



YEDITEPE UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

DISCOVERY OF FUNCTIONAL CHARACTERISTICS OF PLANT CELL
SUSPENSION-DERIVED EXTRACELLULAR VESICLES ON THE IN VITRO
CEREBROVASCULAR INFLAMMATION

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ABSTRACT

DISCOVERY OF FUNCTIONAL CHARACTERISTICS OF PLANT CELL SUSPENSION-DERIVED EXTRACELLULAR VESICLES ON THE IN VITRO CEREBROVASCULAR INFLAMMATION

Extracellular vesicles (EVs) are essential biomolecule carriers responsible for intercellular communication. They play vital roles in many physiological events. Thanks to their bilayer lipid membrane, biomolecules can be protected from the harsh extracellular environment. One of living organisms in which EVs consistently function is plants. Plant-derived EVs (PDEVs) have a role in physiological events and defense against pathogens. Besides, these nanovesicles showed therapeutic potential in antioxidant, anti-inflammatory, and immunomodulatory activities. On the other hand, cerebrovascular inflammation (CVI) is a chronic inflammatory disorder characterized by cerebrovascular dysfunction, proinflammatory phenotype, and vascular hyperpermeability. Based on their therapeutic activities, PDEVs can become a promising tool for cerebrovascular disorders. Our study aims to investigate the functionality of plant cell suspension extracellular vesicles (PCSEVs) and their therapeutic effects on the *in vitro* CVI model. To that end, these nanovesicles were purified from the media of *Stevia rebaudiana*, *Vaccaria hispanica* and characterized. The CVI pathology was modelled and the effects of PCSEVs were investigated. In the results, first, the PCSEVs were successfully purified and characterized in the sense of physical and molecular properties. Besides, the CVI pathology was successfully modelled in the cells and it exhibited critical features of its pathologies. Lastly, it was observed that PCSEVs led to a protective response in the CVI model, and these effects mainly occurred by modulating adhesion molecule expression via the NRF2 pathway. In addition, further analysis revealed that these effects of PCSEVs were mediated through the phosphorylation of key signaling pathways. However, further studies are needed to transform these *in vitro* results into clinical applications. Future studies should include *in vivo* experiments in animal models to confirm the efficacy and safety of the PCSEVs. By demonstrating the therapeutic effects of PCSEVs on cerebrovascular inflammation, our study paves the way for innovative, plant-derived nanomedicine solutions for vascular diseases.

ÖZET

BİTKİ HÜCRE SÜSPANSİYON KÜLTÜRÜNDEN ELDE EDİLEN EKSTRASELÜLER VEZİKÜLLERİN İN VİTRO SEREBROVASKÜLER İNFLAMASYON ÜZERİNDEKİ FONKSİYONEL ÖZELLİKLERİNİN KEŞFEDİLMESİ

Hücre dışı veziküller (EV), hücreler arası iletişimden sorumlu olan biyomoleküler taşıyıcılarıdır. Birçok fizyolojik olayda hayati rol oynamaktadırlar. Çift katmanlı lipit zarları sayesinde biyomoleküller zorlu hücre dışı ortamdan korunabilmektedir. EV'lerin sürekli olarak fonksiyon gösterdikleri organizmalardan biri de bitkilerdir. Bitki kaynaklı EV'ler (PDEV'ler), fizyolojik olaylarda ve patojenlere karşı savunmada rol oynamaktadırlar. Ayrıca, bu nanoveziküller antioksidan, anti-inflamatuar ve immünomodülatör aktivitelerde terapötik potansiyel göstermişlerdir. Öte yandan, serebrovasküler inflamasyon (CVI), serebrovasküler disfonksiyon, proinflamatuar fenotip ve vasküler hiperpermeabilite ile karakterize kronik bir inflamatuvar hastalıktır. Terapötik aktivitelerine dayanarak, PDEV'ler serebrovasküler bozukluklar için umut verici bir araç olabilir. PCSEV'lerin işlevselliğini ve *in vitro* CVI modelindeki terapötik etkilerinin araştırılması amaçlanmaktadır. Bu amaçla, bu nanoveziküller *Stevia rebaudiana*, *Vaccaria hispanica* besiyerlerinden saflaştırılmış ve karakterize edilmişlerdir. CVI patolojisi modellenmiş ve PCSEV'lerin etkileri araştırılmıştır. Sonuçlara bakıldığında, ilk olarak, PCSEV'ler başarıyla saflaştırılmış ve fiziksel ve moleküler özellikleri bakımından karakterize edilmişlerdir. Bunun yanında, CVI patolojisi hücrelerde başarıyla modellenmiş ve hatta hastalığın özelliklerini taşıdığı belirlenmiştir. Son olarak, PCSEV'lerin CVI modelinde koruyucu bir yanıtı açtığı ve bu etkilerin NRF2 yolağıyla adezyon moleküllerinin ekspresyonu üzerinde gerçekleştiği belirlenmiştir. Ek olarak, PCSEV'lerin bu etkilerinin temel sinyal yollarının fosforillenmesi yoluyla gerçekleştiği ortaya konulmuştur. Bu *in vitro* sonuçların klinik uygulamalara dönüştürülmesi için daha fazla çalışmaya ihtiyaç vardır. Gelecekteki çalışmaların, PCSEV'lerin etkinliğini ve güvenliğini doğrulamak için hayvan modelleri üzerinde *in vivo* deneyleri içermesi planlanmaktadır. PCSEV'lerin serebrovasküler inflamasyondaki yenilikçi ve terapötik etkilerinin gösterilmesiyle, vasküler hastalıklarda bitki kaynaklı nanomedikal çözümlerin önü açılmaktadır.

DEDICATION



Dedicated to my lovely wife...

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LIST OF ABBREVIATIONS

μ	Micron
$^{\circ}\text{C}$	Degree Celsius
SD	Standard Deviation
SEM	Standard Error of Mean
AFM	Atomic Force Microscopy
AMPK	AMP-activated Protein Kinase
AVs	Apoptotic Vesicles
BBB	Blood Brain Barrier
BSA	Bovine Serum Albumin
CECs	Cerebral Endothelial Cells
CNS	Central Nervous System
CVI	Cerebrovascular Inflammation
HMOX1	Heme Oxygenase 1
ILVs	Intraluminal Vesicles
JAM	Junctional Adhesion Molecule
MAPK	Mitogen-activated Protein Kinase
MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain Phosphatase
MVBs	Multivesicular Bodies
MVs	Microvesicles
NO	Nitric Oxide
NQO1	NADPH Quinone Dehydrogenase 1
OGD	Oxygen-Glucose Deprivation
PAMP	Pathogen-associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cells
PCSEVs	Plant Cell Suspension-derived Extracellular Vesicles
PDEVs	Plant-derived Extracellular Vesicles
PPARG	Peroxisome Proliferator-activated Receptor Gamma
PSCs	Plant Suspension Cells
ROS	Reactive Oxygen Species

1. INTRODUCTION

1.1. EXTRACELLULAR VESICLES

In multicellular organisms, intercellular communication is essential for successfully continuing physiological functions. Molecular-based signals such as hormones and neurotransmitters take part in the occurrence of various biological events. Regulation of cell or tissue differentiation, tissue repair, and immune responses are the main examples of these events [1]. The signal molecules, uniquely produced by the cells, are transferred to the extracellular milieu and each other in specialized manners. These biomolecules used in paracrine, endocrine, or juxtacrine signaling might be simple gas molecules as well as highly complex vesicle structures. The complex communication tools, which are involved in several physiological-pathological events and are increasingly important every day, are called extracellular vesicles (EVs) [2]. It has been determined that these bilayer lipid vesicles are utilized by numerous organisms, from simple microorganisms like bacteria to higher eukaryotes like mammals or plants. It has recently been understood that EVs, described as cellular wastes for many years after their discovery, have functional effects in the transmission of biomolecules to target cells [3]. These signal molecules protected from the external milieu are mostly composed of nucleic acids, proteins, and lipids. However, the potential of the vesicular carriers, even the contents of which have not been properly elucidated, remains hidden.

1.1.1. Classification of Extracellular Vesicles

The term “extracellular vesicle” is generally used to describe the entire vesicular structures that cells utilize for communication among themselves. However, it is easily understood that the vesicle assemblage cannot be defined homogeneously under a single connotation [4]. Undoubtedly, this heterogeneous distribution among vesicular structures might be due to structural and molecular factors. A practical one of these factors that can enable observational distinction is dimensional differences, and they play a leading role in making similar classifications. Consequently, classes of EVs, divided into three groups previously, have increased significantly in recent years due to classification studies in vesicular structures [5]. Considering the tendency of changes in these distinctions, it is possible to observe that molecular features, rather than structural ones, become the determining factors.

The vesicle classes in the mentioned classification consisted of 30-200 nm-sized exosomes, 100-1000 nm-sized microvesicles, and 0.5-5 μ m-sized apoptotic vesicles [6]. In contemporary classifications of EVs, two novel groups called exosome-like vesicles (20-50 nm) and membrane particles (50-80 nm) are intended to be included in these groups [5]. Even though this classification is the most detailed distinction that can be made nowadays, it does not offer anything beyond the revealed theoretical differences. Moreover, pieces of evidence that exosome-like vesicles have actual differences from exosomes rather than methodological variations do not appear convincing. Therefore, collecting exosomes, exosome-like vesicles, and membrane particles under the name of exosomes would not be wrong in terms of current literature. This is because we cannot purify these vesicles homogeneously in practice, even if we can theoretically classify them, including those that we may not identify today [7]. As a result, the classification of extracellular vesicles within the scope of the dissertation will be considered as microvesicles, apoptotic bodies, and exosomes.

Another factor that supports the classification of EVs according to their dimensional differences is the production pathways of vesicles [8]. Moreover, the fact that different types of EVs have distinct biogenesis pathways supports this separation with dimensional characteristics. Among these types, the biogenesis of apoptotic bodies is considered the least complex mechanism. In the final stages of apoptosis, programmed cell death, the vesicular structures formed by enclosing the cellular contents with membrane fragments are called apoptotic vesicles (AVs) or apoptosomes [9,10]. Regarding the biogenesis mechanism of these vesicles, AVs are thought to have a relatively limited role in physiological events. Nevertheless, in recent studies, it has been proven that AVs are not only cell parts and that apoptotic mechanisms have decisive functions in other intercellular interactions, especially in immune cells [11,12]. Another type of EVs, called microvesicles (MVs), have characteristics that can be naturally distinguished from AVs in terms of functions and biogenesis mechanisms. On the other hand, the overlap of microvesicles with exosomes in terms of some physical and functional properties makes it difficult to distinguish from each other. Accordingly, differences in the biogenesis mechanism are critical in proving that MVs are not just one of the other large-sized vesicular structures. The release of MVs into the extracellular environment by budding is a distinctive factor in the classification of EVs [9,13]. Because, according to the literature, it is known that MVs have active roles just as

exosomes in physiological and pathological events [14]. Similar to the condition in MVs, the particular biogenesis mechanisms of exosomes also contribute to the classification of these vesicular structures. Exosomes majorly emerge with the cellular endosomal system. Intraluminal vesicles (ILVs), which are formed by budding from the endosomal membrane to the vesicle lumen during the maturation process of multivesicular bodies (MVBs), form exosomes in the continuation of this process [15]. One of the mechanisms that function in the selection of proteins carried in the ILVs and deciding vesicular fate ending up either lysosome or cell membrane is the Endosomal Sorting Complexes Required for Transport (ESCRT) pathway [16]. However, in studies on the biogenesis pathways of exosomes, it was discovered that proteins such as ALIX and TSG101, which are involved in the ESCRT pathway, do not have an active role in the transmission of proteolipid structures into the vesicles. Therefore, it was postulated that the exosome biogenesis might also be ESCRT-independent and alternative mechanisms [15].

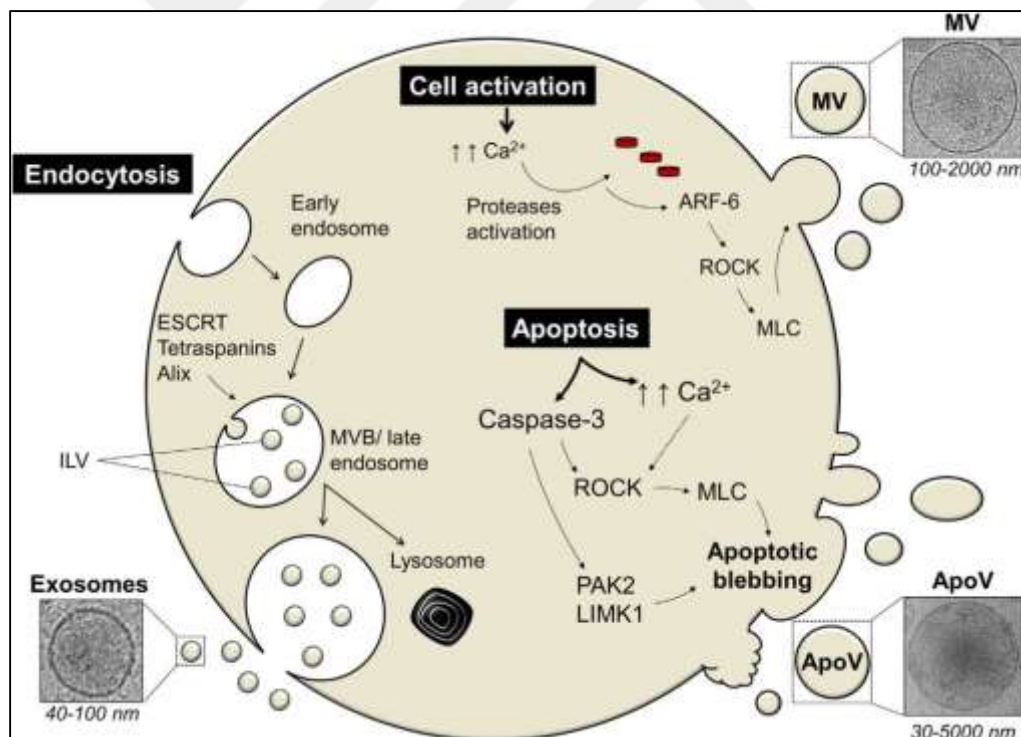


Figure 1.1. Biogenesis mechanisms of EVs according to various types of vesicles [17].

Studies have also indicated that ceramides and tetraspanins have regulatory functions in alternative biogenesis pathways [18]. Especially, as a result of research on sphingomyelinases, it was suggested that the ceramide-producing mechanism has a vital link

with exosome biogenesis [19]. Thus, it is understood that exosomes have differences from other vesicle types in the sense of physical aspects thanks to their distinctive biogenesis pathways. Moreover, as stated in Minimal Information for Studies of Extracellular Vesicles (MISEV), vesicular structures must be observed while cells release exosomes to identify these vesicles as exosomes [20]. In the classification of EVs, the differences in dimensional characteristics, as well as the generation and release pathways in cells, have an important contribution.

1.1.2. The Molecular Composition of Extracellular Vesicles

One of the factors that enable EVs to have decisive roles in intercellular communication is their biomolecular cargo carried in the vesicle structure. However, the structures of lipids and receptors in the membrane layers are quite influential to maintain the activities of molecules in nanovesicles and to reach targeted cells or microenvironments. Accordingly, the lipid, protein, and nucleic acid structures that form the extracellular vesicles have a role both in the formation of the double-layered membrane structure and in the physiological functions of the vesicles [21]. With omics studies conducted on vesicular contents, many novel findings about molecular and physical characteristics of vesicular structures have been discovered. However, at this point, it should not be forgotten that the purification methods used in EV studies reveal significant differences in the exploration of these vesicular characteristics. These differences arising from the purification methods may sometimes be due to varied fractions of EVs depending on the used methodology, or non-EVs originated impurities. Therefore, it is quite useful to be cautious in analyzing the results of the omics studies of extracellular vesicles in the literature [22].

1.1.2.1. Proteins

Protein molecules have a key place in the functions of EVs like in cellular mechanisms. These molecules being crucial for intercellular communication are transferred into the target cells by EVs in the form of enzymes, signal molecules, or receptors without exposing the deteriorating effects of the outer environment [23]. For instance, as a result of having receptor function in the vesicular surface, they are also responsible for delivering these biological signals to target cells and transmitting them accurately. In the literature, it has been found that EVs can carry cytological information such as inflammation responses belonging to parental cells [24]. On the other hand, it is stated that the majority of protein

molecules are also common markers of vesicular structures. Moreover, it is known that these proteins are involved in the biogenesis mechanisms and release of EVs [25]. If protein contents of EVs are evaluated in terms of their cellular localization, cytoplasm, and plasma membrane-derived proteins are generally more abundant in the vesicles than intracellular organelles. EVs include tetraspanin proteins, major histocompatibility complex (MHC) molecules, proteins related to endosomal trafficking, and finally various stress proteins, which are found more abundantly than cells and are mostly used as markers [25]. Besides, it was stated in the literature that some cytosolic and housekeeping proteins may also be used similarly. In addition to these common proteins, EVs also carry specific proteins belonging to their parental cells. An important example is that EVs released from endothelial cells contain VEGFA protein, which is a critical regulatory molecule in angiogenesis [26]. On the other hand, recent studies have suggested that some of these common proteins may be specific to EV subgroups. For example, while the cluster of differentiation-63 (CD63) proteins is found only in some EV subgroups, no such subpopulation segregation appears to be made in CD9 and CD81 proteins [27]. Another major marker for EVs is proteins that have a role in the biogenesis of vesicles. It was discovered that they originate from different stages in the biogenesis mechanisms of EVs. The fact that these are the decisive factors in the classification of vesicles is also reflected in the protein cargoes of EV groups. As a result, while effector proteins such as RAB5, RAB11, RAB31, and RAB27 function in the biogenesis of MVBs, are involved in exosomes [28,29], there are proteins such as ARF6, ARF1, and Rac1 that play a key role in the biogenesis and cargo selection of microvesicles [13]. These differences in the protein content of EVs are critical in the classification of vesicle groups, as well as provide cytological information about the biogenesis mechanism of EVs. Besides, the proteins carried by EVs contribute to the functional properties of the vesicles in various ways. One of these groups is heavily glycosylated proteins. In studies, it was found that EVs have generally conserved lectin-binding motifs [30]. These motifs might be varied depending on the changing conditions in the parental cells. Furthermore, it was determined that lectins in EVs contribute influential functions to vesicles by playing a role in surface receptor interactions and also have regulatory effects on the internalization of vesicles [31]. For example, it was determined that galectin-5 on the surface of EVs released from reticulocytes is vitally needed for EV uptake of macrophages [32]. Once again, it was proven that heparin treatment inhibits the vesicle uptake concerning glycosylation motifs on the surface of EVs [33]. Another point that affects EV functions on the cellular mechanism

is the receptors and growth factors of vesicular structures. These proteins are involved in the pathways in which EVs interact with membrane fusion or juxtacrine signaling [34]. Especially, the role of receptors is quite indispensable in the juxtacrine functions of EVs. In studies, it was determined that EVs are highly effective through many signaling pathways that can be activated extrinsically. For example, MHC molecules, transferrin receptors, and tetraspanins on the surface of EVs interact with cells and activate pathways such as integrin, calcium, and mitogen-activated protein kinase (MAPK) signaling [35]. For instance, EVs are effective in surface interactions in the immune system, including the generation of proinflammatory responses [36]. In vesicular functions, proteins carried in EVs, along with receptor molecules, play crucial roles in cellular interactions. These are mostly composed of growth factors, transcription factors, cytokines, and chemokines. Transcription factors unable to be released outside of cells on their own serve as a remarkable example of the necessity of EVs for intercellular communication [37,38]. Because, when these proteins are taken up by target cells, they might affect the gene expression of cells by functioning fundamental role in transcription. In another study, it was reported that platelet EVs also induce angiogenesis in endothelial cells via vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) [26]. The striking findings at this point are that growth factors are released in a membrane-bound manner, unlike standard release pathways. Thus, it is known that EVs allow for the transposition of growth factors in a target-specific and protected manner [39]. This phenomenon is more comprehensible for the transposition of cytokines and chemokines. EVs have great importance in releasing soluble factors such as cytokines, especially ones that do not have signal peptides at the amino end [40]. Major examples are cytokines like IL1A, IL1B, and IL18. Besides, it was reported that IL18 and IL32 cytokines are released by EVs as an activation response in cells [41,42]. In chemokines, just like cytokines, IL18 is released, while common or specific types can be produced and released due to environmental factors. On the other hand, it is a crucial issue to be able to determine protein contents in a reproducible way along with the functions of the proteins in EVs. Because, EV generation and release in parental cells are rapidly affected by environmental stressors or cellular stimuli, and even by active cellular topography [22]. Thus, it is clear that parental cells have an influential role in the protein content of EVs, even if the purification methods are the same. Thus, with improvements in future proteomics studies, these controversial issues need to be resolved.

1.1.2.2. Lipids

It is an indisputable fact that lipids have an essential role in the biogenesis and continuation of EV functions. Until recently, lipid functions such as forming the structures of vesicle membranes have been emphasized. Furthermore, in studies, it was proven that lipids in EVs also had effects on the physiological roles of these vesicles [43] and the number of these studies is increasing daily. Although the vesicular lipid content may vary the same as in the proteins, it was found that EVs could contain some specific lipid groups than parental cells [44]. In the study, it was determined that lipids such as ganglioside types like GM3 and GM1, hexosylceramide, lactosylceramides, and sphingomyelin are more abundant in EVs compared to parental cells. However, in another study, EVs released from various tissues differ according to lipid types and amounts in common cellular characteristics [45–47]. These studies have discovered information about the roles of lipids in EV stability along with insights into how pathological conditions are reflected in lipid compositions [48]. Nevertheless, as mentioned previously, lipids are not only present in the structure of the vesicular membrane. In studies, it was determined that cholesterol, which is found in the vesicle membrane, has a role in its release pathway from cells [49]. Ceramide, another lipid involved in the release of EVs, has a decisive role in the formation of ILVs in MVBs. Moreover, this mechanism involving ceramides, which emerged under the catalysis of nSMase 2, was named as one of the ESCRT-independent mechanisms [50]. Until now, it has been found that lipids in EV membranes have a role in the biogenesis mechanisms and mostly in their structural properties. Although relatively few studies have been reported, it is known that EVs can transfer functional lipids in membrane-bound and free forms to target cells and tissues [51]. This may be because studies on compositions of EVs showed more interest in other biomolecules than lipid in the first place. Nonetheless, there are many lipids, which have vital importance, still waiting to be discovered in future.

1.1.2.3. Nucleic Acids

Nucleic acids in EVs have critical functions in terms of information transferred to target cells. The genetic materials consist of many different types of nucleic acids, including DNA fragments, mRNA, tRNA, and small RNAs [52]. However, as with other biomolecules, it was determined that nucleic acid diversity occurs distinctively in vesicle subgroups. For instance, in recent studies, it was reported that small EVs did not contain double-stranded DNA or DNA-Histone protein complexes [53]. Besides, there are differences in RNA

species of EVs according to cell conditions. It is known that RNAs found free or bound to proteins in EVs are mostly less than 200 nucleotides [54]. Nevertheless, the relative percentages of these RNA species are still controversial. It was stated that rRNA was found in small amounts in EVs, while in others, contrary findings were obtained [55]. Once again, the impact of variations in purification methods on EV characterization becomes evident. According to the results of studies, it was determined that there were high amounts of small RNAs [56]. Besides the RNA diversity in EVs, the vesicular selection and loading of these molecules are quite important. With the novel findings on vesicular selection, the idea of the passive and spontaneous selection of RNA species into vesicles has been rejected. In the study, it was found that RNAs carrying specific nucleotide motifs increased in EVs and this mechanism is conducted by the heterogeneous nuclear RNP complex A2B1 in parental cells [57]. However, miRNA species are not transferred in vesicles solely for the transposition of information. Studies showed that vesicular miRNAs had essential and functional effects on the cell [58–60]. Moreover, these miRNAs were transferred to EVs derived from cells with recombinant miRNA plasmids and led to critical functions after uptake by recipient cells [61,62]. Thus, it is understood that these miRNAs in EVs have influential roles in intercellular communication. Another group of RNAs that function in the transmission of cellular information is mRNAs. However, mRNAs have decisive differences in terms of size and function in comparison with miRNAs. In the study, the lengths of vesicular mRNAs derived from peripheral blood mononuclear cells (PBMCs) were evaluated [63]. According to the results, it was determined that the lengths of vesicular mRNAs ranged from 33 to 19419 nucleotides. Besides it was observed that the nucleotide average of vesicular mRNAs was 3037 nucleotides in cells and 2799 nucleotides in EVs. Nonetheless, the study found that small-sized mRNAs were more abundant than long ones. The reason why vesicular mRNAs differ from miRNAs in their functional properties is that they continue the translational process rather than transcription in the target cell. In this way, it is known that critical pathological or pathological effects might occur when vesicular mRNAs are translated into proteins in the recipient cells. In another study, the vesicular mRNAs derived from cells expressing the luciferase gene were examined [64]. According to the results, it was proved that these modified EVs transferred luciferase-related mRNAs to target cells and these cells showed luciferase activity over 24 hours. Thus, it was determined that the EVs were taken up by the target cells and their vesicular mRNAs could enter the translation process. On the other hand, there is less information in the literature regarding the existence

and functions of DNAs in EVs compared to RNAs. In previous studies, traces of nucleic acid types such as single-stranded, double-stranded DNA, and mitochondrial DNA were found. It was determined that they ranged from about 100 base pairs to 2.5 kb pairs [65]. Although these genetic materials also provided information about parental cells, the functional properties and information of DNA in EVs are rather limited. In some studies, it was reported that stained DNA molecules in EVs reach the cytosol or even the nucleus of the target fibroblast cells [66]. From these points of view, it is understood that more information is needed regarding the physiological functions of nucleic acids in EVs.

1.1.3. Physiological Functions of Extracellular Vesicles

Investigating the molecular composition of EVs enables access to important pieces of information about biomolecular messages in vesicles and their physiological functions. The fact that these vesicular structures not only carry signal molecules but also accurately convey them to target cells makes EVs functional and remarkable. Studies on the functionality of EVs date back to the early 1980s. Thanks to the study conducted in 1982 revealed the interactions between prostate EVs and sperms, it pioneered the re-investigation of vesicular functions [67]. In recent years, it has even been shown that the physiological effect of EVs, derived from the same cells but in various conditions, could be varied [68]. Besides the discovery of functional effects of mammalian-derived EVs (MEVs), the effects of non-mammalian ones have also been discovered in the studies until today. In literature, it has been proven that EVs have essential roles in the interaction of endothelial cells in the blood-brain barrier (BBB) and immune cells peripheral systems.

1.1.3.1. Extracellular Vesicles in Endothelial System

As in many systems in the body, various effects of EVs stand out in physiological and pathological events in the endothelial tissue. The prime examples of these are the regulation of activation, inflammation, and permeabilization of endothelial cells [69,70]. Therefore, it is understood that EVs have a key role in the development of diseases caused by endothelial dysfunction. However, in the literature, EVs also have effects such as endothelial repair and regeneration [71,72]. In this respect, it seems that EVs are a double-edged sword. One of the important views regarding the detrimental effects of EVs is that endothelium-derived vesicles disrupt the regulation of mechanisms related to vasodilation by inhibiting the nitric oxide (NO) pathway. In studies, it is thought that EVs reduce the availability of NO by

suppressing eNOS. In the study conducted by Densmore et al., the effects of HUVEC cells-derived EVs on the vasodilation of endothelial cells were investigated [73]. According to the findings, it was proven that these vesicles inhibited endothelial vasodilation by decreasing eNOS activity. Besides the study showed that HUVEC-derived vesicles did not affect oxidative stress mechanisms while functioning about the vasodilation mechanism in the endothelial cells. While another study conducted in similar years confirmed these results, it also reported that these results were due to the NADPH oxidase-mediated oxidative stress mechanism [74]. In another study, it was determined that there was a negative correlation between EVs and the rate of vasodilation in the blood [75]. These differences in the studies may be caused by using different cell lines or the impurities arising from the purification methods. Another remarkable point is that the health status of a person has a decisive effect on the functions of EVs. While no pathological effects were found in EVs taken from healthy individuals in the studies, proinflammatory activity was discovered in EVs taken from individuals with various diseases [76]. Moreover, in studies, it was determined that EVs originating from endothelial tissue or peripheral blood caused inflammation as a result of vesicular proinflammatory effects on endothelial cells [77]. Accordingly, the continuation of inflammatory conditions in endothelial cells led to adhesion and infiltration of monocytes and also multiple pathological conditions. In another study, it was observed that the elevated number of systemic neutrophils could lead to changes in BBB structure and functions [78]. Although it was not known whether EVs have any causative role in this case, it was determined in subsequent studies that EVs released from neutrophils negatively affected endothelial cells. In the study, 24 hours after the EV administration, neutrophil-derived vesicles increased the vascular permeability of endothelial cells in BBB through the ubiquitin-protease mechanism and tight junctions. Accordingly, it has been seen as one of the ways neutrophils are utilised to cross the BBB in infections and related diseases. In some cases, it was reported that these immune-derived EVs directly induced inflammation in endothelial cells [79]. Thus, the activated endothelial cells both enhance the leukocyte adhesion and cause infiltration of immune cells. In connection with this, it was determined that THP-1 monocytic cells also released proinflammatory EVs upon treatment with lipopolysaccharides (LPS). Moreover, no such effect was found in the LPS-negative control group. On the other hand, EVs released from LPS-treated cells were found to enhance adhesion proteins such as intercellular adhesion molecule-1 (ICAM1), vascular cell adhesion molecule-1 (VCAM1), and E-selectin (ELAM1) through nuclear factor kappa B (NFkB)

activation in recipient endothelial cells. Thus, it is understood that EVs have decisive roles in the formation of endothelial pathologies.

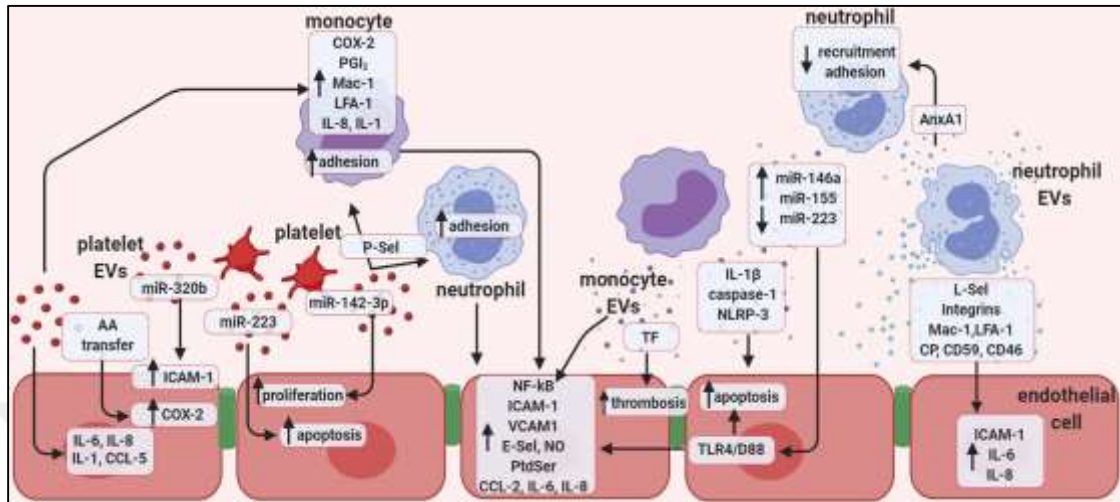


Figure 1.2. Proinflammatory roles of EVs in endothelial-immune cell interactions [69].

EVs, however, are known to have quite beneficial effects on the endothelial system. In studies, the therapeutic and regulatory effects of endothelial or immune-derived EVs released on endothelium stand out. In the study, it was found that endothelial-derived EVs had regenerative and anti-apoptotic effects [80]. According to the findings, EVs released from healthy endothelium inhibited camptothecin-induced apoptosis of the endothelial cells. In another study conducted by the same group, it was determined that the regeneration capacity of endothelial cells cultured under hyperglycemic conditions decreased [81]. Moreover, the pathological effect in the study emerged with the decrease of miR-126 in hyperglycemic endothelial cells. As a result of all these, it is understood that both the repair and regeneration capacity of endothelial cells enhanced by transferring EVs among healthy cells. On the other hand, EVs released from endothelial cells are thought to have various physiological effects other than their pathological effects [82]. Therefore, the utilization of EVs in the communication between endothelial systems has an influential role in the emergence and recovery of various pathologies and physiologies.

1.1.3.2. Extracellular Vesicles in Central Nervous System

Another tissue closely related to physiological and pathological events occurring in the brain endothelium is the central nervous system (CNS). Regarding functional characteristics of EVs, their regulatory roles in the intercellular communication of CNS are generally known.

Considering that the mobility of some cell groups in the nervous system is quite limited, the importance of EVs in intercellular communication increases [83]. Mainly, the functions of EVs in the CNS have been examined under two groups. The first of these are EVs derived from cell groups other than the CNS and their related functions. The second one is the functions of CNS-derived EVs again on resident cells in the nervous system. The main reason for the emergence of the first case is that BBB is not quite successful in restricting the passage of peripheral-derived EVs [84]. Accordingly, it is highly natural that these EVs may migrate to the CNS in various physiological and pathological conditions. Although the direction of these transpositions is not fully known in the literature, its importance for intercellular communication in the CNS is apparent. In studies, it was found that many substances originating from outside the CNS were transmitted to the brain with the help of these vesicular structures [85]. Moreover, it was proven that besides these functions, EVs could carry genetic information and induce recombination in target cells [86]. However, as expected, these transfers also had negative effects. In a related study, it was stated that various cognitive or neuronal disorders could occur as a result of the transfer of EVs to the CNS in pathological conditions such as viral infections [87].

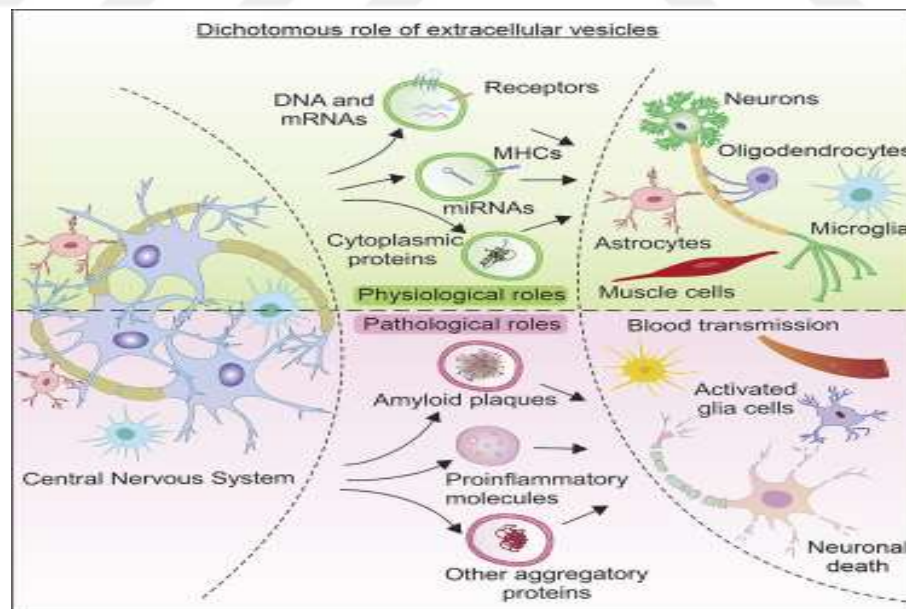


Figure 1.3. Contrasting functional roles of EVs in the central nervous system [83].

In the study conducted in 2018, plasma-derived EVs proved to be an inducing factor in enhanced BBB permeabilization in children with neurocognitive disease [88]. According to the findings, it was observed that plasma-derived EVs disrupted the function of zonula

occludens-1 (ZO1), an essential tight junction protein of endothelial cells in the BBB. In the second case, as in other systems, the paracrine effects of EVs originating in the CNS were mentioned. Interestingly, CNS-derived EVs are also known to cross the BBB and transmit information to peripheral systems. Nevertheless, not much information has been obtained regarding the direction of this communication. Previously, it was thought that EVs could be used jointly in the communication of cell groups in the CNS. One of these communication methods is the synaptic activity between neurons and the information transfer between synapses via EVs. In the study, it was determined that the induction of glutamatergic synaptic activity in neurons had regulatory effects on the release of EVs [89]. Another example among different resident cells in CNS is the functions of microglia-derived EVs on both neuronal and other microglial cells. In another study in 2012, it was discovered that EVs released from microglial cells induced synaptic activity in target neurons through sphingolipid metabolism [90]. Furthermore, microglia-derived EVs led to this phenomenon by increasing the amount of ceramide and sphingosine in neurons. On the other hand, these vesicles regulated the homeostasis and inflammation of microglial cells. For instance, it was found that microglia-derived EVs carried IL1B in vesicular structures and increased the amount of interleukin signaling in cerebrospinal fluid, thus contributing to inflammation in the CNS [91]. As a result of all these examples, it has been discovered that CNS-derived EVs have a wide variety of physiological and, in some cases, pathological effects. In conclusion, it is understood that EVs have an invariable place in terms of the healthy continuation of functions in the CNS.

1.1.4. Plant-derived Extracellular Vesicles

As in mammalian cells, intercellular communication is vital for the life cycles of other higher eukaryotes. However, EV studies have long been predominantly focused on mammalian-derived ones, receiving more attention than those on other organisms. Over the past 20 years, studies have begun on EVs from various non-mammalian sources. In this period, EVs released from plants began to receive relatively great interest. It was thought that the relationship between MVB and plasma membrane in plant cells could be completed by releasing these vesicular structures out of cells. Accordingly, plant-derived EVs (PDEVs) were mentioned for the first time in 1967 [92]. The study aimed to examine the ultrastructural characteristics of cells in carrot culture and to observe alterations in the growth cycle. Therefore, release mechanisms and vesicular forms of EVs were indirectly

suggested. Undoubtedly, the most important factor in this regard was the proposition of view based on the relationship between MVB and plasma membranes. About one year after the study, the internal vesicles produced in cells were visualized in the extracellular or intercellular space, similar to the structures in MVBs [93]. Thus, these two studies proved at the paradigm level that plant cells could also produce and release vesicular structures. Although a correct prediction was made by the link established here, proof of vesicular release in plant cells via MVBs was expected until 2017 [94]. Up to now, while PDEV studies have lost momentum, the working ground has shifted from plant culture to fruit juice isolation [95,96]. When PDEVs are considered in terms of their physical characteristics, it has been determined that they do not have quite different properties. Besides it was proven in literature that there were also apoptotic bodies and MVBs as vesicular structures released from plants. However, pieces of evidence for plasma membrane vesicles did not exist. In this respect, PDEVs appear to be generally similar to mammalian ones. In literature, alterations in the working ground of PDEVs make vesicular classification difficult. It is one of the factors that complicate this classification, especially in the isolation of plant organs such as fruit and leaves. Accordingly, this issue was properly addressed in the study conducted by Woith et al [97]. At this point, the term plant nanovesicles was proposed because PDEVs cannot be separated from intracellular vesicles resulting from cellular lysis. On the other hand, there is practically no such contamination in EVs collected from plant tissue culture, which is similar to the culture conditions in mammalian cells. Therefore, it is understood that PDEVs should originate from plant tissue culture to identify similarities with MEVs. Another structural element is the bilayer lipid membrane structure in MEVs. Similarly, studies have shown that PDEVs have a bilayer lipid structure [98]. When considering the difference between PDEVs and MEVs, another prominent problem is that plant cells have a cell wall structure. While there is no direct evidence and findings in the literature, there are various opinions and comments in this regard. However, the related studies repeatedly proved that plant cells could also release EVs [99]. Besides, another view on this subject suggested that EVs were released directly into the environment by passing through the paramural area, thanks to the high permeability of the cell wall compared to the selectively permeable cell membrane. The study revealed that, unlike the cell membrane, the cell wall was freely permeable to most molecules [100]. Undoubtedly, more detailed information about the physical sizes of EVs and their mechanism of passage through the cell wall is needed. In the study conducted in 1981, proteins in different kilodaltons were tested

to measure the permeability of the cell wall [101]. According to the findings, it was determined that the pores in the wall structure were at least 35 angstroms due to the ability of bovine serum albumin (BSA), the largest protein among the groups to pass through the cell wall. However, it was apparent that the sizes of microvesicles and even exosomes far exceed these measurements. Although the cell wall has a significant effect on the release and regulation of EVs, its molecular relationship with these vesicular mechanisms has remained a mystery. Another notable issue to be investigated regarding PDEVs is the cargo contents of vesicular structures and the discovery of these biological molecules carried in PDEVs is also quite important. Moreover, comparing vesicular contents with other sources might reveal the coverage of conserved molecules between PDEVs and mammalian-derived ones. For instance, one of the studies reported that some CD markers were preserved in PDEVs [102]. On the other hand, the literature on the compositions of EVs is quite limited, and relevant pieces of information are usually obtained from studies whose main scope is not characterization. According to the information obtained from these studies, it was found that PDEVs also contained their unique proteins, lipids, nucleic acids, especially miRNAs. Although there was no direct study on the protein content of PDEVs, some studies were carried out for characterization purposes. One of the studies was performed with *Citrus limon*-derived nanovesicles (CLNVs) [103]. Proteins obtained from CLNVs were compared with the ExoCarta database and it was found that 56.7% of vesicular proteins overlapped with the database. However, these proteins were mostly associated with functional groups related to EV markers or biogenesis in mammalian cells. In the following years, proteomic results were included in the characterization sections of some studies. When the proteomic content of EVs isolated from grape and ginger was examined, it was observed that these proteins acted as vesicular markers. More importantly, it was also revealed that there was a difference in vesicular protein contents derived from these two plants [96,104]. Accordingly, proteins derived from grape-derived EVs (GPDEVs) are more membrane-associated, while those from ginger-derived EVs (GDEVs) contain more cytosolic proteins. Considering all of these findings, it is understood that PDEVs and proteomic data prove the existence of EV markers, but do not have a functionally mammalian related group. Although these proteins may have effects on plants themselves rather than on mammals, it seems that no significant steps were taken in this direction. As with most EVs, lipids have great importance in PDEVs. Moreover, it is quite understandable that these PDEVs have novel characteristic functions due to having plant-derived lipids. Studies have shown that there are significant differences

between the lipid structures of EVs derived from different sources [105]. MEVs have high amounts of phosphatidylcholine (PC) and certain amounts of cholesterol in their lipid structure. On the other hand, phospholipids and glycerol molecules were found in PDEVs, while cholesterol was found to be quite low. Besides PDEVs appear to differ not only from mammalian ones but also within themselves. These differences are mostly caused by the presence of PC and phosphatidylethanolamine (PE) molecules in vesicular structures. When the lipid structures of GPDEVs and GDEVs were examined, it was found that they contained high levels of phosphatidic acid (PA) [106]. As one of the simplest glycerophospholipids, PA has a quite fundamental role in cellular regulation. Cell proliferation, cytoskeletal organization, and vesicle trafficking can be given as examples of functions that PA takes part in. More specifically, it was found to control membrane fission as a lipid mediator and influence the proliferation of mammalian cells via the mTOR pathway [107]. Thus, the role of PA in EV internalization and cytoskeletal reorganization appears essential. Although PDEVs also carry nucleic acids, particularly miRNAs, they differ significantly from MEVs. The first publication on the DNA content of PDEVs was published in 2019 [102]. In studies, DNA was isolated from watermelon-derived EVs (WDEs) and it was suggested to be present in high amounts. According to the findings, it was stated that DNAs, which were claimed to be of plastid origin, had a role in horizontal gene transfer with vesicular carriers. Again, as in MEVs, PDEVs also contain large amounts of small RNAs used in intercellular communication. More importantly, these small RNAs are suggested to be more successful in cross-kingdom interactions than protein molecules. In the study conducted in 2016, at least 125 different miRNAs were detected in the analyses performed with GDEVs [104]. Moreover, it was predicted that 124 of the miRNAs detected in silico analyses could affect the expressions of human genes. Thus, substantial findings were reached about the interaction potential of miRNAs at the molecular level. As a result of another study on EVs isolated from various plant sources, 418 conserved miRNAs were identified and their bioinformatics analyses were performed [108]. According to the results, it was discovered that these miRNAs had the potential to regulate activities related to the generation of immune responses. Finally, it should be emphasized that a significant part of these studies was done using ultracentrifugation without the necessary controls. Accordingly, validation of the results is a critical factor in determining the functions of PDEVs. As a result of all the findings, PDEVs, despite the elevated interest in recent years, is a subject with significant potential that still needs to be investigated at key points.

1.1.5. Functions of Plant-derived Extracellular Vesicles

With the identification of PDEVs and the analysis of their molecular structures, studies in this field have begun to diversify. In some studies, EVs have become a current issue as drug carriers due to their non-toxic and non-immunogenic properties. Accordingly, the investigation of the effects of PDEVs on mammalian cells has gained momentum in recent years. For these studies, samples were collected from a wide variety of plant sources, mostly fruits, and EV purification was performed. Moreover, these PDEVs were tested in various *in vivo* and *in vitro* disease models to investigate their therapeutic effects. Anti-inflammatory, antioxidant, and regenerative effects of PDEVs have been found in 19 studies conducted since 2013. In more than half of these studies, high-purity methods such as sucrose gradient or ultrafiltration were not used for the purification of EVs [109–118]. Furthermore, almost none of the studies used controls suitable for EVs. Besides, intracellular vesicles and contamination are expected to be present in the environment, as all of the studies with fruits are directly broken down by a blender. Therefore, these criteria should be approached to make reliable investigations about the functional properties of PDEVs. From this point of view, it is understood that only 47% of the studies meet these conditions and can be analysed reliably. Considering these, the first study in this group was conducted in 2013 [96]. The activity of GPDEVs on the intestinal tract and how they respond in case of an inflammatory pathology were investigated. In the study, nanovesicles were isolated purely by the sucrose gradient method. In the findings, it was stated that the GPDEVs crossed the mucus barrier and were taken up by intestinal cells. Furthermore, they induced the proliferation of Lgr5⁺ stem cells via the Wnt/ β -catenin pathway. Afterwards, the effects of GPDEVs were investigated by gavage administration in dextran sulfate sodium (DSS)-induced colitis model in mice. In the study, it was reported that GPDEVs had protective effects in the intestinal cells against DSS-induced damage. Thus, it was determined that GPDEVs had therapeutic effects and might have influential functions not only *in vitro* but also *in vivo*. Another related study was conducted with GDEVs in 2015 [119]. In the study, GDEVs were also purified with a sucrose gradient similar to the others [104]. Liver damage induced by the administration of certain concentrations of ethyl alcohol as a liquid diet and gavage was modelled in mice. Consequently, it was found that GDEVs had preventive effects against occurring liver damage. Besides, it was proven that GDEVs inhibited the generation of reactive oxygen species (ROS) by inducing NFE2 like BZIP transcription

factor 2 (NRF2) activation. As a result of investigating the contents of GDEVs, it was found that shogaol molecules were carried inside the vesicles. Thus, the issue of whether phytochemicals can be found in PDEVs became a current issue for the first time in the literature. Another finding on this subject came from EVs originating from strawberries, yet it cannot be considered as strong evidence as in GDEVs due to the purification methods [118]. Thus, the therapeutic potential of GDEVs and interactions among species was re-proved, just like GPDEVs.

