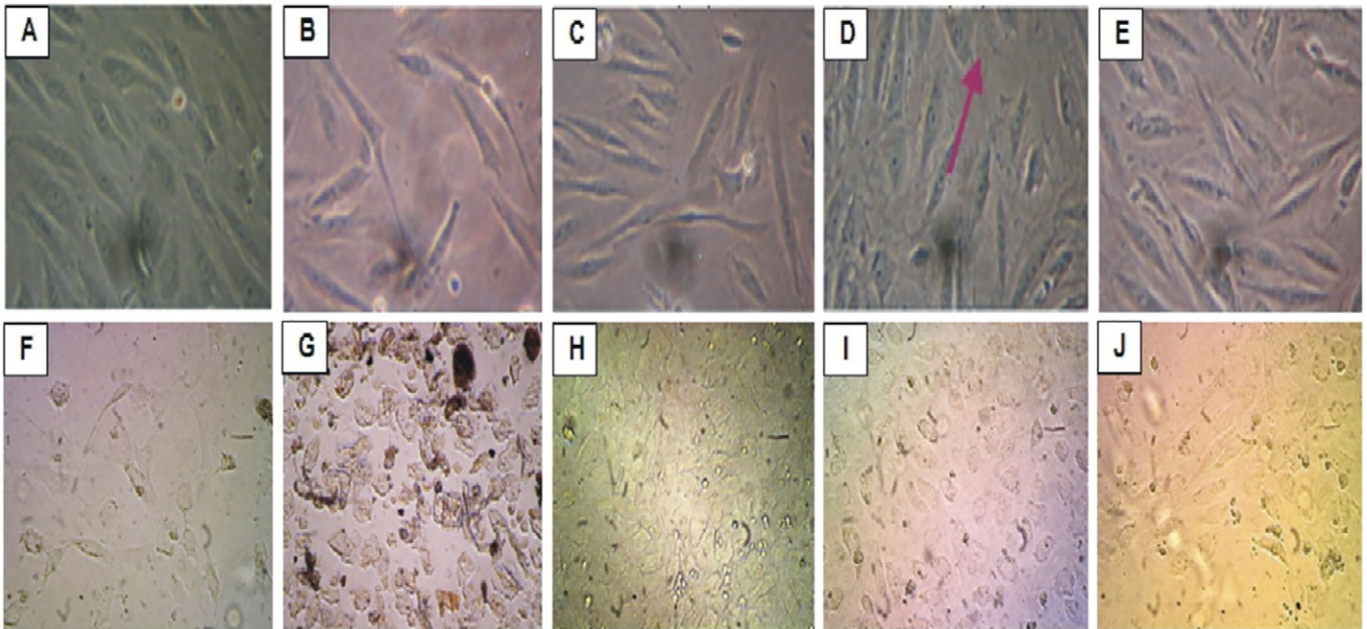


Fig. 2. The morphology of endothelial cells exposed to combination of shear stress and 5 mM (B–E) and 22 mM glucose for seven days (G–J) compared with glucose exposure only (A & F). Duration of shear stress is 5 (B & G), 8 (C & H), 12 (D & I), and 15 (E & J) minutes.

Exposure to 22 mM of glucose for 7 days led to even more shrinkage ECs. Treatment of shear stress for either 5, 8, and 12 min did not affect the morphology of the ECs. On minute 15 of the treatment, the cell density decreased and there were empty spaces among ECs that were previously seen solid.

3.2. Effects of glucose and shear stress on the structure of actin filaments

Immunohistochemical staining of actin filaments exposed to 5 mM of glucose for 7 days without the treatment of shear stress exhibited features of regular filaments and polymerization. Treatment of shear stress for 5 min triggered elongation of actin filaments in accordance with the shape of the ECs cytoplasm and led to the emergence of irregular filaments. Some, but not all, ECs exhibited these changes. On minute 8 of

the treatment, almost all ECs underwent morphological changes, in which they were elongated with actin filaments undergoing changes in accordance with those of the cells' cytoplasm. Exposure to shear stress caused many actin filaments to break and ECs' cytoplasm to stretch. On minute 12 of treatment with shear stress those changes became more dominant. The feature of the ECs' actin filaments on minute 15 did not differ from that of minute 12, as seen in Fig. 3.

