

Uncovering Caffeine Shielding Effect Against High Blood Pressure-Induced Shear Stress on Endothelial Cells

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ABSTRACT: Shear stress plays a critical role in endothelial cell physiology by regulating tissue growth and repair. In this research paper, we investigate the effects of caffeine on shear stress-induced damage to endothelial cells using an *in vitro* blood flow model. We optimized the experimental parameters to accurately mimic high blood pressure conditions *in vitro* and found that 3.0 N/m² shear stress significantly decreases endothelial cell viability compared to the 1.2 N/m² shear stress sample. However, adding 2 μ M caffeine improved cell viability by approximately 60%, indicating a protective effect against turbulent shear stress. Interestingly, the lowest cell viability was observed in samples exposed to 30 μ M caffeine pre-treatment and turbulent shear stress. Our results suggest that low concentrations of caffeine can protect endothelial cells from shear stress-induced damage. Further studies are necessary to determine the optimal caffeine concentration and duration of treatment for protecting endothelial cells *in vitro* and *in vivo*. Overall, our study provides insights into the effects of shear stress on endothelial cell viability and highlights the potential protective effects of caffeine against turbulent shear stress, which may have implications for preventing cardiovascular diseases.

KEYWORDS: Biomedical and Health Sciences; Caffeine; Blood Pressure; Endothelial cell; Shear Stress Protection.

■ Introduction

The endothelium, an essential organ comprising over a trillion endothelial cells, releases glucose and oxygen to maintain regular blood circulation and prevent health issues such as atherosclerosis and heart diseases.¹ The endothelial cells have a critical role in regulating tissue growth and repair by extending and remodeling the network of blood vessels. Without them, a life-support system that extends into every region of the body would be impossible.²

The vascular system, lined by endothelial cells, is responsible for transporting blood and regulating the flow of materials and white blood cells.³ Arteries and veins develop from small vessels constructed solely of endothelial cells, and signals from the endothelial cells to surrounding connective tissue and smooth muscle continue to be crucial in controlling the vessel's form and function.

The mechanical forces to which vascular endothelial cells are exposed, such as stretching, tension, compression, and shear stress, modulate their functional properties through mechanotransduction, which converts mechanical forces into molecular and cellular responses.⁴ Blood flow regulates the internal diameter of arteries by contracting and relaxing vascular smooth muscle cells and rearranging the vessel wall (ECs and extracellular matrix). In both cases, the endothelium acts as a mechanical sensor, and cell components like glycocalyx and extracellular matrix interact with the cytoskeleton and are activated by mechanical deformation.⁵

In summary, the endothelium is crucial in maintaining a healthy cardiovascular system. Therefore, understanding the mechanisms of endothelial cell regulation and response to

mechanical forces is essential for preventing and treating cardiovascular diseases.

Numerous studies have identified endothelial dysfunction as a contributing factor to cardiovascular events. This condition, a form of non-obstructive coronary artery disease, causes major blood arteries on the surface of the heart to contract instead of dilating, leading to symptoms such as chronic chest pain. Women are more commonly affected by this illness, and many individuals may feel frustrated by the lack of diagnosis and treatment available in clinics.⁶

The vascular endothelium plays a crucial role in maintaining cardiovascular health, with abnormalities in this system linked to various ailments, including atherosclerosis, aging, hypertension, obesity, and diabetes. Endothelial dysfunction is characterized by an imbalance in vasodilation and vasoconstriction, elevated reactive oxygen species and proinflammatory factors, and deficiency of nitric oxide bioavailability.⁷

The coordinated contraction and relaxation of vascular smooth muscle cells support the stable flow of blood throughout the human body. In addition, endothelial cells and extracellular matrix organization play a critical role in regulating blood flow. Specifically, endothelial cells are essential for sensing mechanical blood pressure and maintaining stable blood flow.⁸

Shear stress, the mechanical force of flowing blood on the surface of endothelial cells, triggers intracellular signals through mechanotransduction. This phenomenon affects extracellular matrix remodeling, cell behavior, and the expression of several vascular-related molecules. Typically, endothelial cells sense shear stress in the circulation and maintain the homeostasis of endothelial cell health by regulating shear stress. Nitric oxide,

a molecule released by endothelial cells, helps to lower shear forces, which are controlled by the near-smooth muscle cells.⁹