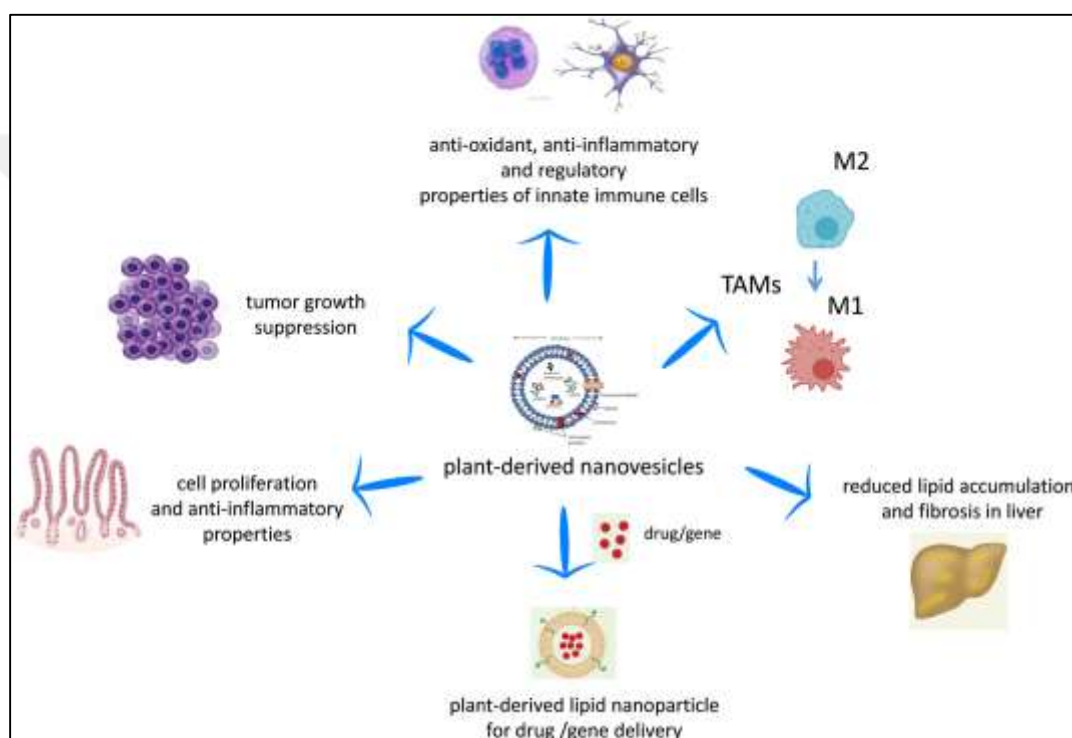


Figure 1.4. Functional characteristics of PDEVs in various pathological conditions [106].

In 2019, a new characteristic related to the functions of PDEVs was discovered [120]. In the study, the anti-melanogenic properties of EVs isolated from the roots and leaves of *Dendropanax mobifera* were investigated. These EVs were obtained using ultrafiltration as another efficient method. As a result of *in vitro* and *in vivo* studies, a significant anti-melanogenic effect was observed for leaf-derived EVs (LEVs), while the same effect was not found for root-derived EVs (RDVs). In the study, it was not specified what caused this function in leaf-derived ones, while it is not known what caused these variations in terms of the characteristics of the two different tissues. As a result, the utilization of PDEVs as natural cosmetic products was also evaluated in the study. These results have been crucial for future

studies in this direction. Another interesting feature of PDEVs in terms of their functional characteristics is their anti-cancer activity. In the study conducted in 2015, the potential of EVs derived from lemon juice on several cancers and healthy cell lines was investigated [103]. In the study, the sucrose cushion method, which is methodologically based on the similarity of sucrose and EV density, was used in the purification of vesicles. According to the measurements at the 48th hour of the treatment, antineoplastic effects of *Lemon citrus*-derived EVs (LCDEVs) were found. Moreover, the results of *in vivo* experiments were also consistent with *in vitro* experiments. In the following years, anticancer effects were observed in the vesicles obtained by different plant sources and purification methods [121,122]. In these studies, it was determined that the potentials of the vesicular structures purified from different tissues were at different levels, as seen in the *Dendropanax mobifera*-derived vesicles. Accordingly, the antineoplastic potential of PDEVs is quite critical in terms of being new biotechnological drug candidates that might be used in cancer treatment. Moreover, studies on this subject have continued actively. Some much so that in February 2021, a study was conducted on the effects of *Asparagus officinalis*-derived EVs on hepatocellular carcinoma [123]. According to the results, it was proven once again that PDEVs had antineoplastic potential. The last study on the functions of PDEVs, it was aimed to characterize cabbage-derived EVs (CDEVs) and investigate their biological activities [124]. In the study, considering that the combination of size-exclusion chromatography and ultrafiltration methods was used together, it was understood that PDEVs were obtained in a quite pure form. Furthermore, the effects and therapeutic potential of these vesicles on mammalian cells were also evaluated. As a result of the study, it was discovered that CDEVs had immunosuppressive properties, as well as suppressing apoptosis in keratinocytes and fibroblasts. Accordingly, it was suggested that CDEVs had significant potential as a therapeutic that could be used in a wide range of diseases. In conclusion, it is understood that PDEVs also have influential therapeutic effects on mammalian cells. In this doctoral project, it is anticipated that much more novel information will be discovered about the functional properties of PDEVs.

1.2. CEREBROVASCULAR INFLAMMATION

1.2.1. Fundamentals of Cerebrovascular Inflammation

The impact of the immune system in the pathogenesis of cerebrovascular diseases has become more and more important with the findings of the studies conducted over the years. Within this period, it was revealed that the excessive activation of the immune system and the formation of a proinflammatory microenvironment due to the stimuli of various signals could have both an initiating and deteriorating role in cerebrovascular diseases [125]. Cerebrovascular inflammation (CVI), which is identified as inflammation arising from immune or endothelial activation in the brain endothelium, includes the production and release of numerous chemokines, cytokines, reactive oxygen species, and second messenger molecules. In the CVI, a considerable majority of these mediator molecules are generated by cerebral endothelial cells (CECs) and immune cells in peripheral blood [126,127]. Furthermore, CECs are involved in not only the generation of some of these mediator signals but also the conversion and augmentation of the signals coming from the peripheral blood to the central nervous system (CNS). Thus, it is inevitable that CVI influences various physiological and pathological events having crucial importance to vascular and cognitive health. Unsurprisingly, the elevated levels of CVI lead to an undesirable destructive state for brain endothelium. Generally, this pathological state is characterized by excessive production of cytokines and chemokines, infiltration of immune cells into the CNS, oedema, breakdown, and escalating of BBB permeability [128]. Therefore, cell death, ischemia, vascular occlusion, and other secondary problems are expected to occur in brain endothelium and, similarly, pathological CVI are generally more associated with cerebrovascular diseases.

1.2.2. Cell-Mediated Mechanisms in Cerebrovascular Inflammation

1.2.2.1. Cerebral Endothelial Cells

While various studies related to the CVI focus on the pathological role of effector T lymphocytes or microglial cells, in subsequent studies, it was found that CECs had critical impacts on the various phases of the inflammation process through modulating gene expressions and adhesion molecules. Besides, it could be stated that perhaps the most important characteristic of CECs in cerebrovascular disease is the formation of the BBB

comprised through the integration of the capillary and post-capillary venules. The BBB is defined as a semi-permeable and considerably selective cellular wall structure formed by the majority of CECs [128]. In these endothelial cells fulfilling the preventive barrier function, it was observed that fenestrated vascular structures decreased, while tight junctions and adherens junctions between the microvascular endothelial cells enhanced. Adherens junctions are mainly generated by establishing the adhesion of proximate cells inter se with the interactions of CDH5 and the adhesion of CECs-matrix through CDH5 signaling over RhoA and actin cytoskeleton proteins [129]. Besides catenin molecules have an essential role in the regulation of the BBB permeability and the association of cadherin molecules with other extracellular and cytoplasmic components. Even though the majority of cadherin proteins in the BBB are CDH5, other types of cadherins are expressed at the least [130]. On the other hand, tight junctions in conjunction with adherens junctions constitute a zipper-like paracellular structure to completely seal the intercellular gap of the CECs and prevent any bi-directional leakage. Tight junctional structures in the CECs are much more involved in the BBB organization than the fenestrated capillary structures presented distinctively in the mucosal layer of several regions such as endocrine organs and choroid plexus. In addition to their properties related to the prevention of paracellular transition, the continuity of these cellular junctures along the BBB is one of the features distinguishing tight junctions from other types of cellular connections [131]. Tight junctions are mainly emerged by the presence of 3 multiprotein complexes. These are claudins, occludins, and junctional adhesion molecules (JAMs). Claudins are multiprotein complexes that make up the majority of main structures in the sealing role of tight junctions. Besides these integral proteins have modulator functions in the regulation of the paracellular pathway. Due to the distinctness in gene expression depending on the tissues, they also have unique patterns of claudin proteins, being either constitutive or inducible states in the tight junctions. For instance, some subtypes of claudin complexes such as claudin-5 and claudin-3 are constitutively expressed, while other subtypes such as claudin-1 must be stimulated to be upregulated. Furthermore, it is known that claudin-5 is the most expressed among these claudin species under physiological conditions [132]. Occludin proteins belonging to the tetraspanins of integral membrane proteins are another protein type presenting in tight junctional structures. They are suitable platforms for signalization mechanisms in which CECs are stimulated through the cytoplasmic domains of occludin proteins, as well as being structurally involved in tight junctions like other junctional proteins. Although their functions in tight junctions are not

known completely, it was stated that their interactions with ZO1 and F-actin are crucial for the continuity of tight junctions [133]. Finally, the last protein group joining the structure of tight junctions are junctional adhesion molecules (JAMs). Similar to the occludins, JAMs have both the intracellular and the extracellular domains responsible for transmitting signals in the interactions of tight junctions. Being a member of the immunoglobulin G (IgG), JAMs are thought to have a key role in tubule formation and leukocyte interactions, as well as having all types in the tight junctions of the endothelium. In the studies, it was proven that as a result of the lessening in the JAMs expression like claudin-5 or occludins, CECs activation and breakdown of the BBB occurred [134,135]. Moreover, unlike the infiltration of peripheral immune cells into the other tissues, the infiltration of immune cells in CVI takes part from the post-capillary venules to the CNS due to this barrier system reinforced by the tight junctions. Besides the fact that CECs physically contribute to the structure of the BBB, astrocytes, pericytes and perivascular macrophages are required to ensure the consistency of the function and barrier structure of the BBB. Under physiological conditions, CECs have notable roles in regulating hemodynamic functions, generating mediator signals to maintain homeostasis or responding to pathogenic signals, and regulating the permeability of the BBB [136]. The interactions of peripheral immune cells with CECs emerging in the CVI do not actualize under these conditions. Nevertheless, the interactions concerning the recruitment of peripheral immune cells by CECs eventuate as a result of endothelial activation and starting to express the leukocyte-associated adhesion molecules. The rearrangement or disruption of junctional protein expression ensues through stimulation of the myosin light chain kinase (MLCK) signaling or RhoA/Rho kinase pathway in activated CECs related to the transition of peripheral mediator signals or effector immune cells. In the study conducted in 2011, it was observed that cortactin deficiency elevates the basal permeability of endothelial cells, yet reduces the migration of the TNF-induced peripheral immune cells. Therefore, it was stated that there was a connection between the escalated permeability of CECs and the migration of leukocytes in an inflammatory response, but not at the molecular degree [137]. Another one of the characteristic features of CECs in the regulation of CVI is that they are one of the cellular systems to detect pathological mediator signals. Nevertheless, considering that the antigen-presenting capacity of the CECs is rather weak, it is thought that they serve a function in indirectly converting and transmitting these signals rather than directly introducing pathogenic molecules [138]. A great variety of recognition receptors, including toll-like receptors (TLRs) and nucleotide oligomerization

domain-like receptors (NLRs), are present on the cell surface or some of them inside the cell in the perception of these signals. Furthermore, it is known that the specific activation of the receptors by these signals, namely first messengers, may lead to the production of mediator molecules such as proinflammatory cytokines and chemokines in CECs, the recruitment of leukocytes, and the hyperpermeability of the BBB [139]. Consequently, it is understood that the endothelial activation of the CECs is a contributing factor in revealing the pathological aspect of the CVI. However, it was revealed that the endothelial activation of the CECs did not occur in the same manner in all cases. Some types of endothelial activation, it was characterized by the stimulation of the RhoA/Rho kinase pathway and elevation in the amount of cytosolic free calcium via G protein-coupled receptors (GPCRs) pathways. In the other activation type, the stimulation of CECs is characterized by the induction of transcriptional and translational machinery by the mediator signals, including cytokines and chemokines, as the cellular response to the activation stimuli [140]. Hence, the phenomenon reduced to two different activation types were separately examined in the following section.

The type I activation of CECs emerging in the CVI has characteristic cellular events such as activation of the Rho signaling pathway and elevated levels of free calcium in the cytoplasm. Being a member of the GTPase family, most of the Rho signaling pathway consists of RhoA, the small GTPase protein, and one of its downstream molecules, namely the Rho-kinase enzyme. Furthermore, there are two signaling mechanisms in which RhoA activated by internal or external stimuli is involved in the activation of CECs in the CVI. One of them is the control of CECs permeability via the Rho signaling pathway, and the other is the reduction of NO bioavailability through the suppression of eNOS activity [141]. Rho-kinase enzyme, the downstream target of Rho, leads to actomyosin interaction causing endothelial contraction by elevating phosphorylation of myosin light chain (MLC) both directly with its kinase activity and indirectly through myosin light chain phosphatase (MLCP) inhibition [142]. Thus, the resulting actomyosin contraction and consequently the tension in the cytoskeleton increases the paracellular permeability of CECs. The Rho signaling pathway in endothelial cells has an essential role in the inflammation response in terms of its role in the CECs activation and its impact on the BBB permeability. In the study conducted in 2017, it was observed that silica nanoparticles elevated the BBB permeability via the Rho signaling mechanism [143]. Accordingly, it was proven that inhibition of the Rho signaling mechanism to lessen the paracellular permeability counteracted the destructive effects of

nanoparticle exposure. Hence, the decisive and regulatory role of Rho signaling in BBB permeability was demonstrated. In another study conducted in 2014, it was observed that there was an excessive increase in vascular permeability in the BBB and endothelial ROS in mice with the oxygen-glucose deprivation (OGD) model [144]. According to the results, it was stated that the application of Rho-kinase inhibitors exactly 4 hours after the initiation of the OGD model induced significant improvements in BBB integration and various endothelial functions. In the study conducted in 2018, it was observed that EphA4 activation, forming through the EphrinA1 signaling mechanism, downregulated the expression of ZO1 and claudin-5 in CECs presenting in the BBB structure [145]. Furthermore, it was understood that Rho-kinase inhibitors applied in the study similar to relevant studies made improvements in the BBB permeability and dysfunction through the Rho signaling pathway and consequently had favourable results in terms of CVI. Therefore, it can be seen how the Rho signaling pathway contributes to the escalation of paracellular permeability in type I endothelial activation. Another mechanism that induces type I endothelial activation occurs through the $\text{PLC}\beta/\text{Ca}^{+2}/\text{MLCK}$ signaling. This signaling pathway is activated by the binding of a specific ligand to GPCRs, as is the case with the Rho signaling pathway. After the ligand-receptor interactions, $\beta\gamma$ subunits of the G protein activate RhoA through the Rho-guanine nucleotide-exchanging factor (Rho-GEF), while the α subunit of the G-protein induces the intracellular release of calcium from the endoplasmic reticulum by activation $\text{PLC}\beta$ and inositol (1,4,5)-triphosphate [$\text{Ins}(1,4,5)\text{P}_3$]. With the escalation in cytoplasmic free calcium, it binds to the cytoplasmic adaptor protein calmodulin (CaM) and forms the Ca^{+2} -CAM complex serving an essential function such as the MLCK activation [146]. Eventually, the activated MLCK enzyme phosphorylates the MLC and causes the actomyosin contractions as previously described, and subsequently induces the paracellular permeability of CECs. Studies conducted in recent years have proven that the elevated free calcium in the cytoplasm leads to type I endothelial activation and BBB permeability [147].

Type II endothelial activation, the second type of activation in the CECs, also has a decisive role in the regulation of CVI. One of the striking differences between type II endothelial activation is that the active cellular state and receptor desensitization last longer than type I. These activities of CECs having critical roles in the production and release of proinflammatory mediators are quite often closely related to the second type of endothelial activation. Unlike the occurrence of type I endothelial activation in a short time, it is seen

that the mediator signals in type II stimulate gene transcription and protein synthesis for a longer time [148]. Thus, cytokines, chemokines, and other proinflammatory mediator molecules released from the CECs regulate effectually the intercellular communication in both paracrine and autocrine manner. One of the examples of type II endothelial activation is the stimulation of the TNF signaling pathway in the CECs having an essential function in the emergence of inflammatory response. This signaling mechanism is activated by the binding of TNFA, the first messenger inducing proinflammatory cellular profile, to TNFR1 and TNFR2 receptors on the surface of CECs. As a result of receptor activation, the cytoplasmic signal transducers allowing the transmission of extracellular signal activate 3 effector signal mechanisms consisting of caspase, MAPK, and NFkB signaling pathways. Of these, MAPK and NFkB pathways lead to pathological consequences such as allowing the interaction of the CECs with effector leukocytes and bringing microvascular endothelial cells into a proinflammatory and hyperactive state [149]. In recent studies, it was proven that TNFA activated the signal transduction mechanism of the CECs, allowing leukocyte adhesion through upregulation of the ICAM1 and VCAM1 expressions and also elevated endothelial contraction and vascular permeability through the same pathways [150]. Type II endothelial activation also has a functional role in CVI due to infection of blood-borne pathogens. These pathogens are detected by pathogen recognition receptors in the CECs, just as in innate immune cells, and one of them is TLRs. TLRs are receptors that allow the recognition of structurally preserved molecules, namely pathogen-associated molecular patterns (PAMPs) sharing widely in pathogens but not in host cells. Therefore, TLRs found in endothelial cells function as primary sensors of innate immunity against pathogenic organisms. Even though there are 13 different types of TLRs, it was determined that the CECs express some types of TLRs such as TLR2,3,4 and 6 [151]. Moreover, the reason for TLR diversity is that the receptor types are specifically activated by their characteristic pathogenic ligand molecules. After the pathogenic signal molecules stimulate TLRs, the conserved toll/interleukin-1 receptor (TIR) domain mediates the signal transduction by allowing the interaction among cytosolic adapters such as MyD88, TRIF, and TRAM. As a result of the functions of these adapter proteins and subsequent activation of effector proteins, the stimulation of NFkB and MAPK leads to the expression of proinflammatory mediators [152]. The studies showed that the activation of TLR2 or TLR6 activates the MAPK signaling pathway and elevates endothelial permeability by inhibiting the expression of junctional proteins [151]. In another study, it was observed that excessive expression of

TLR4 in the CECs led to brain oedema due to the induction of astrocyte swelling. Finally, it was found that the treatment with a therapeutic agent over TLR-4 signaling in the *Klebsiella pneumoniae-induced* mouse model attenuated both the hyperactivation of the signaling pathway and the destructive symptoms of pneumonia [153]. Another characteristic of type II endothelial activation is the critical function in the regulation of chemokine signals. These chemotactic cytokines, allowing the immune cells to migrate to the inflammatory tissues, have essential roles in physiological surveillance and inflammatory response through the CECs. By binding to GPCR family receptors on cell surfaces, chemokines activate the substantial pathways in the target cells such as Rho signaling, Akt signaling, and MAPK signaling pathway [154,155]. Just like TLRs, the CECs also provide infiltration of leukocytes from post-capillary venules to the CNS by expressing particular chemokines and chemokine receptors. In the study conducted in 2015, the LPS-induced CVI was modelled in CXCL1 and CXCR2 knockout mice [156]. Although CXCL1, CXCL2, and CXCL5 were upregulated in the wild-type mice group, it was observed that CXCL1 and CXCR2 deficiency inhibited neutrophil infiltration and the recruitment of leukocytes into the CNS. Thus, the vital role of astrocytic CXCL1 and endothelial CXCR2 molecules in the CVI was revealed. In another study conducted in 2012, it was stated that as a result of the interaction of the CXCR4 receptor involved in the adhesion of leukocytes and CXCL12 chemokine in the CECs, endothelial activation occurred, and consequently, the expression of adhesion proteins such as ICAM1 and VCAM1 elevated [157]. In conclusion, it is understood that the CECs have quite influential functions in CVI.

1.2.2.2. T Lymphocytes in Peripheral Blood

Another cell group that is highly influential in CVI pathology is T lymphocytes in peripheral blood. This immune cell group is involved not only in cerebral tissue but also in almost all inflammatory events. T lymphocytes, which are an essential component of adaptive immunity, have a major role in events such as eliminating infected host cells, activating other immune cells, and generating an inflammatory response [158]. Besides having many subtypes with a wide variety of functional and cytological characteristics, T lymphocytes also have specific functions in inflammation and endothelial dysfunction events occurring in the cerebral endothelium. T lymphocytes originating from hematopoietic stem cells circulate in the peripheral lymphoid organs ready for activation after their selection in the thymus. When they become active at some point in their life cycle, their numbers increase

rapidly and they pass into the effector phase and fulfil their functions [159]. T lymphocytes provide mobile transportation in the circulatory system to perform these functions in different tissues. T lymphocytes, which constitute approximately 70-80% of the mononuclear cells in the peripheral blood, reach the cerebral tissues through this circulation [160]. However, even under physiological conditions, T lymphocytes are found in the brain parenchyma or the perivascular area. Besides it is known that there is a certain number of T lymphocytes in the cerebrospinal fluid in terms of the continuity of surveillance activities in the CNS [161]. Despite these sporadic transpositions, T lymphocytes do not interact under physiological conditions due to the lack of expression of endothelial adhesion molecules. Besides various molecules released from endothelial cells in the BBB to prevent non-specific and undesired conditions suppress leukocyte activation. On the other hand, cerebral inflammation, which occurs for various reasons, causes the activation of T lymphocytes along with other related cells. Subsequently, T lymphocytes have a role in the CVI by interacting with cells residing in cerebrovascular tissue. One of the important events in which T lymphocytes have a role in the inflammation of the cerebrovascular tissue is the adhesion between leukocytes and CECs. Besides TNFA released from activated T lymphocytes contributes to non-specific inflammation in exposed CECs, as well as increases the expression of adhesion molecules such as VCAM1 and ICAM1 [162]. Thus, more T lymphocytes come to the inflammation area and increase BBB permeability. Accordingly, in studies, some molecules were tried within the scope of T lymphocyte-based treatment possibilities in CVI models and significant results were obtained. For instance, in the study conducted in 2019, the potential effects of endothelial-leukocyte interaction in the CVI animal model were examined [163]. Leukocyte adhesion, infiltration, and activity were evaluated in the BTBR animal model using a cathepsin B inhibitor, CA-074Me for 7 days. According to the results, the adhesion of leukocytes was suppressed and their migration to the CNS was decreased with the treatment of cathepsin B inhibitor. Besides the decrease in the endothelial ICAM1 and CD11b in the absence of cathepsin B was determined to be one of the reasons for this. In another study, it was stated that the inhibition of the protein kinase CK2 suppresses the leukocyte-endothelial adhesion, thus reducing the ischemia damage [164]. Therefore, it is known that T lymphocytes affect the emergence and progression of CVI. In literature, the regulatory role of T lymphocytes in CVI was determined in many cerebrovascular diseases. In conclusion, T lymphocytes appear to be a cell population with quite decisive functions in CVI pathology.

1.2.3. Cellular Protective Pathways Against Cerebrovascular Inflammation

The inflammation response emerged in the cerebrovascular tissue naturally stimulates a protective counter-response in the CECs. The purpose of this response is to limit and control the inflammatory response in endothelial cells. Thus, endothelial cells benefit from various cellular protective pathways to accomplish these objectives. Furthermore, it is known in the literature that these pathways in the CECs respond to cellular stress conditions and neutralize them while also serving to protect endothelial functions [165]. To that end, endothelial cells activate a series of protective pathways against these harmful stimuli, including inflammation and oxidative stress. Upon examining the characteristic features of these pathways, it is seen that they fundamentally try to protect cellular homeostasis and endothelial functions and neutralize the sources of cellular stress. In addition, these protective pathways actively benefit from biochemical messengers that regulate cellular communication [166]. Therefore, the endothelial cells contribute to intercellular coordination against inflammatory conditions in both themselves and the cells in the cerebrovascular tissue. In this respect, these pathways activated against inflammation eliminate the stress condition of cells, prevent endothelial cell dysfunction and contribute to the preservation of cellular homeostasis.

Sirtuin proteins are one of the important factors playing a role in these protective pathways in the CECs. Sirtuin enzymes are a protein family acting as NAD⁺-dependent deacetylases and also have functions related to cellular stress and aging. SIRT1, an important member of this family, serves the protective functions in endothelial cells [167]. Therefore, SIRT1 has notable importance in maintaining endothelial homeostasis by exhibiting both anti-inflammatory and regulatory effects on signaling pathways in endothelial cells. Activation and upregulation of SIRT1 protein expressed in endothelial cells are regulated according to various cell signals, environmental stimuli and energy levels of cells. Due to the NAD⁺-dependent activities of SIRT1 enzyme in particular, pathways and regulatory molecules increasing NAD⁺ levels play a crucial role in SIRT1 activation [168]. Furthermore, it is known that the SIRT1 protein negatively regulates proinflammatory pathways such as NFκB during the formation of the inflammation response in endothelial cells [169]. In literature, it is well documented that the NFκB pathway, which plays a regulatory role in CVI pathology, upregulates the expressions of cytokines and adhesion molecules [149,150]. However, the SIRT1 enzyme suppresses its activity by deacetylating the RelA/p65 subunit and also the

inflammatory response [169]. Therefore, the SIRT1 activity limits the cellular damage caused by inflammation in the cerebrovascular tissue and contributes to the protection of endothelial functions. Further, the SIRT1 protein acts as an initiator in the activation of the NRF2 pathway, being an important protective pathway. In literature, SIRT1 enzymes reduce the interaction between NRF2-inhibitor KEAP1 and enable the activation of NRF2 proteins [170]. Thus, the activated NRF2 pathway enables the transcription of various protective enzymes of cells and the emergence of a protective response. As a result of all these, it is understood that SIRT1 expression has a protective effect against cerebrovascular pathology by slowing down vascular aging and preventing pathological processes such as endothelial dysfunction. Therefore, the effects of the SIRT1 protein on cellular defense mechanisms are quite important in terms of CVI pathology.

Another cellular protective mechanism in endothelial cells is the regulation of peroxisome proliferator-activated receptor gamma (PPARG) activity. It is one of the nuclear receptors known for its anti-inflammatory effects in endothelial cells. When activated, the PPARG protein supports vascular health by regulating gene transcription in endothelial cells and limits inflammatory responses [171]. Therefore, it is a regulator playing a critical role in maintaining the homeostasis of cells, suppressing inflammation, and regulating metabolic processes. Furthermore, it is known that the inflammatory response occurring in endothelial cells stimulates the production of ligands being natural activators of PPARG [172]. In particular, the 15d-PGJ2 molecule, a prostaglandin derivative produced under oxidative stress, is a strong agonist of PPARG [173]. It activates the PPARG protein against the inflammatory response in endothelial cells. Thus, PPARG achieves its anti-inflammatory effect by suppressing the expression of proinflammatory genes after endothelial activation. This mechanism relies on PPARG inhibiting the activity of proinflammatory transcription factors such as NFkB and AP1. Specifically, PPARG binds to NFkB protein, preventing it from binding to DNA and thereby suppressing the transcriptional activation of downstream genes [174]. Further, PPARG prevents the spread of inflammation in the cerebrovascular tissue by increasing the production of inhibitory molecules such as Ikb, which suppress inflammatory signals. On the other hand, PPARG proteins have a notable regulatory function on lipid metabolism [175]. In this respect, they reduce the accumulation of free fatty acids in endothelial cells, making them more resistant to inflammation and activation signals. As

a consequence of all these effects, the protective properties of PPAR γ proteins are quite influential as one of the mechanisms supporting endothelial functions.

One of the influential pathways in the protection of endothelial functions in CECs is the NRF2 pathway. The roles of the NRF2 pathway in the formation of protective responses such as anti-inflammatory and antioxidant in endothelial cells are well documented in the literature [176]. It protects endothelial functions and cellular homeostasis by limiting the damage caused by cellular stresses as a consequence of the pathway. The NRF2 pathway is activated when endothelial cells are exposed to stress factors such as inflammation, contributing to the prevention of cell damage and the preservation of vascular health [177]. Under normal conditions, the NRF2 protein is inactive in the cytoplasm, bound to the inhibitor protein called Kelch-like ECH-associated protein 1 (KEAP1). These stress factors lead to the KEAP1 protein in endothelial cells undergoing conformational changes and NRF2 being released in the cytoplasm. The released NRF2 protein translocates to the nucleus as a transcription factor and initiates the transcription of antioxidant and cytoprotective genes by binding to enhancer sequences called antioxidant response elements (ARE) [178]. Important proteins such as Heme Oxygenase 1 (HMOX1), and NAD(P)H Quinone Dehydrogenase 1 (NQO1) are among these downstream target genes. These proteins play crucial roles in neutralizing ROS and maintaining cellular redox balance. On the other hand, the anti-inflammatory effect of the NRF2 pathway is also associated with its suppression of the NF κ B pathway. Studies in the literature have determined that the NRF2 pathway interacts with the NF κ B pathway and suppresses the inflammatory response in TNF α -induced endothelial activation [179]. It is among the important findings that the HMOX1 enzyme, whose expression level increases as a result of the NRF2 pathway activation, reduces endothelial inflammation. These findings demonstrate that these enzymes protect endothelial cells from injuries originating from oxidative stress and regulate inflammatory responses [180]. In the NRF2 pathway, another protein contributing significantly to its anti-inflammatory effect on endothelial cells is the NQO1 enzyme. Studies have determined that NQO1 activity has an anti-inflammatory effect in LPS-induced microglia cells. The study has shown that the NQO1 response is induced in cells, especially through the activation of the AMPK pathway, and that the production of cytokines such as TNF α and IL1 β is reduced [181]. In this respect, it is understood that the NQO1 enzyme is a multifunctional anti-inflammatory and antioxidant enzyme. Thus, as a result of all these

effects, the NRF2 pathway reduces inflammation and its destructive effects in endothelial cells while preserving the integrity of the vascular wall. Nevertheless, the importance of the NRF2 pathway is not limited to controlling cellular stress. It is also critical for maintaining long-term vascular health and preventing inflammation-related disorders. Consequently, the NRF2 pathway contributes to the sustainability of vascular homeostasis by enabling endothelial cells to struggle with oxidative and inflammatory stress.

1.3. AIM OF THE STUDY

Although they are not directly related to the CVI in the literature, there are some studies on the functional activities of PDEVs. However, one of the important problems in these studies is to investigate the purity of EVs and whether the effect is due to EVs. Another is the methodological problems that arise with the use of complex materials such as plant explants or fruit as a vesicle source. Accordingly, this Ph.D. study will aim to answer the following points.

1. Investigation of the presence of EVs in media derived from plant cell suspension culture and whether they are functional or not.
2. Investigation of differences and similarities in the properties of plant cell suspension-derived EVs (PCSEVs) from different media sources.
3. Investigation of possible effects of PCSEVs on cerebrovascular tissue-associated cell lines.
4. Investigation of the interactions of PCSEVs in the *in vitro* model of CVI and whether they have a possible therapeutic potential.

2. MATERIALS AND METHODS

2.1. PHYSICAL AND MOLECULAR CHARACTERIZATION OF PCSEVS

2.1.1. Purification of Extracellular Vesicles

The purification of PCSEVs to be used in the study was made from conditioned media collected from the suspension culture of plant cells under strictly standardized conditions. To keep the molecular and functional properties of PCSEVs in a stable range, it is critical to perform the media collection and purification in a standard manner. To purify PCSEVs from the plant cell suspension culture-derived medium, differential centrifugation and ultrafiltration techniques were used together [120]. In the study, an ultrafiltration-based isolation process was performed as shown in Figure 2.1 to obtain purified PCSEVs. Within the characterization experiments, samples taken from the critical steps of the purification as the stock, concentrate, filtrate, and final solutions were compared. As a beginning, the media were centrifuged at 3600 g for 15 minutes at 4°C to remove cell debris and large particles. The collected supernatant was passed through a 0.45 µm syringe filter (#FJ25ASCCA004FL01, GVS, Italy) to remove large microparticles. The collected 40 mL *Vaccaria hispanica* and *Stevia rebaudiana* cell suspension media were loaded into an Amicon 50 kDa ultrafiltration column (#UFC905096, Merck Millipore, Germany) and concentrated with centrifugation at 3600 g for 10 minutes at 10°C. Subsequently, the concentrated PCSEVs were completed up to 15 mL with nuclease-free water to remove impurities and concentrated again under the same conditions. In the final step, the collected PCSEVs were diluted to 3 mL with nuclease-free water and passed through 0.22 µm syringe filters (#FJ25ANCCA002DD01, GVS, Italy). Sterile PCSEV samples were aliquoted and stored at -80°C.

2.1.2. Nanoparticle Tracking Analysis of PCSEVs

Nanoparticle tracking analysis (NTA) was performed to determine the size distribution and nanoparticle concentration of the purified PCSEVs [182]. NTA technique was performed on the Nanosight NS300 (Nanosight Ltd, UK) instrument equipped with 488 nm and 532 nm laser modules. Regarding the analysis accuracy of the NS300 device, PCSEV samples were diluted with nuclease and vesicle-free water to ensure that the measurement range was

between $\sim 10^7$ - 10^9 particles/mL. The samples were prepared in the required dilutions of 1 mL and loaded into the device with the help of a syringe. Depending on the analysis procedure, 10 videos of 30 seconds were taken and the videos were analysed via NTA 3.4 software [183].

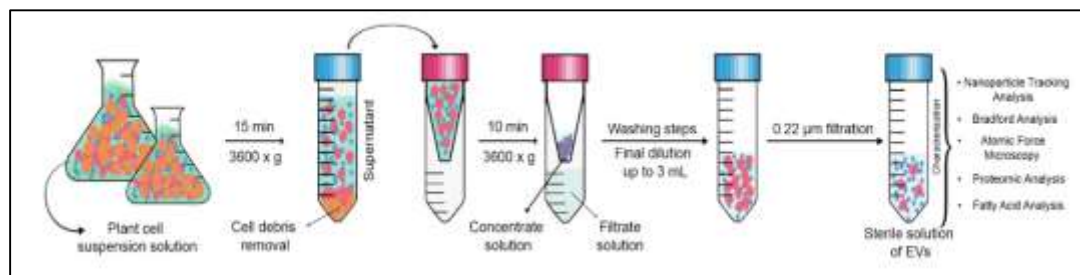


Figure 2.1. Schematic illustration of the purification procedure of PCSEVs collected from plant cell suspension culture.

2.1.3. pH analysis of PCSEV solutions

To analyse the changes in pH of the solutions during the purification of PCSEVs from the collected medium, the pH of the samples collected from certain stages was measured with the Mi-150 pH meter (Milwaukee, USA). These identified samples were collected from the stock, filtrate, concentrate, and final steps.

2.1.4. Protein-based quantification of PCSEVs

Bradford analysis was performed to quantify the purified PCSEVs by protein-based measurement methods [184]. In the analysis, a calibration curve of BSA protein with 6.25 12.5 25, 50, and 100 $\mu\text{g/mL}$ standards was drawn. 40 μL of standards and samples were added to a 96-well plate and mixed with 200 μL Bradford reagent (Bio-Rad, Hercules, CA). Subsequently, the 96-well plate was incubated for 3 minutes on a shaker at room temperature. After incubation, the absorbance was measured at 590 nm in an ELISA plate reader ELX800 (BioTek, USA) and statistical analyses of the measurements were performed using Microsoft Excel software.

2.1.5. Atomic force microscopy analysis of PCSEVs

Morphological analyses and validation of size distributions of purified PCSEVs were performed by atomic force microscopy technique. First, the vesicle samples were diluted with nuclease-free water to a concentration of 1×10^7 p/mL which is suitable for analysis. Subsequently, the diluted samples were dropped on an ultra-flat silicon wafer (Ted Pella Inc., USA) at 10 μ L volume and left to dry overnight at room temperature. After the samples were prepared, they were analysed using an NSC36B probe in the XE-100 atomic force microscope (Park Systems, Korea). Images taken from the device were analysed with XEI software.

2.1.6. Fatty acid methyl ester profiling of PCSEVs and PSCs

To profile the fatty acids in the membrane structure of the purified PCSEVs and plant suspension cells (PSCs), fatty acid methyl ester (FAME) compounds were analysed by gas chromatography (GC) method. Preparation and transesterification of samples to FAME compounds were performed according to the manufacturer's instructions. Subsequently, FAME compounds were inserted into GC vials and analysed on the Agilent Tech GC-Midi 6890 N instrument. Fatty acid types corresponding to the obtained GC peaks were manually searched and confirmed in The Sherlock database (The Sherlock Microbial Identification System, Anatune, UK).

2.1.7. Proteomic analysis of PCSEVs and PSCs

The proteomic analysis method was used to investigate the protein composition of the PSCs and PCSEVs. For analysis, proteins derived from the PSCs and purified PCSEVs were isolated by mixing with RIPA buffer, and then they were prepared for mass spectrometry analysis. Accordingly, in-gel digestion and mass spectrometry data acquisition procedures were performed by following the method in a previous study in detail [185]. To analyse the peptide spectrum collected at the end, Proteome Discoverer 2.2 software (Thermo Fisher Scientific, USA) was used. The obtained final data were searched in the Uniprot/Swissprot database for protein identification [186].

2.1.8. Bioinformatic analysis

The accession numbers from the proteomic analysis were compared with the Uniprot/Swissprot database and information on protein names, entry numbers, and gene names. Since proteins belonging to *Stevia rebaudiana* and *Vaccaria hispanica* plants were in the UniProt database, the higher taxa, asteroideae, and pentapetalae, respectively, were used in protein identification. However, this approach resulted in the same proteins from different species being found in the results. To overcome the problem, the organisms most associated with proteins in the results were used while performing gene ontology (GO) enrichment analysis. Thus, *Helianthus annuus* for *Stevia rebaudiana* and *Spinacia oleracea* for *Vaccaria hispanica* were considered to be used in GO enrichment analysis. In terms of bioinformatics analysis, InteractiVenn, a web-based tool was used for the representation and analysis of the Venn diagram [187]. In the analysis, common or vesicle-specific proteins between PCSEVs and PSCs were determined with the Venn diagram. Afterwards, to perform GO enrichment analysis, entry numbers of the proteins derived from PCSEVs were uploaded to the STRING database [188]. Analysis results were grouped separately in biological process (BP), molecular function (MF), and cellular component (CC) gene datasets. In the analysis, GO terms with an FDR value less than 0.05 were ranked according to their strength (log10 equivalents of the Fold Enrichment values), and the most significant GO terms were identified. From this point of view, GO terms enriched in EVs were ranked according to their strengths in BP and MF gene sets with a lollipop chart. Moreover, the FDR values were reflected in the data points in the graph according to the colour scale. In addition, GO terms enriched in the CC gene set were prepared by heat-map representation by comparing all groups with each other. GO terms, which were significant in all gene sets in the analysis of SDEVs, were prepared by performing network analysis in ShinyGO v0.81 [189]. Finally, Microsoft Excel software was used while performing all the calculations in the intermediate steps of these analyses.

2.1.9. Fluorescence labelling and cellular uptake analysis of PCSEVs

To prove that the limiting membrane structures and functionality of PCSEVs were preserved, uptake analysis was performed *in vitro* cell culture [182]. First, PCSEVs had to be labelled with a fluorescent label so that the cellular uptake of the vesicles could be monitored. In this context, PCSEVs were labelled using a green fluorescent dye, 5 μ M

CMFDA, by incubating at 37° for 30 minutes. Thereafter, the vesicle-dye mixture was made up to 15 mL to reduce the free dye concentration to an experimentally ineffective 100 nM, and the PCSEVs were again concentrated to 200 µL via Amicon 50 kDa ultrafiltration column (Merck Millipore, Germany). At this point, to re-determine the vesicle concentrations and control the fluorescence labelling, PCSEVs were re-analysed in the Nanosight NS300 equipped with a 532 nm laser module, following the protocol in the 3rd subtitle.

After the quantification and fluorescence labelling control with the NTA technique, Jurkat cells were treated with fluorescence-labelled PCSEVs at a concentration of 3.43×10^9 particles for SDEVs and HDEVs. Besides, Jurkat cells were also treated with 3.43×10^9 and 6.86×10^8 nanoparticles of HDEVs to investigate the effect of different nanoparticle quantities on the uptake efficiency. In treatment, cells were first labelled with 5 µM CMTPX fluorescent dye by incubating at 37° for 30 minutes. Subsequently, cells were transferred to a 48-well plate as 1.2×10^5 cells in a volume of 200 µL and mixed with 100 µL of CMTFA-labelled PCSEVs. After 2 hours of incubation at 37°C, cells were centrifuged at 200 g for 4 minutes, washed, and resuspended in 1% Bovine Serum Albumin (BSA)/PBS solution. The prepared cells were analysed in the flow cytometry S3e Cell Sorter (Bio-Rad, Hercules, CA), and uptake of PCSEVs by the cells was observed.

2.2. CELL CVI MODELING AND PCSEV FUNCTIONALITY

2.2.1. Cell Lines and Culture Conditions

Within the scope of the study, T lymphocytes (#TIB-152, ATCC, USA) and human brain endothelial cells (HBECs) (#36201-01, Celprogen, USA) were cultured *in vitro* as part of cell culture experiments. In the study, HBECs were used as an *in vitro* model of cerebral-endothelial tissue and T lymphocytes, namely Jurkat cells were used to model peripheral-derived interactions in the emergence of the CVI in cell culture. While culturing HBECs and Jurkat cells, RPMI 1640 (#11875093, Gibco) basal media completed with 10% Fetal Bovine Serum (FBS) (#10500064, Gibco) and 1% Penicillin-Streptomycin-Amphotericin B (PSA) (#15240062, Gibco) were used. In the trypsinization process, 0.10% trypsin/EDTA (#25200056, Gibco) will be used for the passage and harvesting of adherent cells. Finally,

both cell lines were cultured in a humidified cell culture incubator at 37°C and 5% CO₂ (Thermo Fisher Scientific, USA).

2.2.2. Monolayer Culture Model

To model the CVI pathology in HBECs similar to its state in the cerebrovascular tissue, the monolayer culture model was used. In the model, HBECs were seeded at a concentration of 40,000 cells/cm² and allowed to become the monolayer culture. However, to prove that the elevated cell density does not cause any toxicity in the model, the cell viability analysis was performed with the fluorescent viability dye [190]. In the experiment, after the cells became the monolayer, the CVI model was induced by the RPMI 1640 medium containing 750 nM Ionomycin (#11932, Cayman Chemicals) and 40 nM Phorbol 12-myristate 13-acetate (PMA, #P1585, Sigma-Aldrich). At the 3rd and 24th hour of incubation, the cells' media were changed with the serum-free RPMI 1640 medium containing 1 µM CMTPIX fluorescent dye and incubated at 37°C for 30 minutes. After the incubation, the cells were washed with RPMI 1640 medium. Finally, the fluorescent images of cells were taken with the AxioVert A1 Inverted Fluorescent Microscope (Carl Zeiss, USA).

2.2.3. Cytotoxicity Analysis

To investigate the possible toxicity of CVI-induction on HBECs, an MTS assay, which is a non-radioactive cell proliferation assay, was performed [191]. After the CVI induction, at the 24th hour of the incubation, the cells' media were changed with 200 µL of MTS (#G1111, Promega) solution, which was diluted 5 times in the 4.5 g/L D-glucose (#G5767, Sigma-Aldrich) containing PBS solution. The cells were incubated for 1 hour at the incubator and absorbance values were measured with the ELISA plate reader (#ELX800, BioTek, USA). To confirm the accuracy and reliability of the results, the CVI model's effect was evaluated by trypan blue staining, a method different from metabolism-based assays [192]. In the assay, after 16-18 hours of CVI induction, the cells were collected and washed with the serum-free RPMI 1640 medium. Finally, the cell pellet was resuspended with 200 µL of the fresh medium. For the cell counting, 10 µL of the single-cell suspension was loaded into the cell counting slide, and the numbers of cells were counted with the TC20 Automated Cell Counter (Bio-Rad, USA). For the results, the mean values of cell numbers were used for the analysis.

2.2.4. Mitochondrial Membrane Potential Analysis by Rhodamine 123

Mitochondrial membrane potential analysis was performed by staining Rhodamine 123 dye to investigate the possible effects of the CVI model [193]. In the experiment, after the CVI induction, the cells were stained with the RPMI 1640 medium containing 1 $\mu\text{g/mL}$ Rhodamine 123 (#R302, Thermo Fisher Scientific, USA) and 1 $\mu\text{g/mL}$ Hoechst 33342 (#H3570, Sigma-Aldrich). Subsequently, the CVI model was induced for 3 hours and washed with RPMI 1640 medium. The fluorescent images of cells were taken with the AxioVert A1 Inverted Fluorescent Microscope (Carl Zeiss, USA). Afterwards, the Fiji software was used for the intensity analysis of the fluorescent images [194]. In the analysis, the fluorescent signals of cells were divided by the number of cells and the value of relative fluorescence intensity was calculated and compared between groups. In the analysis, to calculate the total cell number, the nucleus number of Hoechst-stained cells needed to be counted. To that end, the result images were converted to an 8-bit grayscale format to standardize the image data. For the counting of cell nuclei, first, the threshold function was applied to make the nuclei more prominent in the images. Subsequently, the particle analysis was performed to overlay the area of cell nuclei with the mask option. The number of cells was calculated using the StarDist plugin from these mask images [195]. Besides, the fluorescence images of Rhodamine 123 dye were also converted to 8-bit grayscale format and the fluorescent signals were filtered with the threshold function. Thus, the intensity values originating from the cells were measured via the area parameter. As a result of these analyses, the values of relative fluorescent intensity per cell were calculated and statistically compared.

2.2.5. Intracellular Calcium Analysis by Fluo-4 Staining

The fluorescence dye-based calcium assay was performed to investigate whether the CVI induction increases the intracellular calcium concentration by Fluo-4 staining. In the analysis, HBECs were prepared as the monolayer culture and the CVI induction was performed for 3 hours. After the incubation, the cells were stained with the RPMI 1640 medium containing 5 μM Fluo-4 acetoxymethyl (AM) ester dye (#F14201, Thermo Fisher Scientific, USA), 0.2% (w/v) Pluronic F127 (55401892, BASF, USA) and 1 $\mu\text{g/mL}$ Hoechst 33342 for 45 minutes in the incubator. Subsequently, the staining solution was changed with the growth medium and the cells were incubated for 15 minutes to allow complete de-

esterification of intracellular AM esters. In the end, the fluorescent images of cells were taken with the AxioVert A1 Inverted Fluorescent Microscope (Carl Zeiss, USA). To analyse the intensity values of the fluorescent images, the calculation of relative fluorescence intensity per cell was performed as described in section 2.2.4.

2.2.6. Gene Expression Analysis by Relative rt-PCR assay

The real-time reverse transcription polymerase chain reaction (rt-PCR) was performed to investigate the gene expression in CVI-induced HBECs. While designing and performing the relative rt-PCR assay, the MIQE guideline was followed [196]. In the experiment, after the CVI induction, the cells were collected and their RNAs were isolated with a column-based RNA purification kit (#740955, Macherey-Nagel). Subsequently, cDNA templates were synthesized by using a high-capacity cDNA reverse transcription kit (#4368814, Thermo Scientific) from purely obtained RNAs. The rt-PCR reactions were taken place with iTaq Universal SYBR Green Supermix (#1725122, Bio-Rad, USA) in the CFX96 Touch real-time PCR detection system (Bio-Rad, USA). In the experiment, the ATP5F1B gene was used as a reference gene. All information such as the gene names and the primer sequences is given in the related tables. The information on real-time reaction setup and condition was in Tables 2.1 and 2.2.

Table 2.1. Rt-PCR reaction setup per well.

Component	Volume	Final Concentration
iTaq Universal SYBR Green Supermix (2x)	5 μ L	1x
Forward Primer	0.5 μ L	0.5 μ M
Reverse Primer	0.5 μ L	0.5 μ M
Nuclease-free Water	2 μ L	-
cDNA Template	2 μ L	1 ng/ μ L
Total Volume	10 μ L	

Table 2.2. Rt-PCR reaction conditions.

Step	Cycles	Temperature	Time
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Initialization	1	95°C	02:00
Denaturation	35	95°C	00:05
Annealing/Extension		60°C	00:30
Melt Curve	60	0.5°C	00:05
Cooling	1	4°C	∞

2.2.7. Gene Expression Analysis by Agarose Gel Electrophoresis

To validate the rt-PCR results, the PCR and agarose gel electrophoresis were performed. In the analysis, cDNA templates were taken from the gene expression analysis by relative rt-PCR assay. As an enzyme, Maximo Taq DNA Polymerase (#S102, GeneON, Germany) was used in the reactions. All the information about the reaction setup and condition is given in Table 2.3. and 2.4. In the analysis, the ATP5F1B gene was used as a loading control.