Exposure to 22 mM of glucose for 7 days resulted in reduced actin filaments and the more globular ones, especially around the nuclei. Treatment of shear stress for 5 min had more effects on changes in the shape of ECs cytoplasm. Starting on minute 8, the longer the exposure to shear stress, the more the number of elongated ECs and the more visible features of globular actin were. On minute 12 of exposure to shear stress, damage to actin filaments was clearly visible in which they were broken

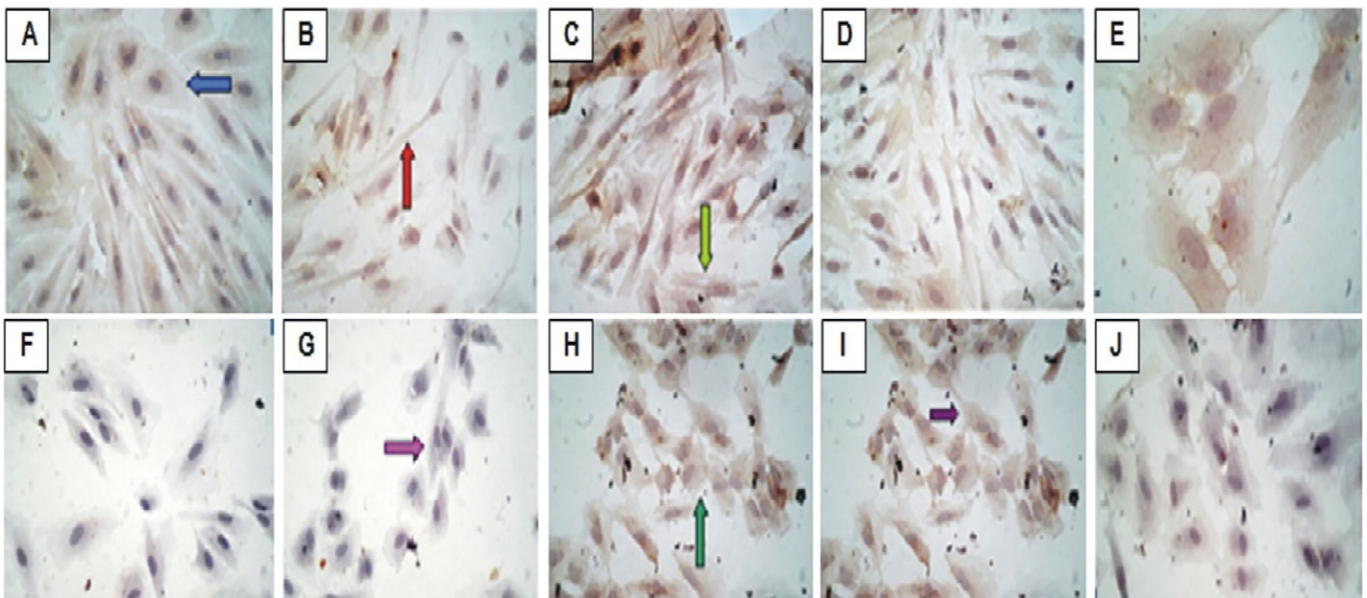


Fig. 3. The structure of actin filament from endothelial cells exposed to combination of shear stress and 5 mM (B–E) and 22 mM glucose for seven days (G–J) compared with glucose exposure only (A & F). Duration of shear stress is 5 (B & G), 8 (C & H), 12 (D & I), and 15 (E & J) minutes.

in close proximity to the cell membrane. The features of actin filaments exposed to shear stress for 15 min did not differ from that of 12 min (Fig. 3).

3.3. Effects of glucose and shear stress on the structure of VE-cadherin

Fig. 4 present the structure of VE-cadherin in each experimental group. In normal condition, those interconnecting filaments were intact. Exposure to high levels of glucose and shear stress caused the breakdown of the filaments. There was a decline in scores with significant differences between treatments with 5 mM and 22 mM of glucose.

4. Discussion

The relatively straight regions in the arteries, with high hemodynamic shear stress (>10 dynes/cm²), show higher resistance to disease development compared with bifurcation regions or sections with high curvature where flow separation and recirculation result in low-average shear stress or low-oscillatory shear index. The hemodynamic stress stimuli are transmitted through the ECs surface layer, the glycocalyx layer, to reach the ECs surface and generate an intracellular response [12–14]. Previous in vitro studies showed that ECs exposed to high glucose (30 mmol/l) for 24 h causes the appearance of large intercellular gaps between the ECs, and thereby the cells anatomically appear to have no intercellular membrane and have loss intercellular adhesion. Cells treated with physiologic D-glucose showed no effect on membrane and hence adhesion [15]. In this study, exposure to 5 mM of glucose for 7 days resulted in ECs having regularly arranged cobble stone-like shape (fusiform). Exposure to 22 mM of glucose for 7 days led to even more shrinkage ECs. These morphological changes constitute ECs adaptation to high level of glucose, leading to skeletal reorganization. Shrinkage structure actually show a tendency toward oxidative damage to membrane and cell junctions.