Caffeine is a widely consumed vasoactive substance that has been found to affect the widths of blood vessels, with different effects depending on its concentration. At high concentrations, caffeine can decrease the lumen of blood vessels, causing a rise in blood pressure. This is supported by research from the University of South Australia, which found that high doses of caffeine can increase calcium loss in urine, leading to a decrease in the amount of calcium that protects endothelial cells from shear stress, ultimately increasing the risk of high blood pressure.¹⁰ However, low concentrations of caffeine have been shown to have a protective effect on endothelial cells by increasing the amount of calcium in rabbit aortic endothelial cells, thereby protecting them from shear stress.¹¹

Caffeine is a vasoconstrictor, which means that it decreases the size of blood vessels and can raise blood pressure. This effect is thought to be mediated by caffeine's interaction with various receptors in the brain, including adenosine receptors in the kidney. Given that 80% of adults in the United States have a daily intake of 200 to 300 mg of caffeine, it is important to understand the potential effects of caffeine consumption on cardiovascular health. A recent meta-analysis suggests that long-term coffee consumption may increase systolic blood pressure.¹²

Turbulent flow is a form of fluid (gas or liquid) movement characterized by random fluctuations and mixing, in contrast to the smooth, layered movement of laminar flow. Turbulent flow occurs in many systems, including blood flow in arteries, oil pipelines, air and ocean currents, and pumps and turbines.¹³ Turbulence is often accompanied by eddies and swirls, which cause the mixing of the fluid layers. Two primary factors can cause turbulent flow: the introduction of perpendicular velocities and obstacles or sharp corners, such as those found in a faucet. Understanding turbulent flow is critical for many engineering and medical applications, as it can impact materials' transport and biological tissues' health.

The role of caffeine in cardiovascular health has been debated for years. While some studies suggest that caffeine can increase the risk of heart disease, others propose that caffeine has beneficial effects on the endothelium, the inner lining of blood vessels. Recent research has shown that caffeine can increase calcium concentration in rabbit aortic endothelial cells, which mediate muscle relaxation after shear stress. In light of this finding, we hypothesize that caffeine may protect endothelial cells under high-shear stress, potentially preventing cell death. Our null hypothesis is that increased calcium concentration in caffeine will not affect endothelial cells' response to high-shear stress. By examining the effects of caffeine on endothelial cells, we aim to shed light on the potential cardiovascular benefits or risks associated with caffeine consumption.

■ Methods

Cell culture and maintenance:

To culture HUVEC cells using RPMI1640 cell culture media. The medium was then supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution. After preparation, the cell culture medium was stored at 4°C

until use. HUVEC cells were obtained and cultured in a sterile laminar flow hood using aseptic techniques. The cells were seeded into six-well culture plates at a density of 5x10⁵ cells per flask and then incubated in a humidified incubator at 37°C and 5% CO₂. After incubation for 24 hours, the medium was replaced with RPMI1640 cell culture medium supplemented with 10% FBS and 1% penicillin-streptomycin solution. The cells were then observed and monitored for adherence and proliferation under an inverted microscope. The medium was changed every two to three days, and the cells were passaged using trypsin-EDTA solution when they reached 70-80% confluency.

Preparation of the Ibidi pump system:

The Ibidi pump system was assembled according to the manufacturer's instructions. First, the pump was connected to a media reservoir filled with HUVEC cell culture media supplemented with fetal bovine serum (FBS) and antibiotics. The system was then primed with the media to ensure that there were no air bubbles in the tubing. Next, HUVEC cells were trypsinized and resuspended in the HUVEC media. The cells were then seeded into the chamber slide and protected in a humidified incubator with 5% CO₂ at 37°C. After 24 hours, the media in the chamber slide was replaced with fresh media, and the chamber slide was placed on the pump. The tubing was then connected to the inlet and outlet ports on the chamber slide, and the flow rate was set to the desired speed.