Table 2.3. PCR reaction setup per well.

Component	Volume	Final Concentration
10X Reaction Buffer	1 µL	1x
dNTP-Mix	0.2 µL	0.2 mM
Forward Primer	0.5 µL	0.5 µM
Reverse Primer	0.5 µL	0.5 µM
Maximo Taq DNA Polymerase	0.04 µL	0.02 U/µL
Nuclease-free water	5.71 µL	-
cDNA Template	2 µL	1 ng/µL
Total Volume	10 µL	

Table 2.4. PCR reaction conditions.

Step	Cycles	Temperature	Time
Initialization	1	95°C	03:00
Denaturation	35	95°C	00:15
Annealing		60°C	00:30
Extension		72°C	00:30

Final Extension	1	72°C	05:00
Cooling	1	4°C	∞

The visualization of the PCR reactions was performed using an agarose gel electrophoresis technique to analyse the gene expression in HBECs [197]. In the experiment, the 2% agarose gel solution was prepared in 1X Tris-Acetic acid-EDTA (TAE) buffer (#880-540 LL, Wisent, Canada), and SYBR Green I nucleic acid dye (#S9430, Sigma-Aldrich) was mixed at a ratio of 1/20,000. Meanwhile, 10 μ L of the PCR product was mixed with 2 μ L of 6x TriTrack DNA Loading Dye to prepare the samples for the electrophoresis. In the agarose gel, GeneRuler 50 bp DNA Ladder was used for the approximate sizing of the DNA bands. The gel electrophoresis was carried out at 80V for 45 min. After the electrophoresis, DNA bands were visualized on the ChemiDoc XRS+ System (Bio-Rad, USA). The densitometric analysis of DNA bands was performed using ImageLab Software (Bio-Rad, USA).

2.2.8. Dose Analysis and Optimization of CVI Induction

To select the feasible dose in the CVI induction, PMA/Ionomycin treatment was modified in terms of the dose. In the experiment, the monolayer culture model was prepared and the HBECs were treated with PMA/Ionomycin at doses of 40 nM/750 nM, 16 nM/250 nM, and 16 nM/125 nM for 3 hours. After the incubation, the cells were harvested and the gene expression analysis was performed as described in section 2.2.6. To validate the relative rt-PCR results, gene expression analysis by agarose gel electrophoresis was performed as described in section 2.2.7. In the analysis, the CVI model induced under different conditions was evaluated through IL8 and PAI1 gene expression. All the information such as the primer sequences and the product lengths is given in Table 2.5.

Table 2.5. Primer sequences used in the analysis of CVI doses.

Gene Name	Strand	Sequences (5'-3')	Product Length	Reference
ATP Synthase F1 Subunit Beta (ATP5F1B)	F	GTCTTCACAGGTCATATGGGGA	122 bp	[198]
	R	ATGGGTCCCACCATATAGAAGG		
C-X-C Motif Chemokine Ligand 8 (IL8)	F	ACTGAGAGTGATTGAGAGTGGAC	112 bp	[199]
	R	AACCCTCTGCACCCAGTTTTTC		
	F	CAGCCAGATGCAATCAATGCC	131 bp	

Monocyte Chemoattractant Protein-1 (MCP1)	R	TGGAATCCTGAACCCACTTCT		
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2.2.9. Gene Expression Analysis of CVI Model in HBECs

The effects of the PMA/Ionomycin treatment on CVI-related markers in the HBECs were determined with the gene expression analysis. In the experiment, first, the monolayer culture model was prepared and the cells were treated with the RPMI 1640 medium containing 16 nM PMA and 125 nM Ionomycin for 3 hours, which will be called the CVI induction for the following protocols. After the incubation, the gene expression analysis was performed as described in section 2.2.6. The information such as gene names and primer sequences is given in Table 2.6.

Table 2.6. Primer sequences used in the analysis of gene expression analysis.

Gene Name	Strand	Sequences (5'-3')	Reference
ATP Synthase F1 Subunit Beta (ATP5F1B)	F	GTCTTCACAGGTCATATGGGGA	[198]
	R	ATGGGTCCCACCATATAGAAGG	
C-X-C Motif Chemokine Ligand 8 (IL8)	F	ACTGAGAGTGATTGAGAGTGGAC	[199]
	R	AACCCTCTGCACCCAGTTTTTC	
Interleukin 6 (IL6)	F	ACTCACCTCTTCAGAACGAATTG	
	R	CCATCTTTGGAAGGTTTCAGGTTG	
Monocyte Chemoattractant Protein-1 (MCP1)	F	CAGCCAGATGCAATCAATGCC	
	R	TGGAATCCTGAACCCACTTCT	
Plasminogen Activator Inhibitor 1 (PAI1)	F	GCCAGTGGAAGACTCCCTTC	
	R	GGGCGTGGTGAAGTCACTATAG	
Forkhead Box Protein O1 (FOXO1)	F	GGGTAGTGAGCAGGTTACACTT	
	R	TGGACTGCTTCTCTCAGTTCCT	
Peroxisome Proliferator Activated Receptor Gamma (PPARG)	F	ACCAAAGTGCAATCAAAGTGGA	
	R	ATGAGGGAGTTGGAAGGCTCT	
Sirtuin 1 (SIRT1)	F	AAGGCCACGGATAGGTCCAT	
	R	TCAGGTGGAGGTATTGTTTCCG	
NFE2 BZIP Transcription Factor 2 (NRF2)	F	TTCCCGGTCACATCGAGAG	
	R	TCCTGTTGCATACCGTCTAAATC	
	F	AAAGAAGCCTGTGGGCGAA	

Marker Of Proliferation Ki-67 (MKI67)	R	TTGAGCACTCTGTAGGGTCG	
BCL2 Apoptosis Regulator (BCL2)	F	CTGGTGGACAACATCGCCCT	
	R	TCTTCAGAGACAGCCAGGAGAAAT	
BCL2 Associated X, Apoptosis Regulator (BAX)	F	TGCAGAGGATGATTGCCGCCG	
	R	ACCCAACCACCCTGGTGTGG	
AKT Serine/Threonine Kinase 1 (AKT)	F	GAAGCTGCTGGGCAAGGGGCA	
	R	GTGGGCCACCTCGTCCTTGG	[199]
Vascular Cell Adhesion Molecule 1 (VCAM1)	F	GGGAAGATGGTCGTGATCCTT	
	R	TCTGGGGTGGTCTCGATTTTA	
Intercellular Adhesion Molecule 1 (ICAM1)	F	TTGGGCATAGAGACCCCGTT	
	R	GCACATTGCTCAGTTCATACACC	
Endothelial Leukocyte Adhesion Molecule 1 (ELAM1)	F	CAGCAAAGGTACACACACCTG	
	R	CAGACCCACACATTGTTGACTT	
Vascular Endothelial Growth Factor A (VEGFA)	F	AGGGCAGAATCATCACGAAGT	
	R	AGGGTCTCGATTGGATGGCA	
Platelet And Endothelial Cell Adhesion Molecule 1 (PECAM1)	F	AACAGTGTTGACATGAAGAGCC	
	R	TGTAAAACAGCACGTCATCCTT	
Von Willebrand Factor (VWF)	F	CCTTGACCTCGGACCCTTATG	
	R	GATGCCCCGTTACACCACT	
Kinase Insert Domain Receptor (KDR)	F	GGCCCAATAATCAGAGTGGCA	
	R	CCAGTGTCAATTCGATCACTTT	
Angiopoietin 2 (ANGPT2)	F	AACTTTCGGAAGAGCATGGAC	
	R	CGAGTCATCGTATTCGAGCGG	
Zona Occludens 1 (ZO1)	F	ACCAGTAAGTCGTCCTGATCC	
	R	TCGGCCAAATCTTCTCACTCC	
Claudin 1 (CLDN1)	F	CCTCCTGGGAGTGATAGCAAT	
	R	GGCAACTAAAATAGCCAGACCT	
Occludin (OCLDN)	F	GACTTCAGGCAGCCTCGTTAC	
	R	GCCAGTTGTGTAGTCTGTCTCA	
Matrix Metalloproteinase 3 (MMP3)	F	GACAAAGGATACAACAGGGACC	
	R	AGCTTCAGTGTTGGCTGAGT	
Matrix Metalloproteinase 9 (MMP9)	F	AGACCTGGGCAGATTCCAAAC	
	R	CGGCAAGTCTTCCGAGTAGT	

2.2.10. Western Blot Analysis of CVI Model in HBECs

The western blot analysis was performed to inspect the effect of CVI induction on the protein expressions of HBECs. In the experiment, the CVI induction was performed and the cells were treated for 3 hours in the incubator. After the incubation, the HBECs were harvested and prepared for protein isolation. To obtain a cell lysate, RIPA buffer (89900, Thermo Scientific) including protease inhibitor cocktail (ab201111, Abcam) and sodium orthovanadate was used as a cell lysis solution. The cell pellets were resuspended with 100 μ L RIPA buffer and incubated on ice for 30 min with interval vortexing every 5 min. At the end of the incubation, the cell lysates were centrifuged at 20000 g for 10 minutes at 4°C to remove the insoluble particles in the solution. The supernatants were aliquoted and stored at -80°C. Subsequently, the protein concentration of samples was determined by using a Pierce BCA protein assay kit (23225, Thermo Scientific). In the analysis, the samples were diluted 5 times and the assay was performed according to the manufacturer's instructions.

In the western blot analysis, firstly, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using 4-20% Mini-PROTEAN TGX protein gels (4561094, Bio-Rad, USA) in the reduced and denatured condition. To prepare the samples, 10 μ g of samples were mixed with 4x Laemmli sample buffer (1610747, Bio-Rad, USA) including 10% beta-mercaptoethanol (β ME), and incubated for 5 minutes at 95°C in a thermal cycler. Then, the gel was run at 120 volts for 15 minutes and continued at 200 volts for approximately 40 minutes. After the separation of proteins, the proteins in the polyacrylamide gel were transferred to 0.45 μ m nitrocellulose membrane by performing an immunoblotting technique. Therefore, the transfer of proteins was performed at 250 mA for 1 hour in the Tris-glycine-based transfer buffer (1610771, Bio-Rad, USA). Subsequently, the membrane was immersed in a blocking buffer consisting of a 5% blotting-grade blocker in tris-buffered saline containing 0.1% Tween-20 (TBS-T) and incubated overnight at 4°C with gentle shaking. After the blocking step, the membrane was cut according to the molecular weights of target proteins and was probed with primary antibodies diluted in the blocking buffer according to Table 2.7. After the incubation, the membranes were washed 3 times for 5 minutes with TBS-T solution. For the secondary antibody treatment, the membranes were probed for 1 hour at room temperature (RT) with horse radish peroxidase (HRP)-conjugated secondary antibodies (ab191866, Abcam) diluted in the blocking buffer according to Table 2.7. Subsequently, the membranes were washed 3 times with TBS-T and

2 times with TBS for 5 minutes. For the imaging of membranes, an Immobilon ECL Ultra chemiluminescence kit was used and the prepared ECL solution was diluted 5 times before the analysis. After 20 seconds of incubation with HRP substrate solution, the membranes were exposed for 1 min and images were taken with ChemiDoc XRS+ System (Bio-Rad, USA). The densitometric analyses of protein bands were performed by using ImageLab Software (Bio-Rad, USA).

Table 2.7. Antibody information used in the western blot analysis of CVI model.

Antibodies	Cat. No	Dilution Ratio
PPARG	81B8 / Cell Signaling	1:1000
SIRT1	NBP-49540 / Novus Biologicals	1:5000
NRF2	NBP-32822 / Novus Biologicals	1:10,000
PAI1	BS-6562R / Thermo Scientific	1:5000
FOXO1	18592-1-AP / Proteintech	1:10,000
ANG2	Ab155106 / Abcam	1:5000
ACTB	20536-1-AP / Proteintech	1:10,000
Anti-Rabbit HRP	Ab191866 / Abcam	1:10,000

2.2.11. Immunocytochemistry Analysis of the CVI model in HBECs

To analyse the effects of CVI induction on the protein expression and localization of HBECs, an immunocytochemistry (ICC) analysis was performed [200]. For the analysis, sterilized coverslips were placed into the 6-well plates and coated with cell basement membrane solution (ACS-3035, ATCC) and incubated for 30 minutes in the incubator. Subsequently, the HBECs were seeded onto the coated coverslips and, after 48 hours, the CVI model was induced. After the incubation, the cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) solution for 15 minutes at RT. The fixed HBECs were washed with PBS twice and permeabilized with PBS containing 0.1% Triton-X 100 for 5 minutes at RT. Then, the cells were washed with PBS again and blocked with PBS-T containing 1% bovine serum albumin (BSA) (37520, Thermo Scientific) for 30 minutes at RT. At the end of incubation, the cells were incubated overnight with primary antibodies diluted in the blocking solution according to Table 2.8. On the second day of analysis, the

cells were washed thrice with PBS-T and incubated with secondary antibodies diluted in the blocking solution according to Table 2.8 for 1 hour at RT (dark). After the incubation, the cells were stained with DAPI solution (1:1000 diluted in PBS) for 5 minutes and washed thrice with PBS. In the end, the fluorescent images of cells were taken with a confocal microscope (LSM700, Zeiss, Heidelberg, Germany) and intensities were calculated with ZEN microscopy software. To analyse the intensity values of the fluorescent images, the calculation of relative fluorescence intensity per cell was performed as described in section 2.2.4.

Table 2.8. Antibody information used in the ICC analysis of CVI model.

Antibodies	Cat. No	Dilution Ratio
IL8	STJ96518 / St. John's Laboratory	1:200
KI67	51-9007231 / BD Biosciences	
F-actin	Ab176153 / Abcam	1:1000
VCAM1	13662S / Cell Signaling	1:200
ELAM1	NBP1-45545 / Novus Biologicals	
KDR	Ab11939 / Abcam	
ZO1	13663S / Cell Signaling	
CLDN1	Ab15098 / Abcam	
OCLN	13409-1-AP / Proteintech	
CDH5	PA5-19612 / Thermo Scientific	
Anti-Rabbit 488	Ab150077 / Abcam	1:500
Anti-Mouse 488	Ab150117 / Abcam	
Anti-Rabbit 555	406412 / BioLegend	
Anti-Mouse 555	405324 / BioLegend	

2.2.12. Inflammatory Cytokine Analysis by Cytometric Bead Array

To investigate the effects of CVI induction on inflammation-related cytokines of HBECs, an inflammatory cytokine analysis was performed using a Cytometric bead array kit (551811, BD Biosciences). In the experiment, the CVI model was induced by PMA/Ionomycin treatment and the HBECs were incubated for 3 hours. After that, the supernatants of cells were collected to be used in the experiment. To begin with, the supernatants were centrifuged at 300 g for 5 minutes to remove live and dead cells and also

centrifuged at 1000 g for 5 minutes to remove cell debris. In the experiment, all analyses were performed by following the manufacturer's instructions. Accordingly, firstly standards and antibody capture bead mixture were prepared and 50 μ L of capture bead mixture was added to the tubes. Then, 50 μ L of undiluted supernatants were added to this bead solution. Lastly, 50 μ L of PE detection reagent was added to the mixture. All the samples were mixed and incubated for 3 hours at RT (dark). At the end of incubation, 1 mL of wash buffer solution was added to the samples and centrifuged at 200 g for 5 minutes. Finally, the supernatants were discarded and 300 μ L of wash buffer was added to each tube and analysed by flow cytometry S3e Cell Sorter (Bio-Rad, Hercules, CA). In the analyses, the FCAP array software was used and the protein concentration of target proteins in the supernatant was calculated using standard curves obtained from the standards and compared with each other.

2.2.13. Tube Formation Analysis of the CVI Model in HBECs

The possible angiogenic effects of CVI induction on HBECs were investigated by performing a tube formation analysis. In the experiment, the wells of 96-well plates are coated with 60 μ L of 10 mg/mL cell basement membrane solution and incubated for 30 minutes at 37°C. After the incubation, the cells were seeded in each well with 20,000 cells in 100 μ L growth medium and the RPMI-1640 medium containing PMA/Ionomycin as stated in the CVI modelling. At the end of the 6-hour incubation at 37°C, the images were taken with the AxioVert A1 Inverted Fluorescent Microscope (Carl Zeiss, USA). For the analysis, the ImageJ software and its angiogenesis macro were used [201]. In the analysis, angiogenesis parameters such as total length, number of nodes, number of branches and total meshes area were used to compare the angiogenic potential between groups.

2.2.14. Gene Expression Analysis of T Lymphocyte Activation

The inflammatory conditions in the cerebrovascular tissue affect not only the CECs but also the immune cells. Furthermore, one of the critical characteristics in CVI pathology is the leukocyte adhesion to the endothelial cells and their infiltration to the parenchymal regions of the brain. In the study, it was decided to use Jurkat cells as T lymphocytes. However, to be certain that the results of the experiment with Jurkat cells are reliable, it is necessary to set up an experiment to determine whether these cells are not auto-activated and can respond to activation signals. To that end, Jurkat cells were prepared at a density of 1.5 million

cells/mL and activated by CVI induction for 3 hours. After the incubation, the gene expression analysis was performed following the protocol described in section 2.2.6. All the information about gene names and primer sequences is given in Table 2.9.

Table 2.9. Primer sequences used in the analysis of Jurkat activation.

Gene Name	Strand	Sequences (5'-3')	Reference
ATP Synthase F1 Subunit Beta (ATP5F1B)	F	GTCTTCACAGGTCATATGGGGA	[198]
	R	ATGGGTCCCACCATATAGAAGG	
Interleukin 2 (IL2)	F	AGAACTCAAACCTCTGGAGGAAG	[199]
	R	GCTGTCTCATCAGCATATTACACAC	
Interferon Gamma (IFNG)	F	TCGGTAACTGACTTGAATGTCCA	
	R	TCGCTTCCCTGTTTTAGCTGC	
C-X-C Motif Chemokine Ligand 8 (IL8)	F	ACTGAGAGTGATTGAGAGTGGAC	
	R	AACCCTCTGCACCCAGTTTTTC	
Interleukin 4 (IL4)	F	CGGCAACTTTGTCCACGGA	
	R	TCTGTTACGGTCAACTCGGTG	
NFE2 Like BZIP Transcription Factor 2 (NRF2)	F	TTCCCGGTCACATCGAGAG	
	R	TCCTGTTGCATACCGTCTAAATC	
Sirtuin 1 (SIRT1)	F	AAGGCCACGGATAGGTCCAT	
	R	TCAGGTGGAGGTATTGTTTCCG	
Peroxisome Proliferator Activated Receptor Gamma (PPARG)	F	ACCAAAGTGCAATCAAAGTGGA	
	R	ATGAGGGAGTTGGAAGGCTCT	
BCL2 Apoptosis Regulator (BCL2)	F	CTGGTGGACAACATCGCCCT	
	R	TCTTCAGAGACAGCCAGGAGAAAT	
BCL2 Associated X, Apoptosis Regulator (BAX)	F	TGCAGAGGATGATTGCCGCCG	
	R	ACCCAACCACCCTGGTGTGTTGG	
AKT Serine/Threonine Kinase 1 (AKT)	F	GAAGCTGCTGGGCAAGGGGCA	
	R	GTGGGCCACCTCGTCCTTGG	
Marker Of Proliferation Ki-67 (MKI67)	F	AAAGAAGCCTGTGGGCGAA	
	R	TTGAGCACTCTGTAGGGTCG	
Lymphocyte Function-Associated Antigen 1 (LFA)	F	CAGGCTATTTGGGTTACACCG	
	R	CCATGTGCTGGTATCGAGGG	

2.2.15. Functionality Analysis of the CVI Model

2.2.15.1. *T Lymphocyte Adhesion Assay*

The adhesion of T lymphocytes to CECs is one of the important characteristics of endothelial activation. To investigate the adhesion of T lymphocytes on HBECs in the CVI model, the leukocyte adhesion assay was performed [202]. In the experiment, HBECs were seeded in 12-well plates and the CVI model was induced as previously determined. During the 3 hours of incubation, Jurkat cells were centrifuged at 200 g for 5 minutes and resuspended with the serum-free RPMI 1640 medium containing 1 μ M CMTPX fluorescent dye at a density of 1 million cells/mL and incubated at 37°C for 30 minutes. Subsequently, the cells were washed with the serum-free medium and were ready to be transferred to the HBECs' culture plate. Upon completion of the 3-hour incubation, HBECs were washed once with the growth medium and incubated with RPMI 1640 medium containing 1 μ g/mL Hoechst 33342 dye at 37°C for 20 minutes. After the incubation, the staining solution was aspirated and Jurkat cells were transferred as 1 mL of homogeneous cell suspension to HBECs' culture plate. Jurkat cells and HBECs were incubated at 37°C for 30 minutes. Subsequently, media containing the non-attached Jurkat cells was discarded, and the cell surface was washed twice with the serum-free medium. Finally, the fluorescent images of cells were taken with the AxioVert A1 Inverted Fluorescent Microscope (Carl Zeiss, USA). To analyse the intensity values of the fluorescent images, the calculation of relative fluorescence intensity per cell was performed as described in section 2.2.4.

2.2.15.2. *In Vitro Endothelial Permeability Assay*

Another characteristic condition case observed in the CVI pathology is the increased permeability of the BBB structure. Being one of the cell groups that form the neurovascular unit, CECs also play essential roles in the BBB physiology and integrity. To investigate the effect of CVI induction on the permeability of the monolayer culture of HBECs, a transwell-based *in vitro* permeability assay was performed [203]. In the experiment, first, HBECs were seeded on 24-well-inserts and the CVI model was induced as previously determined. After the 3-hour incubation, 4 kDa FITC-conjugated dextran solution was added to the media at a final concentration of 50 μ g/mL. The fluorescent molecule was incubated with the monolayer culture of HBECs at 37°C for 4 hours. After the incubation, 10 μ L of medium samples were taken from the bottom of the well to evaluate the endothelial barrier ability

and permeability. The fluorescence measurement of these samples was performed by using Varioskan Microplate Reader (Thermo Scientific) via μ Drop plates at 490/520 nm.

2.3. INVESTIGATING EFFECTS OF THE PCSEVS ON CVI MODEL

2.3.1. Cell Viability Analysis of PCSEVs on HBECs

To investigate the possible toxicity of PCSEVs and determine their effective doses, the MTS assay was performed. To begin with, the monolayer culture of HBECs was prepared and treated with various doses of the PCSEVs. These doses were prepared from stock solutions with 10-fold serial dilution just before the cell treatment. The stock doses of the PCSEVs were 1.37×10^9 particles/mL for SDEVs and 1.1×10^{10} particles/mL for HDEVs. After 24-hour incubation, the experiment procedure was performed as described in section 2.2.3. At the end of the experiment procedure, the measured absorbance values were analysed and compared.

2.3.2. Gene Expression Analysis of CVI in PCSEVs-treated HBECs

In the PMA/Ionomycin induced endothelial activation model of HBECs, biochemical molecules or treatments that have protective or therapeutic potential are expected to demonstrate some functional effects on CVI pathology. Accordingly, the effects of the PCSEVs on the CVI model of HBECs, when applied as a protective agent, were analysed with findings from the experiments of *in vitro* endothelial activation. For this purpose, firstly, it was investigated that the PCSEV treatment affected the expression of CVI-related gene groups in PMA/Ionomycin-induced HBECs. In the experiment, the monolayer culture model of HBECs was prepared and the pre-treatment of the PCSEVs was performed. Therefore, HBECs were treated with SDEVs at a concentration of 1.37×10^9 and 1.37×10^8 particles/mL and HDEVs at a concentration of 1.1×10^{10} and 1.1×10^9 particles/mL. The PCSEVs-treated HBECs were incubated for 24 hours and then, the CVI induction was performed for 3 hours. After the incubation, the cells were harvested and the gene expression analyses were performed as described in section 2.2.6. All the information about gene names and primer sequences is given in Table 2.6.

2.3.3. Immunocytochemistry Analysis of CVI in PCSEVs-treated HBECs

To investigate the potential effects of the PCSEVs on the protein expressions of CVI-induced HBECs, the ICC analysis was performed again. In the experiment, the HBECs were seeded and prepared in the monolayer form. Upon the completion of the monolayer culture of cells, the HBECs were treated with SDEVs and HDEVs with doses of 1.37×10^9 and 1.1×10^{10} particles/mL, respectively. After 24 hours of incubation, the ICC analysis was performed according to the previously used protocol described in section 2.2.11. In the end, the experimental results were compared both visually and quantitatively as abovementioned.

2.3.4. Inflammatory Cytokine Analysis of CVI in PCSEVs-treated HBECs

The bead-based inflammatory cytokine analysis was performed to investigate the effects of CVI induction on the PCSEVs-treated HBECs. In the experiment, the monolayer culture of HBECs was prepared and the cells were treated with SDEVs and HDEVs with doses of 1.37×10^9 and 1.1×10^{10} particles/mL, respectively. After 24 hours of incubation, the CVI model was induced by PMA/Ionomycin treatment and the cells were incubated for 3 hours. Then, the inflammatory cytokine analysis was performed according to the protocol described in section 2.2.12. Consequently, all the results were analysed and compared quantitatively.

2.3.5. T Lymphocyte Adhesion Assay of CVI in PCSEVs-treated HBECs

To investigate the effects of the PCSEVs on adhesion interactions between HBECs and T lymphocytes under induction of the CVI model, the leukocyte adhesion assay was performed. In the experiment, HBECs were prepared as a monolayer form and the cells were treated with SDEVs and HDEVs with doses of 1.37×10^9 and 1.1×10^{10} particles/mL, respectively. After 24 hours of incubation, the CVI model was induced by PMA/Ionomycin treatment and the cells were incubated for 3 hours. Then, the leukocyte adhesion assay was performed as described in section 2.2.15. As a result, all the findings were analysed.

2.3.6. Gene Expression Analysis of the PCSEVs-treated HBECs

To discover how the PCSEVs affect the gene expression of HBECs used in the CVI model, the gene expression analysis was performed by using the rt-PCR analysis. In the experiment, the monolayer culture of HBECs was prepared and treated with the PCSEVs described in

section 2.3.2. After 24 hours of incubation, the treated cells were collected and their gene expressions were analysed as described in section 2.2.6. All the information about gene names and primer sequences is given in Table 2.10.

Table 2.10. Primer sequences used in the analysis of the PCSEVs-treated HBECs.

Gene Name	Strand	Sequences (5'-3')	Reference
ATP Synthase F1 Subunit Beta (ATP5F1B)	F	GTCTTCACAGGTCATATGGGGA	[198]
	R	ATGGGTCCCACCATATAGAAGG	
Heme Oxygenase-1 (HMOX1)	F	AAGACTGCGTTCCTGCTCAAC	[199]
	R	AAAGCCCTACAGCAACTGTCTG	
NAD(P)H Quinone Dehydrogenase 1 (NQO1)	F	GAAGAGCACTGATCGTACTGGC	
	R	GGATACTGAAAGTTCGCAGGG	
Kelch-like ECH-associated protein 1 (KEAP1)	F	CTGGAGGATCATACCAAGCAGG	
	R	GGATACCCTCAATGGACACCAC	
NFE2 Like BZIP Transcription Factor 2 (NRF2)	F	TTCCCGGTCACATCGAGAG	
	R	TCCTGTTGCATACCGTCTAAATC	
Sirtuin 1 (SIRT1)	F	AAGGCCACGGATAGGTCCAT	
	R	TCAGGTGGAGGTATTGTTTCCG	
Peroxisome Proliferator Activated Receptor Gamma (PPARG)	F	ACCAAAGTGCAATCAAAGTGGA	
	R	ATGAGGGAGTTGGAAGGCTCT	
Marker Of Proliferation Ki-67 (MKI67)	F	AAAGAAGCCTGTGGGCGAA	
	R	TTGAGCACTCTGTAGGGTCG	
BCL2 Apoptosis Regulator (BCL2)	F	CTGGTGGACAACATCGCCCT	
	R	TCTTCAGAGACAGCCAGGAGAAAT	
AKT Serine/Threonine Kinase 1 (AKT)	F	GAAGCTGCTGGGCAAGGGGCA	
	R	GTGGGCCACCTCGTCCTTGG	

2.3.7. Time and Dose-Dependent Expression Analysis of the PCSEVs in HBECs

In the study, the effects of the PCSEVs on the gene expression of HBECs were successfully explored. However, analysis of these gene expressions was performed with the treatment of two close doses at a single time point. Thus, to investigate the effects of the dose and time parameters on the expression of anti-inflammatory-related genes, dose and time-dependent

expression analyses were performed by using the rt-PCR analysis. In the experiment, first, the HBECs were seeded on 12-well plates and prepared as a monolayer culture. For the dose-dependent analysis, the cells were treated with several doses of the PCSEVs. The HBECs were treated with SDEVs at a concentration of 1.37×10^9 , 1.37×10^7 , and 1.37×10^5 particles/mL and HDEVs at a concentration of 1.1×10^{10} , 1.1×10^8 , and 1.1×10^6 particles/mL. Subsequently, all these treatments lasted 24 hours. For the time-dependent analysis, the cells were treated with 1.1×10^{10} particles/mL of HDEVs for 7, 24, and 48 hours. After these incubations, the treated cells were collected and their gene expressions were analysed as described in section 2.2.6. All the information about gene names and primer sequences is given in Table 2.10.

2.3.8. Western Blot Analysis of PCSEVs-treated HBECs

Investigating the potential effect of the PCSEVs on cytoprotective pathways of HBECs against CVI induction has notable importance in the sense of the therapeutic roles of these vesicular structures. To that end, in the study, it was investigated how the PCSEVs affect the expression of target proteins in these protective pathways in the CECs. In the experiment, the monolayer culture of HBECs was prepared and treated with the PCSEVs described in section 2.3.2. After 24 hours of incubation, the treated cells were collected and their protein expressions were analysed as described in section 2.2.10. All the information about antibody names and dilution ratios is given in Table 2.11.

Table 2.11. Antibodies used in the western blot analysis of the PCSEVs-treated HBECs.

Antibodies	Cat. No	Dilution Ratio
NRF2	81B8 / Cell Signaling	1:10,000
NQO1	NBP-49540 / Novus Biologicals	1:5000
ACTB	NBP-32822 / Novus Biologicals	1:10,000
Anti-Rabbit HRP	Ab191866 / Abcam	1:10,000

2.3.9. Capillary Electrophoresis Analysis of Phosphorylated Proteins of PCSEVs-treated HBECs

In the study, investigating the protective effects of PCSEVs on endothelial cells is as valuable as the cytoprotective cellular pathways through which these nanovesicles exert these effects. For this purpose, some upstream signaling pathways playing crucial regulatory roles in cells were selected and the phosphorylation status of target proteins was analysed after the PCSEV treatment on HBECs. Therefore, the interactions between these pathways and the effects of PCSEVs were investigated in the experiment. In the analysis, a monolayer culture model was formed using the HBECs and the cells were treated with the PCSEVs as previously described in section 2.3.2. After 24 hours of incubation, the treated cells were collected and the protein concentrations were measured as previously described in section 2.2.10. In the phosphorylation analysis, the levels of target proteins in HBECs were analysed with a capillary electrophoresis system. In the experiment, the sample preparation and all the analyses were performed according to the study in the literature [204]. Consequently, the area data in the electropherogram of capillary electrophoresis results were used in the analysis and signal-to-noise ratio values were used when selecting the relevant bands of target proteins. The band densities of the target proteins were normalized with GAPDH protein. Furthermore, in the analysis of phosphorylation levels between groups, the densities of phosphorylated proteins were normalized by dividing by total protein expressions. In the end, the effects of PCSEVs on phosphorylations of these pathways were compared using the fold change data. All the information about antibody names and dilution ratios is given in Table 2.12.

Table 2.12. Antibodies used in the capillary electrophoresis analysis of the PCSEVs-treated HBECs.

Antibodies	Cat. No	Dilution Ratio
ERK 1/2	4695S / Cell Signaling	1:50
AKT	9272S / Cell Signaling	
P38	8690S / Cell Signaling	
Phospho-Erk 1/2	9101S / Cell Signaling	
Phospho-Akt	9271S / Cell Signaling	
Phospho-p38	4511S / Cell Signaling	

GAPDH	60004-1-Ig / Proteintech	1:400
Anti-Rabbit HRP	Ab191866 / Abcam	1:200
Anti-Mouse HRP	Ab205719 / Abcam	1:400

2.4. STATISTICAL ANALYSIS

Statistical analyses were used to evaluate the significant differences and reproducibility among the experimental groups. The significance analyses of all the results were performed with one-way ANOVA and the two-tailed t-test in the GraphPad 9 software. Besides, the Microsoft Excel software was used only in the demonstration of the GO enrichment analysis. In the study, all experiments were repeated with biological replicates in addition to technical replicates. Differences among experimental groups will be considered statistically significant if the p-values are less than 0.05, 0.01, or 0.001.

3. RESULTS

3.1. MULTIPARAMETER CHARACTERIZATION OF THE PCSEVS

Investigating the extracellular vesicles obtained from plant suspension media from many perspectives allows us to understand their genuine characteristic without the interference of intracellular vesicles. To reach this goal in our study, the characteristic features of PCSEVs were investigated physically and molecularly. In the physical analyses, the size distributions and nanoparticle quantities of PCSEVs were determined by the NTA technique as shown in Figure 3.1. The size distribution result of SDEVs showed that the stock solution had three main populations with different nanoparticle quantities (Figure 3.1a).

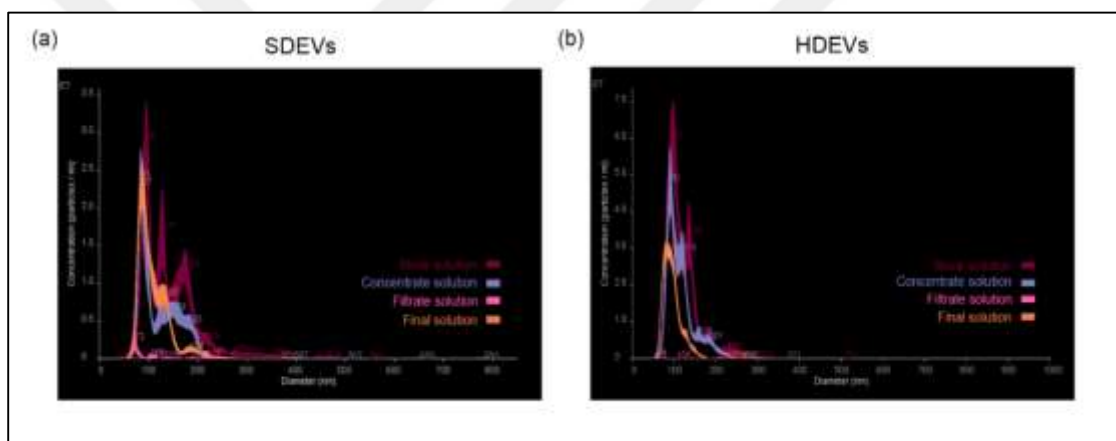


Figure 3.1. The size distribution analysis of PCSEVs. The vesicle samples taken from the critical steps of the purification of the SDEVs (a) and HDEVs (b) were represented as different colours.

With the purification process, these two large populations were reduced and a single smaller main population remained in the concentrate group. Similarly, PCSEVs in the final group, obtained by washing and filtration, were observed as a single population within the expected size range. In the filtrate group, the presence of nanoparticles was quite low as expected. On the other hand, the size distribution of HDEVs showed that the stock solution had two main populations whose size ranges were narrower than the SDEVs (Figure 3.1b). As the purification process continued, the size of nanoparticle populations decreased gradually and showed a more homogeneous distribution. However, unlike in the SDEVs, it was also

observed that the nanoparticle quantities of the final group in HDEVs decreased stepwise in the main population.

The nanoparticle quantities of the samples collected from the critical steps were compared as shown in Figure 3.2 to analyse the effects of the purification process. The results of SDEVs (Figure 3.2a) showed that the quantity of stock solution was 9.35×10^9 nanoparticles. After the first separation in the protein column, the quantities of filtrate and concentrate groups were 6.81×10^8 and 4.71×10^9 nanoparticles, respectively. On the other hand, in the HDEV results (Figure 3.2b), the quantity of stock solution was measured as 5.34×10^{10} nanoparticles. After the column application, the quantities of filtrate and concentrate groups were 5.85×10^8 and 1.88×10^{10} nanoparticles, respectively. As a result of the purification process, the quantities of the final groups were measured as 2.24×10^9 and 9.59×10^9 nanoparticles in SDEVs and HDEVs, respectively. Besides the comparison between groups indicated that the nanoparticle quantities of the concentrate solutions were significantly higher than those of the filtrate group for both plants. Furthermore, in the HDEVs, a significant difference was observed between the concentrate and final groups.

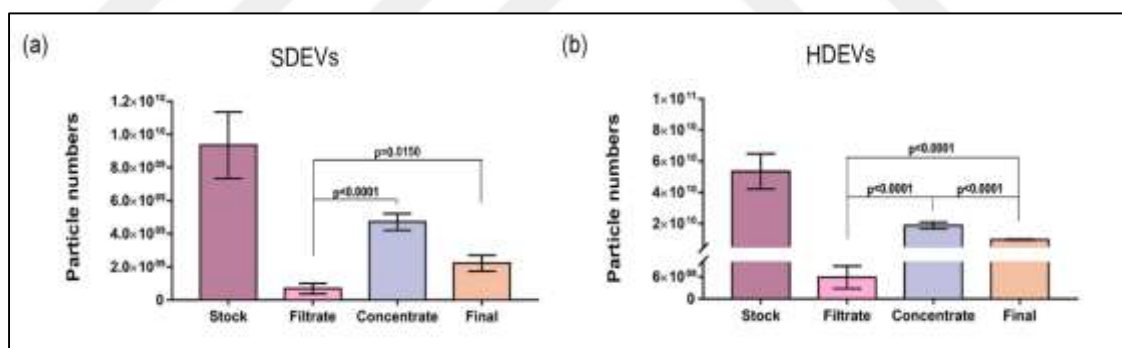


Figure 3.2. The nanoparticle quantity comparison of the PCSEVs. The nanoparticle concentrations of samples collected from the purification steps of the SDEVs (a) and HDEVs (b) were shown in the bar graphs. All the values were represented as means $\pm$ SD.

In the study, the effect of the purification process on the size distribution of PCSEVs was another decisive factor in the vesicle characterization. Therefore, the size distributions of the PCSEVs were compared as shown in Figure 3.3. Firstly, the vesicle size of the filtrate group could not be measured reliably due to the detection of the insufficient number of nanoparticles. The numbers of the nanoparticles were 1.06 and 0.93 particles per frame for SDEVs and HDEVs, respectively (Figure 3.3a and b). Upon examining the other groups, it

was observed that the column application did not affect the vesicle sizes of the PCSEVs for both plants. Furthermore, there were no significant differences between the stock and concentrate groups. Besides, another important parameter in size distribution analysis is the D90 value, which means that 90% of the nanoparticles in the total volume are smaller than the specified nanometer size. The results indicated that the D90 values of the PCSEVs in the final groups were 153 and 145 nanometers, respectively. Therefore, in the final groups, the size distributions of the SDEVs and HDEVs were less than 200 nm in size.

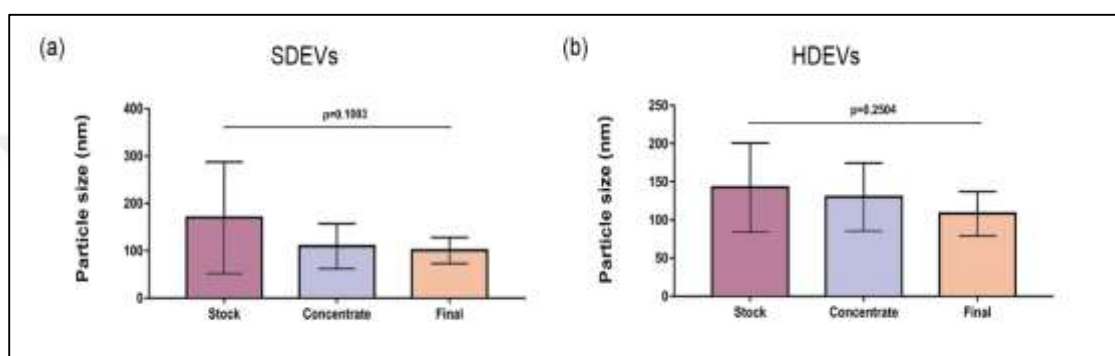


Figure 3.3. The size distribution comparison of the PCSEVs. The size distributions of samples collected from the purification steps of the SDEVs (a) and HDEVs (b) were shown as the bar graphs. All the values were represented as means $\pm$ SD.

In the characterization of PCSEVs, pH is a notable parameter that shows the relationship between the purification process and plant-based residual contaminants. Furthermore, being able to perform the purification of the PCSEVs at a neutral pH is advantageous for the isolation process and vesicle integrity. Thus, the pH measurement was performed with samples collected from the critical steps (Figure 3.4). According to measurement in SDEVs, the pH of the stock solution was 5.87. After the column application, the pH value of the filtrate group was still 5.88, while that of the concentrate group was shifted to a neutral pH of 7.09. The pH value of the final group was 7.08. On the other hand, the results of HDEVs showed a similar tendency to the SDEVs. In the stock solution, the pH value was measured as 5.52. After the ultrafiltration step, the pH values of the filtrate and concentrate groups were 5.96 and 7.05, respectively. Lastly, the pH value was measured as 7.07 in the final group of the HDEVs. Therefore, the results showed that the column application increase significantly the pH values in the concentrate group, while the pH of the filtrate groups remained at similar levels as the stock solutions.

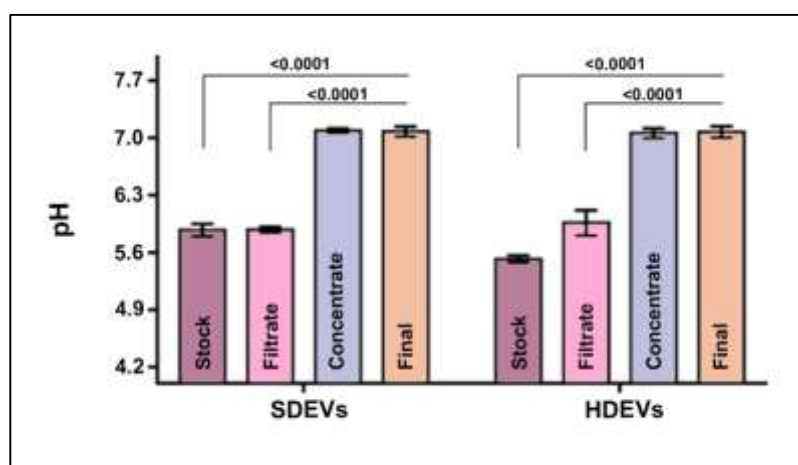


Figure 3.4. The comparison of pH values of the PCSEVs. The pH values of the SDEVs and HDEVs collected from the purification steps were shown as the bar graphs. All the values were represented as means $\pm$ SD.

One of the proposed methods in literature to evaluate the protein contaminants in EV solution is the determination of the nanoparticle/protein ratio. To perform this method, the total protein assay was performed and the nanoparticle quantity per μg protein was calculated as shown in Figure 3.5. In the results, the nanoparticle quantities per 1 μg of SDEVs and HDEVs were calculated as 1.91×10^9 and 5.08×10^8 nanoparticles/ μg . Thus, according to the literature criteria, it was observed that the purity of SDEVs was significantly higher than that of the HDEVs.

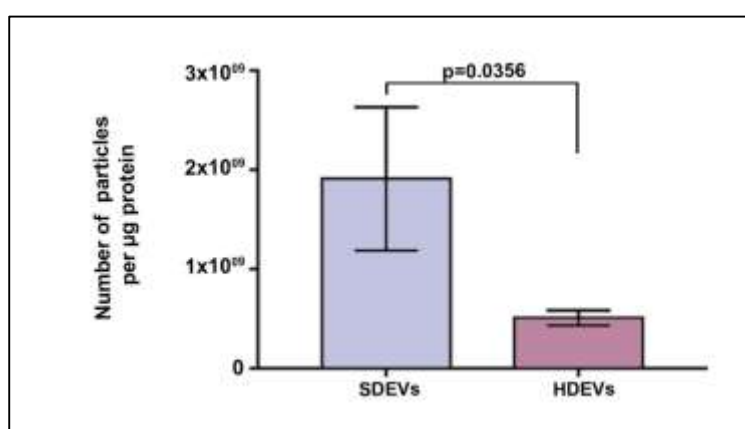


Figure 3.5. The comparison of particle-to-protein ration of the PCSEVs. The purities of the SDEVs and HDEVs were evaluated by calculating the nanoparticle quantity per μg protein just as in the literature. All the values were represented as means $\pm$ SD.

To confirm the size distribution results and examine morphologically the vesicular structures, the PCSEVs in the final group were analysed by atomic force microscopy (AFM) technique. Both wide and close-range images were examined and 3D topography analyses were also performed as shown in Figure 3.6. In the analysis, both SDEVs and HDEVs were detected by AFM imaging (Figure 3.6a and b). The results showed that the PCSEVs have a nearly spherical shape and their sizes are less than 200 nm. Furthermore, the analysis of the surface topography indicated that the SDEVs were smaller and more homogeneously distributed than the HDEVs. Upon examining the wide-range images (far left), it was observed that they were dispersed over the surface rather than aggregated. In the 3D plots, the height of SDEVs was measured as 4 nm at most, while this value increased to 12 nm in HDEVs. Besides, in terms of vesicle sizes, structural data obtained from the AFM analysis were consistent with the size distribution data of the NTA technique.

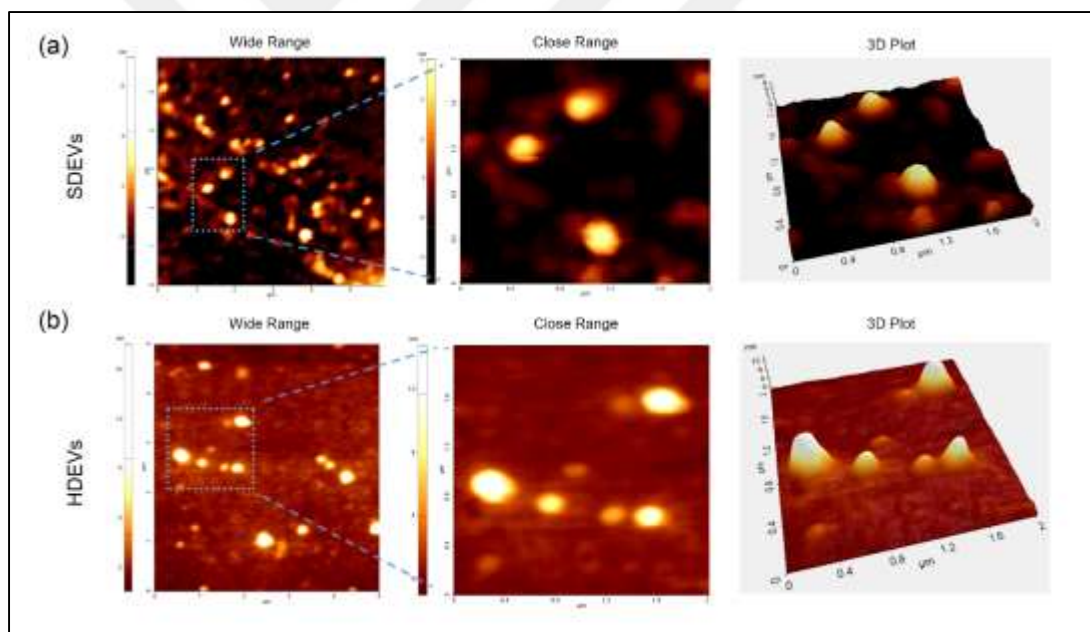


Figure 3.6. The atomic force microscopy analysis of the PCSEVs. While performing microscopic analysis of SDEVs (a) and HDEVs (b), firstly wide-range images were taken (far left) and close-up images (middle) were taken from this wide-range area (far left). On the far right, the 3D representation of close-up images was presented.