Endothelial cells actin filaments consist of three structures constituting an organization of cortical actin filaments, stress fibers, and junctional-associated actin filament system. On day 7, exposure to

high levels of glucose (22 mM) resulted in a feature of increased depolymerization of actin filaments, which was likely to be globular, and a slight feature of interwoven actin filaments. Nuclei were enlarged, leading to a reduced ratio of nucleus to cytoplasm, indicating a sign of apoptosis of endothelial cells. Thus, presence of hemodynamic force of shear stress brings about changes in the structure and pattern of endothelial cells, the dynamics of which were time- and force-dependent. Our finding indicated that the presence of shear stress in ECs exposed to physiologic level of glucose caused transformation of endothelial cells from cuboidal (cobblestone) to polygonal (elongated) morphology and the arrangement pattern was in the same direction as the shear stress. Previous studies showed that ECs tend to elongate when pro-angiogenic cues are present, e.g., shear stress and VEGF. Our finding extended previous studies [16]. When ECs in shrinkage condition due to high level of glucose, shear stress reduced cell density. Decrease in the density of endothelial cells after treatment with shear stress of 10 dyne/cm² was associated with the detachment of endothelial cells from the base, which was related to the function of VE-cadherin. Mechanism of endothelial cell detachment was largely unknown. VE-cadherin is a member of a superfamily of Ca⁺⁺-dependent adhesion receptors known to bind cells together via homotypic interactions. VE-cadherin is crucial for the proper assembly of vascular structure and maintenance of vascular integrity [17,18]. VE-cadherin as transmembrane glycoprotein physically interconnected cell membranes. VE-cadherin extended from the cytoskeletal protein bound portions of cytoplasm to the extracellular domains. Exposure of endothelial cells to high levels of glucose induced the formation of intracellular reactive oxygen compounds disrupting the structure of VE-cadherin. Decrease in scores of VE-cadherin was showed on exposure to 22 mM of glucose for 7 days.

5. Conclusion

Combination of exposure between shear stress and high glucose changes morphology, reduces actin filament and VE-cadherin, of endothelial cells.

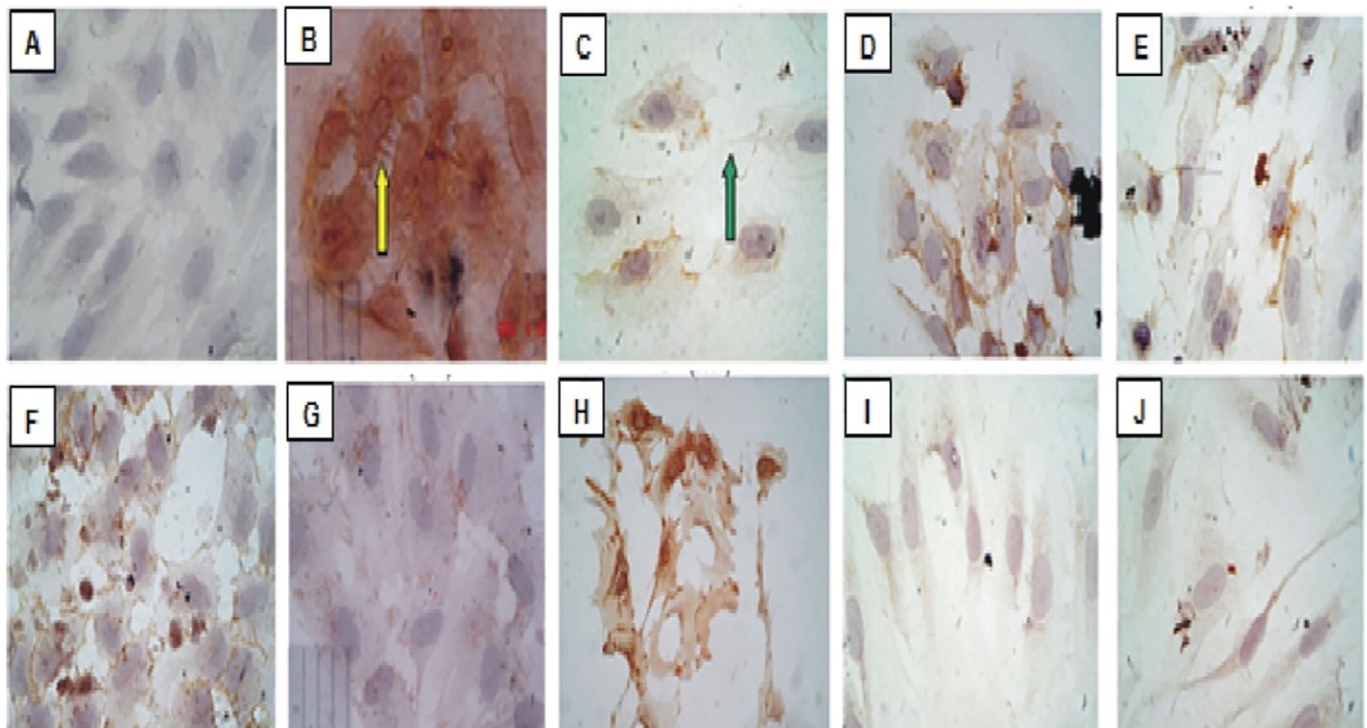


Fig. 4. The expression of VE-cadherin from endothelial cells exposed to combination of shear stress and 5 mM (B–E) and 22 mM glucose for seven days (G–J) compared with glucose exposure only (A & F). Duration of shear stress is 5 (B & G), 8 (C & H), 12 (D & I), and 15 (E & J) minutes.

Declaration of interest

The author(s) declare(s) that there is no conflict of interests regarding the publication of this article.

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