Caffeine treatment under flow conditions:

To perform the experiment, cells were first seeded onto Ibidi µ-Slide I 0.8 Luer chamber slides and allowed to attach and grow for 24 hours. After the attachment period, the cells were exposed to caffeine treatment at 2 mM and 30 mM concentrations for 24 hours. Following the treatment, the Ibidi pump system was set up with the chamber slide to simulate flow conditions. Specifically, the Ibidi pump system was programmed to generate a continuous flow rate laminar and turbulent flow of cell culture media containing caffeine. Time-lapse imaging was performed using an inverted fluorescence microscope with a 20x objective lens, capturing images.

Cell viability imaging:

The AO/PI staining method was used to assess cell viability in this study. Firstly, the cell culture medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS). Next, the staining solution containing acridine orange (AO) and propidium iodide (PI) was prepared by diluting 100 µg/mL AO and 100 µg/mL PI in PBS. The staining solution was added to the cells and incubated for 5 minutes in the dark at room temperature. After incubation, the staining solution was aspirated, and the cells were washed twice with PBS. The cells were then observed under a fluorescent microscope, with live cells appearing green and dead cells appearing red. The number of live and dead cells was counted, and the percentage of viable cells was calculated. The AO/PI staining method was repeated in triplicate to obtain reliable results.

■ Results

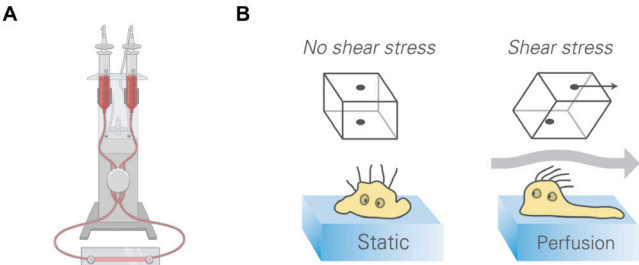


Figure 1: Ibidi pump system induces shear stress through perfusion. (A) Illustration of Ibidi pump system using single channel cell culture slide. (B) The concept of shear stress after perfusion.

In this study, we aimed to generate shear stress within the experimental conditions using the Ibidi pump system and incubator (Figure 1A). The Ibidi pump system can produce a range of pressures to stimulate and generate shear stress, and a glass slide with attached cells is connected to both ends of a tube through which cell media can continuously flow. This system controls the intensity of shear stress and flow rate and can induce shear stress in cells for a desired period by automatically controlling the flow. The incubator maintained a constant temperature and CO₂ level to ensure healthy cell conditions. In addition, real-time monitoring was performed to confirm the health status of endothelial cells, and cell viability was assessed using the Acridine Orange/Propidium Iodide staining technique, which can distinguish between live and dead cells.

The influence of shear stress resulting from blood flow on endothelial cells has been established to affect cellular functions and cell death (Figure 1B). Different incubation time points were tested to optimize the shear stress condition on endothelial cells. In the first trial, the cells were incubated for 24 hours, resulting in the death of most of the endothelial cells due to the high intensity and duration of the blood pressure. An eight-hour incubation time was used in the second trial, but this was also long enough to induce cell death in most cells. The optimized condition was found when the incubation time was set to five hours, with a shear stress rate protection representing normal blood pressure and 3.0 N/m² representing high blood pressure, showing that most cells were alive and dead, respectively. After testing over 50 conditions to optimize the shear stress condition in this experiment, the shear stress rate and time were finally optimized to analyze the effect of the shear stress condition on endothelial cell viability.

Table 1: The type of flow used to test its effect on endothelial cell viability.

Type of flow	Physiological occurrence	Flow rate	Flow direction
Unidirectional Laminar Flow	In most small healthy biological vessels	Constant	Constant
Oscillatory laminar flow	Induced turbulence stimulation using flow chambers	Constant	Periodically changing

We conducted this experiment to compare the impact of laminar flow in standard and high blood pressure on endothelial cells. We chose two types of laminar flow for this purpose: unidirectional laminar flow, which represents nor-

mal blood flow in the human body, and oscillatory laminar flow, which means the abnormal flow that occurs in high blood pressure and induces turbulent flow (Table 1). Physiologically, unidirectional laminar flow occurs in most small healthy biological vessels with constant blood flow and direction. We used oscillatory laminar flow to stimulate turbulent flow in the experiment, which is induced turbulence stimulation using flow chambers with a continuous flow rate but periodically changing direction. We aimed to measure the impact on endothelial cells by causing turbulent flow, which is an abnormal flow that appears in the body, rather than simply adjusting the pressure to measure the viability of endothelial cells.