3.2. FATTY ACID PROFILING OF THE PCSEVS

The fatty acid composition in EV membranes plays a crucial role in determining membrane stability, fluidity, and intercellular communication. This composition also shapes the

biomolecule carrying capacity and the roles of EVs in the immune response. Therefore, an uncommon yet quite insightful analysis in the characterization of PCSEVs is the fatty acid profiling of these vesicular structures. To profile the fatty acid composition of the PSCs and PCSEVs, the gas chromatograph-mass spectrometry (GC-MS) analysis was performed. In the analysis, the fatty acid molecules could be detected in both *S. rebaudiana* cells and SDEVs as shown in Figure 3.7. According to the results, the fatty acid profile of the *S. rebaudiana* cells was mostly composed of unsaturated fatty acids (UFAs) and the most abundant ones were linoleic acid, palmitic acid, and cis-vaccenic acid (Figure 3.7a). Conversely, it was observed that the saturated fatty acid (SFAs) content increased in the fatty acid composition of SDEVs and the most enriched fatty acids were stearic acid and palmitic acid (Figure 3.7b). Therefore, the results indicated that the amount of SFAs was higher in the fatty acid composition of SDEVs compared to *S. rebaudiana* cells. Besides, both of them had in common specific fatty acids in the vesicle membranes. Linoleic acid was specifically found in the *S. rebaudiana* cells, while it was undetectable in SDEVs, where oleic acid emerged as a new fatty acid.

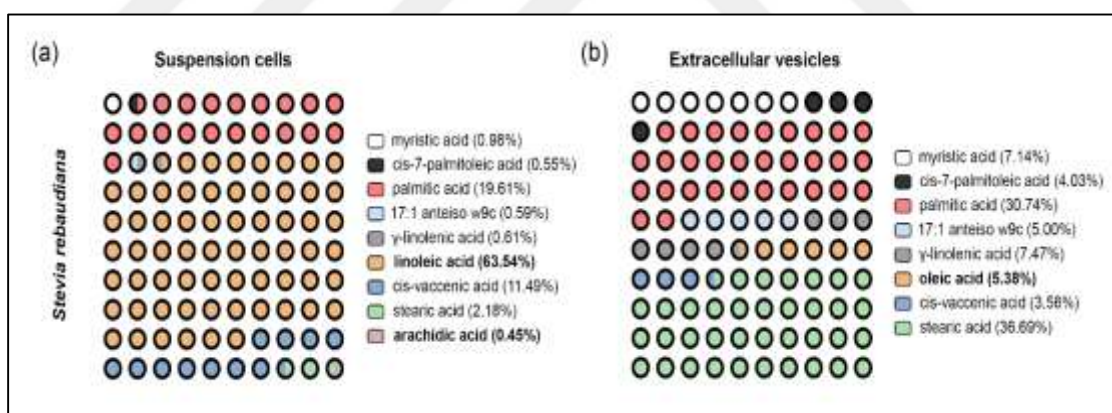


Figure 3.7. The fatty acid composition of the *S. rebaudiana* cells and SDEVs. The fatty acid profile of parental cells (a) and EVs (b) were shown as percentages. Different fatty acids emerging between PSCs and PCSEVs were indicated in bold.

Another prominent finding in the comparison of the fatty acid composition of the *S. rebaudiana* cells and SDEVs was the concentration difference between particular fatty acids. Although some fatty acids are found commonly in PSCs and PCSEVs, the percentage of most of them changed significantly. In the results, whereas the fatty acid percentage of cis-vaccenic acid decreased significantly in the SDEVs, the rest of them as the myristic acid,

palmitoleic acid, palmitic acid, 17:1 anteiso w9c, γ -linolenic acid, and stearic acid increased (Figure 3.8).

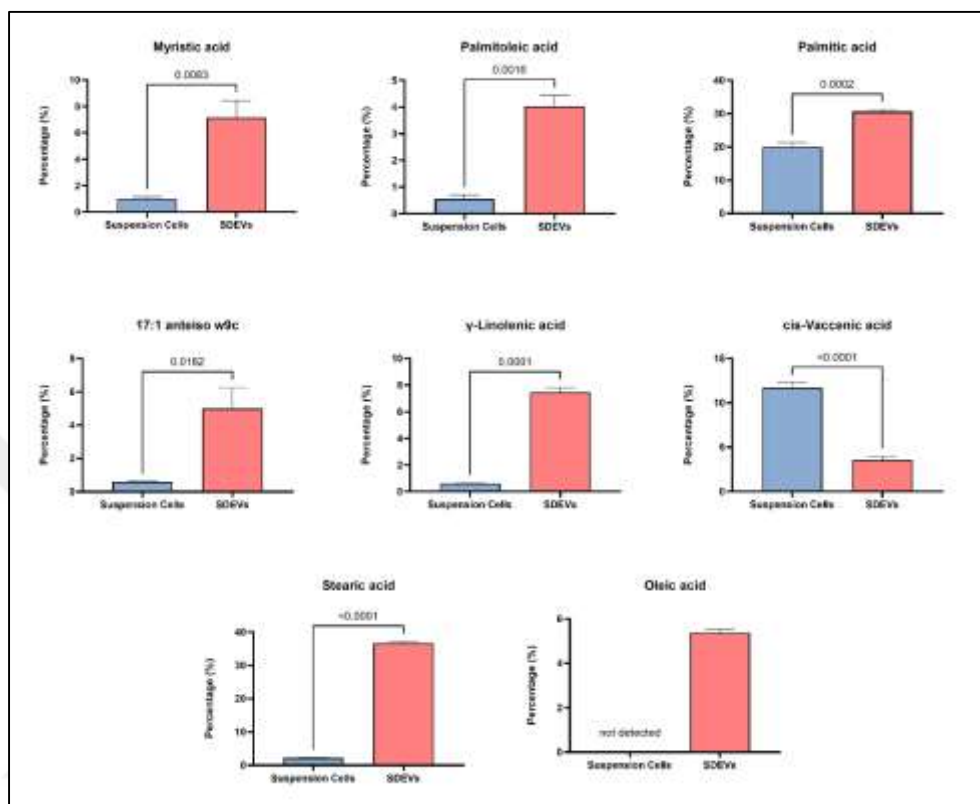


Figure 3.8. The comparison of the fatty acid percentages between the *S. rebaudiana* cells and SDEVs. The fatty acid percentages of the parental cells and SDEVs were shown in the bar graphs. All the values were represented as means $\pm$ SD.

Upon examining the fatty acid composition in *V. hispanica* cells and HDEVs, it was seen that these results also had a similar disposition as shown in Figure 3.9. In the results, the *V. hispanica* cells contained mostly UFAs in their vesicle membranes, consisting of enriched fatty acids such as linoleic acid, cis-vaccenic acid, and palmitic acid (Figure 3.9a). Then again, similar to the SDEVs, the SFA content in the fatty acid profile of the HDEVs increased and the most abundant fatty acids were palmitic acid, stearic acid, and myristic acid (Figure 3.9b). Hence, it was observed that the HDEVs had more SFA molecules than the *V. hispanica* cells. However, it was seen that the *V. hispanica* cells had far more specific fatty acids in comparison with the *S. rebaudiana* cells. Lauric acid, palmitoleyl alcohol, palmitoleic acid, cis-margaroleic acid, linoleic acid, cis-vaccenic acid, and gondoic acid were specifically found in the *V. hispanica* cells, while it could not be detected in HDEVs.

Nevertheless, the 17:1 anteiso w9c fatty acid was found only in the fatty acid profile of the HDEVs. In the analysis, there were noticeable differences in the percentages of particular fatty acids of the *V. hispanica* cells and HDEVs. The results showed that the amounts of all the fatty acids as myristic acid, palmitic acid, 17:1 anteiso w9c, γ -linolenic acid, and stearic acid increased as shown in Figure 3.10.

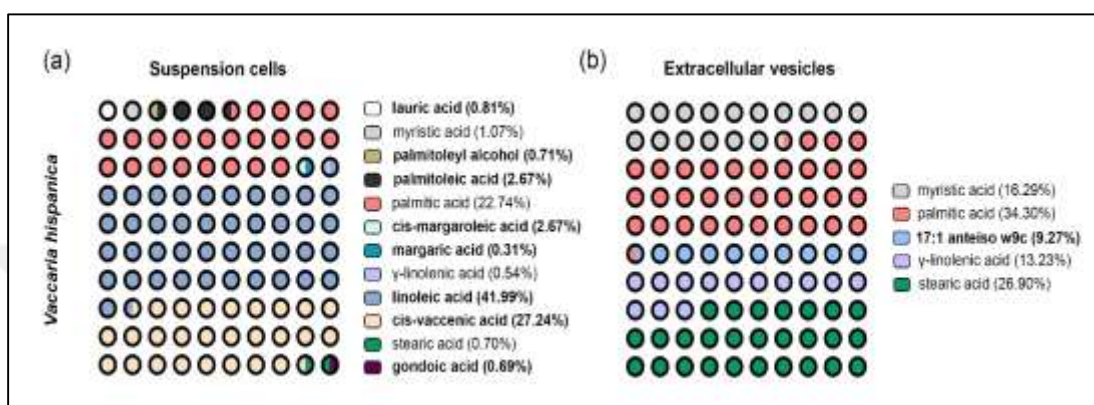


Figure 3.9. The fatty acid composition of the *V. hispanica* cells and HDEVs. The fatty acid profile of parental cells (a) and EVs (b) were shown as percentages. Different fatty acids emerging between PSCs and PCSEVs were indicated in bold.

According to the result of the fatty acid profiling, it was also found that there was a difference between the PSCs and PCSEVs derived from the *S. rebaudiana* and *V. hispanica* plants. Although the same fatty acids were found in both plants, the percentages of the linoleic acid and cis-vaccenic acid vary significantly. Concerning the fatty acid composition of the PCSEVs, more than 50% of the fatty acids in the membranes of both plants consisted of palmitic acid and stearic acid. Moreover, the presence of myristic acid in the PCSEVs increased significantly compared to the cells in both plants. Besides, there were qualitative as well as quantitative differences between the fatty acid composition of the PCSEVs. While oleic acid specifically emerged in SDEVs, 17:1 anteiso w9c fatty acid emerged in HDEVs. Quantitatively, the percentage of myristic acid in HDEVs increased more than 2 times compared to SDEVs. Whereas, the percentage of stearic acid in HDEVs decreased by 10% compared to SDEVs.

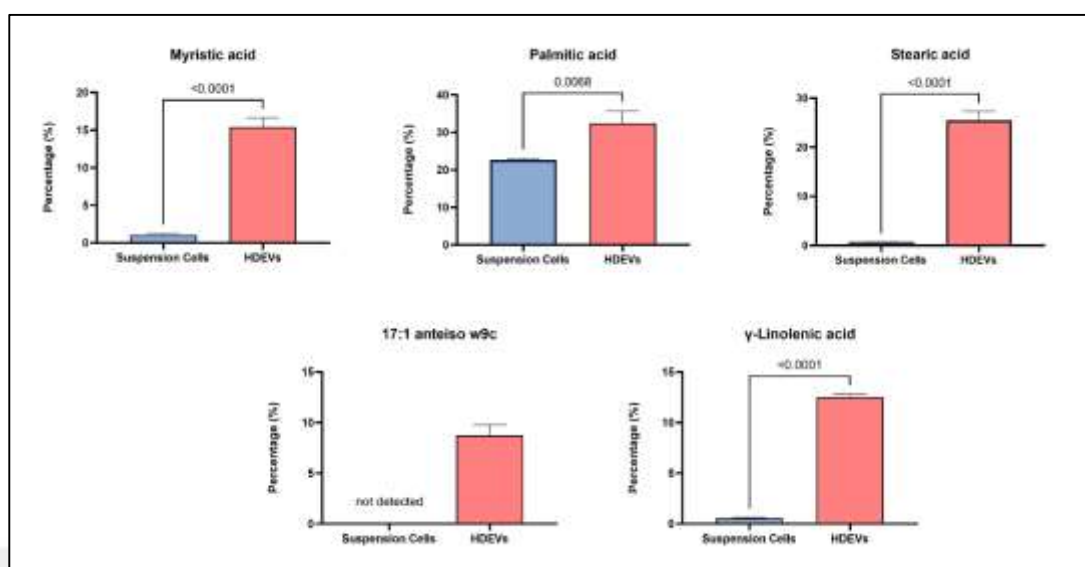


Figure 3.10. The comparison of the fatty acid percentages between the *V. hispanica* cells and HDEVs. The fatty acid percentages of the parental cells and HDEVs were shown in the bar graphs. All the values were represented as means $\pm$ SD.

Comparing the fatty acid compositions of PSCs and PCSEVs in detail, the percentages of some fatty acids found in the plant cells decreased, while others increased noticeably. While the percentage of cis-vaccenic acid, found plentifully in plant cells, decreased to 3.56% in SDEVs, it could not be detected in HDEVs. A similar decline disposition was also observed in the linoleic acid in both plants. However, the percentages of fatty acids found scarcely in both plant cells such as stearic acid, γ -linolenic acid, and 17:1 anteiso w9c increased in the PCSEVs. To analyse the fatty acid composition of PSCs and PCSEVs from a broader perspective, fatty acids were compared by classifying according to the degree of saturation as shown in Figure 3.11. Fatty acids in the cell membrane in both plants consisted of 20% saturated fatty acids (SFAs) and 80% unsaturated fatty acids (UFAs). However, in the PCSEVs, the percentage of SFAs in the vesicle membrane increased to 80%. Besides, there were significant differences in the UFAs between both PSCs. The results showed that MUFA percentages of the *V. hispanica* cells were higher than *S. rebaudiana* cells. In PCSEVs, it was observed that while the percentage of MUFA decreased in HDEVs, it increased in SDEVs. In the other group, although PUFAs in *S. rebaudiana* cells were found at a higher percentage than in *V. hispanica* cells, they were significantly reduced in both PCSEVs.

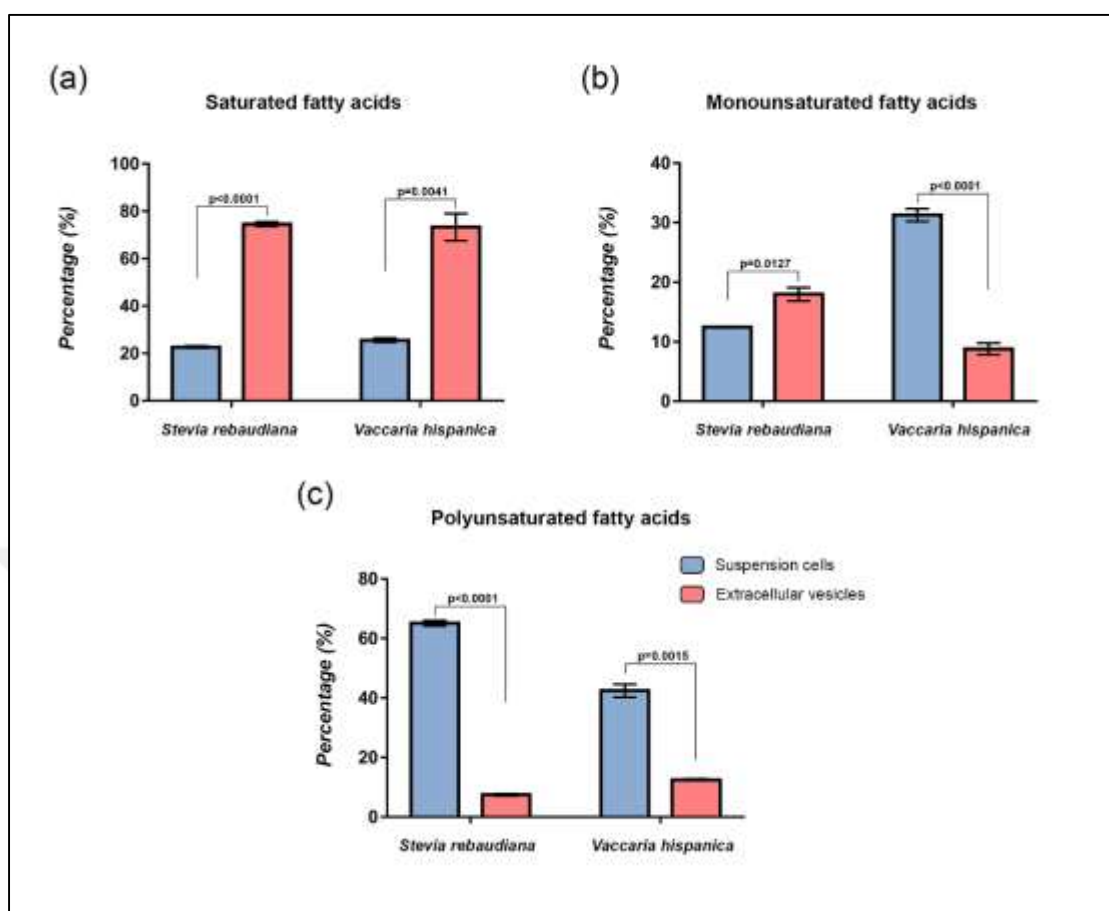


Figure 3.11. The comparison of the fatty acid properties of the PSCs and PCSEVs. The fatty acids emerging in parental cells and EVs were classified and compared according to their degree of saturation/unsaturation (a-c). The percentages of the total saturated and unsaturated fatty acids are shown in the bar graphs. All the values were represented as means $\pm$ SD.

3.3. PROTEOMIC ANALYSIS OF THE PCSEVs

Many proteins carried in EVs play a crucial role in intercellular communication and regulation of various cellular processes. The studying on these proteins and the protein composition of EVs have a great importance in terms of both in the diagnosis of diseases and in discovering the therapeutic effects of the PDEVs. Thus, the proteomic analysis of the purified vesicular structures was essential in the characterization of the PCSEVs. To profile the protein composition of the PSCs and PCSEVs, we performed mass spectrometry and bioinformatics analyses as shown in Figure 3.12 and Figure 3.13. In the experiment, it was investigated whether the detected proteins were enriched in any ontology term by performing

GO enrichment analysis under three gene datasets BP, MF, and CC. In the BP dataset, the results showed that the most enriched GO terms in the protein composition of SDEVs were determined as Arabinan metabolic process, Arabinan catabolic process, Xylan catabolic process, Cell wall polysaccharide catabolic process, and Xylan metabolic process (Figure 3.12a). In the analysis, it was observed that all these GO terms were enriched more than 100-fold. Besides, the important GO terms such as Iron ion homeostasis and Cell wall organization or biogenesis were also determined. In the MF dataset, the most enriched GO terms in the protein composition were determined as Xylan 1,4- β -xylosidase activity, α -L-arabinofuranosidase activity, Xylanase activity, Ferroxidase activity, and Oxidoreductase activity (Figure 3.12b). These GO terms were enriched about 100-fold and more. Furthermore, the quite decisive GO terms were also detected as Peroxidase activity, Antioxidant activity, and Copper ion binding. Lastly, the most enriched GO terms in the CC dataset were Plant-type cell wall, Cell wall, External encapsulating structure, Anchored component of the plasma membrane, and Apoplast, all of which were associated with extracellular characteristics of PDEVs (Figure 3.12c). On the other hand, the important GO terms were also detected in the protein composition of HDEVs as shown in Figure 3.13. In the BP dataset, the results showed that the most enriched GO terms were determined as Hydrogen peroxide catabolic process, Reactive oxygen species metabolic process, Cellular oxidant detoxification, Cellular response to toxic substance, and Detoxification (Figure 3.13a). As a result of the analysis, all these abundant GO terms were enriched more than 30-fold and the decisive GO terms were detected as a Response to oxidative stress and Oxidation-reduction process. The most enriched GO terms in the MF dataset were α -1,4-glucosidase activity, Maltose α -glucosidase activity, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase activity, Peroxidase activity, and Oxidoreductase activity (Figure 3.13b). Furthermore, most of them were enriched more than 100-fold and the important GO terms such as Antioxidant activity, Heme binding, and Oxidoreductase activity. Finally, in the CC dataset, the most enriched GO terms were determined as an Anchored component of membrane, Plant-type cell wall, Anchored component of the plasma membrane, Extracellular region, and Cell wall (Figure 3.13c). Then again, similar to the SDEVs, the enriched GO terms in the CC dataset were related to the extracellular characteristics of PDEVs.

In the GO enrichment analysis of protein composition in PSCs and PCSEVs, enriched GO terms in the CC dataset were shown as heat-maps representation in Figure 3.14. The results showed that the proteins in the PCSEVs were enriched in GO terms such as Plant-type cell wall, Apoplast and Extracellular region in the CC dataset. Upon examining the protein composition of the PSCs, these GO terms were detected in *S. rebaudiana* and *V. hispanica* cells, despite not being enriched just as PCSEVs. On the other hand, it was observed that proteins in PSCs were mostly enriched with GO terms such as Proteasome core complex, Nucleosome, Proteasome complex, DNA packaging complex, and Endopeptidase complex. Vice versa, any of the enriched GO terms in the PSCs could not be detected in the PCSEVs. Therefore, it was found that the GO terms enriched in SDEVs and HDEVs did not demonstrate any commonality with those enriched in PSCs.

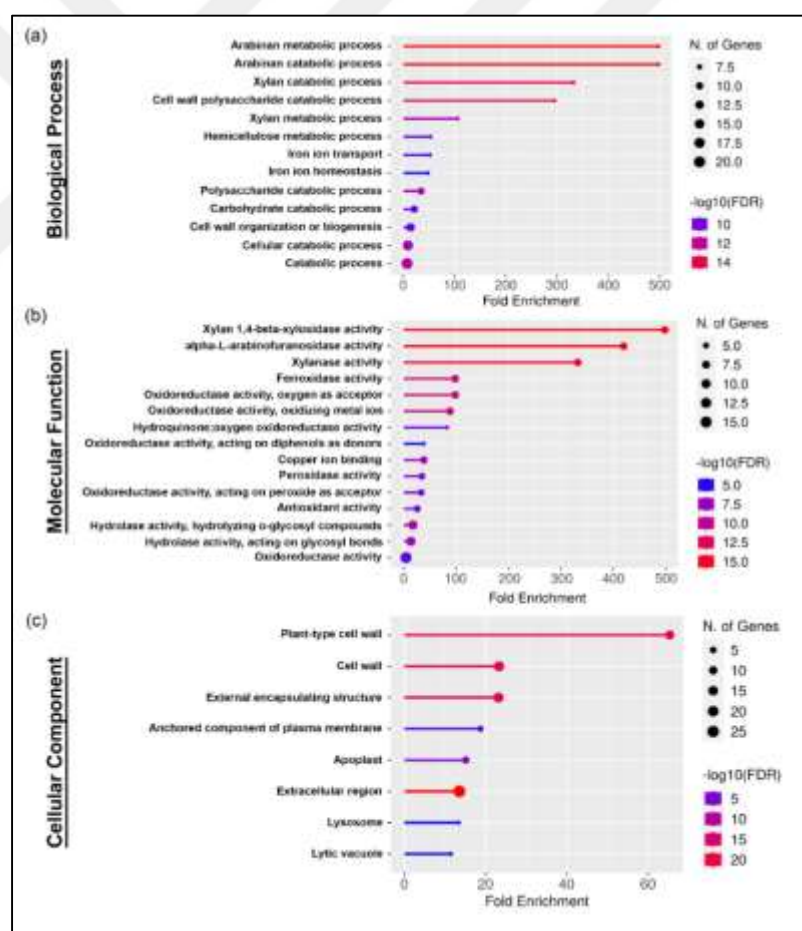


Figure 3.12. The GO enrichment analysis of the SDEVs. Chart view representation of GO-terms in BP (a), MF (b) and CC (c) gene datasets obtained as a result of enrichment analysis of the SDEVs. In chart view, GO-terms were also ordered by strength and colored according to their FDR values.

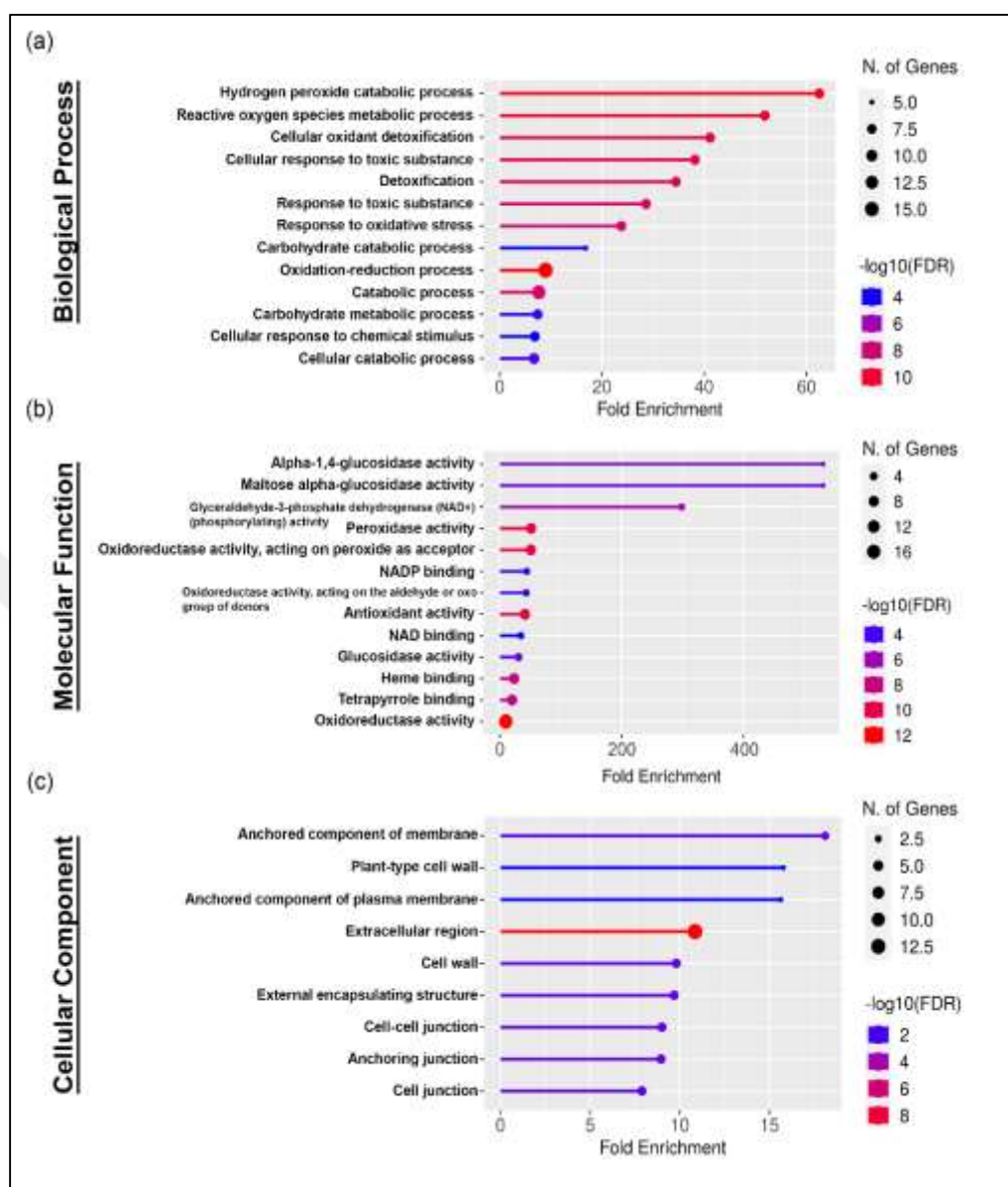


Figure 3.13. The GO enrichment analysis of the HDEVs. Chart view representation of GO-terms in BP (a), MF (b) and CC (c) gene datasets obtained as a result of enrichment analysis of the SDEVs. In chart view, GO-terms were also ordered by strength and colored according to their FDR values.

In the analysis of the proteomic results, investigating the interaction and relationship of GO terms with each other is also critical. Thus, the GO terms in all the gene sets of SDEVs and HDEVs with network analysis were analysed to investigate the interactions of these terms obtained from the enrichment analysis (Figure 3.15). Consequently, the results showed that all the GO terms in the PCSEVs were both associated with extracellular-related terms and highly interacted with each other (Figure 3.15a and b). On the other hand, it was also

investigated which of these proteins detected in proteomic results by Venn analysis were found to be common in the PSCs and PCSEVs, and which were found specifically in SDEVs and HDEVs as shown in Figure 3.16. The results showed that 11 common and 34 specific proteins were detected between SDEVs and *S. rebaudiana* cells (Figure 3.16a). The results showed that the common proteins were mostly associated with the lignin metabolic process and iron ion homeostasis. Nevertheless, the specific proteins in SDEVs were similar to the GO terms in the enrichment analysis since these specific ones comprised 75% of the detected proteins in the SDEVs. In the other group, 8 common and 21 specific proteins were detected between HDEVs and *V. hispanica* cells (Figure 3.16b). In the results, the common proteins were related to the methylglyoxal metabolic process and the ketone catabolic process. Besides, the specific proteins in HDEVs were associated with the antioxidant response of the parental cells. In the analysis, GO terms such as hydrogen peroxide catabolic process and reactive oxygen species metabolic process were detected.

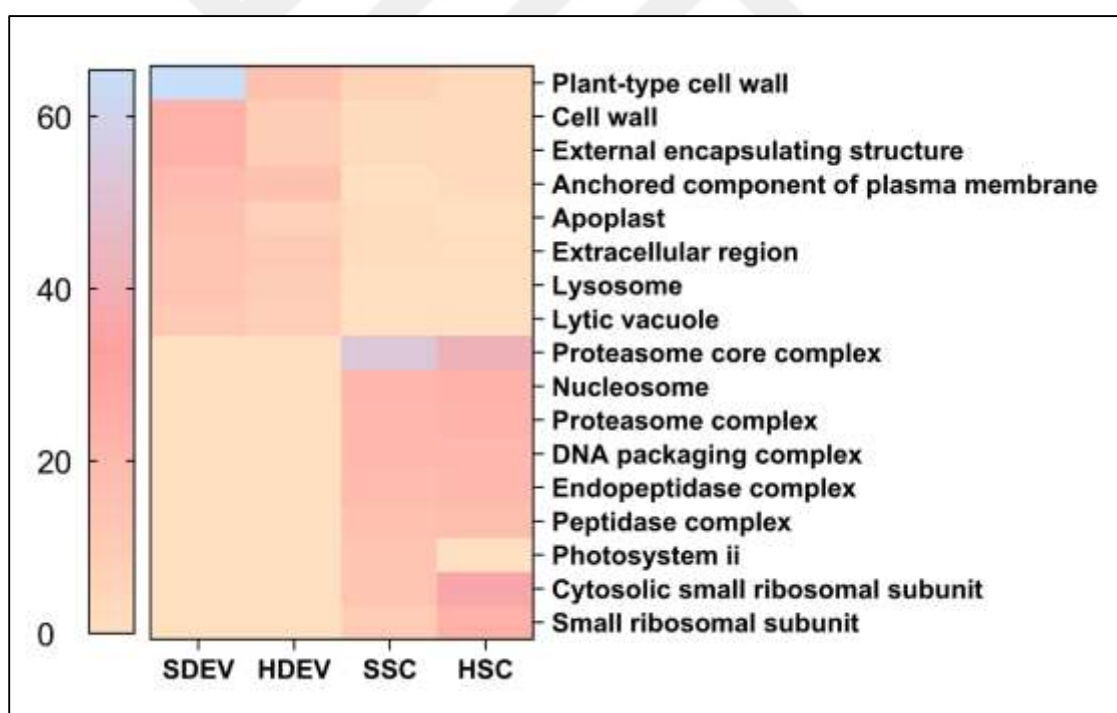


Figure 3.14. The comparison of the GO terms derived from the PSCs and PCSEVs. The enriched GO terms and strengths in the CC gene dataset were represented in the heatmap analysis. The fold of enriched GO terms was shown in color mapping.

(a) Venn diagram showing the overlap of differentially expressed genes between *Stevia rebaudiana* culture cells (orange circle) and SDEVs (green circle). The number of genes unique to culture cells is 475, the number of genes unique to SDEVs is 34, and the number of genes shared between them is 11.

(b) Venn diagram showing the overlap of differentially expressed genes between *Vaccaria hispanica* culture cells (orange circle) and HDEVs (green circle). The number of genes unique to culture cells is 248, the number of genes unique to HDEVs is 21, and the number of genes shared between them is 8.

3.4. FLUORESCENCE LABELING AND UPTAKE ANALYSIS OF PCSEVS

The cellular uptake of PCSEVs is critical for investigating the intercellular communication and biological functions of the vesicular structures. However, in cellular uptake experiments, PCSEVs could not be used because these lipid vesicles do not emit any fluorescent signal in

their natural form. Therefore, the fluorescent labelling of PCSEVs in these experiments allows direct observation of the nanovesicles' entry into the recipient cells. Furthermore, these labelling methods are also important in the sense of causing false-positive results. To monitor the uptake of the PCSEVs by Jurkat cells, the cellular uptake analysis was performed with fluorescently labelled SDEVs and HDEVs. Firstly, the labelling of the PCSEVs with CMFDA dye and removal of free dye was analysed by the fluorescence NTA technique (Figure 3.17). In the results of free dye control, the sample solutions could not be measured and calculated since a sufficient number of nanoparticles was not detected to calculate nanoparticle concentration accurately. Therefore, these samples were compared on the particles per frame (Figure 3.17a). The result showed that very few particles were detected at 5 μ M (8.5 particles) and diluted 500 nM (2.6 particles) concentrations of the free CMFDA dye. After the ultrafiltration of the diluted dye solution, it was observed that the measured nanoparticle number (2.9 particles) did not change significantly. Therefore, all the results indicated that the ultrafiltration process could remove the excessive and non-vesicular dye in the fluorescent labelling of the PCSEVs. Before the fluorescent measurement of CMFDA-labelled vesicles, the possible adverse effects of the dye removal process on the nanoparticle quantities were also investigated (Figure 3.17b and c). In the results of the SDEVs, it was determined that the column treatment only did not cause any significant loss in the nanoparticle concentration. The results of the HDEVs also supported this finding and so their vesicle quantity did not change. On the other hand, to analyse the PCSEVs by fluorescent NTA technique, these vesicles were labelled with CMFDA dye and purified again. These results showed that the nanoparticle concentrations of SDEVs and HDEVs were noticeably reduced compared to the results obtained with the 488 nm laser module.

After the validation of fluorescence labelling of the PCSEVs, the cellular uptake assay was performed by flow cytometry analysis (Figure 3.18). After the analysis, the results showed that all the cells were stained with CMTPX dye so there was no cell death in the Jurkat cells across the groups. Since some fluorescent dyes used to label the EVs could cause the fluorescence spillover, the singlet control was added to the flow cytometry analysis. In the result of singlet control, it was observed that there was no green fluorescent signal that brought about the spillover spread of the CMTPX dye. Besides, the dye removal process needed to be controlled to prevent the emerging false-positive fluorescent signal caused by the excessive non-vesicular fluorescent dye. In the analysis, the staining solution, which did

not contain any PCSEVs, was prepared with nuclease-free water and incubated. Subsequently, the dye removal process was applied as if this solution had the PCSEVs. The obtained solution at the end is named as dye control solution. The results showed that there was a negligible increase in the fluorescent signal of the dye control group. Consequently, these results indicated that neither the spillover spread of the CMTPIX dye nor the excessive non-vesicular dye brought about the false-positive signal that did not originate from the PCSEVs. In the cellular uptake analysis of PCSEVs, the results demonstrated that PCSEVs were successfully internalized by CMTPIX-labelled Jurkat cells after hours of incubation. Moreover, the uptake rate of PCSEVs by the cells exceeded 90%.

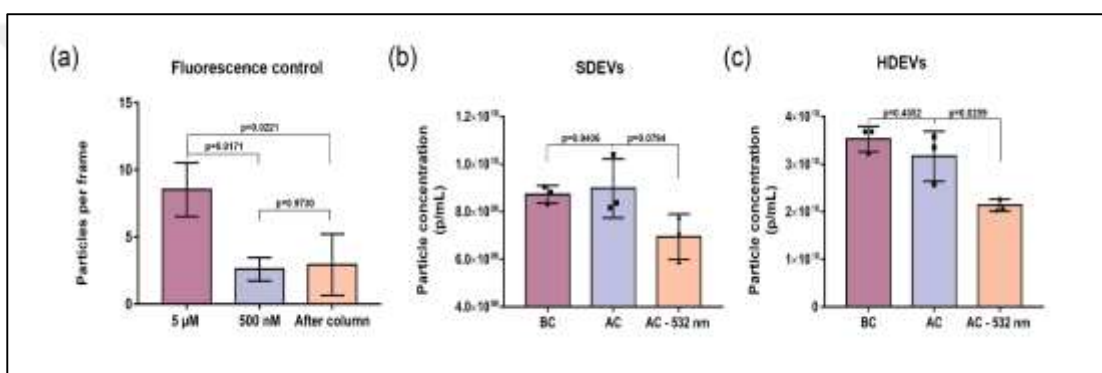


Figure 3.17. The fluorescence labelling validation of the PCSEVs. CMFDA-labeled PCSEVs were analyzed and compared by the fluorescent NTA technique. Control experiments for the fluorescent labelling process were shown (a). Concerning the effects of the ultrafiltration process, fluorescence NTA results were also presented for the SDEVs (b) and HDEVs (c). Besides, in the graphs, BC stood for “before column treatment” and AC stood for “after column treatment”.

On the other hand, the relationship between the different doses of PCSEVs in terms of cellular uptake analysis was investigated (Figure 3.19). Accordingly, the cellular uptake assay was performed using two different doses of the HDEVs, low dose (6.86×10^8 particles) and high dose (3.43×10^9 particles). Rather like the uptake results of PCSEVs, any increases in the green fluorescent signal were not observed in the singlet control and dye control groups. Nevertheless, in the uptake result of different doses, the low-dose HDEVs were taken up by low-dose HDEVs were taken up by 74% of the cells and in high-dose treatment, over 90% of HDEVs were taken up into cells. Therefore, it was found that there was a dose-dependent relationship in the cellular uptake of PCSEVs by Jurkat cells.

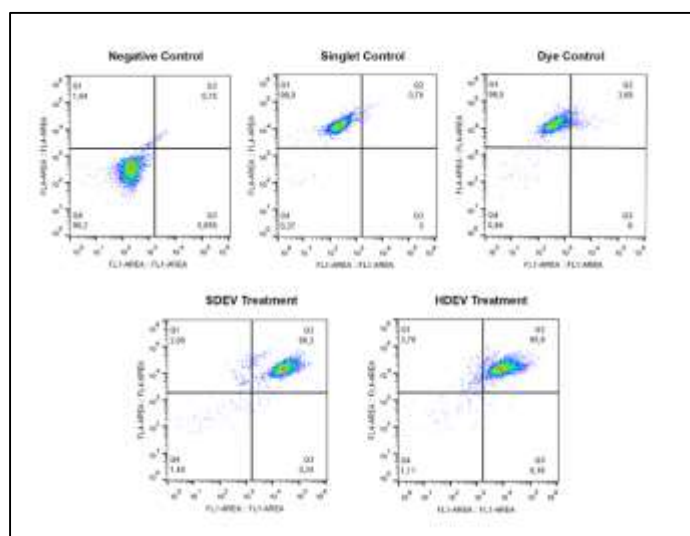


Figure 3.18. The cellular uptake analysis of the fluorescent-labelled PCSEVs. The internalization of the SDEVs and HDEVs by Jurkat cells was analysed by flow cytometry technique after 2 hours of incubation. Cellular uptake results of HDEVs and SDEVs were shown as multivariate density plots.

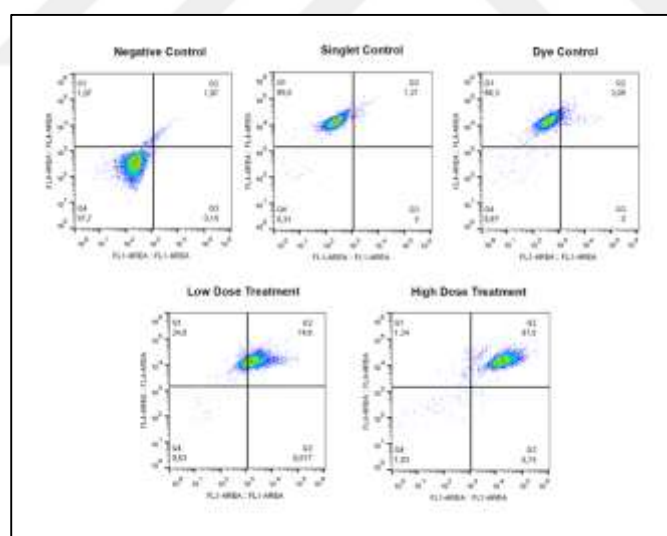


Figure 3.19. The cellular uptake analysis of different doses of the HDEVs. The internalization of the different doses of HDEVs by Jurkat cells was analysed by flow cytometry technique after 2 hours of incubation. Cellular uptake results of low-dose and high-dose groups were shown as multivariate density plots.

3.5. MONOLAYER CELL CULTURE MODEL OF HBECs

In vitro studies related to endothelial cells have great importance in the sense of investigating the barrier functions of brain endothelial cells. One of the common models used in literature is the monolayer culture model. Especially for brain endothelial cells, the model plays a critical role in simulating the structural and functional characteristics of the BBB. However, since the full confluence of the cells *in vitro* could lead to cell death, the monolayer culture model needed to be controlled before starting the CVI-induction experiments. To investigate whether there was a cytotoxic effect caused by high cell density in the modified CVI model in HBECs, the viability of the monolayer culture was determined by cell staining with fluorescence tracker dyes (Figure 3.20).

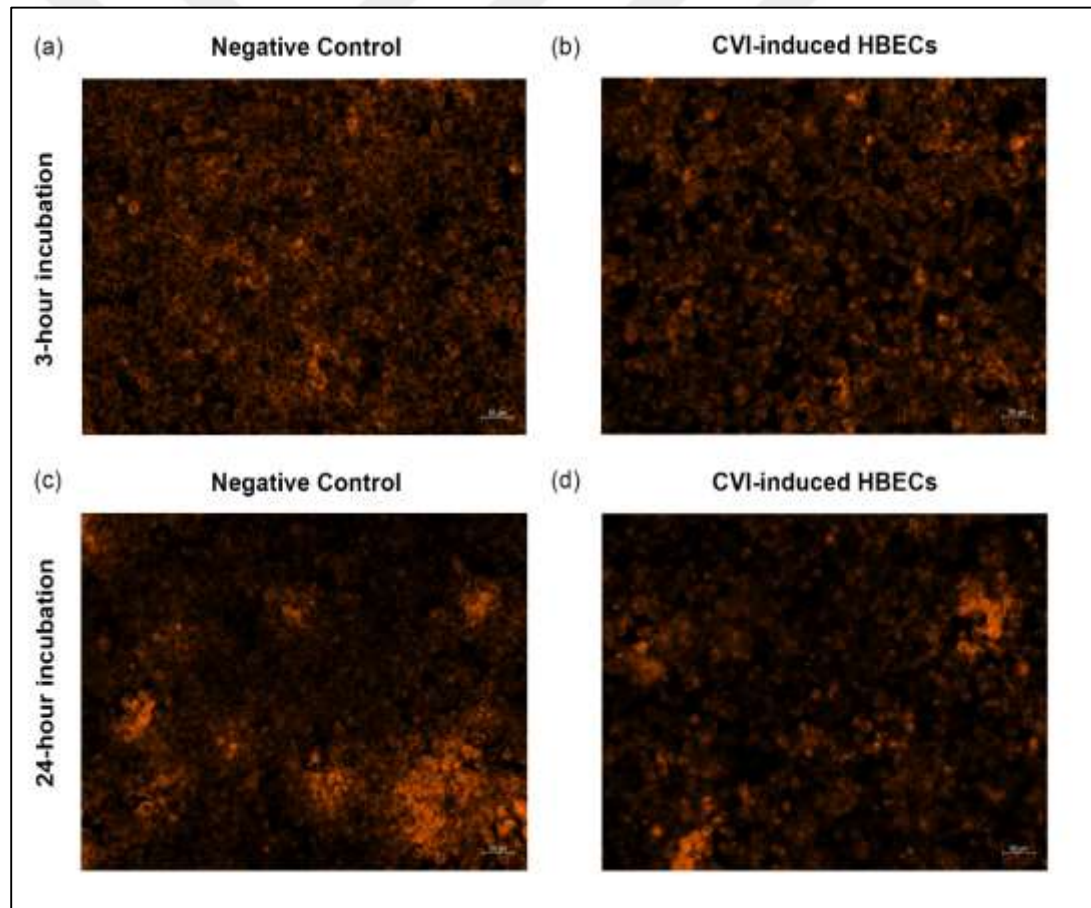


Figure 3.20. The effects of monolayer cell culture model on CVI-induced HBECs. Morphological and cell viability analysis of the cells using CMTPX fluorescence staining was performed at the 3rd (a) and 24th (c) hours. The effect of CVI induction on the same parameters was visualized with a fluorescent microscope monitoring at the 3rd (b) and 24th (d) hours. Scale bar: 50 μ m.

In the results, it was observed that there was no abnormality in the cell viability in the 3rd hour after the formation of the monolayer culture (Figure 3.20a and b). On the 24th hour of the CVI induction, the results showed that the cell viability of cells did not change (Figure 3.20c and d). However, it was found that the CVI induction in the HBECs reduced the intercellular adhesion structures between cells and caused particular gaps in the monolayer culture at 3rd hour. When the CVI induction was extended to 24 hours, these effects did not worsen. Moreover, they remained similar to the results in the 3-hour treatment.

3.6. CELL VIABILITY ANALYSES OF CVI-INDUCED HBECs

The cell viability of HBECs plays a crucial role in the monolayer culture model of CVI pathology. In the monolayer culture model, the viability of cells was controlled by staining with fluorescence cell tracker dye. However, it seems that the metabolism-based viability assay is more convenient for determining the early-stage cell damage. Therefore, the cytotoxic effect of PMA/Ionomycin treatment was evaluated with the MTS assay to model *in vitro* CVI pathology in the HBECs as shown in Figure 3.21. In the results, it was observed that the CVI induction did not demonstrate any cytotoxic effects on the HBECs (Figure 3.21a). On the contrary, the absorbance value of the CVI group significantly increased. Nevertheless, evaluating the cell proliferation by the MTS assay could not be accurate since the metabolism-based assays do not measure specifically cell proliferation.

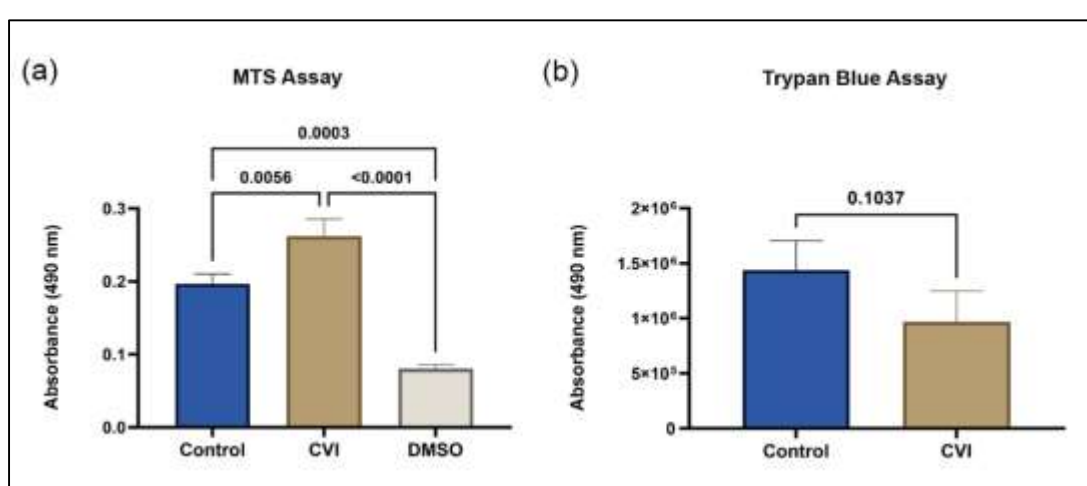


Figure 3.21. The effects of CVI induction on cell viability of HBECs. The effects of PMA/Ionomycin treatment on cell viability were analysed with MTS (a) and Trypan Blue Exclusion (b) assay at the 24th hour. All values were represented as means $\pm$ SD.

To confirm the viability results, these effects of CVI induction were also analysed by a trypan blue exclusion method (Figure 3.21b). However, the results showed that the CVI induction, unlike the results of the MTS assay, did not cause a significant increase in the proliferation rate of HBECs, and even caused a slight decrease in cell proliferation. Thus, it was found that the CVI induction did not have any cytotoxic and proliferative effects on the HBECs despite the significant increase in absorbance values in the MTS assay.

3.7. MITOCHONDRIAL MEMBRANE POTENTIAL ANALYSIS

During the pathogenesis of CVI, cellular energy demand increases and mitochondria can be a source of oxidative stress in the process. This stress can lead to increased production of oxidative stress further exacerbating the inflammatory response. Mitochondrial dysfunction may contribute to the development of cerebrovascular disorders by disrupting the endothelial cell barrier and increasing vascular permeability. Besides, fluorescent dyes that measure mitochondrial potential, such as Rhodamine 123, are widely used to assess mitochondrial function. Therefore, the analysis of mitochondrial potential in the inflammation model was performed with the Rhodamine 123 staining to understand how CVI induction affected the mitochondrial function as shown in Figure 3.22. According to the results, it was observed that the CVI induction had negative effects on the mitochondrial membrane potential (Figure 3.22a and b). In the analysis, the results of Hoechst staining allowed the clear visualisation of cell nuclei in both groups. In the Rhodamine 123 staining, the green fluorescence signal was observed more intensely in the negative control group, while it was significantly reduced in the CVI induction group. In the quantification analysis, the result showed that the fluorescence intensity of Rhodamine 123 was higher in the control group, while it was significantly decreased in the CVI induction group. Besides these effects on the mitochondrial function, it appeared that the CVI induction also caused the morphological changes in HBECs. Consequently, all results indicated that CVI induction affected negatively the mitochondrial membrane potential and its functions.

3.8. INTRACELLULAR CALCIUM ANALYSIS OF CVI-INDUCED HBECs

In the regulation of critical signalling pathways in endothelial cells, intracellular Ca^{+2} levels are one of the key molecules. During cerebrovascular disorders, elevated Ca^{+2} levels cause the activation of many intracellular signalling pathways. In particular, it leads to the type I

activation of CECs and has an aggravating role in the type II endothelial activation. Furthermore, these processes bring about increased permeability, impaired cerebrovascular integrity, and progression of cerebrovascular pathology. Consequently, the elevated intracellular Ca^{+2} levels stand out as a critical marker for the CVI model.

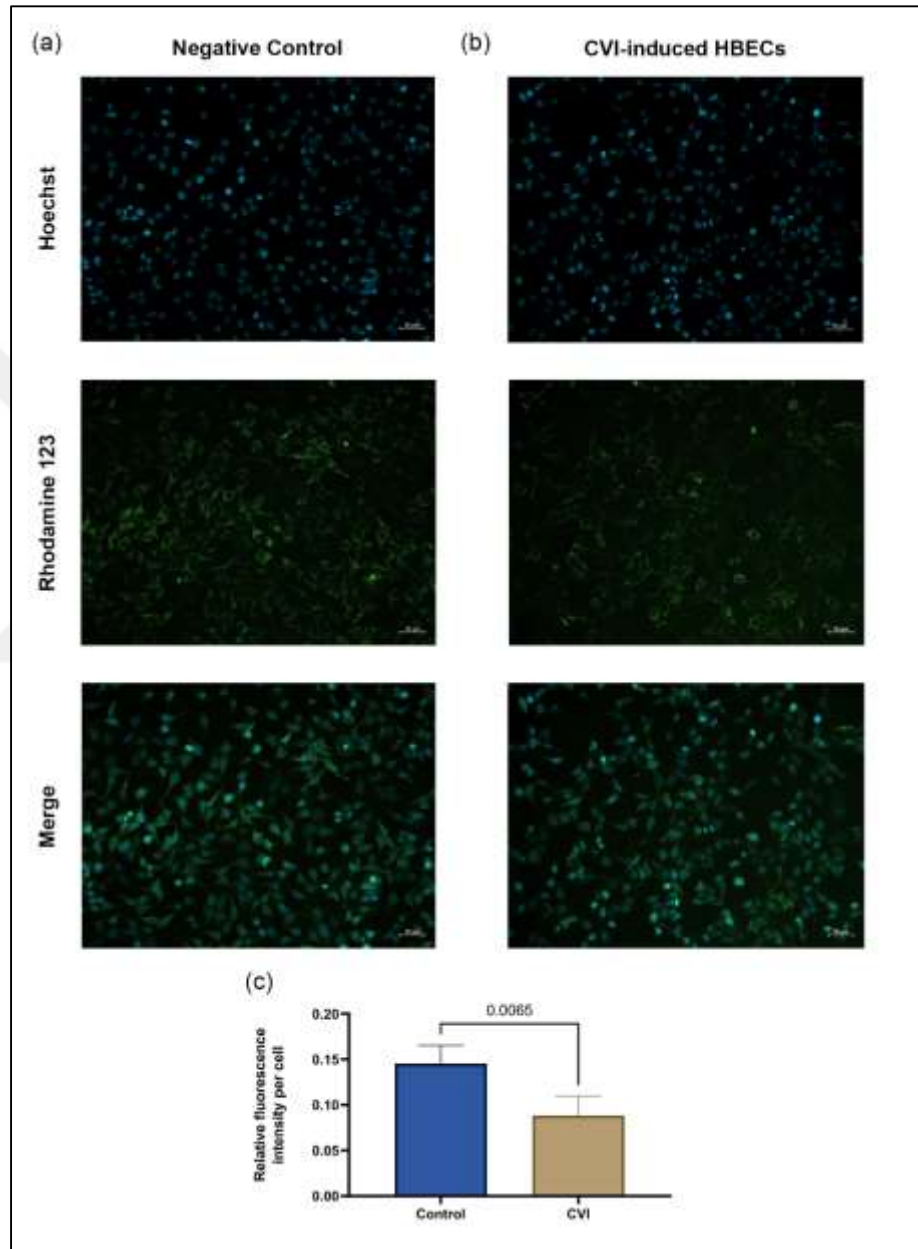


Figure 3.22. The effects of CVI induction on mitochondrial membrane potential of HBECs. In the experiment, the HBECs were stained with Rhodamine 123/Hoechst dye mixture. The fluorescence images between groups (a, b) and intensity analysis (c) were demonstrated after the 24th hour of CVI induction. Scale bar: 50 μm . Relative fluorescence unit values were represented as means $\pm$ SD.

To investigate these effects on CVI-induced HBECs, the intracellular Ca^{+2} levels were measured by Fluo-4 AM staining after the PMA/Ionomycin treatment as shown in Figure 3.23. According to the results, it was found that the CVI induction increased the intracellular Ca^{+2} levels in the monolayer culture of HBECs (Figure 3.23a and b). In the analysis, the results of Hoechst staining allowed the clear visualisation of cell nuclei in both groups. Upon examining the fluorescence signals of Fluo-4 AM dye, it was observed that the green fluorescence intensity was more elevated in the CVI-induced HBECs in comparison with the negative control group. The quantification analysis in Figure 3.23c demonstrated that Fluo-4 AM fluorescence intensity was significantly higher in the CVI induction compared to the control group. Furthermore, the intracellular Ca^{+2} levels based on fluorescent signals of the Fluo-4 AM dye increased 3.6-fold in the CVI-induced HBECs. Thus, the results indicated that the intracellular Ca^{+2} concentration was significantly elevated as a result of CVI induction at the 3rd hour of treatment.

3.9. DETERMINATION OF THE CVI INDUCTION DOSE IN HBECs

In the CVI modelling *in vitro*, HBECs were treated with various doses of PMA/Ionomycin. Therefore, it is of great importance to determine the appropriate dose for CVI induction. To determine the suitable dose of induction, the effects of PMA/Ionomycin at various doses on the CVI-related marker genes were determined by gene expression analysis as shown in Figure 3.24. The CVI model in HBECs was induced with three different doses using PMA/Ionomycin as 40 nM/750 nM (Dose-1), 16 nM/250 nM (Dose-2), and 16 nM/125 nM (Dose-3). In the experiments, it was observed that all three doses of PMA/Ionomycin treatment upregulated the IL8 expression, indicating that the CVI was successfully induced (Figure 3.24a). The results showed that the Dose-1 treatment upregulated the expression of the IL8 gene by 10.92-fold compared to the control group. Furthermore, Dose-1 also provided more elevated gene upregulation by 8.73- and 7.35-fold compared to Dose-2 and Dose-3, respectively (Figure 3.24b). Then again, in the relative rt-PCR results, similar findings about the expression of the IL8 gene were found. The results demonstrated that the IL8 gene was significantly upregulated by 130.91-, 103.81-, and 74.14-fold at the Dose-1, Dose-2, and Dose-3 groups, respectively (Figure 3.24c). Based on these findings, Dose-3 was decided as the most appropriate dose for the CVI induction in further experiments, as it showed the most pronounced effects at the lowest concentration.

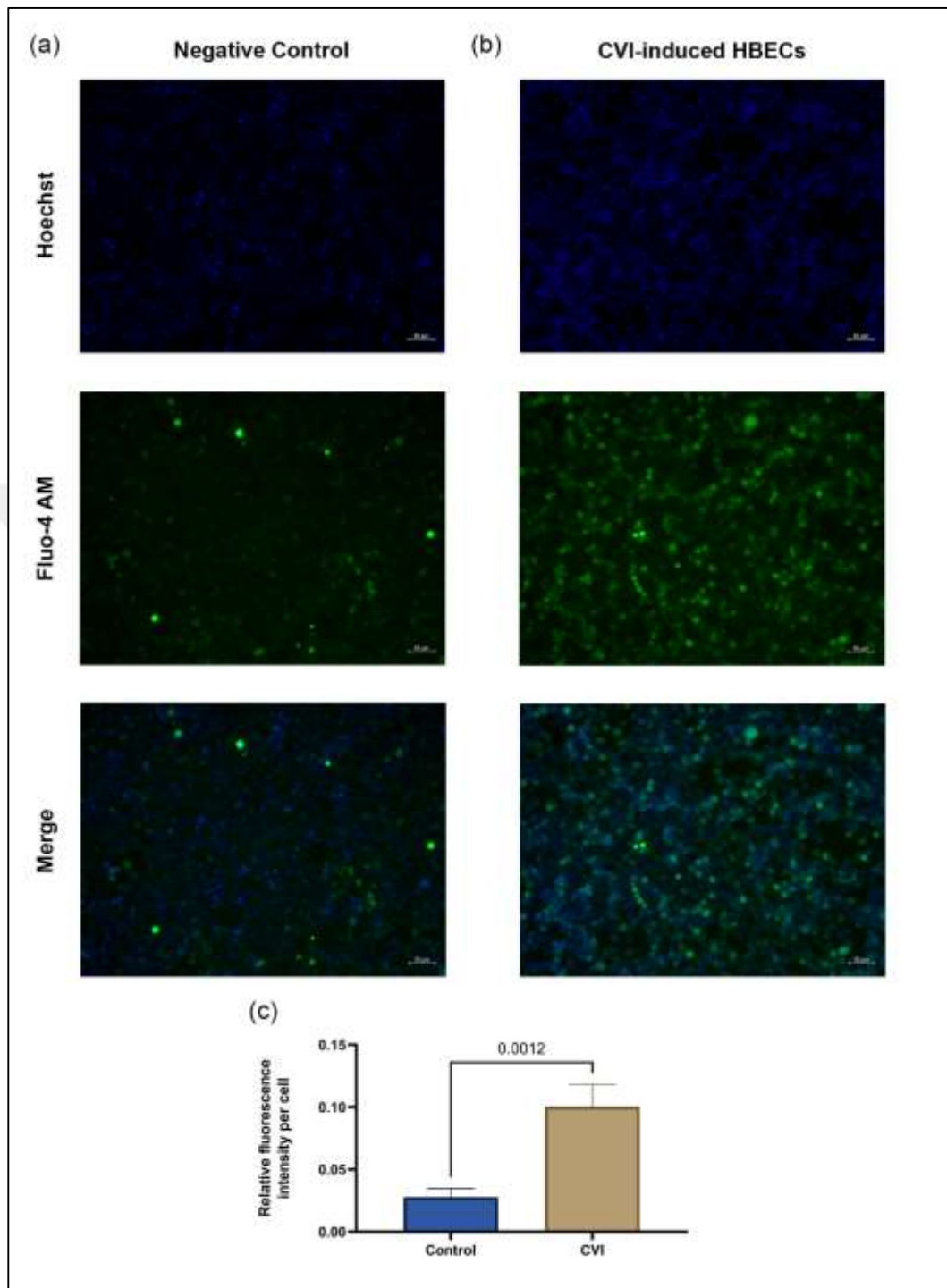


Figure 3.23. The effects of CVI induction on the intracellular Ca^{+2} levels of HBECs. The fluorescence intensity-based Ca^{+2} levels measurement analysis was performed. The fluorescence images between groups (a, b) and intensity analysis (c) were demonstrated after 3rd hour of CVI induction. Scale bar: 50 µm. Relative fluorescence unit values were represented as means $\pm$ SD.

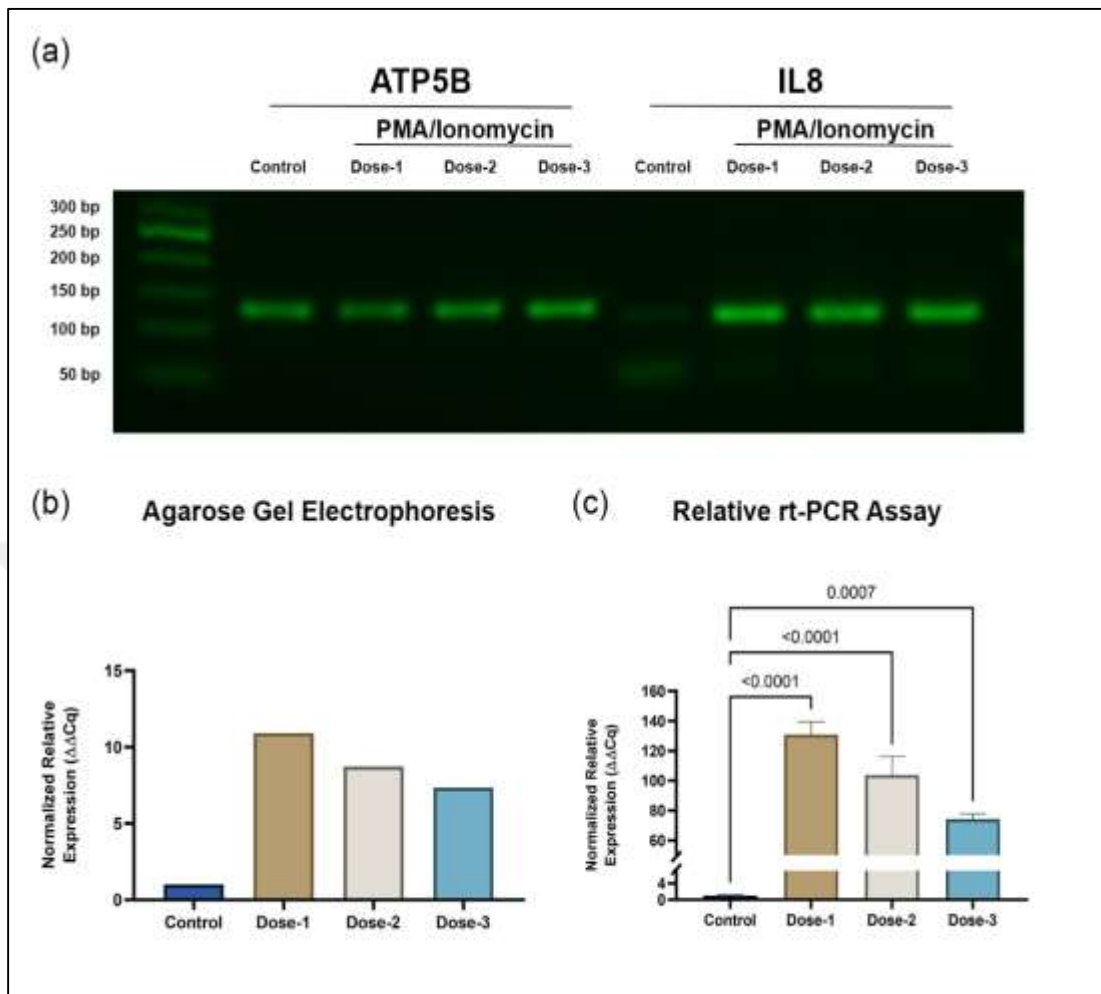


Figure 3.24. The effects of various doses of PMA/Ionomycin on the CVI-related marker gene. The agarose gel electrophoresis image (a) and densitometric analysis (b) were represented. In addition, the normalized expression of the IL8 gene was analyzed by relative rt-PCR technique (c). In the analysis, the results of gene expression were normalized using ATP5F1B as a reference gene. Fold change values were represented as means $\pm$ SEM.

3.10. GENE EXPRESSION ANALYSIS OF CVI-INDUCED HBECS

One of the cellular reactions that CECs elicit from the inflammatory state of cells is the changes in their gene expression profiles. These changes in endothelial cells under the CVI model have critical importance for evaluating inflammatory processes at the molecular level. Therefore, the gene expression analysis provides a valuable guide in determining the stage and severity of the inflammatory response. In the previous experiment, the suitable dose of CVI induction had been determined. However, the effectiveness of the selected dose on other

genes associated with the CVI model needed to be investigated. In the study, the relative rt-PCR analysis was performed to understand whether PMA/Ionomycin treatment changes the gene expressions of HBECs characteristically. According to the results, the CVI induction changed the gene expressions of HBECs related to CVI pathology. Upon examining the heatmap analysis, it was observed that there was an upregulation in the expression of many genes associated with the CVI pathology as shown in Figure 3.25. The results showed that the expression of IL8, MCP1, and PAI1 genes, except the IL6 gene, increased by 24.43-, 3.84-, and 10.13-fold, respectively (Figure 3.26). Besides, in the anti-inflammatory genes, the expression of SIRT1 and NRF2 genes was significantly upregulated, while the expression of the PPARG gene just increased by 1.26-fold (Figure 3.27). In the proliferation- and apoptosis-related genes, the results showed that there was no significant change in the KI67, BCL2, BAX, and AKT genes (Figure 3.28). On the other hand, in the cellular adhesion genes, there was an upregulation by 4.29- and 2.84-fold in the expression of VCAM1 and ELAM1 genes, while the expression of the ICAM1 gene did not significantly change as shown in Figure 3.27. Examining the genes related to angiogenesis, it was found that the VEGFA gene showed an upregulation in expression, whereas the expression levels of PECAM, VWF, KDR, and ANG2 remained unchanged (Figure 3.29). In the genes associated with tight junctions and permeability characteristics of CECs, while any changes were not observed in the expression of CLDN1 and OCLN genes, the expression of the ZO1 gene was significantly downregulated. Besides, the expression of the MMP3 gene significantly increased by 13.47-fold, whereas the MMP gene demonstrated only a modest rise of 1.67-fold. Consequently, it was found that the CVI induction with a selected dose of PMA/Ionomycin treatment could change the gene expression profile of HBECs following cerebral endothelial activation.

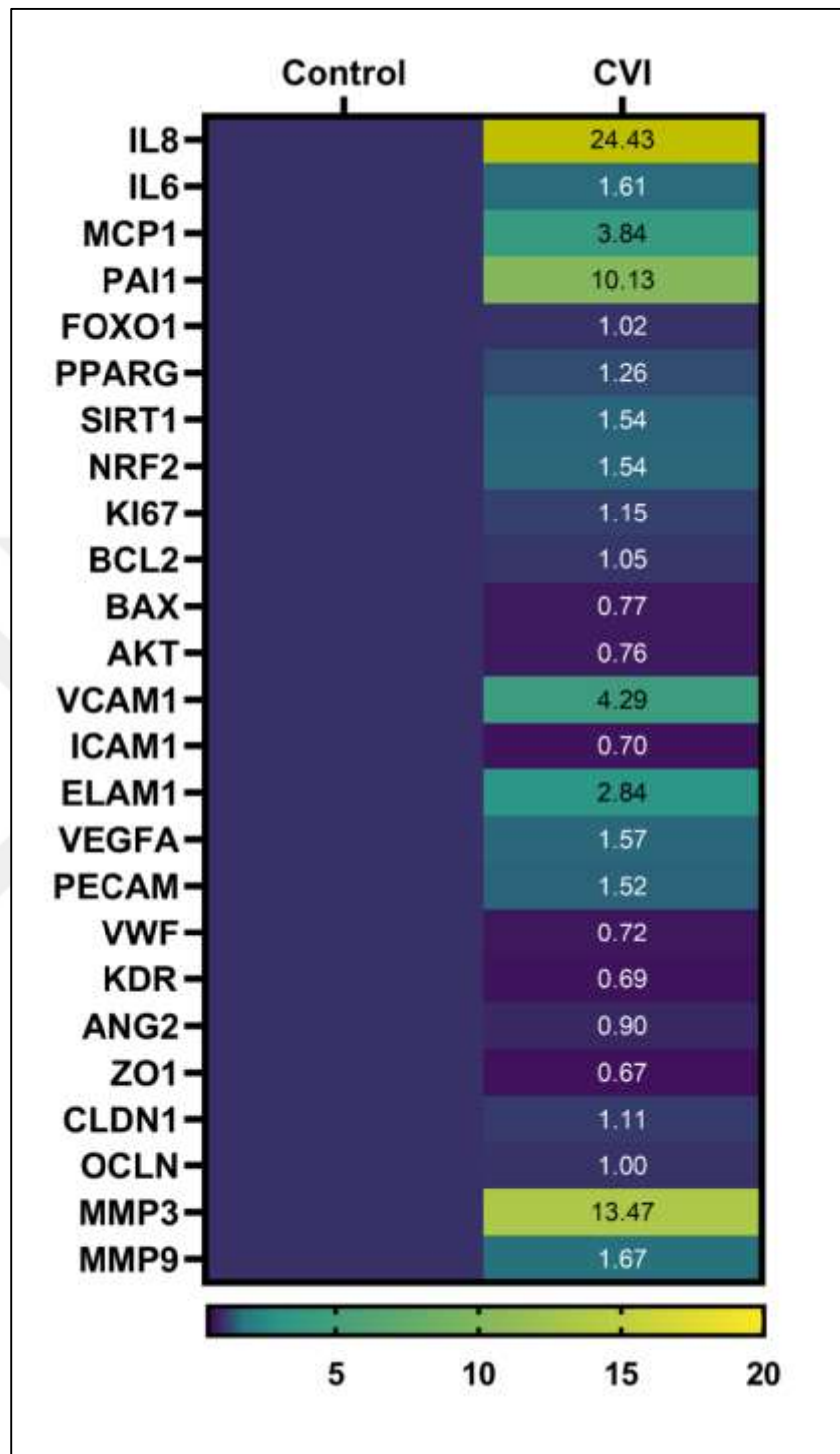


Figure 3.25. The effects of CVI induction on gene expression profile of HBECs. Relative normalized expression of CVI-related marker genes was analyzed by relative rt-PCR analysis. The expression of these genes was represented in the heatmap analysis. Fold change values were represented as means.

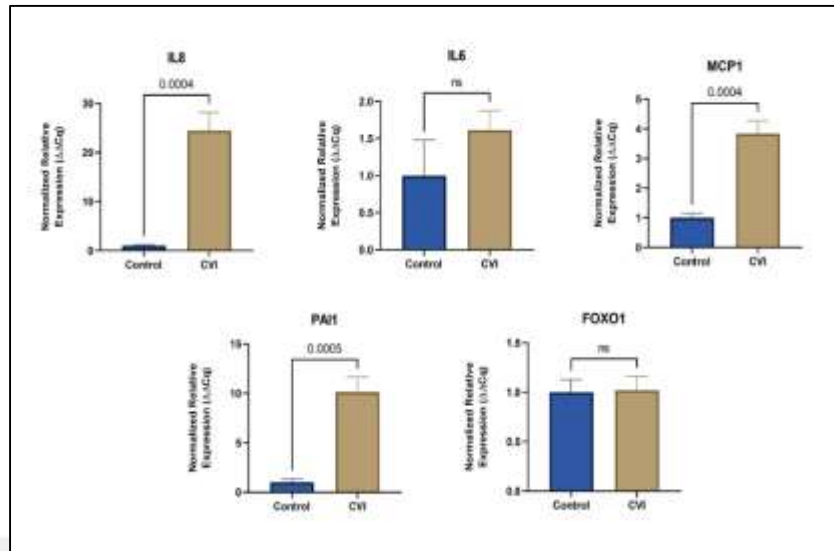


Figure 3.26. The effects of CVI induction on the expression of inflammation-related genes of HBECS. Relative normalized expression of CVI-related marker genes was analyzed by relative rt-PCR analysis. In the analysis, ATP5F1B was used as a reference gene for normalization. Fold change values were represented as means $\pm$ SEM.

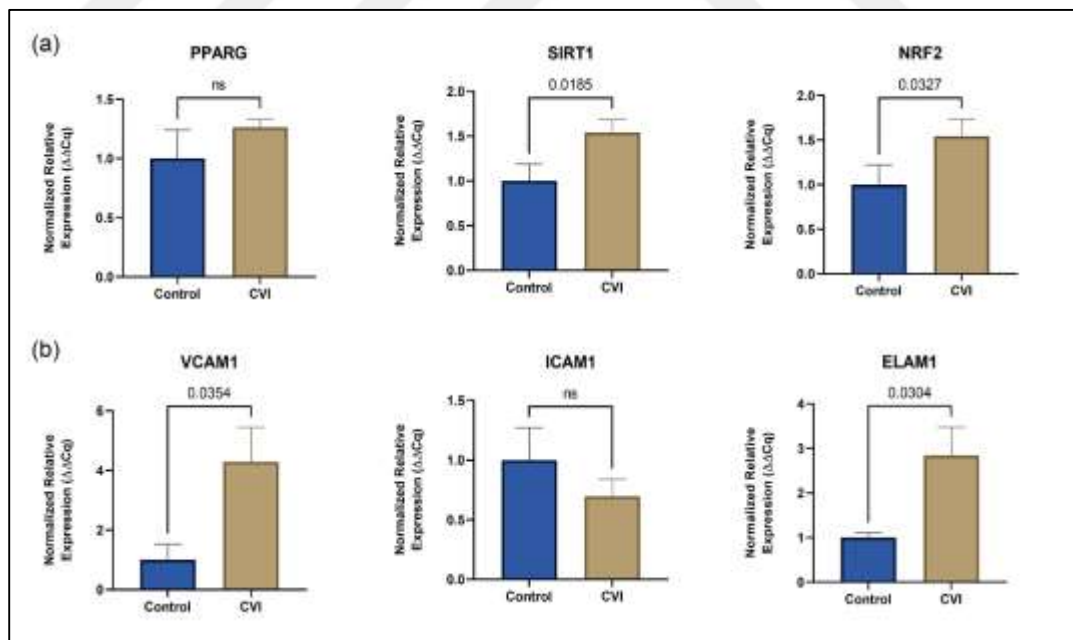


Figure 3.27. The effects of CVI induction on the expression of anti-inflammatory and cellular adhesion-related genes of HBECS. Relative normalized expression of anti-inflammatory (a) and cellular adhesion-related (b) genes were analyzed by relative rt-PCR analysis. In the analysis, ATP5F1B was used as a reference gene for normalization. Fold change values were represented as means $\pm$ SEM.

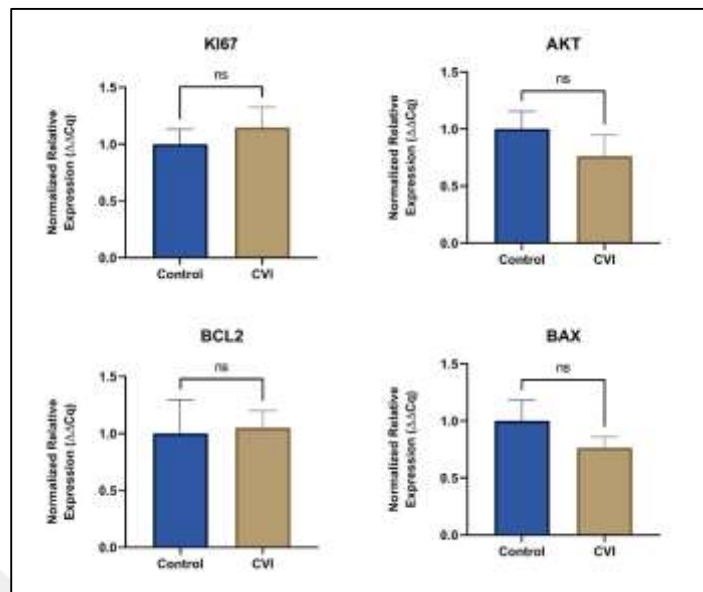


Figure 3.28. The of CVI induction on the expression of proliferation and apoptosis-related genes of HBECs. Relative normalized expression of CVI-related marker genes was analyzed by relative rt-PCR analysis. In the analysis, ATP5F1B was used as a reference gene for normalization. Fold change values were represented as means $\pm$ SEM.

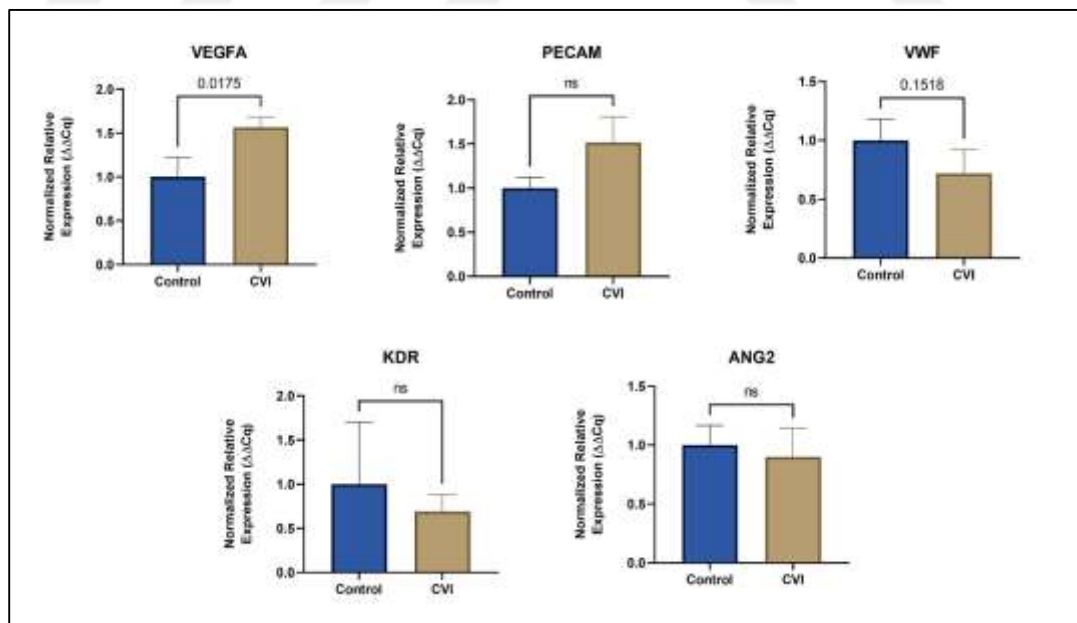


Figure 3.29. The effects of CVI induction on the expression of proliferation and apoptosis-related genes of HBECs. Relative normalized expression of CVI-related marker genes was analyzed by relative rt-PCR analysis. In the analysis, ATP5F1B was used as a reference gene for normalization. Fold change values were represented as means $\pm$ SEM.

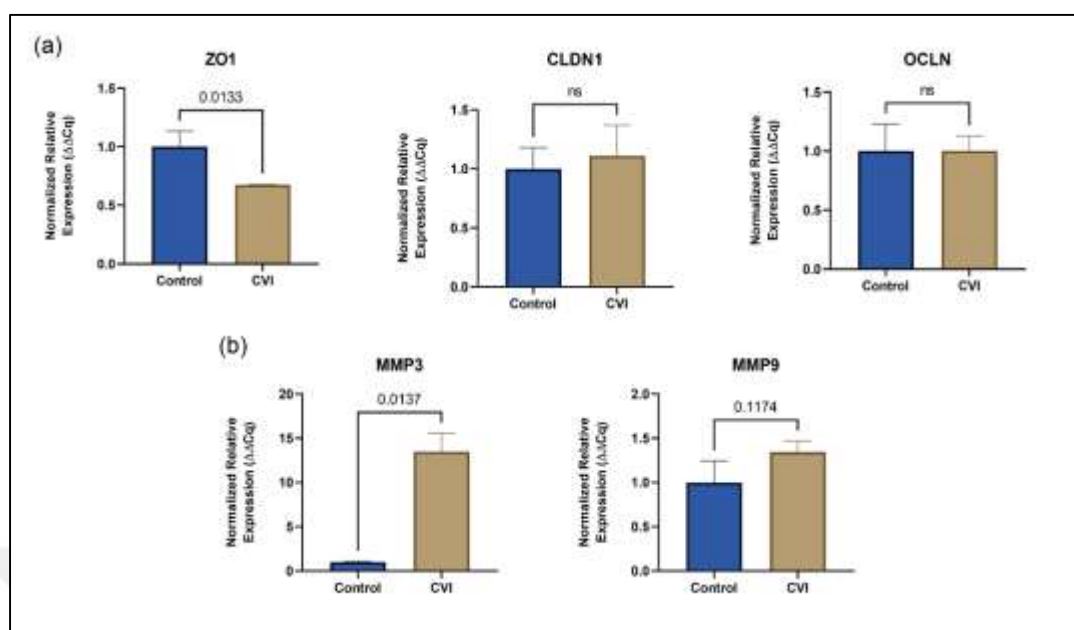


Figure 3.30. The effects of CVI induction on the expression of tight junction and matrix metalloproteinase-related genes of HBECS. Relative normalized expression of tight junction (a) and metalloproteinase-related (b) genes were analyzed by relative rt-PCR analysis. In the analysis, ATP5F1B was used as a reference gene for normalization. Fold change values were represented as means $\pm$ SEM.

3.11. WESTERN BLOT ANALYSIS OF CVI-INDUCED HBECS

In comprehending the effects of inflammation induction in CECs, the CVI model has great importance in evaluating the functional consequences of HBECS' responses to PMA/Ionomycin treatment. Since changes in gene expression may not always be reflected at the protein level, the evaluation of protein levels involved in CVI processes may be a decisive indicator. Therefore, the protein expression-related analysis helps to understand whether the CVI pathology can be modelled in the HBECS. In the experiment, to investigate the effects of CVI induction on protein expressions of HBECS, the western blot analysis was performed as shown in Figure 3.31. In the analysis, the results showed that the CVI induction led to significant changes in the expressions of particular marker proteins in HBECS (Figure 3.31a). According to the results, the expression of PPARG protein increased by 1.55-fold, while the expression of SIRT1 protein did not change. Furthermore, it was observed that the expression of NRF2 protein decreased to 0.62-fold. Then again, any changes were not also observed in the expressions of PAI1 and FOXO1 proteins. Lastly, the expression of ANG2

protein increased by 1.23-fold after the CVI induction. Thus, it was found that the CVI induction led to changes in the expression of CVI-related proteins and contributed importantly to the endothelial activation of HBECs.

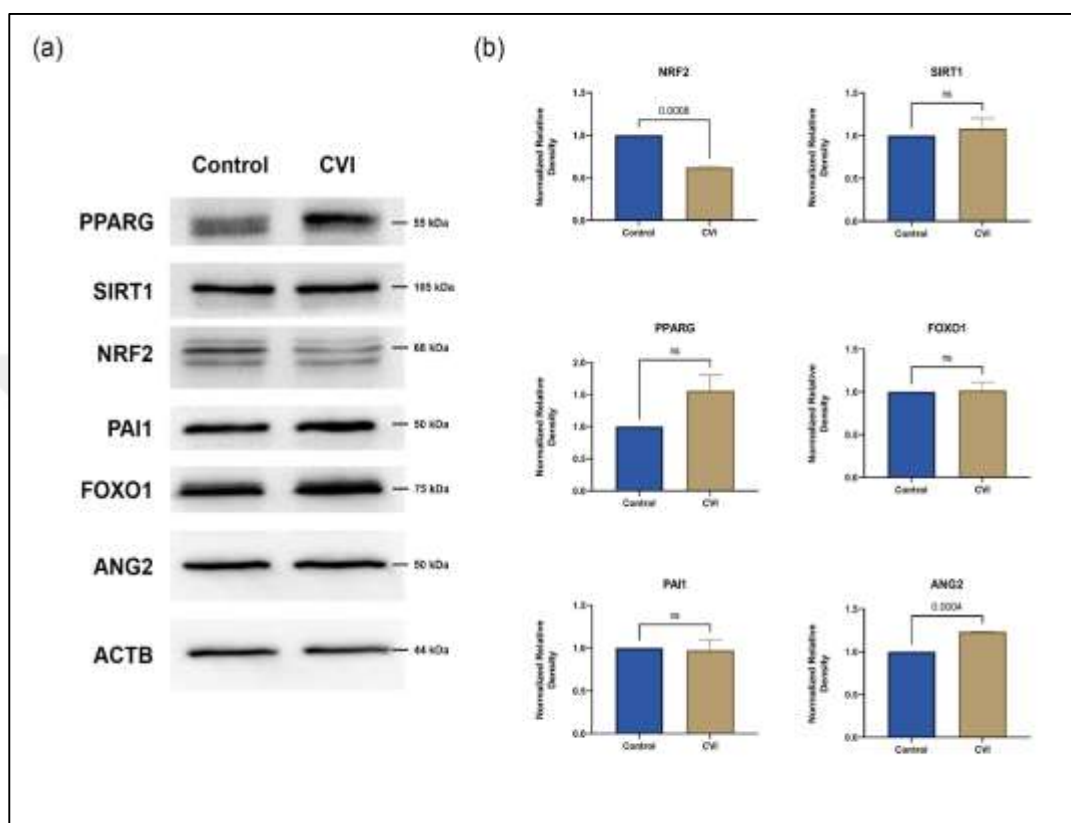


Figure 3.31. The effects of CVI induction on protein expression levels in HBECs. Relative normalized protein expression of CVI-related markers (a) was analyzed by western blot analysis and densitometric results (b) were compared statistically. ACTB expression was used as an internal control. Fold change values were represented as means $\pm$ SD.

3.12. IMMUNOCYTOCHEMISTRY ANALYSIS OF CVI-INDUCED HBECs

Another investigation method to evaluate protein expressions in the CVI model is immunocytochemistry (ICC) analysis. Unlike the outcomes of the western blot technique, the localization of proteins can also be visualized while investigating protein expressions with the ICC method. Therefore, the PMA/Ionomycin induction led to significant changes in the expression of the CVI-related marker proteins in HBECs. During the endothelial activation, IL8 is a cytokine playing an important role in the inflammatory response and its expression level increases. The results showed that the IL8 expression increased

significantly in CVI-induced HBECs as shown in Figure 3.32. According to the images, the IL8 expression was moderate and homogeneously distributed in the negative control, while the fluorescence intensity of IL8 expression rose significantly in the CVI model (Figure 3.32a and b). Further, the quantification result of fluorescence intensity also supported this finding and the intensity of IL8 protein in the CVI model increased by 1.52-fold than negative control (Figure 3.32c). As another characteristic marker for the CVI model, KI67 is a nuclear protein related to cell proliferation and has been used to analyse proliferative activity. According to the results, it was found that the CVI-induced HBEC showed a reduction in the KI67 expression as shown in Figures 3.33a and b. While a noticeable drop in fluorescence intensity of KI67 expression was observed in the CVI group, the negative control group displayed widespread and high intensity in the expression of KI67 protein. The findings were also verified by the result of quantification analysis, which showed that the fluorescence intensity of the CVI group decreased to 0.46-fold according to the negative control (Figure 3.33c). Another characteristic marker supporting essentially cellular movement and cytoskeleton integrity is the F-actin protein. The results demonstrated that the CVI induction led to a moderate reduction in the expression of F-actin protein in HBECs as shown in Figures 3.34a and b. In the result images, the negative control group had intense and distributed fluorescence signals, but a decrease and shrinkage in F-actin filaments were observed in the CVI model. In the quantification analysis, the fluorescence intensity of the CVI group decreased by 1.23-fold compared to the negative control, yet the difference was not statistically significant (Figure 3.34c). Besides, one of the other characteristic features related to the CVI model is the expression of adhesion molecules used as a marker of endothelial cell activation and whose expression rises on the surface of HBECs during the inflammatory response as shown in Figure 3.35 and Figure 3.36. The results demonstrated that the expression of adhesion molecules raised significantly in the CVI model (Figure 3.35a-b and Figure 3.36a-b). While VCAM1 and ELAM1 expressions were at a nominal level in the negative control, a significant surge in these expressions was observed in the CVI-induced HBECs. In the CVI group, the expressions of VCAM1 and ELAM1 increased by 1.44 and 3.85-fold, respectively. The quantification analysis also supported the finding and the fluorescence intensities of these two markers were significantly higher compared to the control groups (Figure 3.35c and Figure 3.36c). Further, KDR or VEGFR2 protein is a receptor playing a crucial role in the proliferation and angiogenic potential of endothelial cells. In the results, the expression of KDR protein was observed in the CVI model as shown

in Figure 3.37. According to the image results, the KDR expression was robust and homogeneous in the negative control, while a slight decrease in fluorescence intensity was observed in the CVI-induced HBECs (Figure 3.37a and b). The quantification analysis demonstrated that although the KDR expression in the CVI group decreased by 1.11-fold compared to the negative control, it was found that the difference was statistically significant (Figure 3.37c). Another characteristically decisive marker in CVI pathology is the expression of tight and adherens junction-related proteins and their regulations. In the experiment, the expressions of ZO1, CLDN1 and OCLN as tight junctions and CDH5 as adherens junctions were analysed. The results showed that CVI induction affects the expression of tight and adherens junction-related proteins in HBECs as shown in Figure 3.38, Figure 3.39, Figure 3.40, and Figure 3.41. According to the results, there was no change in the expression of CLDN1 and CDH5 proteins, while the expressions of ZO1 and OCLN proteins decreased by 3.75 and 1.63 times after CVI induction (Figure 3.38-3.41a and b). Upon examining the fluorescent images of ZO1 and OCLN proteins, it was observed that their expressions were notably reduced in the paracellular junctions in the CVI-induced HBECs. In the quantification results, it was determined that, unlike the CLDN1 and CDH5 proteins, the expression changes in the ZO1 and OCLN proteins were significant (Figure 3.38-3.41c). Consequently, considering all these ICC results and analyses, it was found that the expression of marker proteins in HBECs changed following the characteristic pathology of the CVI model after 3-hour PMA/Ionomycin treatment.

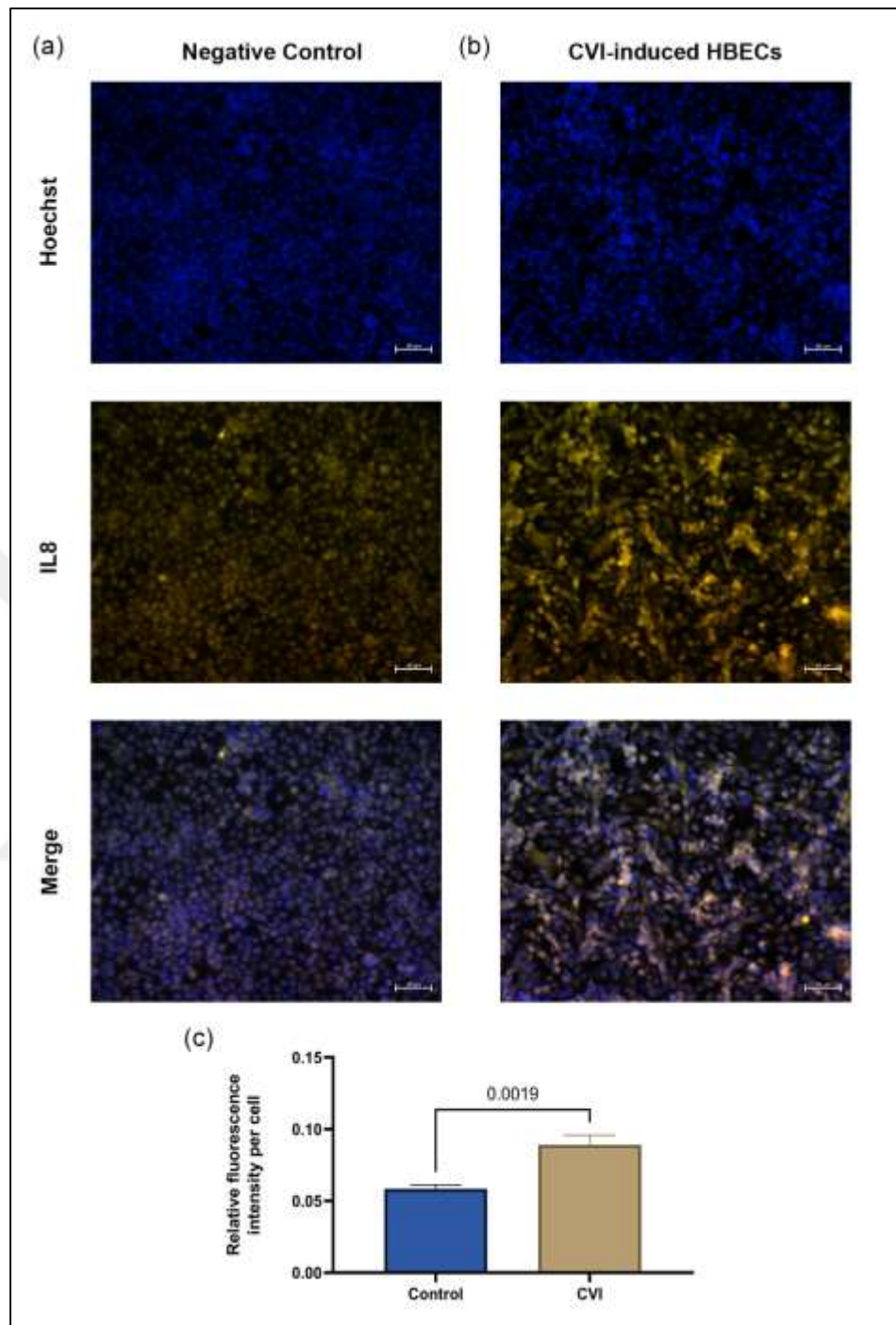


Figure 3.32. The ICC analysis of IL8 protein in CVI-induced HBECs. The fluorescence images between groups (a, b) and intensity analysis (c) were demonstrated after 3rd hour of CVI induction. Scale bar: 50 μ m. Relative fluorescence unit values were represented as means $\pm$ SD.