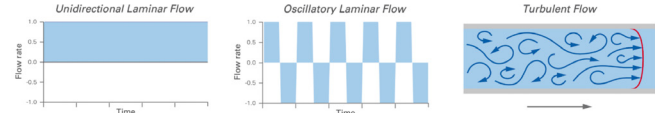


Figure 2: The difference between unidirectional and oscillatory laminar flow in terms of flow rate and time. Oscillatory laminar flow generates the turbulent flow indicated on the right side.

This study aimed to clarify the mechanism of turbulent flow generation. As illustrated in Figure 2, only oscillatory laminar flow can trigger turbulent flow due to its inconstant flow rate. Unlike unidirectional flow with a constant positive flow rate, the oscillatory flow has a positive and negative flow rate depending on the given time, resulting in velocity increases. These increases further cause turbulence. To generate different types of laminar flow, we used two methods: unidirectional laminar flow and oscillatory laminar flow. Our findings show that only oscillatory laminar flow triggers turbulent flow due to its flow rate, and the inconstant flow rate enhances the turbulence flow.

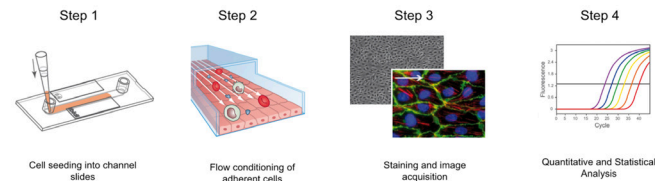


Figure 3: Experimental procedure for evaluating the effect of blood flow on endothelial cell viability. The figure illustrates the four main steps of the experiment, which include (1) preparation of cell-containing slide channels to simulate blood flow, (2) confirmation of cell adhesion through imaging, (3) assessment of cell integrity and viability using Acridine/Propidium iodide staining and (4) fluorescence signal analysis to quantify the number of live and dead cells.

In this study, we established a blood flow mimicking system for high and low blood pressure conditions to investigate the effect of blood flow on endothelial cell viability. The experiment consisted of four steps, as illustrated in Figure 3. Firstly, cell-containing slide channels were prepared to simulate blood flow. In step 2, cell adhesion was confirmed by imaging the cells 24 hours after seeding to ensure a confluent monolayer. In step 3, the overall integrity of the cells was assessed through cell imaging, and cell viability was determined using the Acridine/Propidium iodide staining method. In the fluorescence image, green fluorescence indicates live cells,

cence signal analysis was performed to evaluate cell viability. The number of green and red cells was quantified from the images. These experimental steps were critical in examining the effects of blood flow on endothelial cell viability *in vitro*.

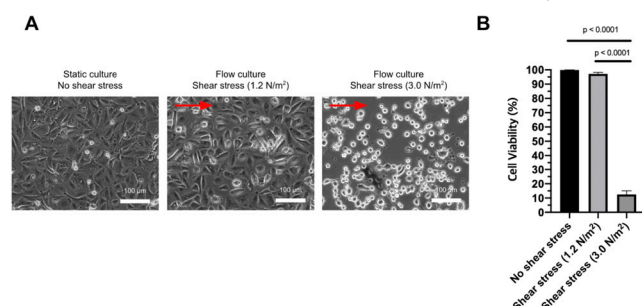


Figure 4: The effect of shear stress on cell detachment. (A) When no shear stress was applied, cells were mostly attached to the slide (left panel). However, when 1.2 N/m² shear stress was applied, more detached cells (white glowing cells) were observed compared to the no-shear stressed sample (middle panel). At 3.0 N/m² shear stress, most cells were detached from the slide, as indicated by the white glowing cells in the image (right panel). (B) Displays the mean and standard deviation of cell viabilities under different shear stress conditions. The highest shear stress level (3.0 N/m²) showed the lowest cell viability, confirming the sensitivity of endothelial cells to blood shear stress. A one-way ANOVA test with Tukey's test was performed to calculate the p-value.