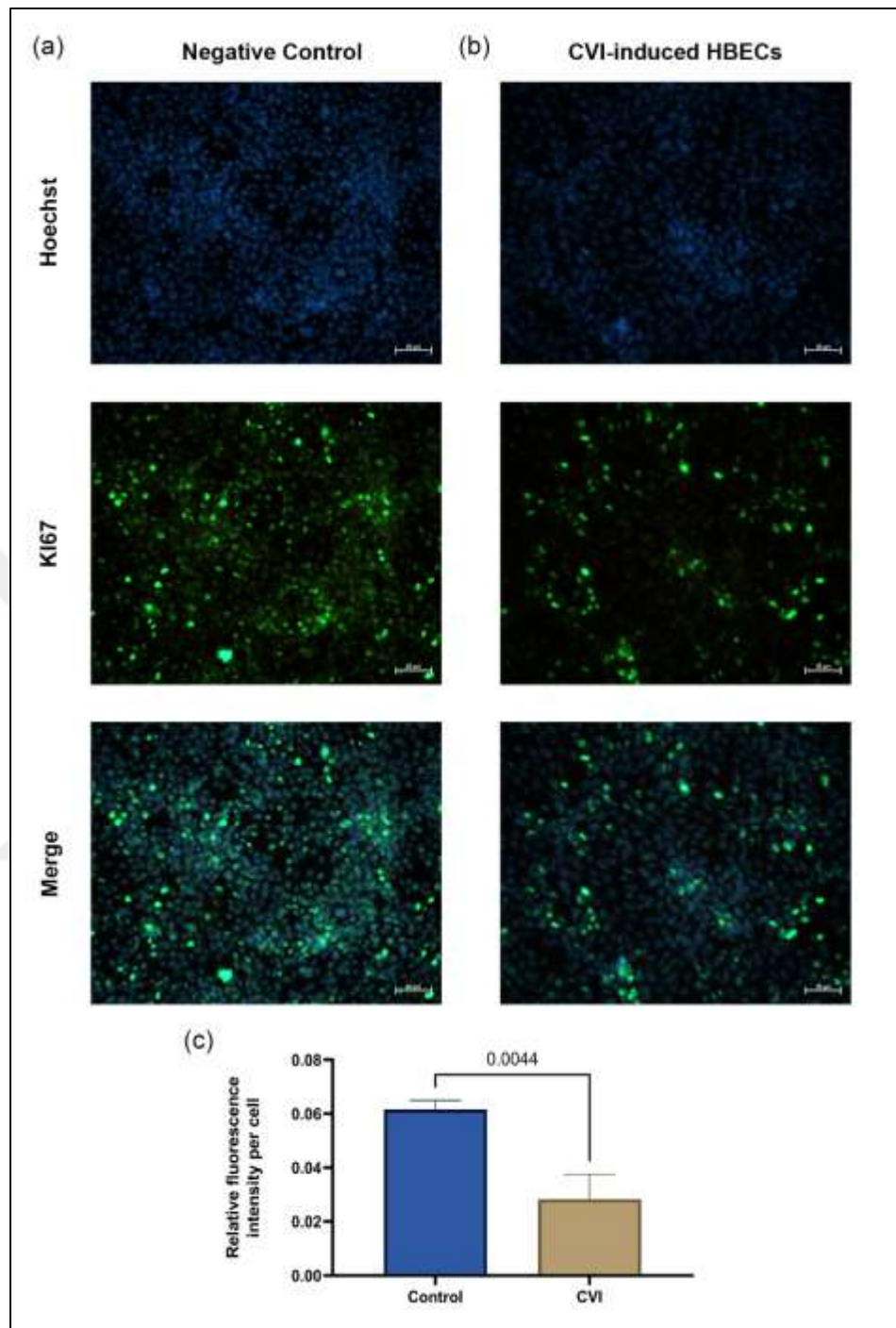


Figure 3.33. The ICC analysis of KI67 protein in CVI-induced HBECs. The fluorescence images between groups (a, b) and intensity analysis (c) were demonstrated after 3rd hour of CVI induction. Scale bar: 50 μ m. Relative fluorescence unit values were represented as means $\pm$ SD.

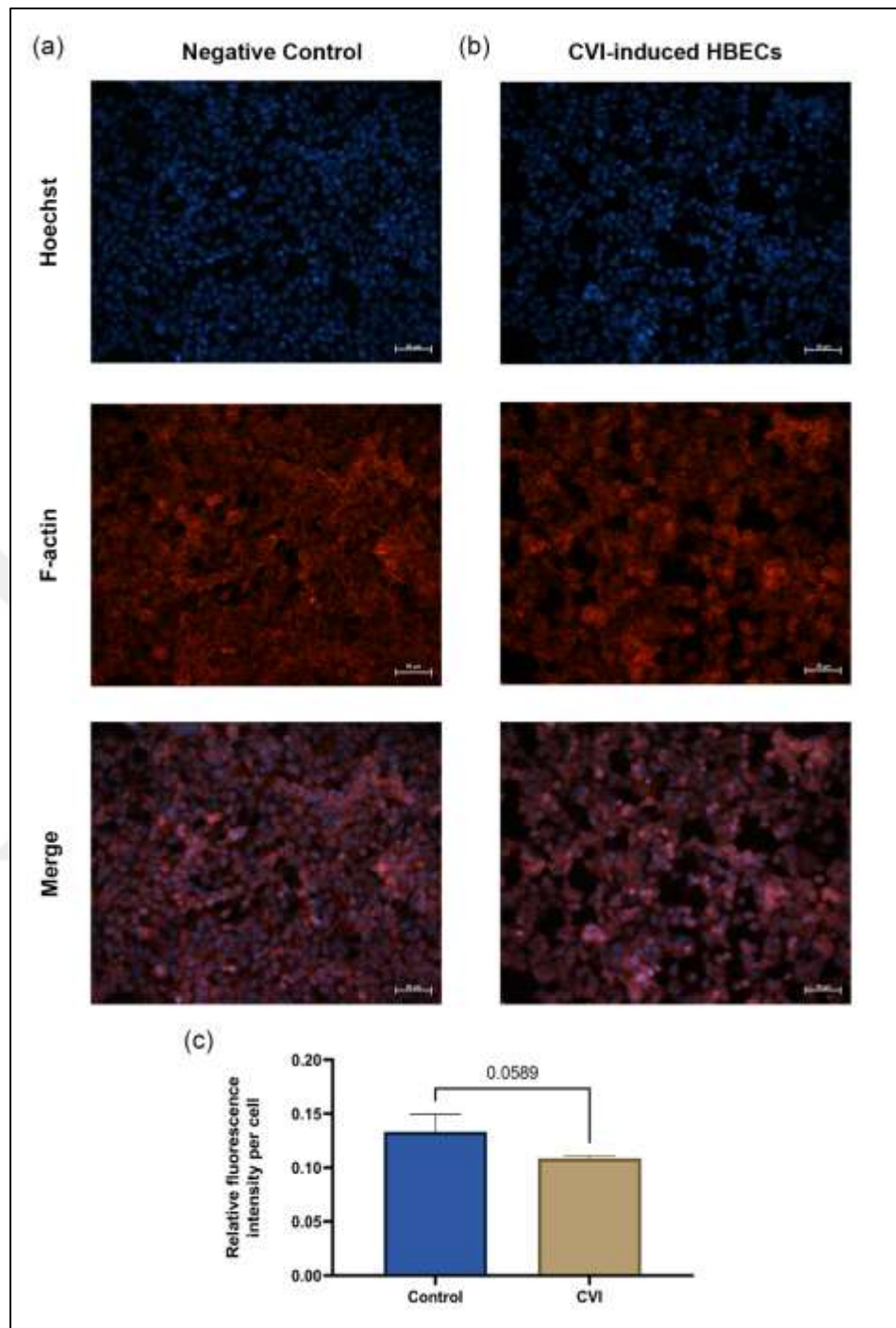


Figure 3.34. The ICC analysis of F-actin protein in CVI-induced HBECs. The fluorescence images between groups (a, b) and intensity analysis (c) were demonstrated after 3rd hour of CVI induction. Scale bar: 50 μm . Relative fluorescence unit values were represented as means $\pm$ SD.

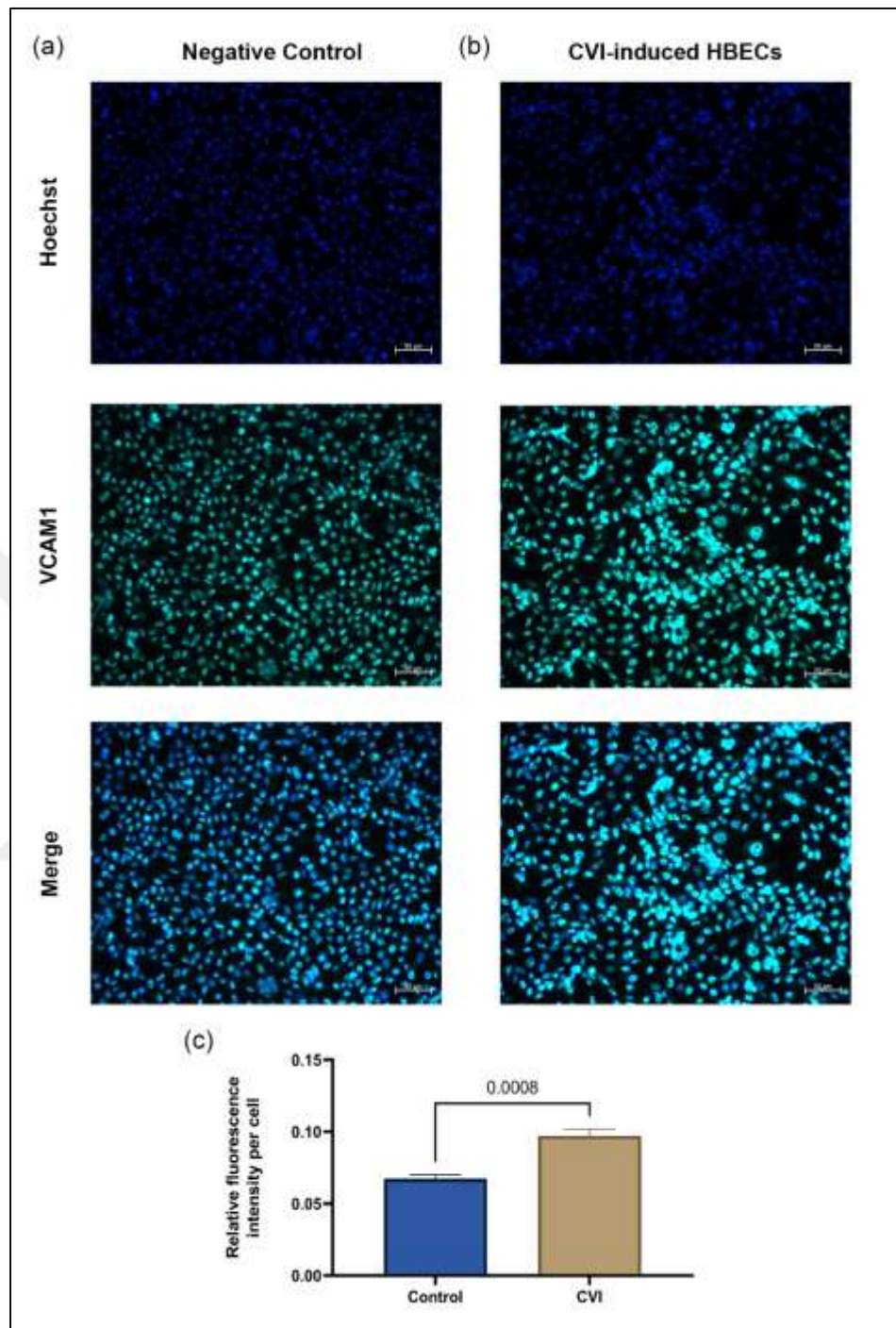


Figure 3.35. The ICC analysis of VCAM1 protein in CVI-induced HBECs. The fluorescence images between groups (a, b) and intensity analysis (c) were demonstrated after 3rd hour of CVI induction. Scale bar: 50 μ m. Relative fluorescence unit values were represented as means $\pm$ SD.

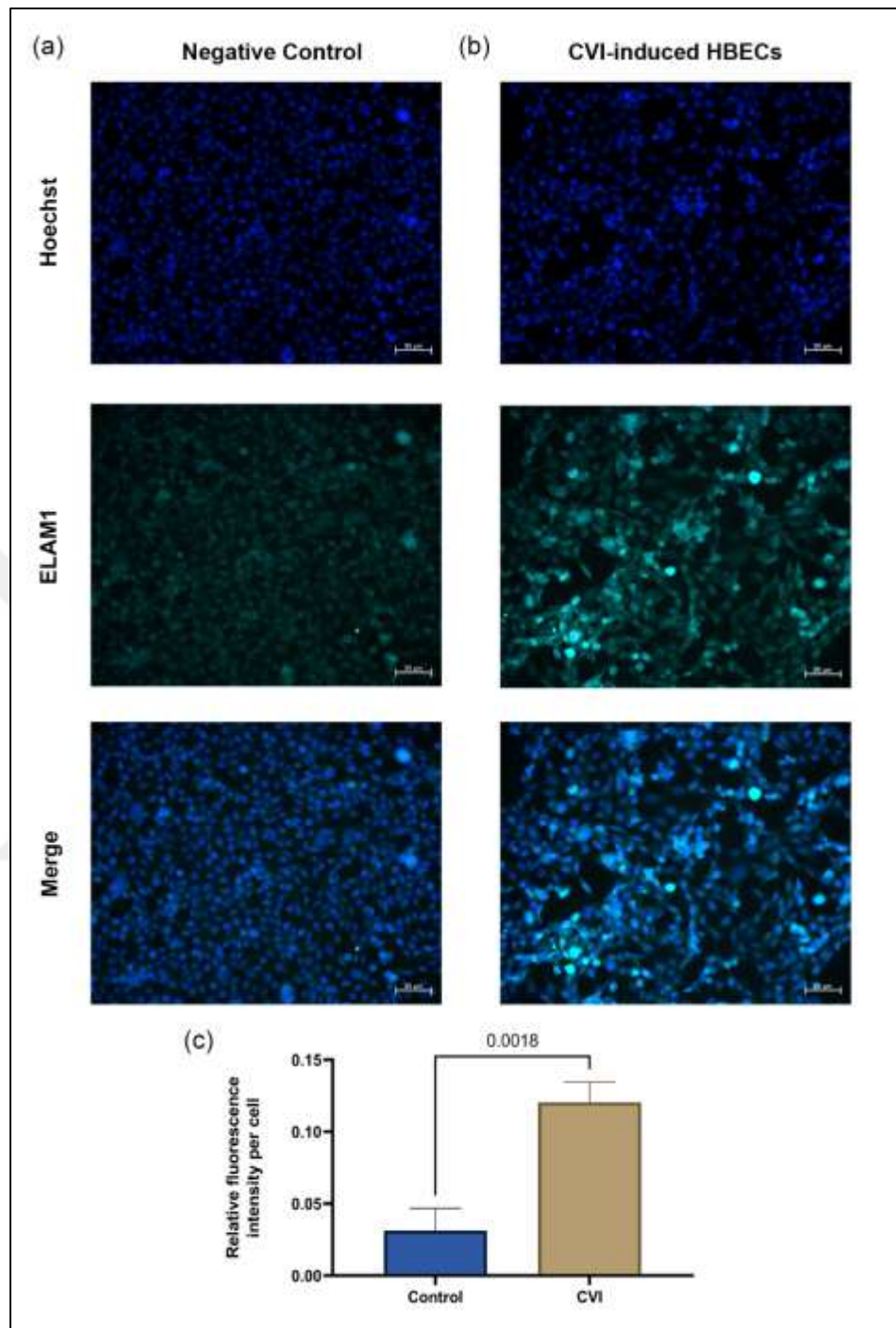


Figure 3.36. The ICC analysis of ELAM1 protein in CVI-induced HBECs. The fluorescence images between groups (a, b) and intensity analysis (c) were demonstrated after 3rd hour of CVI induction. Scale bar: 50 μm . Relative fluorescence unit values were represented as means $\pm$ SD.

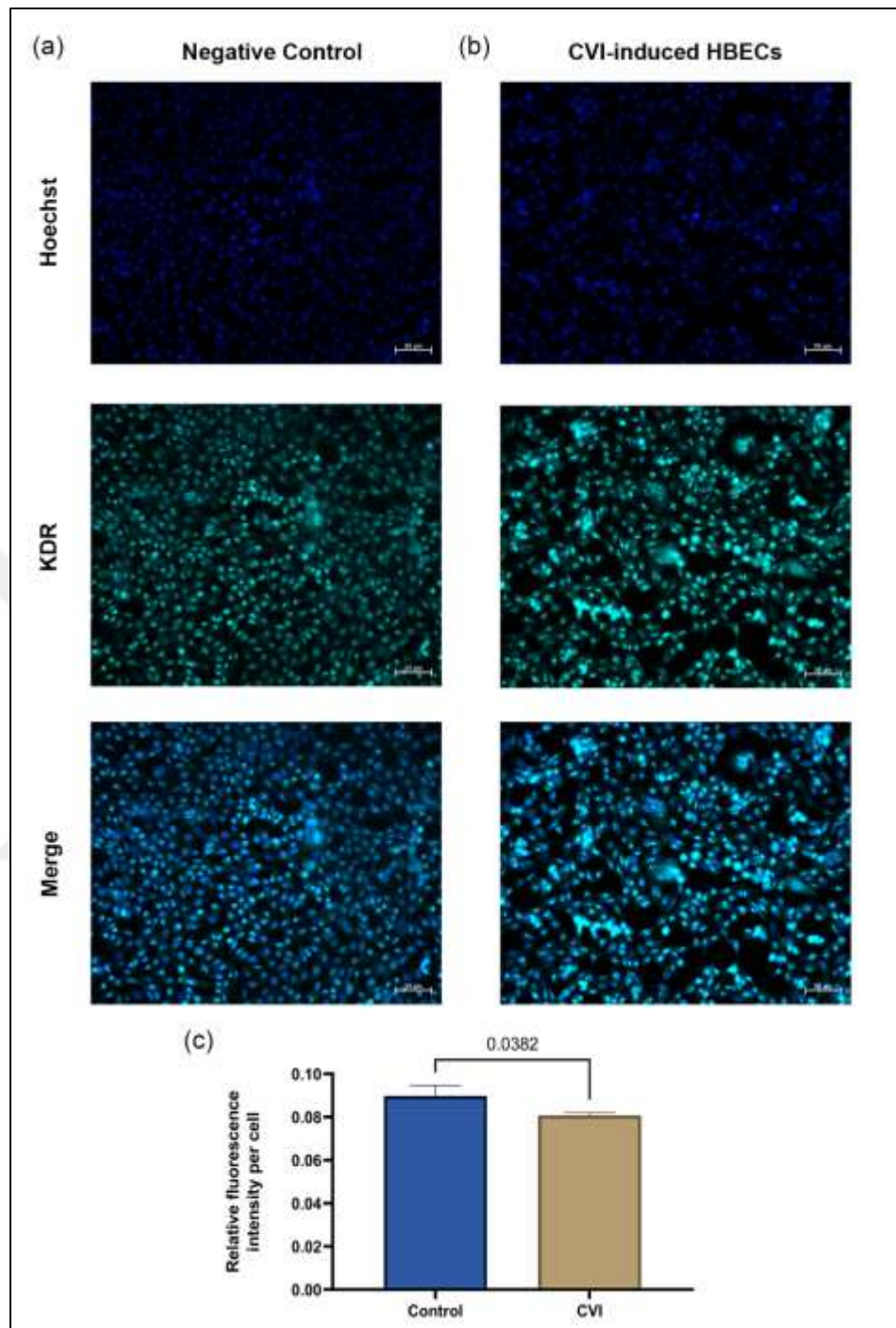


Figure 3.37. The ICC analysis of KDR protein in CVI-induced HBECs. The fluorescence images between groups (a, b) and intensity analysis (c) were demonstrated after 3rd hour of CVI induction. Scale bar: 50 μ m. Relative fluorescence unit values were represented as means $\pm$ SD.

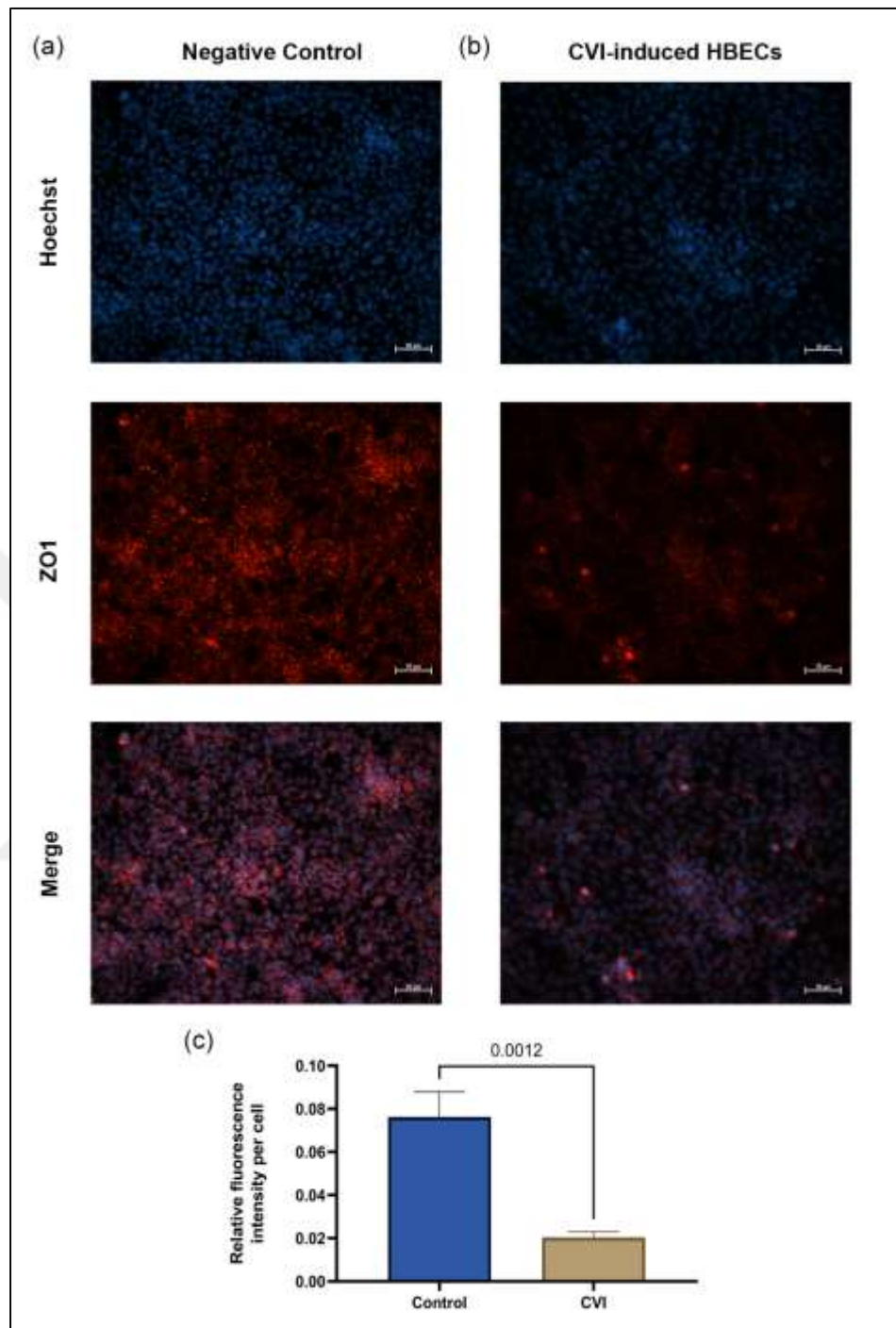


Figure 3.38. The ICC analysis of ZO1 protein in CVI-induced HBECs. The fluorescence images between groups (a, b) and intensity analysis (c) were demonstrated after 3rd hour of CVI induction. Scale bar: 50 μ m. Relative fluorescence unit values were represented as means $\pm$ SD.

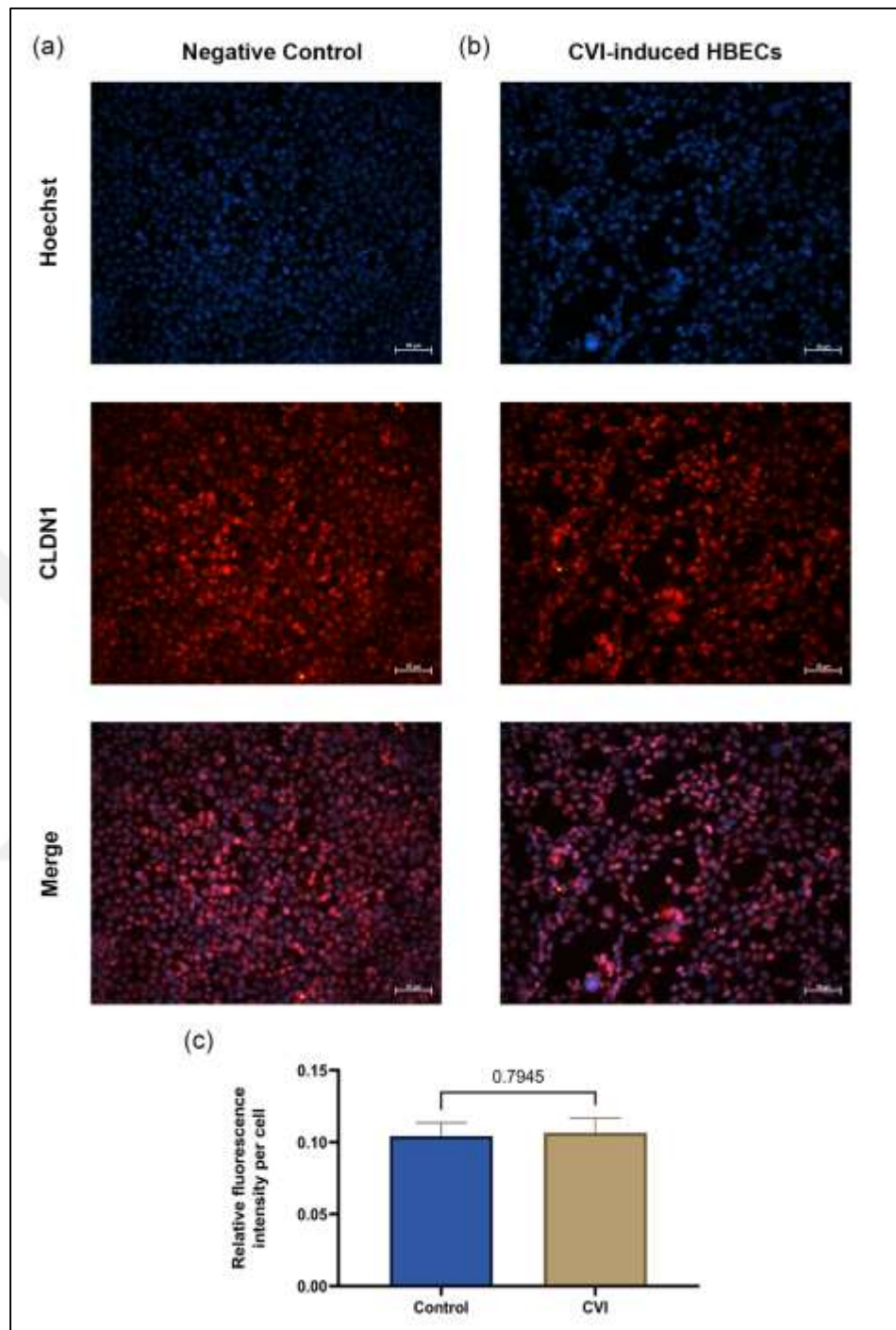


Figure 3.39. The ICC analysis of CLDN1 protein in CVI-induced HBECs. The fluorescence images between groups (a, b) and intensity analysis (c) were demonstrated after 3rd hour of CVI induction. Scale bar: 50 μ m. Relative fluorescence unit values were represented as means $\pm$ SD.

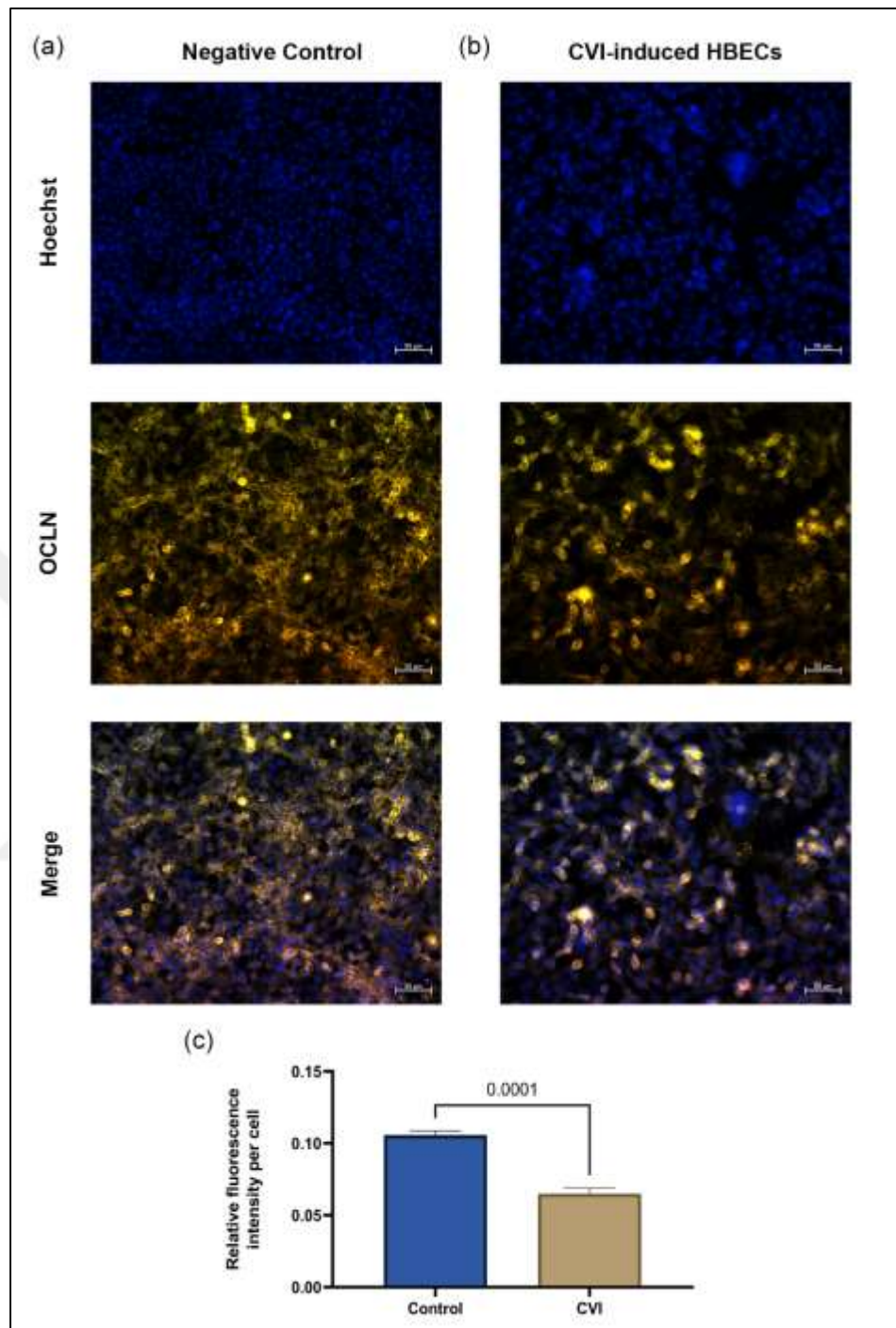


Figure 3.40. The ICC analysis of OCLN protein in CVI-induced HBECs. The fluorescence images between groups (a, b) and intensity analysis (c) were demonstrated after 3rd hour of CVI induction. Scale bar: 50 μm . Relative fluorescence unit values were represented as means $\pm$ SD.

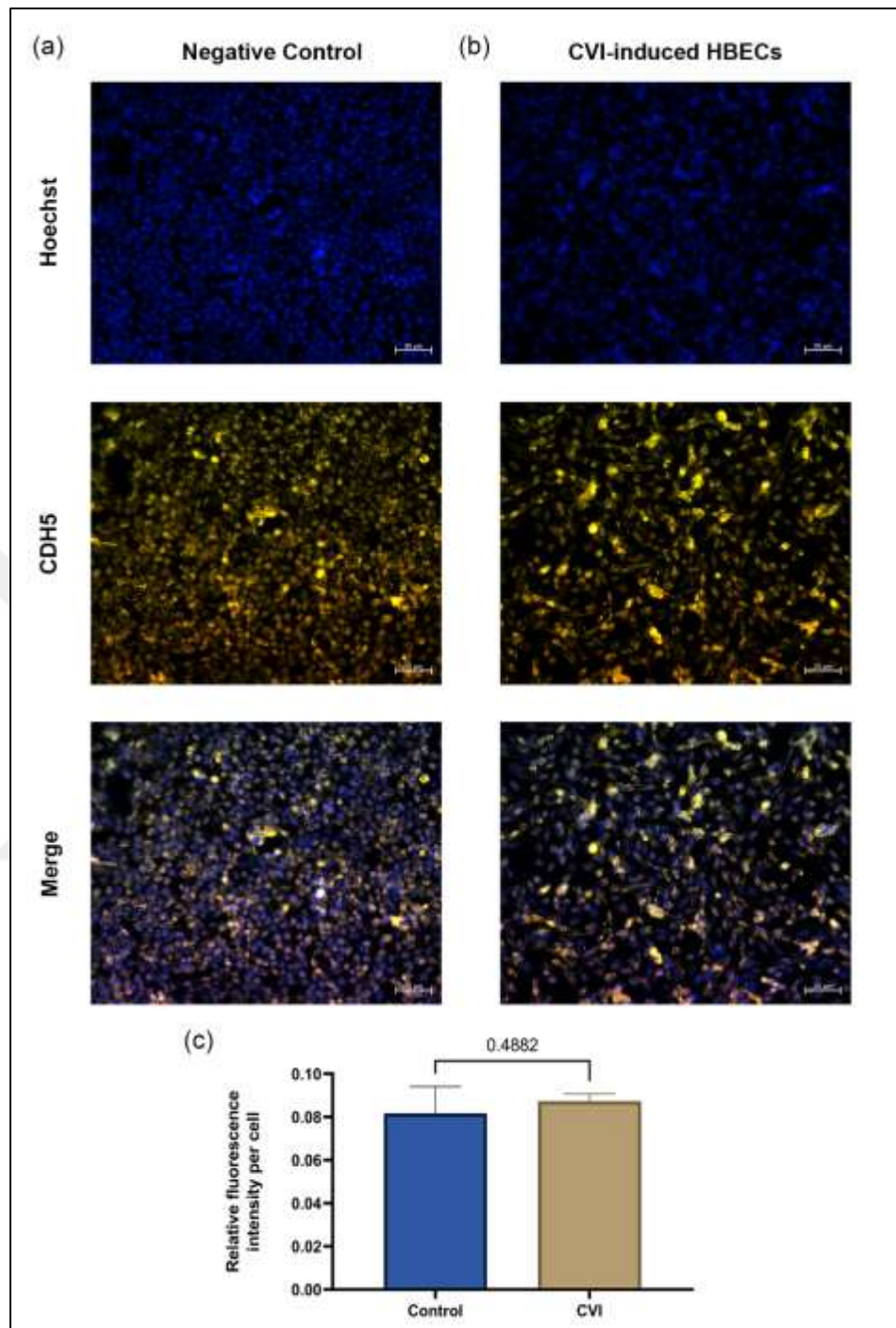


Figure 3.41. The ICC analysis of CHD5 protein in CVI-induced HBECs. The fluorescence images between groups (a, b) and intensity analysis (c) were demonstrated after 3rd hour of CVI induction. Scale bar: 50 μ m. Relative fluorescence unit values were represented as means $\pm$ SD.

3.13. INFLAMMATORY CYTOKINE ANALYSIS OF CVI-INDUCED HBECs

In the pathogenesis of CVI, cytokines and chemokines are the key players, being small proteins that play critical roles in endothelial activation. The dramatic changes in the balance of these molecules may lead to inflammatory responses in cerebrovascular tissue and CVI pathology. Therefore, determining the cytokines and chemokines released from PMA/Ionomycin-treated HBECs into the culture medium is quite important in the sense of the CVI model. In the experiment, to investigate the effects of CVI induction on cytokine and chemokine levels, the bead-based cytometric array (CBA) analysis was performed as shown in Figure 3.42. Further, the methodologic illustration of CBA analysis was demonstrated in Figure 3.42a [205]. According to the results, it was found that IL8 protein showed a significant increase in the CVI model compared to the control group. In the results, IL8 concentration in the culture medium increased by 7.46-fold in the CVI-induced HBECs. On the other hand, it was not observed any significant changes in the other parameters such as IL6 or TNF. Consequently, these findings indicate that the CVI induction significantly increased IL8 production in HBECs, but did not lead to any changes in other cytokines.

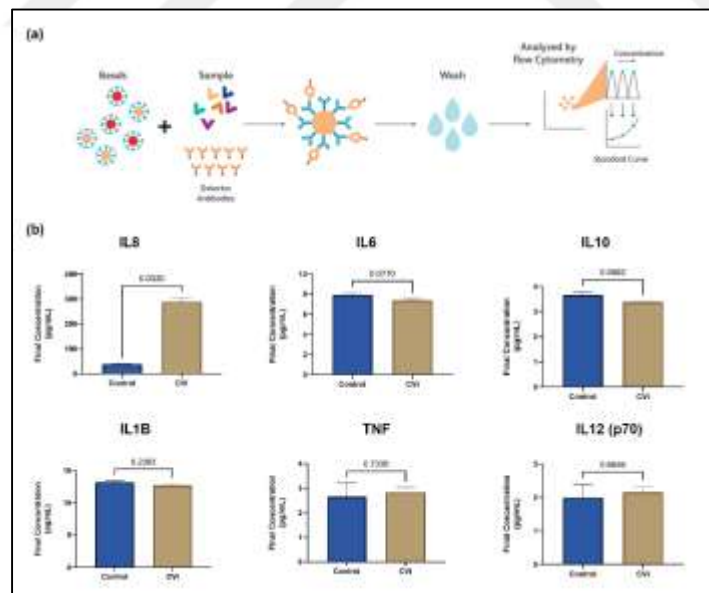


Figure 3.42. The CBA analysis of CVI-induced HBECs. The methodological representation of the analysis was demonstrated (a). The concentration of cytokines and chemokines was measured by CBA analysis and compared statistically (b). The concentration values were represented as means $\pm$ SD.

3.14. TUBE FORMATION ANALYSIS OF CVI-INDUCED HBECs

In CVI pathology, angiogenesis is one of the critical parameters, affecting the functionality and stability of the blood-brain barrier. Therefore, the regulation of angiogenic activity is critical for the sustainability of cerebrovascular physiology. To investigate the relationship between the CVI induction and angiogenesis potential of HBECs, the tube formation analysis was performed. In the experiment, the effects of CVI induction on the angiogenic potential of HBECs were observed with tube formation analysis. The results showed that the angiogenic response of HBECs was suppressed due to the CVI induction as shown in Figure 3.44. According to the results, it was observed that the tubular structures of cells were decreased in the bright-field images at the end of 6-hour incubation (Figure 3.44a and b). Besides, these bright-field images were analysed with angiogenesis macro in ImageJ software to resolve these visual results with quantitative parameters such as total length, number of nodes, branches, and total meshes area (Figure 3.44c-f). In the results, as important parameters in the tube formation assay, the total length, node number, and branch number significantly decreased in the CVI model compared to the negative control. These parameters indicated that the CVI induction affected negatively the tube formation by breaching the network structure of endothelial cells. Nevertheless, any statistically significant difference was not observed in the total mesh area, indicating that CVI induction leads to a change in the morphological parameter, yet not the whole structure homogeneously. Consequently, it was found that CVI induction may decrease the angiogenic potential of HBECs over tube formation-related parameters.

3.15. GENE EXPRESSION ANALYSIS OF T LYMPHOCYTE ACTIVATION

It is known that CECs play crucial roles in the initiation of inflammatory response in the CVI condition, while leukocytes also make important contributions to this response. They have decisive functions, especially in their interactions with the CECs and in the progression of inflammation. Accordingly, the response of T lymphocytes to the activation signal is quite decisive in terms of endothelial-leukocyte crosstalk. Thus, in the study, changes in gene expression of Jurkat cells were analysed when they were activated similar to the CVI model. According to the results, it was found there were significant changes in the expression profiles of activation-related genes in Jurkat cells as shown in Figure 3.44.

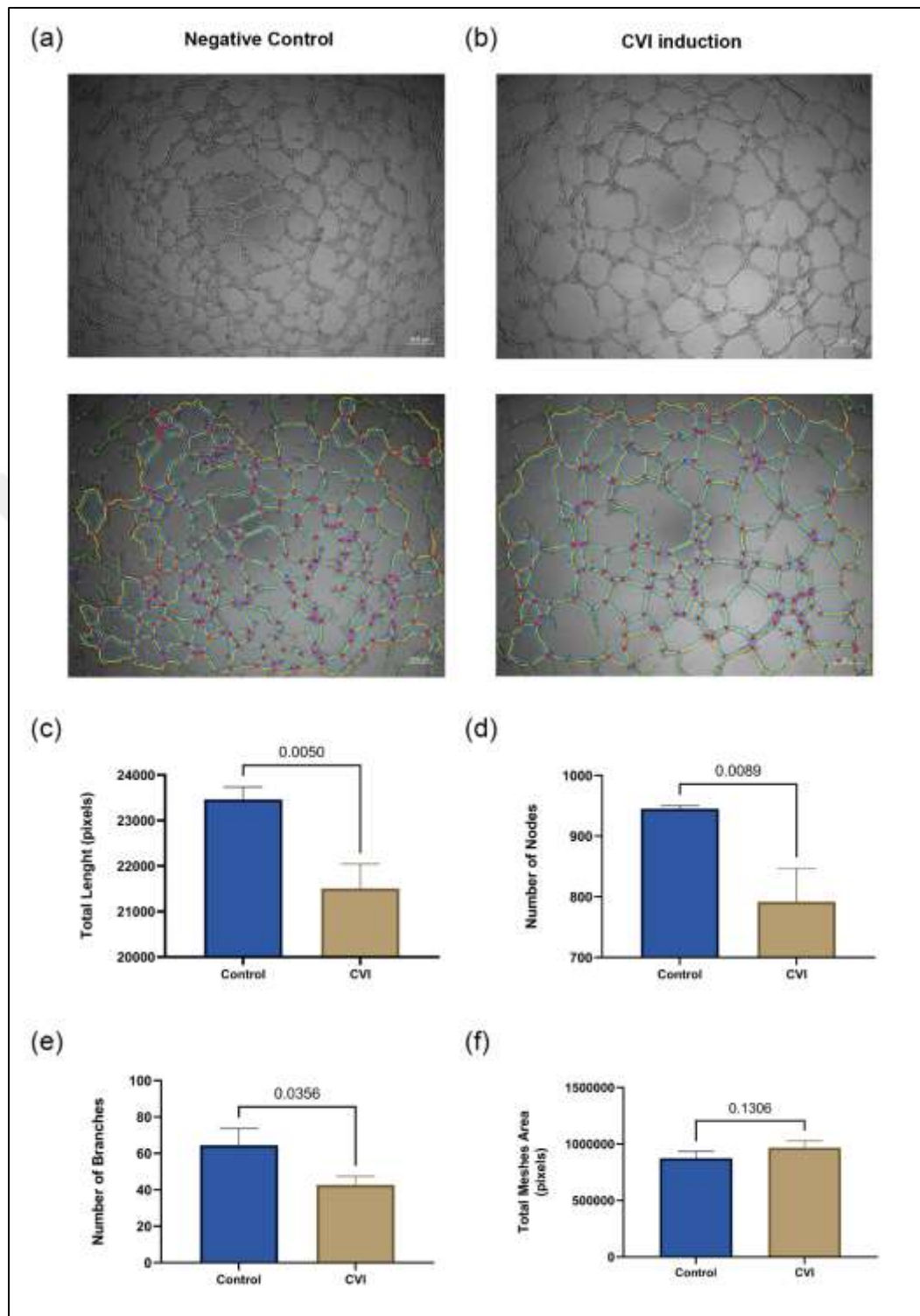


Figure 3.43. The tube formation analysis in CVI-induced HBECs. For the analysis, the bright-field images were taken and analyzed with ImageJ software (a, b). Quantitative parameters such as total length, number of nodes and branches, and total meshes area were also demonstrated (c-f). Scale bar: 100 μ m. All the values were represented as means $\pm$ SD.

Examining the heatmap analysis, there was a noticeable upregulation in the expression of many genes associated with T cell activation (Figure 3.44a). The results showed that the expression of IL2, IFNG, and IL8 genes significantly increased by 39.91-, 32.76-, and 72.74-fold, respectively, in the CVI model (Figure 3.44b). The expression of the IL4 gene only increased 1.12-fold, and it was not found to be statistically significant. In the CVI groups, it was seen that the expression of NRF2 and SIRT1 genes did not change, while the expression of PPARG increased significantly by 2.11-fold in the induction group. On the other hand, the expression of the BAX gene showed a significant decrease to 0.64-fold, while there was not any change in the expression of BCL2 and AKT genes. Being a gene associated with cell proliferation, the expression of the KI67 gene was upregulated by 1.58-fold in the CVI model. Besides, as another critical gene related to endothelial adhesion, the expression of the LFA gene increased significantly by 3.46-fold in the CVI group compared to the control group. Consequently, it was found that the Jurkat cells can respond appropriately to the activation signal and their gene expression matched with the CVI-related activation pattern.

3.16. ENDOTHELIAL FUNCTIONALITY ANALYSIS OF THE CVI MODEL

In CVI pathology, the adhesion interaction between CECs and leukocytes and disruption in vascular permeability has great importance. While the interactions of CECs with leukocytes regulate the inflammatory response, increased permeability of the cells also has a substantial role in the disruption of the BBB. Therefore, the investigation of changes in these adhesion and permeability properties of HBECs seems necessary for the biological convenience of the CVI model. According to the results, it was found that the adhesion capability of HBECs increased substantially, while the endothelial permeability of the cells did not change as a result of the CVI induction as shown in Figure 3.45. In the results, the elevation in the adhesion interactions between HBECs and Jurkat cells was demonstrated in the fluorescence images (Figure 3.45a). In the analysis, the result images were also analysed quantitatively and graphically represented. The results exhibited that the adhesion capability of HBECs significantly increased by 1.66-fold in the CVI group compared to the negative control (Figure 3.45b). On the other hand, it was not found to be significant changes in the endothelial permeability under the CVI induction.

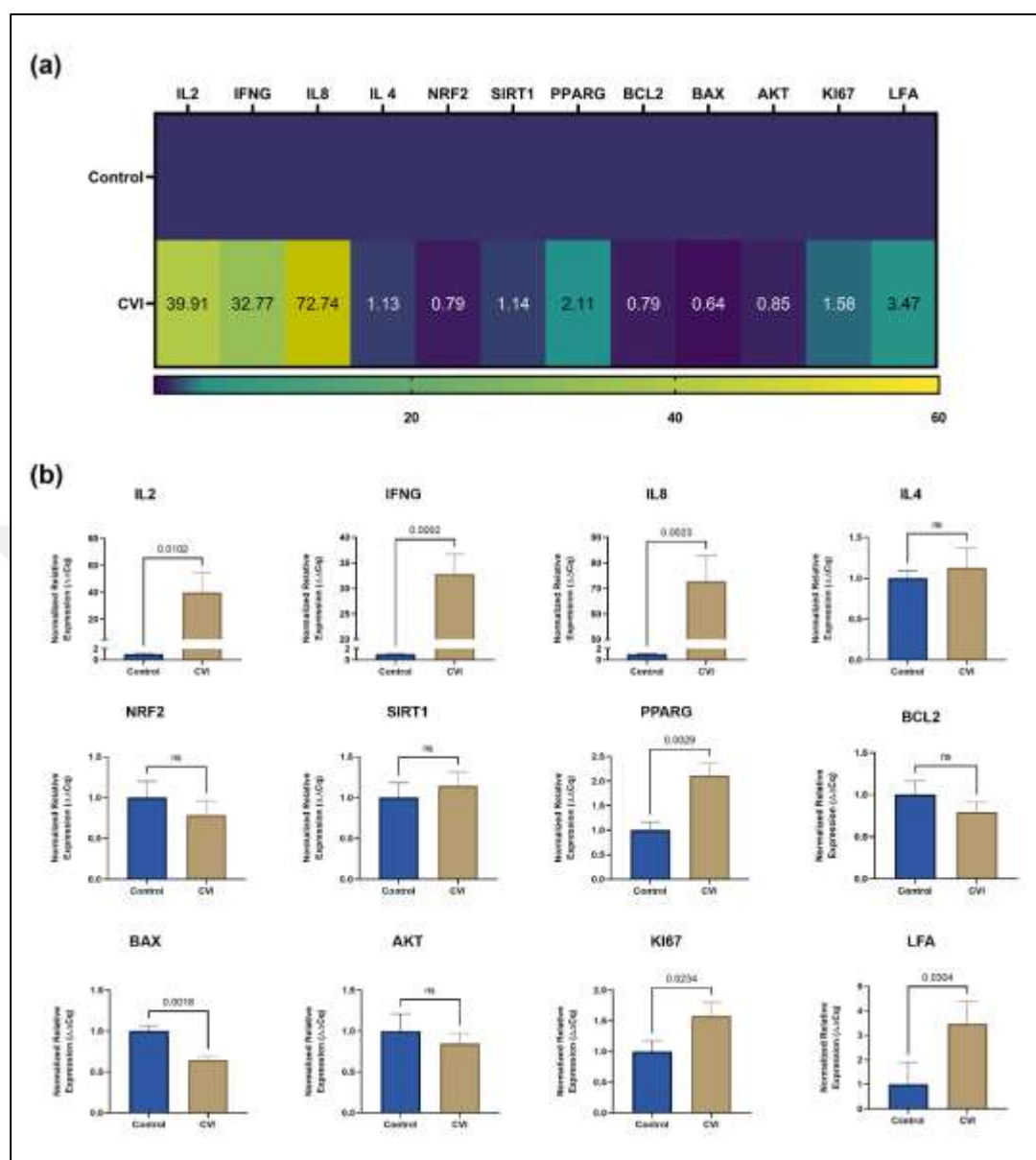


Figure 3.44. The effects of CVI induction on gene expression profile of Jurkat cells. Relative normalized expression of CVI-related marker genes was analysed by rt-PCR and represented in the heatmap analysis (a). The expression of these genes was also represented graphically (b). In the analysis, ATP5F1B was used as a reference gene for normalization. Fold change values were represented as means $\pm$ SEM.

In the results, first, it was observed that the 4 kDa FITC-conjugated dextran molecule passes through the inserts and can be measured successfully from media in the bottom of the well. However, the measurements showed that the fluorescent signal in the media did not increase as a result of the CVI induction. Besides, there was no change between the fluorescent signal

of the control group and the blank group, which did not have any seeded cells. Examining the results, it was found that the monolayer form of HBECs did not reduce the passage of 4 kDa FITC-conjugated dextran with their adherens and tight junctional complexes, but rather the CVI induction did not increase the endothelial permeability.

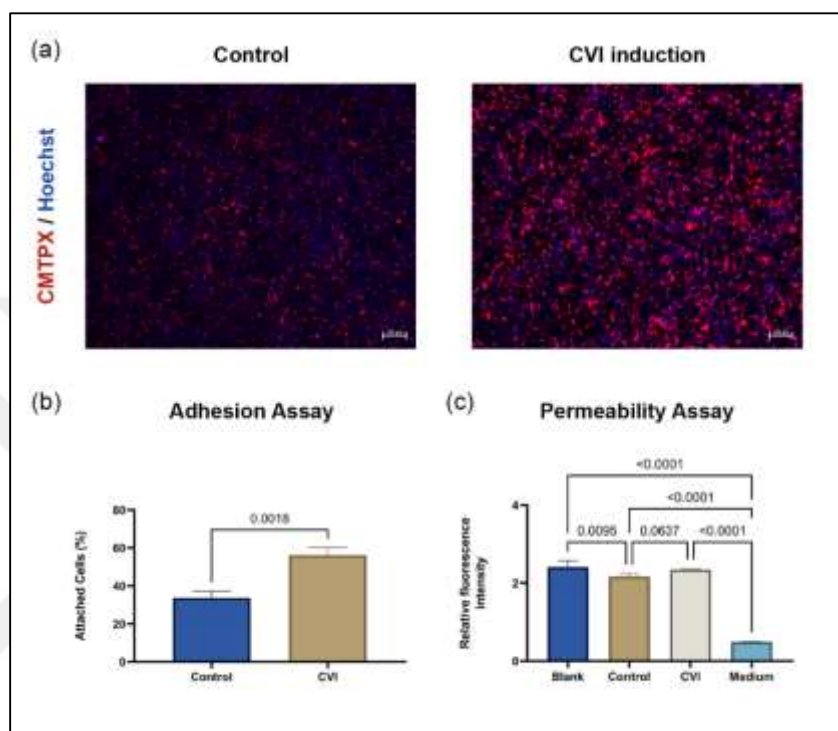


Figure 3.45. The endothelial functionality analysis of CVI-induced HBECs. The changes in the adhesion interactions and endothelial permeability of HBECs after the CVI induction were represented (a). Further, the quantitative analysis of adhesion assay and endothelial permeability was represented graphically (b and c). Scale bar: 100 μ m. In the results, percentage and intensity values were represented as means $\pm$ SD.

3.17. CELL VIABILITY ANALYSIS OF CVI IN PCSEVS-TREATED HBECs

At this point of the study, whether the PCSEVs have any protective effect on HBECs induced with the CVI model was investigated. However, before investigating further biochemical experiments, it must be determined that the PCSEVs had any cytotoxic effects on HBECs under normal physiological conditions. Thus, the possible cytotoxic effects of the PCSEVs on the monolayer culture of HBECs were investigated by MTS assay. According to the results, it was seen that the PCSEVs did not demonstrate any cytotoxic effect on HBECs as shown in Figure 3.46. In the analysis, there were not any significant changes in the cell

viability of HBECs in all concentrations of both SDEVs and HDEVs compared to the negative control. On the other hand, the DMSO group confirmed the cytotoxic effect by causing a significant decrease in cell viability. Consequently, all these results indicated that using the PCSEVs did not adversely affect the cell viability and was safe for further downstream experiments.

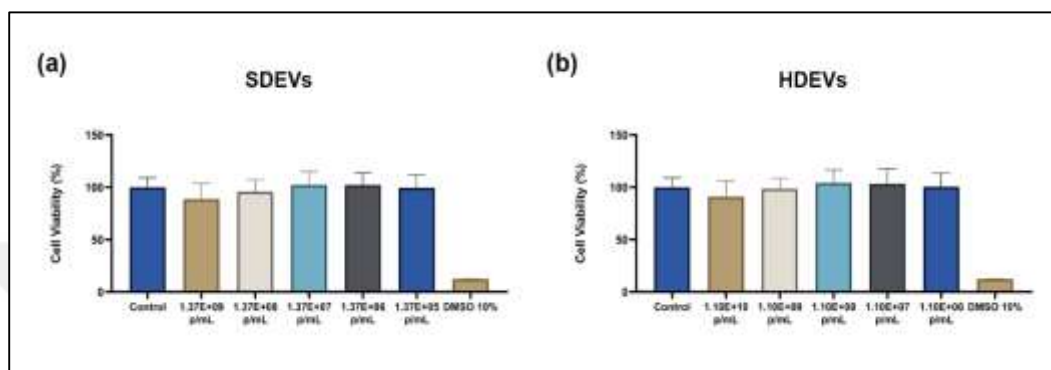


Figure 3.46. The effects of the PCSEVs on cell viability of HBECs. The effects of the SDEV (a) and HDEV (b) treatment on cell viability were analysed with MTS assay after 24 hours of incubation. All values were represented as means $\pm$ SD.

3.18. GENE EXPRESSION ANALYSIS OF CVI IN PCSEVS-TREATED HBECs

For discovering the potential functional effects of the PCSEVs, it is quite critical to investigate the protective effects of these vesicles on the CVI-induced HBECs. Accordingly, the effects of the PCSEVs on the CVI model were investigated in terms of their protective effects. Thus, these effects on the expression of particular genes related to the CVI model in HBECs were evaluated by gene expression analysis with rt-PCR experiments. According to the results, it was found that both SDEVs and HDEVs reduced the expression of CVI-related genes in HBECs, but their effects may vary on different genes as shown in Figure 3.47 and Figure 3.48. In the results of the SDEV treatment, all the changes in the gene expressions were represented in the heatmap analysis (Figure 3.47a). In the analysis, the expression of inflammation-related genes such as IL8, MCP1, and PAI1 did not change significantly in the pretreatment group compared to the CVI model (Figure 3.47b). The pretreatment of SDEV doses did also not affect the expression of the ZO1 gene. Besides, it was observed that both doses of SDEV pretreatment significantly decreased the expression of VCAM1 and ELAM1 genes compared to the CVI groups. Lastly, the expression of the MMP3 gene

showed a significant upregulation in the 1.37×10^9 particles/mL dose, while the 1.37×10^8 particles/mL dose did not lead to any alteration compared to the CVI group. Besides, the HDEV treatment also brought about changes in the gene expressions demonstrated by heatmap analysis in Figure 3.48a. In the results of the analysis, it was observed that the HDEV pretreatment showed a notable decrease in the expression of IL8 and PAI genes by 212- and 30-fold, respectively, at the 1.10×10^{10} particles/mL dose, but this decline in the expression was not found to be statistically significant. Besides, there were not any changes in the expression of PAI1 and ZO1 genes. On the other hand, the expressions of VCAM1 and ELAM1 genes were significantly downregulated by 40- and 12.44-fold, respectively, at both of the HDEV doses. Further, the expression of MMP3 expression did not change significantly in the pretreatment groups compared to the CVI model. Consequently, these results indicated that the PCSEV pretreatment mostly affected the cellular adhesion-related genes and could limit the interaction of HBECs and Jurkat cells in CVI pathology.

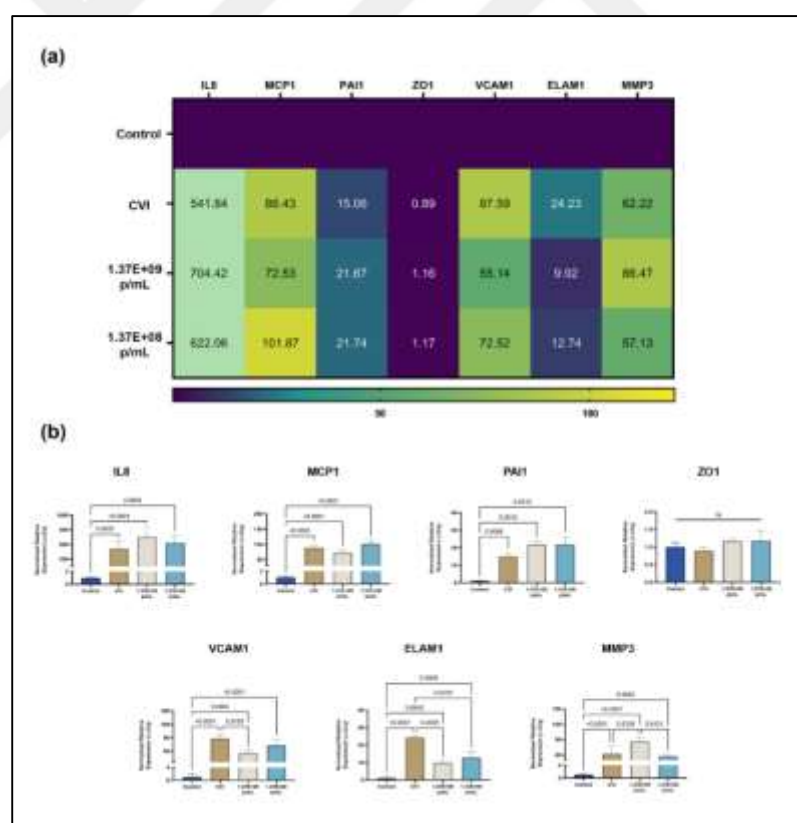


Figure 3.47. The effects of SDEV pretreatment on gene expression of CVI-induced HBECs. Relative normalized expression of CVI-related marker genes was analysed by rt-PCR and represented in the heatmap analysis (a). The expression of these genes was also

represented graphically (b). ATP5F1B was used as a reference gene for normalization. Fold change values were represented as means $\pm$ SEM.

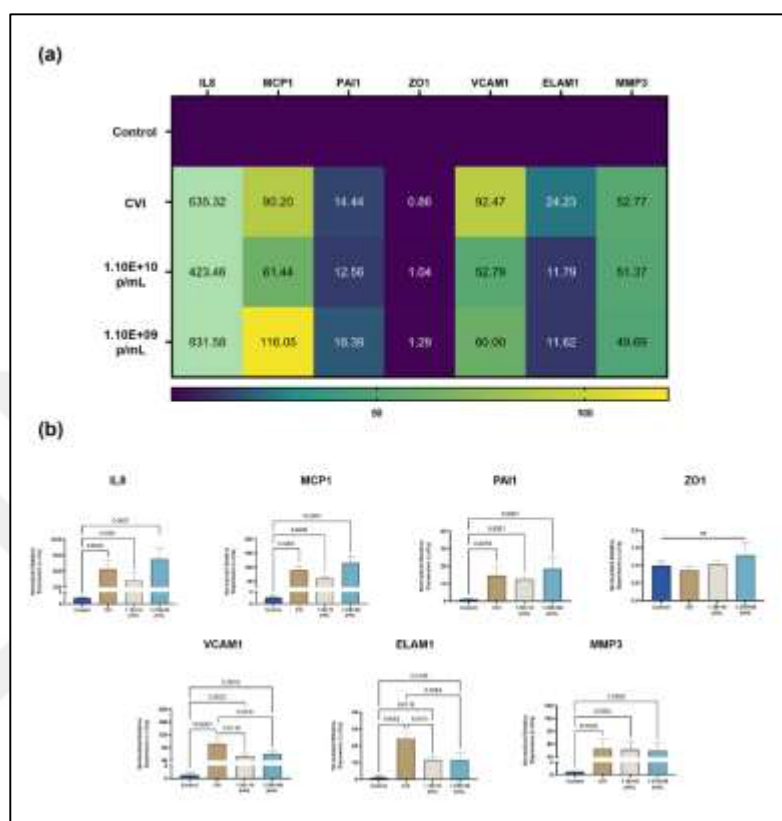


Figure 3.48. The effects of HDEV pretreatment on gene expression of CVI-induced HBECs. Relative normalized expression of CVI-related marker genes was analysed by rt-PCR and represented in the heatmap analysis (a). The expression of these genes was also represented graphically (b). ATP5F1B was used as a reference gene for normalization.

Fold change values were represented as means $\pm$ SEM.

3.19. IMMUNOCYTOCHEMISTRY ANALYSIS OF CVI MODEL IN PCSEVS-TREATED HBECs

In the study, the protective effects of the PCSEVs on the expression of CVI-related genes in HBECs were investigated and it was found that the PCSEVs had protective effects in the sense of the expression of cellular adhesion genes. Subsequently, to confirm these results in protein expressions also, the ICC analysis was performed. According to the results, both PCSEVs demonstrated protective effects against CVI induction in terms of VCAM1 expression as shown in Figure 3.49. In the result of fluorescence images, it was seen that the

CVI induction led to significantly increased VCAM1 expression compared to the control (Figure 3.49a). However, a noticeable decrease in VCAM1 expression was observed in SDEV and HDEV pretreated groups under the CVI induction. Besides, the image results were quantitatively analysed and represented graphically (Figure 3.49b). In the analysis, the results showed that the pretreatment of HBECs with both SDEVs and HDEVs significantly reduced VCAM1 expression by 1.78- and 1.41-fold, respectively, while the CVI induction group caused to increase the its expression by 2.72-fold. Besides, another important marker being closely related to VCAM1 protein was the expression of ELAM1 protein. According to the results, the effects of the PCSEVs on its expression in CVI-induced HBECs were demonstrated similarly with VCAM1 protein as shown in Figure 3.50. Upon examining the fluorescence images, it was observed that the pretreatment of HBECs with the PCSEVs decreased the elevated ELAM1 expression in comparison with the CVI induction group (Figure 3.50a). Besides, the quantitative analysis of images also proved the protective effects of the PCSEVs under the CVI induction (Figure 3.50b). In the analysis, the results showed that CVI induction upregulated the expression of ELAM1 by 3.05-fold. Nevertheless, its expression in HBECs decreased as a result of the pretreatment of SDEVs and HDEVs by 1.89- and 2.31-fold, respectively, compared to the CVI model. In the experiment, the expression of the ZO1 protein was another marker to be analysed as a tight junction-related protein. According to the results, it was found that the PCSEVs supported the paracellular barrier integrity by conserving the expression of ZO1 protein in CVI-induced HBECs as shown in Figure 3.51. In the fluorescent images, it was seen that the expression of ZO1 protein considerably decreased in the CVI induction group, especially at the cell boundaries where they formed the tight junctional complexes (Figure 3.51a). Nevertheless, a noticeable increase in the expression of ZO1 was observed in the PCSEV-pretreated HBECs. In addition, the quantitative analysis of relative fluorescence intensity values was graphically presented (3.51b). In the analysis, the results showed that the pretreatment of both SDEVs and HDEVs significantly increased the ZO1 expression by 2.03- and 1.82-fold compared to the CVI model. Although it seemed as if HDEVs had a more pronounced effect in preserving ZO1 levels, the difference between SDEVs and HDEVs was found to be statistically insignificant. Besides, another important marker in the CVI is the expression of the F-actin protein. Its role in the healthy cytoskeleton and permeability at the BBB maintains functional endothelial cells and preserves the neurovascular unit. According to the results, it was observed that the pretreatment of PCSEVs supported the cytoskeletal structure and F-actin

integrity under the CVI induction as shown in Figure 3.52. In the result of fluorescence images, it was seen that the CVI model led to a decrease in the expression of F-actin protein, while the treatments of SDEVs and HDEVs preserved their expression and supported the cytoskeleton integrity (Figure 3.52a). Furthermore, the quantitative analysis of fluorescence images was also graphically presented in Figure 3.52b. In the results, although CVI induction did not cause a significant decrease compared to the control group, the pretreatment of SDEVs and HDEVs significantly increased the expression of F-actin protein by 1.38- and 1.29-fold, respectively. As a result, these findings indicated that both PCSEVs were effective in supporting cytoskeletal integrity.

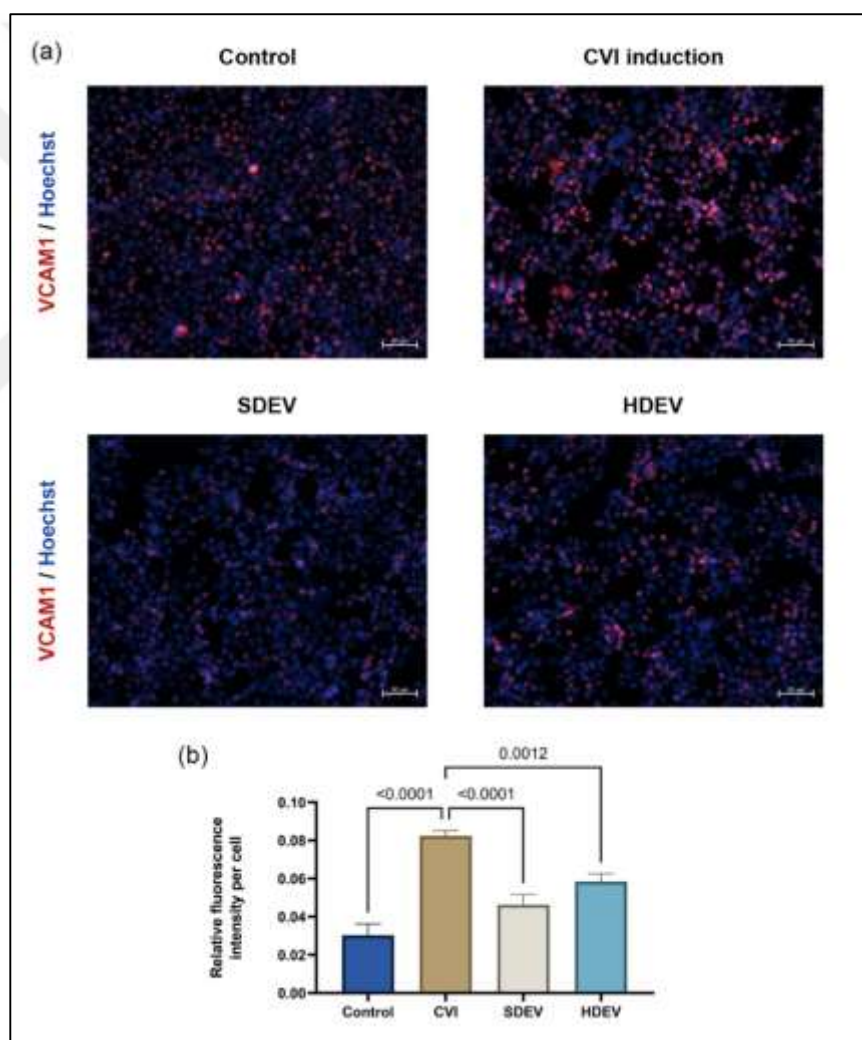


Figure 3.49. The ICC analysis of VCAM1 protein in CVI model in PCSEVs-treated HBECS. The fluorescence images between groups (a) and intensity analysis (b) were demonstrated after 24 hours of the PCSEV pretreatment and 3 hours of CVI induction. Scale bar: 50 μ m. Relative fluorescence unit values were represented as means $\pm$ SD.

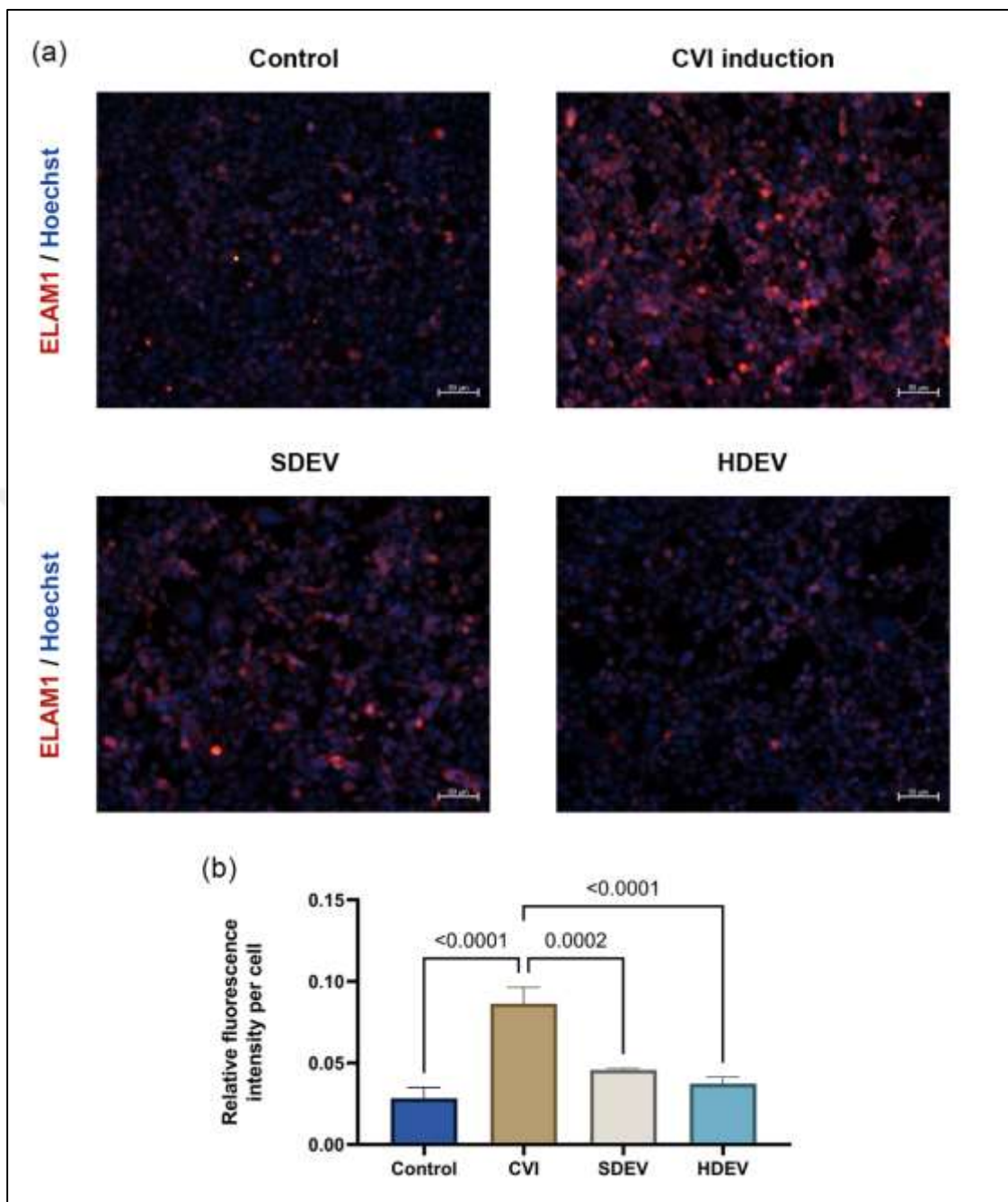


Figure 3.50. The ICC analysis of ELAM1 protein in CVI model in PCSEVs-treated HBECS. The fluorescence images between groups (a) and intensity analysis (b) were demonstrated after 24 hours of the PCSEV pretreatment and 3 hours of CVI induction. Scale bar: 50 μ m. Relative fluorescence unit values were represented as means $\pm$ SD.

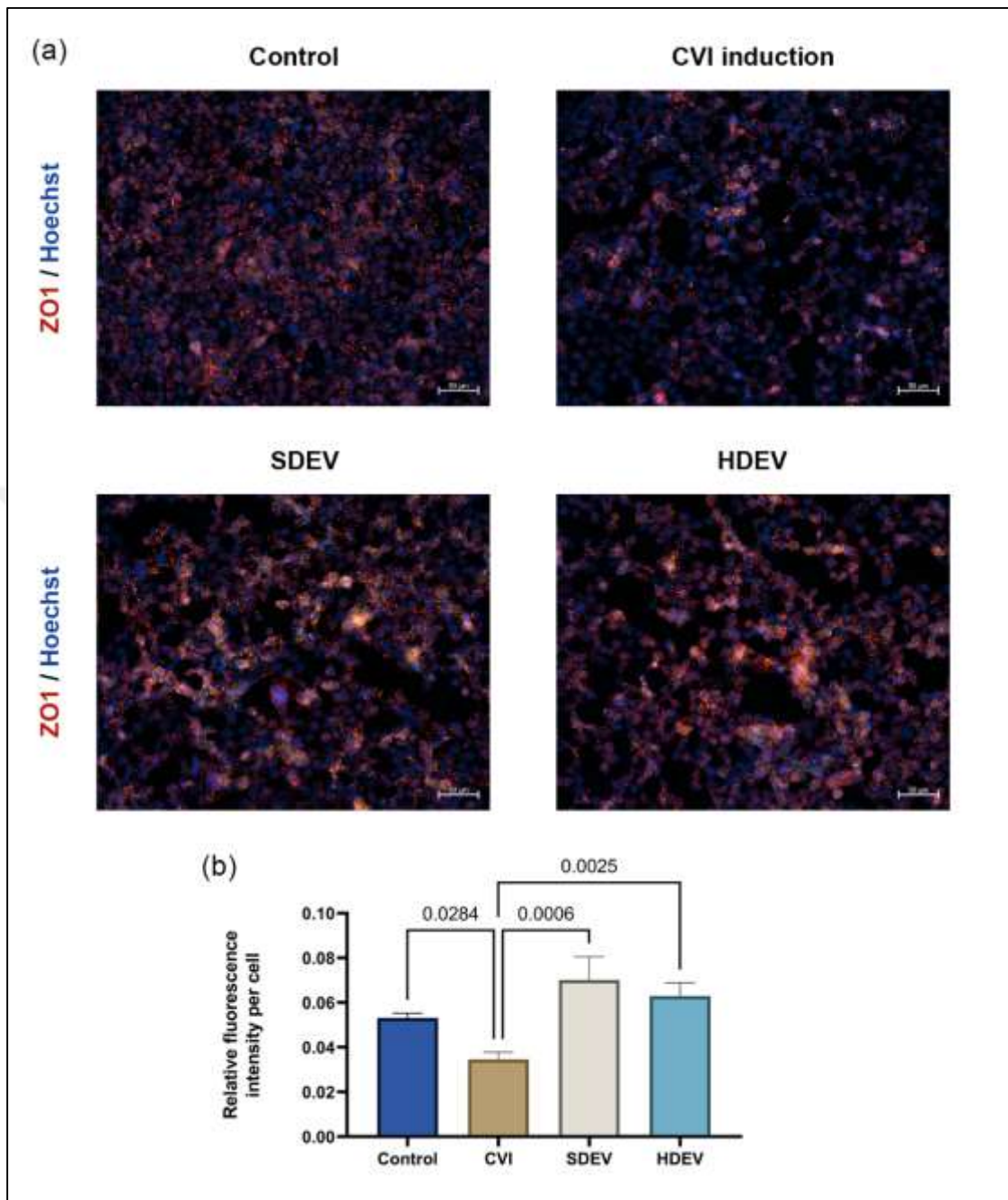


Figure 3.51. The ICC analysis of ZO1 protein in CVI model in PCSEVs-treated HBECs. The fluorescence images between groups (a) and intensity analysis (b) were demonstrated after 24 hours of the PCSEV pretreatment and 3 hours of CVI induction. Scale bar: 50 μ m.

Relative fluorescence unit values were represented as means $\pm$ SD.

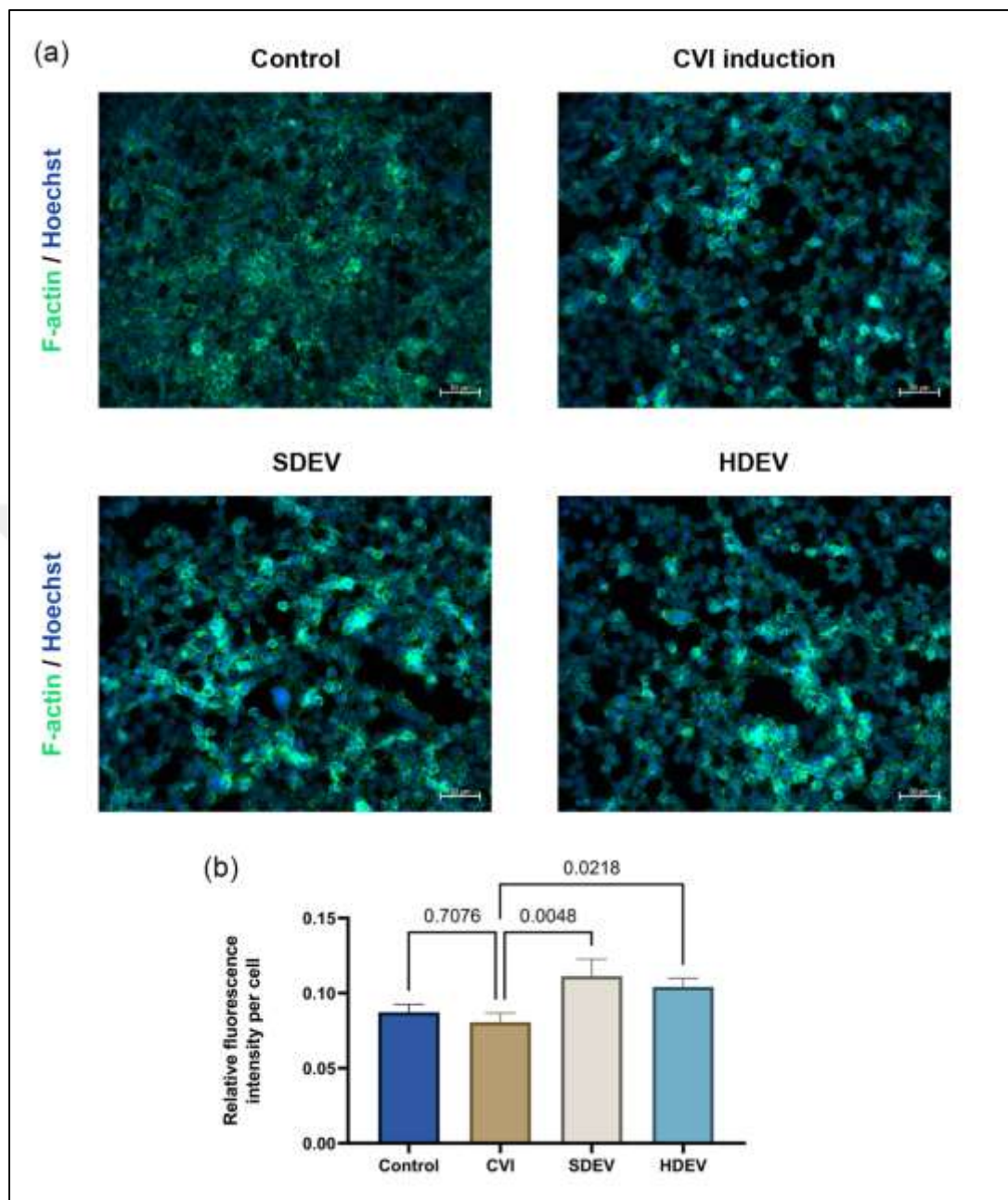


Figure 3.52. The ICC analysis of F-actin protein in CVI model in PCSEVs-treated HBECs. The fluorescence images between groups (a) and intensity analysis (b) were demonstrated after 24 hours of the PCSEV pretreatment and 3 hours of CVI induction. Scale bar: 50 μ m.

Relative fluorescence unit values were represented as means $\pm$ SD.

3.20. INFLAMMATORY CYTOKINE ANALYSIS OF CVI MODEL IN PCSEVS-TREATED HBECS

In examining the effects of the PCSEVs on the CVI model, the analysis of cytokine and chemokine release is quite important aspect of endothelial activation in HBECS. Thus, the CBA analysis was performed to investigate the effects of the PCSEV pretreatment on the CVI induction model on cytokine and chemokine levels as shown in Figure 3.53. According to the results, firstly, it was observed that there is a significant change in the final concentration of only IL8 protein as a result of the CVI induction. Apart from that, no change was observed in the concentrations of IL6, IL10, IL1B, TNF and IL12 proteins released into the media after the CVI induction. Besides, while the HDEV pretreatment did not cause any change in the concentration of IL8 protein, it was seen that the SDEV pretreatment slightly increased the IL8 concentration. Further, there was no significant change in other proteins as a consequence of pretreatment with both PCSEVs. Thus, the results of the analysis indicated that the protective effects of the PCSEVs on HBECS do not occur through inflammation-related cytokines and chemokines.

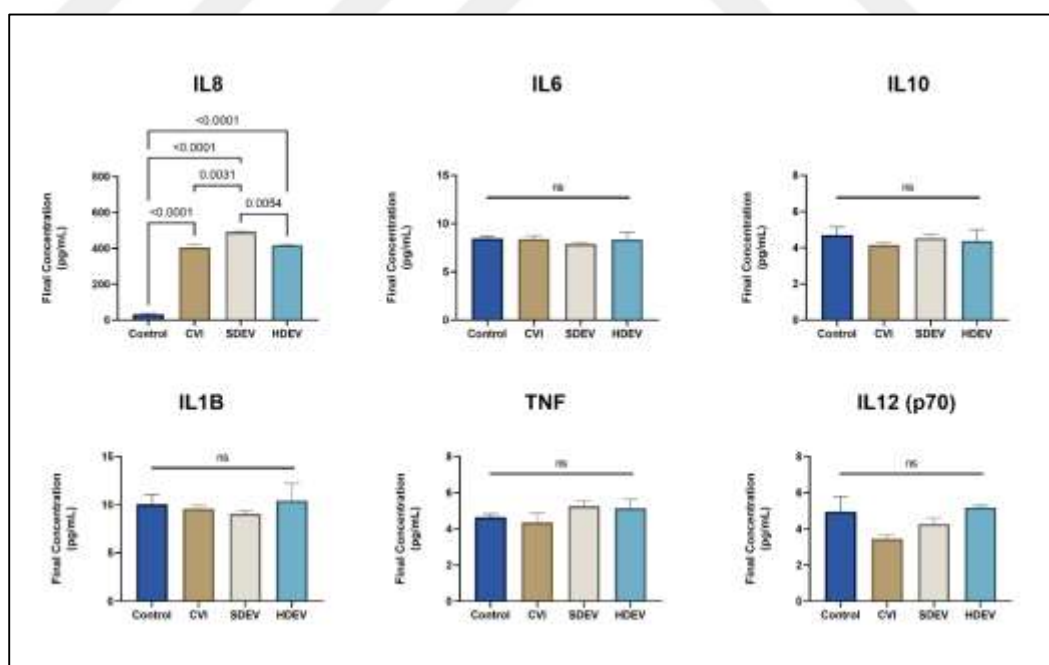


Figure 3.53. The CBA analysis of CVI model in PCSEVs-treated HBECS. The concentration of cytokines and chemokines was measured by CBA analysis and quantitative analysis was graphically represented. The concentration values were represented as means $\pm$ SD.

3.21. T LYMPHOCYTE ADHESION ASSAY OF CVI INDUCTION IN PCSEVS-TREATED HBECs

In the CVI, the interaction of leukocytes with CECs and their recruitment by endothelial cells is quite critical in the sense of preserving endothelial functionality. One of the most important of these intercellular interactions is the leukocyte adhesion to CECs. Accordingly, the T lymphocyte adhesion assay was performed with the Jurkat cells to investigate the changes in the adhesion capability of HBECs pretreated with the PCSEVs under the CVI induction. According to the results, the pretreatment of HBECs with the PCSEVs protected the endothelial cells against elevated adhesion capability as a result of CVI induction as shown in Figure 3.54. In the findings of fluorescence images, the results showed that the number of Jurkat cells adhering to HBECs increased considerably in the CVI induction group (Figure 3.54a). However, upon pretreatment of HBECs with SDEVs and HDEVs, it was seen that there was a noticeable decrease in the number of Jurkat cells adhered to the monolayer form of endothelial cells. Further, the statistical analysis of the fluorescence image results was graphically presented (Figure 3.54b). In the analysis, the results demonstrated that the CVI induction significantly increased the percentage of attached cells to the monolayer form of HBECs. Nevertheless, in the pretreatment of the PCSEVs, it was observed that both types of vesicles reduced significantly the percentage of attached cells compared to the CVI induction group. Furthermore, although both PCSEVs were effective in decreasing the adhesion interaction between the cells, the HDEV pretreatment provided a more significant decline compared to the effect of SDEVs. Consequently, these findings indicated that the PCSEVs could play a protective role in reducing the severity of CVI induction by limiting the recruitment and migration of inflammatory cells into the brain parenchyma.

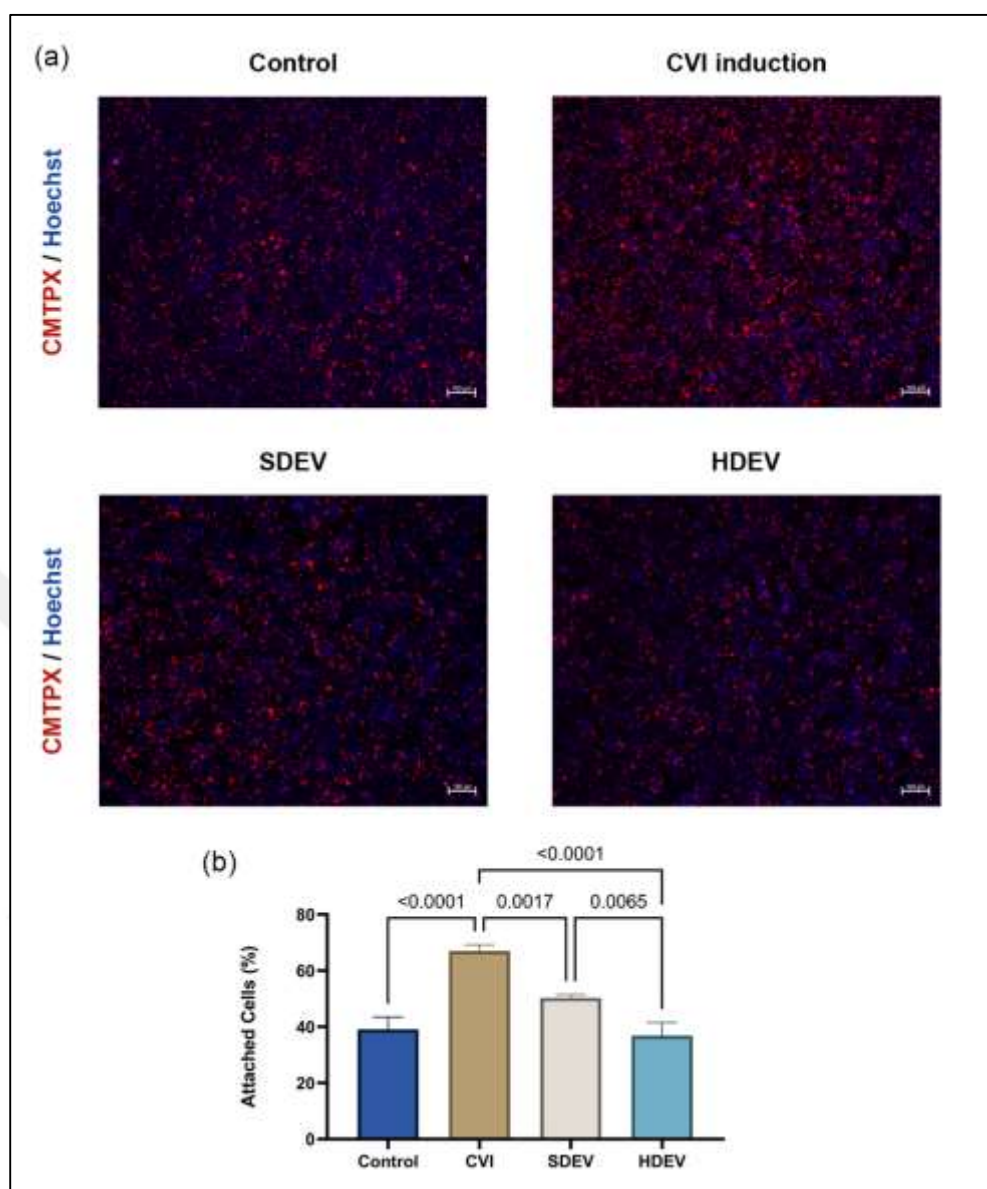


Figure 3.54. The T lymphocyte adhesion assay of CVI model in PCSEVs-treated HBECs. The fluorescence images of the adhesion interactions of HBECs after the CVI induction were presented (a). Further, the quantitative analysis of the adhesion assay was also represented graphically (b). Scale bar: 100 μ m. In the results, percentage values were represented as means $\pm$ SD.

3.22. GENE EXPRESSION ANALYSIS OF PCSEVS-TREATED HBECs

In the study, it was determined that the PCSEVs had a protective effect on HBECs' functions under the CVI induction. However, it has not been known how SDEVs and HDEVs reveal these protective effects on HBECs. Thus, it is quite important to study the information about

the mechanisms of these preventive effects revealed by the PCSEVs. To investigate these effects of the PCSEVs on the expression of anti-inflammation-related genes in HBECs, the gene expression analysis was performed by using the rt-PCR technique. Upon examining the experimental results, it was found that the PCSEVs increased the expression of some protective genes known to have anti-inflammatory and antioxidant properties in endothelial cells as shown in Figure 3.55 and Figure 3.56. In the analysis, the effects of the SDEV treatment on the expression of these genes in HBECs were demonstrated in the heatmap analysis (Figure 3.55a). The results showed the expression of the NRF2 gene increased significantly at the 1.37×10^9 and 1.37×10^8 particles/mL doses of SDEVs by 1.31- and 1.46-fold, respectively, while the expression of PPARG and SIRT1 genes did not change at all (Figure 3.55b). Besides, it was also observed that there was no change in the expression of the KEAP1 gene, which is also related to the NRF2 pathway. On the other hand, both doses of SDEVs showed a significant increase in expressions of HMOX1 and NQO1 genes, which are downstream target genes of the NRF2 pathway. The 1.37×10^9 particles/mL dose upregulated the expression of HMOX1 and NQO1 genes by 13.77- and 3.75-fold, whereas the 1.37×10^8 particles/mL dose increased by 6.92- and 2.56-fold. In the KI67, BCL2 and AKT genes, there were no significant changes in the expression of these genes. Being another important vesicle type in the experiment, HDEVs also affect the critical protective genes in HBECs and these results are shown in the heatmap analysis (Figure 3.56a). In experimental results, it was observed that there was no significant change in the expression of NRF2 and KEAP1 genes as a result of HDEV treatment. However, it was found that the expression of PPARG and SIRT1 gene decreased at the 1.1×10^{10} particles/mL dose of HDEVs. In addition, it was observed that the 1.1×10^{10} particles/mL dose of HDEVs significantly increased the HMOX1 and NQO1 gene by 6.39- and 1.83-fold, respectively. Further, the high dose of HDEVs did not affect the expression of the BCL2 gene, while it decreased the expressions of the KI67 and AKT genes. Considering all these results, it indicated that both PCSEVs affected the NRF2 pathway and emerged the cellular protection response, although it was seen that the effects of both vesicles may differ. Consequently, these findings suggested that SDEVs and HDEVs may play a protective role against inflammation and oxidative stress in the HBECs and activate the anti-inflammatory defence mechanisms such as the NRF2 pathway.

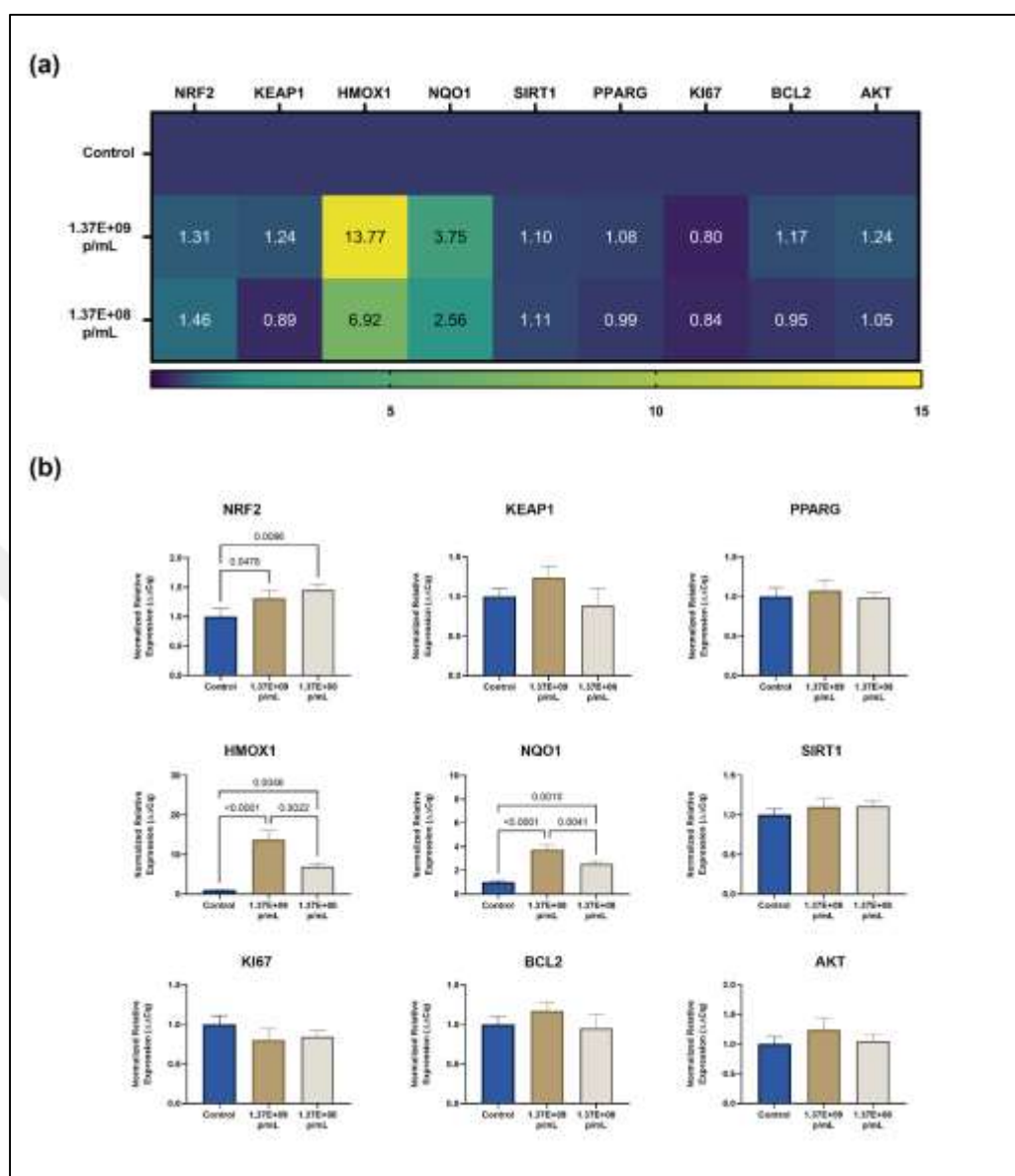


Figure 3.55. The effects of SDEV pretreatment on gene expression of HBECs. Relative normalized expression of anti-inflammation-related marker genes was analysed by rt-PCR and represented in the heatmap analysis (a). The expression of these genes was also represented graphically (b). ATP5F1B was used as a reference gene for normalization. Fold change values were represented as means $\pm$ SEM.