We conducted an analysis to determine the effect of increasing the intensity of shear stress on the viability of cells using the blood flow model. Our study hypothesized that a higher shear stress level would decrease cell viability. First, we divided the cells into three groups and prepared three conditions: no shear stress, 1.2 N/m² shear stress, and 3.0 N/m² shear stress. Then, we incubated the cells in their respective conditions for 5 hours. After 5 hours, we stained the cells using Acridine/Propidium iodide staining solutions and evaluated cell viability based on the fluorescence intensity under a fluorescent microscope.

When no shear stress was applied, we observed that almost no flowing cells were present, and most cells were attached to the slide (Figure 4A). However, when 1.2 N/m² shear stress was applied, more detached cells (white glowing cells) were observed due to the shear stress compared to the no-shear stressed sample (Figure 4A). Lastly, when 3.0 N/m² shear stress was applied, we observed that most cells were detached from the cells, as indicated by white glowing cells in the image (Figure 4A).

Next, we analyzed cell viability. Figure 4B shows the mean and standard deviation of the percentage of cell viability in the three different conditions used in Figure 4A. When no shear stress was applied, the cell viability was near 100 percent, indicating that cells were not damaged (Figure 4B). However, the viability percentage significantly decreased as the 3.0 N/m² shear stress was applied. Additionally, compared to the 1.2 N/m² shear stress sample, the 3.0 N/m² shear stress significantly reduced cell viability (Figure 4B). This result demonstrates the impact of shear stress on endothelial cell viability. In line with our hypothesis, the highest shear stress level (3.0 N/m²) showed the lowest cell viability. This finding confirms that endothelial cells are susceptible to blood shear stress.

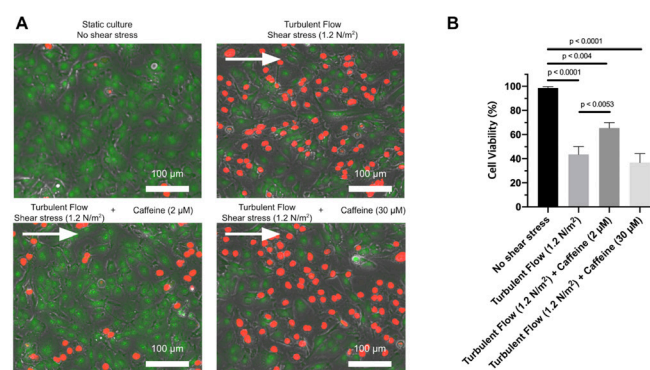


Figure 5: The impact of caffeine on endothelial cell viability under turbulent flow shear stress. (A) Fluorescence images were obtained after applying four different conditions, with red staining indicating significant cell death under turbulent flow shear stress. (B) The mean and standard deviation of the percentage of cell viability in the four different conditions show that the cell viability was near 100% when no shear stress was applied. A one-way ANOVA test with Tukey's test was performed to calculate the p-value.

We conducted an experiment to investigate the effects of turbulent flow on endothelial cells and to explore whether caffeine can protect the cells from such effects. We hypothesized that caffeine would decrease the effect of shear stress on endothelial cell flexibility and protect the cells from turbulent flow-induced cell death. Therefore, we prepared four conditions to measure endothelial cell viability: negative control with no shear stress, turbulent flow (1.2 N/m²) without caffeine, and turbulent flow (1.2 N/m²) with either 2 µM or 30 µM caffeine.

We obtained fluorescence images of the endothelial cells after applying the four conditions and observed that most cells remained alive in the absence of shear stress. However, when turbulent flow shear stress was used, most cells were stained red, indicating significant cell death. Notably, when 2 µM caffeine was pre-applied to the endothelial cells before the turbulent flow, most cells remained alive compared to those without caffeine (Figure 5A). On the other hand, 30 µM caffeine did not show any protective effect against the turbulent flow shear stress (Figure 5A).

We plotted the mean and standard deviation of the percentage of cell viability in the four different conditions, which showed that the cell viability was near 100 percent when no shear stress was applied. As expected, the viability percentage significantly decreased as the 1.2 N/m² shear stress was applied (Figure 5B). However, when 2 µM caffeine was added, the viability increased to about 60 percent, indicating that the cells were protected from the turbulent shear stress (Figure 5B). The lowest cell viability was observed in the samples with 30 µM caffeine pre-treated with turbulent shear stress (Figure 5B). Our experimental results support our hypothesis, but only when 2 µM caffeine was applied to the endothelial cells.

■ Discussion

In this study, we developed an *in vitro* blood flow model to investigate the effects of shear stress on endothelial cell viability. We extensively tested various conditions, including incubation time, shear stress levels, and blood flow types, and optimized the experimental parameters to mimic high blood

pressure conditions *in vitro* accurately. Our findings showed that 3.0 N/m² shear stress significantly decreased endothelial cell viability compared to the 1.2 N/m² shear stress sample. However, adding 2 µM caffeine improved cell viability by approximately 60%, indicating a protective effect against turbulent shear stress (Figure 4B). Notably, the lowest cell viability was observed in samples exposed to 30 µM caffeine pre-treatment and turbulent shear stress (Figure 4B). These results support our hypothesis that low concentrations of caffeine can protect endothelial cells from shear stress-induced damage. However, it is important to note that this effect was observed only by applying 2 µM caffeine. Further studies are necessary to investigate the optimal caffeine concentration and duration of treatment for protecting endothelial cells *in vitro* and *in vivo*.

Before the present study, more research is needed to investigate caffeine's effects on endothelial cells. Although coffee is commonly associated with increased blood pressure due to its caffeine content, the underlying mechanisms remain unclear. To address this knowledge gap, we developed a standard blood flow model *in vitro* using endothelial cells exposed to turbulent and unidirectional flow. By simulating high blood pressure conditions, we sought to test the widely held hypothesis that coffee consumption leads to elevated blood pressure. During the experiment, we observed the impact of turbulent flow on endothelial cell viability and documented changes in endothelial cell morphology through imaging.

Caffeine intake is known to elevate blood pressure at high doses due to its vasoconstrictive effects on blood vessels.¹⁵ Recent research by the University of South Australia investigated the impact of coffee on the body's calcium management system and found that high doses of coffee (800 mg) consumed over six hours doubled the amount of calcium excreted in the urine. These findings suggest that short-term, high-dose caffeine intake affects renal calcium clearance, which can increase blood pressure by exposing endothelial cells to elevated shear stress. However, low caffeine concentrations can stimulate calcium production, which mediates endothelial cell relaxation in response to shear stress.¹⁵ This protective effect may help prevent endothelial cell death under severe shear stress conditions. Our results showed that endothelial cells exposed to low concentrations of caffeine had the lowest number of dead cells. In contrast, those exposed to high concentrations of caffeine exhibited significant cell death. These findings suggest that caffeine has a dual effect on endothelial cells depending on the concentration, with potential implications for cardiovascular health.

■ Conclusion

Our study focused on the effects of caffeine on one particular type of endothelial cell line, which limits the generalizability of our findings to other cell types. Further research is necessary to investigate the effects of caffeine on different endothelial cell lines to confirm the consistency of our results. Moreover, our study did not explore the precise mechanisms underlying the protective effect of low caffeine concentration on endothelial cells exposed to high shear stress produced by turbulent flow. Future research should elucidate this protective mechanism's

molecular and cellular pathways. Additionally, our study did not examine the potential long-term effects of caffeine exposure on endothelial cell viability and function. Thus, more research is needed to evaluate the safety and effectiveness of caffeine as a therapeutic agent for cardiovascular disease. Overall, our study raises important questions regarding the molecular and cellular mechanisms underlying the protective effects of low caffeine concentrations on endothelial cells under high-shear stress conditions.

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