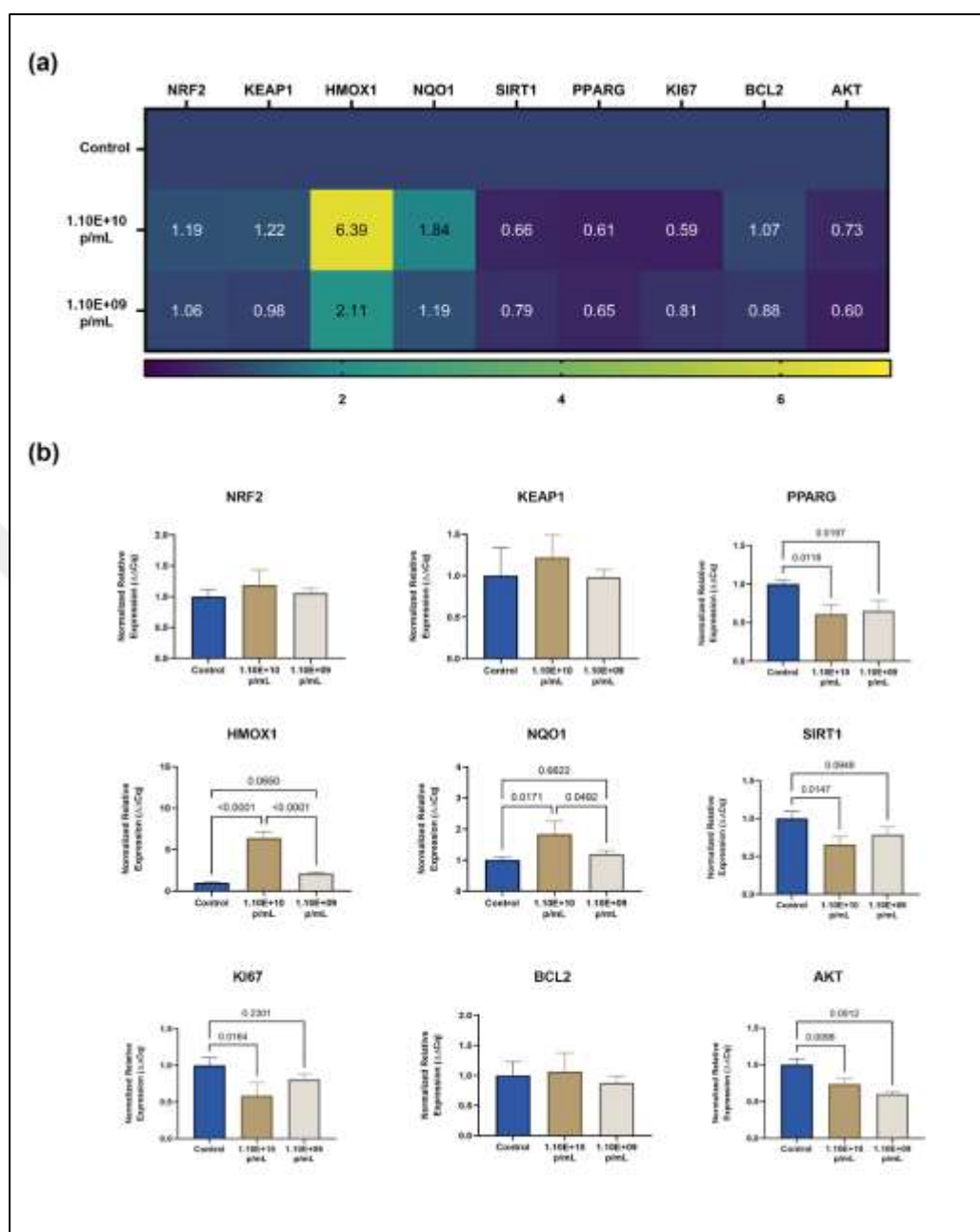


Figure 3.56. The effects of HDEV pretreatment on gene expression of HBECs. Relative normalized expression of anti-inflammation-related marker genes was analysed by rt-PCR and represented in the heatmap analysis (a). The expression of these genes was also represented graphically (b). ATP5F1B was used as a reference gene for normalization. Fold change values were represented as means $\pm$ SEM.

3.23. TIME AND DOSE-DEPENDENT EXPRESSION ANALYSIS OF PCSEVS-TREATED HBECs

In the previous experiment, it was determined that the PCSEVs have a significant effect on the expression of cellular protection-related genes in HBEC under CVI induction. However, to acquire more detailed information about the effects of the PCSEVs, performing time and dose-dependent analyses is of great importance. Thus, in the experiment, the time and dose-dependent expression analysis were performed by using rt-PCR analysis to investigate the effect of these factors on the expression of the HMOX1 gene. According to the results, it was found that both the dose and time factors had effects on the expression of the HMOX1 gene in HBECs as shown in Figure 3.57. In the dose-response analysis, it was proved that the protective effects of both SDEVs and HDEVs are dose-dependent (3.57a and b). In the SDEV treatment, the 1.37×10^9 and 1.37×10^7 particles/mL demonstrated a significant increase by 14.2- and 3.48-fold, respectively, while the 1.37×10^5 particles/mL dose did not lead to any alteration in the expression of HMOX1 gene compared to the control group.

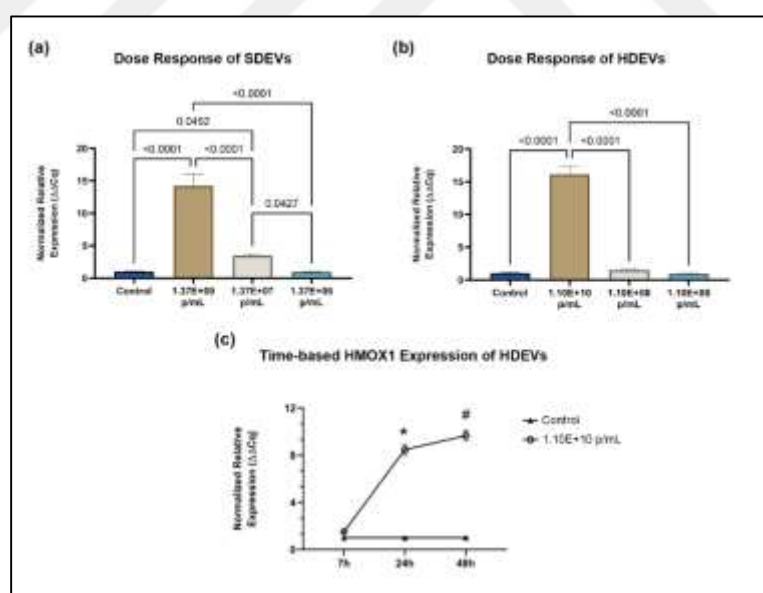


Figure 3.57. The time and dose dependent effects of PCSEVs on HMOX1 expression. Relative normalized expression of HMOX1 genes was analysed by rt-PCR and represented graphically (a, b). The expression of these genes over time was also represented graphically (c). ATP5F1B was used as a reference gene for normalization. In the analyses, the * and # signs indicated a significant difference of less than 0.05 according to control at 24h, and 48h, respectively. Fold change values were represented as means $\pm$ SEM.

Similarly, In the HDEV treatment, the 1.1×10^{10} particles/mL dose significantly increased the expression of the HMOX1 gene. Whereas, at lower concentrations of 1.1×10^8 and 1.1×10^6 particles/mL, no significant change was observed. Besides, it was observed that the treatment time was also an influential factor in the protective effects of the PCSEVs as shown in Figure 3.57c. In the analysis, after the treatment of HDEVs, a significant increase in expression of the HMOX1 gene was observed after 24 hours, and the increase continued for up to 48 hours. Consequently, these findings indicated that the high concentration and long-term effect of the PCSEVs have the potential to preserve endothelial functions.

3.24. WESTERN BLOT ANALYSIS OF PCSEVS-TREATED HBECs

With the gene expression analysis, it was found that the treatment of PCSEVs induced the cytoprotective effect in HBEC via activation of the NRF2 pathway. Nevertheless, it is also necessary to control whether these gene expression results changed significantly in terms of protein expression. To that end, the expression of some proteins related to the NRF2 pathway in the HBECs was investigated after the PCSEV treatment. Upon examining the results, it was observed that the expression of target proteins involved in the NRF2 pathway increased after the PCSEV treatment as shown in Figure 3.58. Furthermore, the protein bands and their alteration between groups were also clearly seen in the membrane images in the western blot analysis (Figure 3.58a). In the analysis, it was found that the treatments of both SDEVs and HDEVs significantly affected the expression of NRF2 protein (Figure 3.58b). However, in this protein, the results demonstrated that there are two isoforms of the NRF2 related to different activated statuses of the protein. According to our results, the expression of the 100 kDa isoform of NRF2 protein demonstrated a significant increase of 1.98- and 1.32-fold at both SDEVs and HDEVs, respectively. On the other hand, the SDEV treatment did not cause any changes in the expression of 68 kDa isoform, while the HDEV treatment led to a significant increase by 1.49-fold in its expression. Besides, examining the results of NQO1 expression, it was found that its expression significantly increased as a result of PCSEV application. In the analysis, the expression of the NQO1 enzyme increased by 2.04- and 2.45-fold at the treatments of SDEVs and HDEVs, respectively. Nevertheless, it was seen that there was no significant difference between the PCSEVs. As a result of these findings, it was understood that the PCSEVs increased the expression of the target protein in the NRF2

pathway and its downstream genes. Thus, it seemed that the PCSEV treatment has a potential antioxidant and cytoprotective effect via the NRF2 pathway.

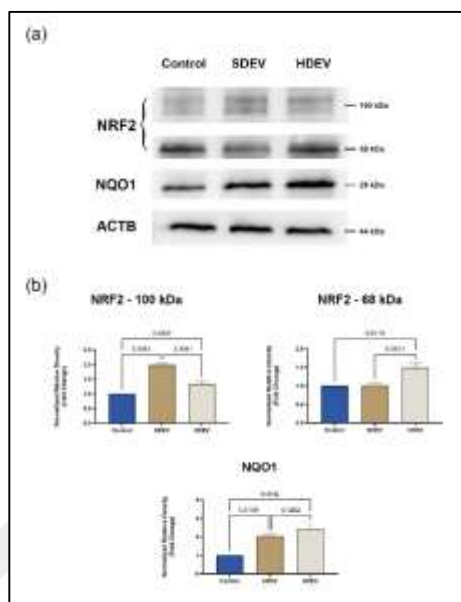


Figure 3.58. The effects of PCSEV treatment on protein expression levels in HBECS. Relative normalized protein expression of target protein related to NRF2 pathway (a) was analysed by western blot analysis and densitometric results (b) were compared statistically. ACTB expression was used as an internal control. Fold change values were represented as means $\pm$ SD.

3.25. CAPILLARY ELECTROPHORESIS ANALYSIS OF PHOSPHORYLATED PROTEINS IN PCSEVS TREATED HBECS

In the study, considerable data has been found that the PCSEVs have a cytoprotective effect on endothelial health and function via the NRF2 pathway. However, it is still unknown how these nanovesicles affect the phosphorylation status and thus the activation of critical upstream pathways while inducing the previously mentioned effects. Therefore, to investigate the potential effects of the PCSEVs on these pathways, the phosphorylation levels of some target regulatory pathways were analysed by western blot analysis. According to our results, it was found that the PCSEVs have significant effects on these pathways in the HBECS as shown in Figure 3.59. In the phosphorylation level of ERK 1/2 (p-ERK 1/2) protein, the results revealed that there was a significant increase of 2.12-fold in the SDEV treatment compared to the control, while the HDEVs did not affect its phosphorylation level

at all. However, the Phosphorylation of Akt (p-AKT) protein was significantly decreased by 3.23- and 3.33-fold in the treatments of both SDEVs and HDEVs. Furthermore, a similar pattern was observed in the p38 phosphorylation (p-p38). Both SDEV and HDEV treatments significantly reduced p-p38 levels by 2.25- and 2.13-fold compared to the control group. Besides, no significant difference between the two vesicle treatments was detected in both p-AKT and p-p38 levels. Consequently, these results suggest distinct modulation of upstream signaling pathways by plant vesicles in endothelial cells.

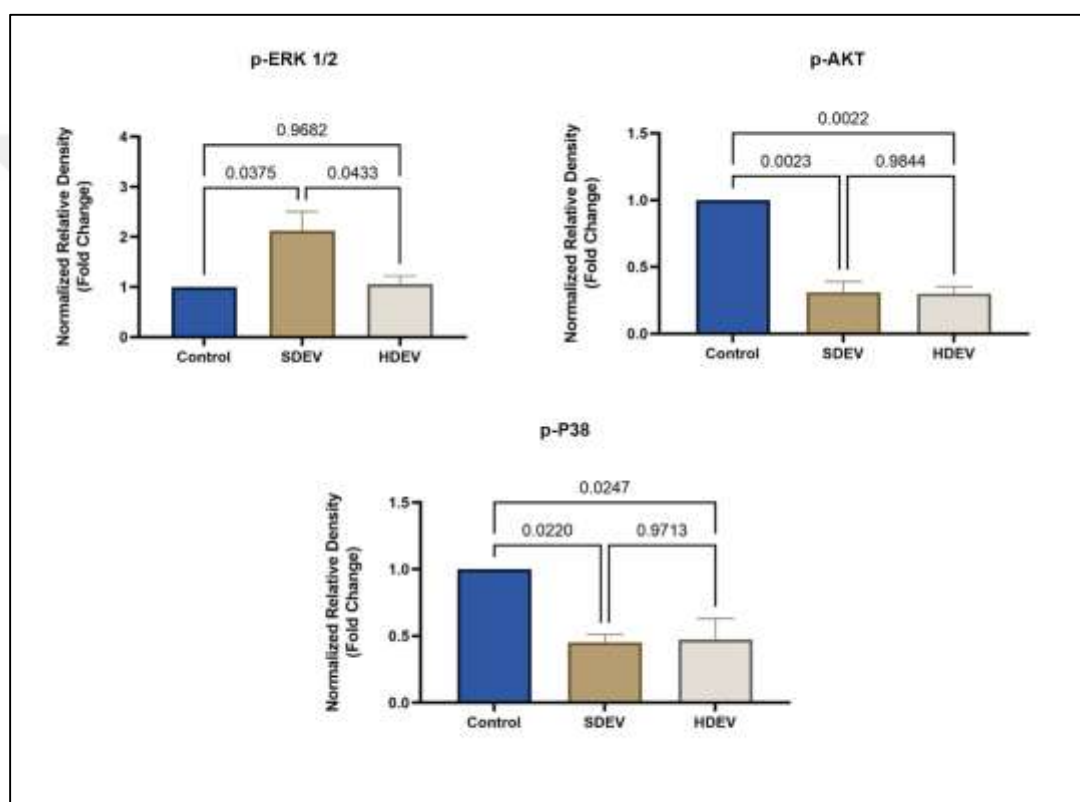


Figure 3.59. The effects of PCSEV treatment on phosphorylation levels of upstream pathways in HBECS. Relative normalized expression of phosphorylated proteins was analysed by Simple Western analysis and densitometric results were compared statistically. ACTB expression was used as an internal control and the phosphorylation levels were normalized with ERK1/2, AKT and P38 total protein levels. Fold change values were represented as means $\pm$ SD.

4. DISCUSSION

4.1. PHYSICAL AND MOLECULAR CHARACTERIZATION OF PCSEVS

Recent studies demonstrated that PDEVs have the potential for therapeutic properties [106]. However, these exciting findings have unfortunately led to ignoring fundamental problems such as their purity, undamaged vesicle structure, and variation in source plants [206]. Moreover, there were hardly any studies in the literature on developing solutions to these bottleneck problems. Thus, in our study, we tried to offer a new solution to these problems by purification of PDEVs from the PCSC-derived medium using an ultrafiltration-based process. In this way, it is thought that problems such as fragmentation of plant cells, internal vesicle contamination, heterogeneity and high variation among source plants are eliminated. Furthermore, considering that the plant tissues were disrupted with a blender in previous studies, the PCSC-derived media are quite pure starting solutions. In our study, both the physical and molecular properties of the PCSEVs purified from these media were characterized in a detailed manner.

In characterizing nanovesicular features, it is crucial to consider findings related to the size distribution and concentration of the PCSEVs. Since there is hardly any study in the literature, the characterization data regarding the PCSEVs were evaluated with findings in studies on PDEVs. In the literature, it was determined that the sizes of extracellular vesicles are generally less than 200 nm [207]. Whereas, in some studies, the sizes of PDEVs were found to be between 200-400 nm [208,209]. In our study, the sizes of PCSEVs decreased toward the end of the purification process and were around 100 nm in the final group. Plausibly, we think that the differences in size distribution resulted from the benefits of the plant cell suspension culture. Therefore, it seems appropriate to describe the obtained vesicle population as small extracellular vesicles according to MISEV guidelines [207]. On the other hand, it is quite difficult to talk about any agreed standard regarding nanoparticle quantities in literature. In PDEV studies, vesicle concentrations were close to or above 10^{11} particles/mL, although the NTA surprisingly was not preferred as a quantification method in several studies. In our study, it was determined that the vesicle quantities in the final groups were between 10^9 and 10^{10} nanoparticles [120,209]. It was thought that the differences between the nanoparticle quantities were caused by the effect of internal vesicles and membrane fragments on the NTA measurements.

Another factor affecting the PCSEV quantities in our study is the ultrafiltration-based purification method. Especially when the filtrate and concentrate groups were examined, it was found that PCSEVs were successfully concentrated by the ultrafiltration column. In MISEV2023, it is also mentioned that low molecular weight cut-off columns such as 10 kDa may also retain the contaminants [207]. In our study, to investigate this phenomenon, we measured the pH of the solutions in the purification steps. Consequently, it was found that the pH values reached a neutral level with the removal of plant-derived contaminants from the stock to the final group. Furthermore, the pH of the filtrate group being the same as the stock group proved that the medium-derived contaminants passed through the column to a large extent. Another purity criterion based on the nanoparticle quantities per μg of proteins is included in the literature [210]. However, it is doubtful that this parameter, which first appeared for mammalian extracellular vesicles, may also be feasible for PDEVs. Because we think that the main contaminants in PEV studies are plant-derived chemicals such as ascorbic acid, and tannic acid rather than proteins. The possible presence of these chemicals is also inevitable to interfere with protein measurement assays [211]. Still, in our study, the purity of PCSEVs was determined according to this criterion. At this point, unfortunately, a more suitable and more accurate alternative is needed to determine the purity of PCSEVs.

Atomic microscopic analysis of the PCSEVs is quite important for the visualization of vesicular structures and validation of NTA results. In our study, these findings were obtained by examining PCSEVs with AFM analysis. In AFM images in the literature, the extracellular vesicles were mostly found to be close to spherical shape [212]. Although the AFM results are generally consistent with the NTA data, it was found that the sizes of extracellular vesicles increase and may even change (cup-shaped structure) in techniques such as AFM or transmission electron microscopy (TEM) [213]. In the studies, it was suggested that these alterations in extracellular vesicles are caused by the drying process in the sample preparation. In our findings, the PCSEVs of both plants have a spherical shape and surprisingly, any deterioration did not occur in their structures such as cup-shaped. However, in the AFM results, the sizes of the PCSEVs increased to the range of 180-200 nm, possibly due to the drying process. Consequently, the obtained findings were consistent with both the literature and characterization data.

One of the decisive findings in the molecular characterization of the PCSEVs is the profiling of fatty acid composition. Thanks to this analysis, many novel findings regarding the

vesicular membrane structure will be obtained. In the literature, no studies could be found for the profiling of fatty acids of the PSCs. Nevertheless, studies have been found to investigate the fatty acid profiles of these plants. In the study of the *S. rebaudiana* plant, it was found that the fatty acid composition consisted of mostly UFAs and to a lesser extent SFA species such as palmitic acid and stearic acid [214,215]. Moreover, studies regarding the *V. hispanica* plant also showed a similar distribution to the results of studies regarding the *S. rebaudiana* plant [216]. Looking at our findings, it was found that the fatty acid compositions of PSCs were mostly similar to the literature. However, unlike the literature, a significant presence of cis-vaccenic acid was detected in both plants. We think that these differences in cis-vaccenic acid were due to the PEV source used in the purification. Although there were studies related to the lipid contents of PDEVs in the literature, no studies on the fatty acid compositions could be found. In our study, the fatty acids of the PCSEVs were profiled for the first time. Our findings showed that the membranes of the PCSEVs were highly enriched in SFA, unlike the PSCs. With this sharp increase in the SFAs, the PCSEVs were expected to have a more rigid and less permeable membrane than the PSCs [217,218]. Indeed, we think that this finding is also consistent with the inherent characteristics and functions of PDEVs. Eventually, more comprehensive information about the fatty acid composition of PDEVs will be obtained with new studies in the future.

Protein compositions of the PCSEVs also provide valuable information about the structural, functional, and cellular origins of vesicles. Although there are many studies in the literature on the protein composition of PDEVs, there is only one study related to PCSEVs [206,219,220]. In one of these studies, proteomic analysis of PDEVs obtained from the leaves of *Arabidopsis thaliana* was performed. In the results, proteins from many cellular components, including the endoplasmic reticulum, were detected in the proteomic analysis of PDEVs obtained by cell lysis. Moreover, these types of proteins were found in PDEVs obtained by apoplastic wash [221]. In the literature, the apoplastic wash method was presented as an alternative for obtaining PDEVs instead of the disruption of plant tissues as a whole. Nevertheless, the apoplastic wash method has also disadvantages such as damaging the plasma membrane of the cells or causing stress by submersion and pressure during the infiltration. On the other hand, it was reported in MISEV2023 that proteins associated with the intracellular organelles should not be enriched in small extracellular vesicles [207]. We think that the most apparent reason for these findings is the release of intracellular vesicles

and organelles-related proteins due to the lysis of plant cells. In the literature, it was reported that cytosolic proteins may also be found in abundance in PDEVs obtained from some plant sources [222]. In our findings, it was found that the protein composition of SDEVs and HDEVs was mostly related to proteins associated with the extracellular structures and functions. Furthermore, the close association of all GO terms detected in the SDEVs and HDEVs suggested that the protein composition of the extracellular vesicles might be chosen in a controlled manner. Accordingly, we think that the purification of PDEVs without lysis of plant cells contributes quite positively to obtaining these results. Besides, another factor that negatively affects the proteomic analysis in PEV studies is the presence of non-vesicular proteins. It seems quite possible that non-vesicular proteins may interfere with the proteomic results due to giving false or irrelevant results in the analysis when they are abundant in the PEV solution. Considering our findings, it was proven that the presence of non-vesicular proteins in the PCSEVs is quite low. Therefore, it is thought that the protein composition of PCSEVs is not affected by the non-vesicular proteins in our proteomic analysis. Eventually, the presence of enriched GO terms such as response to oxidative stress and peroxidase activity revealed whether the PCSEVs may also have therapeutic effects.

A functionally important feature in the characterization of extracellular vesicles is their successful uptake by recipient cells. Since the cellular uptake process of PDEVs is visualized with the help of a fluorescent tracker dye, the results should be evaluated quite carefully. In the literature, it was seen that the studies on the cellular uptake of PDEVs were relatively high [120,223–225]. In most of the studies, lipophilic dyes such as PKH dyes were preferred as fluorescent markers. However, in recent studies, negative findings were discovered on the use of these lipophilic dyes in the tracking of extracellular vesicles [226,227]. These findings are mostly related to leakage of dye from the vesicle membrane or failure to be removed efficiently. Besides, another finding discovered in the studies is that the uptake efficiency varies depending on the incubation time and nanoparticle quantities [120]. In our findings, firstly, it was found that the PCSEVs of both plants were successfully labelled with CMFDA dye and after the removal process, the free dye did not interfere with fluorescent NTA measurements. Related to these findings, it was seen that both HDEVs and SDEVs were taken up by the Jurkat cells after 2 hours of incubation. Besides, it was found that the difference between HDEVs and SDEVs was due to the nanoparticle concentrations used in the experiment. On the other hand, the absence of any increase in the fluorescent signal of

the dye control group indicates that the CMFDA dye was successfully removed. This finding was also consistent with the absence of any signal from the fluorescent control samples in the NTA analysis. We think that this finding is caused by the activation and fluorescence signal emission mechanism of the CMFDA dye. Besides, the use of CMFDA dye in our study had crucial contributions to the cellular uptake assay, unlike the adverse outcomes of lipophilic dyes reported in the literature [226,227]. Thus, CMFDA dye appears to be a powerful alternative to lipophilic dyes in the cellular uptake experiment.

4.2. IN VITRO MODELING OF CEREBROVASCULAR INFLAMMATION

In studies conducted in cell culture, cell confluence is one of the critical factors, affecting the cellular characteristics of *in vitro* culture models. Due to the mechanisms such as cell-cell contact inhibition, the cell confluence is also an important parameter in CVI pathology intended to be modelled in our study. In the literature, it is seen that especially in studies on endothelial cells, the monolayer culture technique, which has high confluency and a slowed-down division rate, is successfully performed [228,229]. Nevertheless, since the high density of cells can result in cell death in the case of prolonged contact inhibition, the viability and functionality of the cells is also a critical point [230]. In these studies, any results associated with the cytotoxicity were not reported on the endothelial cells in the monolayer culture model. In other studies, it is observed that the expression of tight junction molecules decreases and several gaps occur between the cells as a result of PMA/Ionomycin treatment [229,231]. In our results, it was found that the monolayer culture model and PMA/Ionomycin treatment did not cause any cytotoxic effects on the HBECs. Furthermore, as consistent with the literature, alteration in cells' phenotypes and decreased intercellular junctional interactions were determined in the CVI model. Consequently, it is understood that the monolayer culture model is suitable in terms of its resemblance to the targeted endothelium tissue in the CVI model.

Besides the effects of the monolayer culture technique, the effect of the CVI model on the cell viability of HBECs is also quite important point. The CVI induction model is aimed to stimulate a cellular dysfunction in the HBECs similar to the formation of endothelial activation in cerebrovascular tissue, but not to cause any cytotoxic effects. In the literature, it seems that there is no study on the effect of a PMA/Ionomycin-induced CVI model on the proliferation and viability of the HBECs due to the relatively small number of studies on

CECs [232,233]. On the other hand, there are some studies in the literature indicating that PMA/Ionomycin induction increases the proliferation of T lymphocytes [234,235]. In our results, it was observed that the absorbance values of the MTS assay increased as a result of CVI induction. One of the possible inferences of this result was that CVI induction stimulated the proliferation of HBECs. However, these results were not compatible with the microscopic images taken from the monolayer culture model. Accordingly, to confirm the MTS results, the effect of CVI induction on the cells was also evaluated with a trypan blue assay. According to the experiment results, it became evident that the findings of the MTS assay are completely contradictory. Upon examining the literature, it is understood that the absorbance increase in the MTS assay is related to the effect of high Ca^{+2} levels caused by PMA/Ionomycin induction on mitochondrial enzymes [236]. Besides, it is thought that the slowdown in cell proliferation after PMA/Ionomycin treatment is more closely related to the inflammation state in the endothelial cells [237]. Consequently, it is understood that the CVI model performed at high doses and prolonged hours does not show any cytotoxicity on the monolayer culture model of HBECs and is suitable enough to use in our study.

The functions of mitochondria also play a crucial role in the activation or dysfunction of HBECs. In this respect, the effect of modelled CVI pathology on the mitochondria of HBECs is also one of the attractive topics in the study. On the other hand, it could not be found any studies in the literature on the effects of CVI induction on the mitochondrial membrane. Nevertheless, a study investigated the effects of endothelial activation models induced by various molecules on mitochondrial membranes using the Rhodamine 123 dye [238,239]. In the study, it was investigated that the hydrogen peroxide treatment caused a cytotoxic effect on endothelial cells. Subsequently, it was observed that Rhodamine 123 dye left the cells due to emerging damage in the mitochondrial membrane. Therefore, it was understood that there is a relationship between the emerging cytotoxicity and the mitochondrial membrane integrity. Furthermore, in another study conducted with microglia, Rhodamine 123 staining was used to examine the inflammatory status in the cells and the contribution of mitochondrial dysfunction to proinflammatory processes was emphasized [240]. In our results, it was observed that the CVI induction led to a significant decrease in the mitochondrial membrane potential of HBECs. Therefore, it is thought that this decline negatively affects endothelial functions by impairing mitochondrial functions in the CVI model. Furthermore, it is found that our experimental results on mitochondrial membrane

potential are consistent with the literature. Consequently, it is determined that the modified CVI model is compatible with the endothelial activation characteristic similar to the cerebrovascular tissue.

One of the important features in both activation and dysfunction of endothelial cells is the elevated intracellular Ca^{+2} levels. Since endothelial cells use calcium ions as a secondary messenger, they regulate the intracellular concentration with very subtle mechanisms. Furthermore, it is known that the increase in intracellular Ca^{+2} levels is directly related to CVI pathology. In literature, there are many studies examining the relationship between PMA/Ionomycin induction and an increase in intracellular Ca^{+2} concentration [241–244]. In these studies, it was found that PMA/Ionomycin treatment at various doses and times increased the intracellular Ca^{+2} levels in both endothelial cells and T lymphocytes. Furthermore, studies in the literature reported that increased Ca^{+2} concentration within endothelial cells caused CVI-related conditions such as endothelial dysfunction, hyperpermeability, and severe inflammation [245–247]. In our results, it was also found that the CVI induction significantly increased the intracellular Ca^{+2} levels in HBECs. As a result of the series of reactions triggered by this Ca^{+2} elevation, it is understood that the *in vitro* CVI model has a noticeable similarity to the cellular pathology in the cerebrovascular tissue. Comparing with the studies in the literature, it is also determined that the close relationship between the increase in Ca^{+2} concentration of HBECs and the CVI model is consistent with data in the literature. Therefore, it is understood that the *in vitro* CVI model in HBECs is consistent and compatible with the CVI pathology.

In modelling CVI pathology in CECs, changes in gene expression of HBECs and their conformity with activation characteristics have great importance. However, in the literature, it is seen that endothelial cells are induced with various PMA/Ionomycin doses in some studies. For instance, some studies used 40 nM/1.34 mM PMA/Ionomycin as a high-dose treatment, while others utilized 16 nM/250 μM PMA/Ionomycin as a low-dose treatment [248,249]. Furthermore, considering that the cell types in the studies were also different, it was necessary to determine an appropriate dose to be used in the CVI model. In this context, its appropriate dose was decided based on the changes in the expression of the IL8 gene in HBECs. According to the results, it was observed that the expression of the IL8 gene decreased significantly as the PMA/Ionomycin dose was diluted. Nevertheless, the expression of the IL8 gene increased significantly compared to the control group even at the

lowest dose. In the study, to confirm the accuracy of the results of the rt-PCR technique, the gene expression analysis by agarose gel electrophoresis was performed and the obtained results. However, in the experiment, lower doses below the Dose-3 were not investigated because they were well below the limits in the literature. Consequently, it was decided to use the 16 nM/250 μ M PMA/Ionomycin treatment for the CVI induction used throughout the study. Therefore, it is understood that experimental results are compatible with the data in the literature in terms of changes in the expression of the IL8 gene and the determined dose.

In the literature, only a few studies have investigated PMA/Ionomycin induction in endothelial cells [250,251]. Upon examining the results, it was observed that some genes related to endothelial activation were upregulated following PMA/Ionomycin induction. Furthermore, other studies also reported the effects of this treatment on various cell types. In these studies, it was demonstrated that the PMA/Ionomycin treatment induced the upregulation of genes related to proinflammatory responses and cellular adhesion [252–255]. Besides, genes associated with anti-inflammatory and antioxidant activities such as SIRT1 and NRF2 have more controversial results. In literature, it has been well-known that the protective genes in our study naturally increase in response to both inflammatory response and treatment with protective molecules [249,256]. Thus, the expression levels may vary depending on the experimental conditions. Another influential group of studies was about the expression of genes related to cell survival and proliferation. In these studies, it is seen that the effects of PMA/Ionomycin treatment on these genes vary between cell types. In studies conducted with T lymphocytes, the expression of survival and proliferation genes was upregulated [257,258], whereas the PMA/Ionomycin treatment was known to induce apoptosis in some cell lines [259,260]. Further, the expression of genes related to the angiogenesis process also plays an important role in the emergence and progression of CVI pathology. In the literature, it was stated that the increased angiogenic response associated with endothelial activation elevated the permeability of the BBB and thus led to the CVI condition [261]. Besides, one of the important gene groups is the junctional complexes related genes, which are found between endothelial cells and have a decisive role in sealing the paracellular cleft. Thus, they are directly related to the permeability of the BBB, which is a notable consequence of CVI pathology. In the literature, it is known that the expressions of tight junction complexes are regulated at the post-translational level rather than directly

downregulated at the transcriptional level [262,263]. Nevertheless, some studies reported that inflammatory stimulation can significantly decrease the expression of the ZO1 gene in endothelial cells [264]. Lastly, the gene expression of matrix metalloproteinases (MMPs), being another substantial gene group, is also quite decisive in CVI pathology. Many studies in the literature have proven that MMPs have a detrimental effect on endothelial functions in cerebrovascular disorders [265,266]. It was reported that the expression of the MMP3 gene, which is expressed at a very low level in CECs in healthy conditions, was upregulated as a result of cerebrovascular dysfunction [267]. In our results, changes in the expression of the analysed gene groups were similar to expression patterns of genes associated with endothelial activation. In the study, it was observed that the expression of IL8 and MCP1 genes responded to the activation signal as consistent with the literature, while the expression of the IL6 gene did not increase at the same rate. It is thought that this finding is due to the differences in the gene expression profile of HBECs and HUVEC cell lines used in most of the studies. On the other hand, it was observed that the protective genes such as PPARG, SIRT1 and NRF2 increased in the CVI induction. It appeared that these findings were related to the cells' response to PMA/Ionomycin induction. As another finding consistent with the literature, there was a significant upregulation in the expression of cellular adhesion genes such as VCAM1 and ELAM1, which is quite resembling a response in the cerebrovascular tissue. Besides, in the angiogenesis-related genes, it was observed that PMA/Ionomycin induction upregulated the expression of the VEGFA gene, but did not affect the expression levels of other related genes. Accordingly, it is thought that this finding arises from the specific effect of the CVI model on the expression of the VEGFA gene rather than directly affecting the angiogenesis process. Lastly, in the analysis of tight junction and MMP-related genes, it was observed that the expression of the ZO1 gene was downregulated, while the expression of the MMP3 gene was upregulated as a result of CVI induction. Consequently, it is determined that the PMA/Ionomycin treatment induces the expected alterations in the expression of CVI-related genes in HBECs.

Through the analysis of gene expressions in the CVI model performed on HBECs, it was found that the cells demonstrated the CVI-related markers in the gene expressions. However, besides these results, it is quite essential to evaluate the protein expressions of the relevant genes and their resemblance to the endothelial activation condition in the cerebrovascular tissue. In the literature, there are studies investigating the effects of PMA/Ionomycin

induction on the expressions of several marker proteins. However, the number of studies conducted with endothelial cells is very few, and mostly, these studies have been conducted by investigating immune cells such as T lymphocytes [256]. In some studies, it was observed that the expression of SIRT1 protein increased as a result of PMA/ionomycin treatment [249]. Besides, there is no study in the literature examining the effects of CVI modelling on the expression of PPARG, PAI1, and FOXO1. On the other hand, considering the expression of NRF2 protein, it is seen that there are some controversial points in the literature. First, according to the manufacturers, the predicted molecular weight of the NRF2 proteins is estimated as 68 kDa. However, in some studies, it was claimed that this estimated molecular weight belonged to the inactive form of the NRF2 protein and that the active form of the proteins was 100 kDa, and this result was proven in the study [268]. In this case, it is thought that the expression of NRF2 protein increases as a result of the activation of endothelial cells with the CVI induction and that the expression of the form at 68 kDa decreases accordingly. Furthermore, it is found in the literature that the expression of NRF2 protein in cells elevates after the PMA/Ionomycin induction [256]. Finally, in another study, it was stated that the expression of ANG2 protein was increased as a result of CVI induction [251]. In our results, firstly, it is found that the changes in expressions of NRF2 and ANG2 are consistent with the literature, but the expression of the SIRT1 protein is different from the studies in the literature. Thus, it is thought that the inconsistency in the result of SIRT1 is mainly due to the differences in cell types. Because, in these studies, the expression of the SIRT1 protein was studied in the HUVEC cell line. It was revealed in the literature that there are differences in the activation characteristics of endothelial cells originating from different tissues [269]. Apart from these, we consider the increased expression of PPARG protein as a cellular response to CVI induction, but, surprisingly, there was no increase in the expressions of FOXO1 and PAI1 proteins. At least theoretically, an increase in the expression of these inflammation-related proteins was expected. Consequently, it is determined that some critical proteins in HBECs related to CVI pathology changed significantly as a result of PMA/ionomycin treatment.

In the investigation of protein expressions of cells about the modelling of CVI pathology in HBECs, ICC analysis was used as another important experimental method. In this respect, it was investigated how the expression of marker proteins altered in the CVI model. In the literature, no studies could be found related to the expression of CVI-associated protein

markers in HBECs via ICC analysis following the PMA/Ionomycin induction. Therefore, many CVI-related proteins analysed via the ICC technique in our study were visualised in HBECs for the first time in the literature. However, there are few studies about endothelial activation or dysfunction in CECs induced by various cytokines [270,271]. In these studies, only the tight junction-related proteins were analysed by using the ICC technique. These studies reported that the expression of ZO1 and Occludin proteins decreased noticeably after the induction of inflammatory stimulation in CECs. Upon examining our results, it was also found that the CVI model induction similarly demonstrated a significant decrease in the expression of these genes. Nevertheless, it was observed that there was no change in the expression of adherens junction proteins such as CDH5 according to the results. In the literature, it was stated that this finding is due to the destruction of adherens junctions in chronic and more detrimental inflammatory conditions. Consequently, it is understood that the obtained results from the ICC analysis are supportive in terms of the accuracy of the CVI model in HBECs.

In terms of the characteristic features of the CVI model in HBEC cells, both transcriptional and translational results obtained in the study were consistent with each other. However, the synthesis of these marker proteins by endothelial cells as well as their successful release from the cells to the extracellular environment is quite critical. In literature, studies on the analysis of cytokines released into the extracellular environment from PMA/Ionomycin-induced cells have mostly used PBMCs or T lymphocytes instead of CECs [232,257]. Accordingly, in these studies, it was observed that decisive inflammatory factors such as IFNG and TNFA were produced and released into the culture medium by PBMCs. In our results, it was found that there was a significant increase in only the IL8 protein level among the cytokines in the CBA kit and there was no change in the IL6 levels in the CVI model in HBECs. Furthermore, it indicates that these results are consistent with the findings in the gene expression analysis in terms of the expressions of these two proteins. Thus, with all these results, the effects of the CVI model on the protein expressions of HBECs were investigated and it is understood that these results are consistent with the activation characteristics in the cerebrovascular tissue.

Angiogenesis is one of the key factors influencing the stability and functionality of BBB in CVI-related disorders. In these conditions, the angiogenic capability of cells has a dual effect on cerebrovascular physiology and inflammation. Uncontrolled and abnormal angiogenic

activity could cause hyperpermeability and chronic inflammation, while angiogenesis is needed for tissue remodelling and oxygenation. Thus, the maintenance of cerebrovascular physiology depends on the control of angiogenic activity. However, any study could not be found in the literature on the effects of the CVI model on the angiogenesis process in HBECs. Nevertheless, there are some studies in the literature that were conducted using endothelial cells from other tissues or only by treated PMA solution [272–274]. The results of these studies found that both PKC pathway activation and TNFA treatment-induced inflammation in endothelial cells and increased angiogenesis. On the other hand, in another study using only PMA, it was stated that it could increase the angiogenesis potential in the early stages of treatment and had an inhibitory effect on the angiogenesis in prolonged exposures via the upregulation of Thy-1 protein [275]. In our results, it was observed that PMA/Ionomycin treatment reduced the angiogenesis potential of cells. Since only PMA treatment was examined in the studies, it is thought that the inhibition result may arise from the dual effect of PMA/ionomycin. Moreover, at this point, the use of umbilical cord-derived endothelial cells could be another important reason. Furthermore, elevated angiogenic potential as a result of PMA/Ionomycin treatment seems to be more convenient for the CVI model. Therefore, these results indicate that it is necessary to investigate in more detail whether PMA/Ionomycin treatment suppresses the angiogenesis potential of HBECs.

Examining the tissue characteristics of endothelial activation pathology in CVI, it is understood that endothelial cells are not the only ones responsible for the occurrence and progression of this pathology. At this point, it seems that immune cells in peripheral blood also have quite important roles in the pathology. Therefore, incorporating immune cells into the *in vitro* CVI model is considered essential. In our study, it was evaluated whether the T lymphocytes were suitable for use in the CVI model. For this purpose, the gene expression analysis of Jurkat cells in both the growth phase and the activation phase was performed. In the literature, the activation characteristics of Jurkat cells were evaluated with various doses and treatment times of PMA/Ionomycin molecules and significant patterns of T lymphocyte activation were found [248,276]. It is well documented in the literature that various cytokines and chemokines with proinflammatory properties are the fundamental factors regulating the cellular immune response in cerebrovascular tissue [277]. These studies discovered that the expression of these proinflammatory agents increased as a consequence of the activation of T lymphocytes. On the other hand, there are other studies in the literature

showing that genes with protective properties such as NRF2 and PPARG mostly have an antagonistic impact on the activation of T lymphocytes [278,279]. Especially, one of these important studies reported that the NRF2 activation could suppress the inflammatory response of the cells. Another group of genes involved in T lymphocyte activation are the genes associated with cell survival and proliferation. Some studies related to these genes demonstrated that they have essential roles in supporting the survival of leukocytes and especially the proliferation of the cells [280]. Lastly, LFA protein is one of the substantial markers expressed in T lymphocytes in response to adhesion interactions of CECs in the leukocyte recruitment process. In the studies, it is also well reported that increased expression levels in the activation of the cells [163]. In our results, the characteristic changes in the gene expression of Jurkat cells were activated by PMA/Ionomycin induction. Upon examining the proinflammatory genes, it was observed that there was a significant increase in their expressions. Furthermore, the elevation in the expression of IL2 and IFNG genes indicates that a strong inflammatory response was induced in the cells. The increase in the expression of the IL8 gene also points out the capacity of Jurkat cells to attract other leukocytes playing important roles in the inflammatory microenvironment. Besides, the absence of significant changes observed in the expression of cellular protective genes such as NRF2 and SIRT1 suggests that inflammation does not suppress the expression of these genes but their activation remains limited. Nevertheless, the increase in the expression of the PPARG gene indicates that activation in the cells can also induce the protective feedback mechanisms aimed at limiting inflammation. Also, the decline in the expression of the BAX gene, one of the genes associated with apoptosis, suggests that PMA/Ionomycin stimulation increases the survival capacity of Jurkat cells. Being another upregulated gene, the KI67 gene is a proliferation marker and the increase in its expression indicates that the stimulation triggered a proliferative response in the cells. Lastly, the increase in the expression of the LFA-1 gene suggests that the adhesion capacity of the cells and their ability to adhere to HBECs are elevated. Therefore, these findings are consistent with studies in the literature. Consequently, it has been understood that Jurkat cells are suitable for use in the CVI model and can be successfully activated if they are induced.

In the homeostasis continuity of vascular system and playing crucial roles in inflammatory processes, the endothelial cells in the cerebral tissue have substantial cellular functions. The most eminent examples are cellular barrier structure and adhesion interactions with

leukocytes. In particular, changes in permeability and adhesion properties of endothelial cells during inflammation have great importance in the inflammatory response reaching a detrimental and chronic level. Studies in the literature reached valuable findings on elevated permeability and adhesion interactions in CECs caused by PMA/Ionomycin induction [281]. In our results, it was observed that the percentage of Jurkat cells adhering to the CVI-induced HBEC increased significantly in the CVI model. So, this increase demonstrates that the CVI condition increases the expression of adhesion molecules such as VCAM1 on the endothelial surface and causes T lymphocytes to bind more effectively to CECs in the cerebrovascular tissue. Studies in the literature also emphasize that adhesion molecules upregulated in inflammatory conditions facilitate the binding and accumulation of leukocytes to the cell's surface, and this is consistent with our results [282]. In the endothelial permeability analysis, an opposite result was found from the findings in the literature. Nevertheless, it is thought that this finding is related to the assay methodology, independent of the inflammation or activation level. In studies conducted on endothelial permeability, it was stated that no significant change was observed due to selecting to use low kDa indicator molecules such as 4 kDa FITC-conjugated dextran [203]. In our study, these results were both confirmed and they indicated that the endothelial permeability analysis should be performed using higher molecular weights such as 70 kDa types of these indicators in the experiments.

4.3. INVESTIGATING THE PROTECTIVE EFFECTS OF PCSEVS ON CVI MODEL IN HBECs

At this point of the study, it was managed that the CVI pathology in HBECs can be modelled *in vitro* in many characteristics similar to that in cerebrovascular tissue. Naturally, one of the important aims of the study was to investigate the potential effects of PCSEVs on the CVI model. Therefore, the effects of the PCSEVs on the CVI model are included in this section. Therefore, it was considered that the cytotoxicity of the PCSEVs should be evaluated on HBECs. In addition, with the MTS experiment, the effective doses of the PCSEVs planned to be used in further experiments were determined. Upon examining the literature, suspension culture media of plant cells has been used as a source in very few studies on the PCSEVs and their isolation has mostly been made from plant parts or fruits in studies [283]. Therefore, the cytotoxicity results of studies are predominantly associated with these types of vesicles called edible nanovesicles. In these results, it was determined that the PCSEVs

do not have any cytotoxic effect on various cells. Considering our results, it was determined that the PCSEVs did not have any cytotoxic effects on HBECs at all doses, similar to the literature. As a result, it was deemed appropriate to use the two highest doses of the PCSEVs for investigating their effects in the CVI model.

The effects of the PCSEVs on the CVI model were first evaluated through the expressions of selected marker genes in HBECs. Although there are no studies in the literature on the effects of the PCSEVs on the CVI model, many studies have been conducted on the therapeutic effects of plant vesicles on various forms of inflammation [106]. Almost all of these studies emphasize that plant vesicles have either antioxidant or anti-inflammatory effects. According to our results, we also discovered that the PCSEVs have a protective effect against CVI induction on HBECs. Upon examining the changes in the expression of inflammatory genes in the study, it was determined that there were insignificant changes in the expression of IL8, MCP1 and PAI1 genes in the SDEV treatment. However, in the results of HDEVs, it was observed that there was a notable decline in the expression of both MCP1 and IL8 genes at high doses. In this respect, the decrease in the expression of inflammatory genes in the CVI model after HDEV treatment suggests that the PCSEVs have the potential to regulate chemokine and cytokine signaling and may alleviate the inflammatory response. Besides, it is known that the CVI model leads to overexpression of cellular adhesion molecules in endothelial cells [277]. However, the expression levels of genes such as VCAM1 and ELAM1 were significantly reduced in both SDEVs and HDEVs as a result of the PCSEV treatment. These findings demonstrate that the increased endothelial cell-immune cell interactions during inflammation are limited and the PCSEVs are effective in protecting endothelial functions. Further, it was observed that there were no significant changes in the expression of ZO1 and MMP3 genes after the PCSEV treatment. Therefore, it indicates that the PCSEVs do not have a direct effect on tight junction proteins and matrix remodelling processes under the CVI induction or that these target proteins are subject to a more complex regulation. Considering these results, they are consistent with studies in the literature reporting anti-inflammatory properties of the PCSEVs and suggest that these nanovesicles may play a protective role in inflammation-induced vascular deterioration.

The protective effects of the PCSEVs with anti-inflammatory properties on the expression of related genes in the CVI model were determined. To confirm these effects at the protein level, the effects of the PCSEVs were evaluated with ICC and CBA analyses. Examining the

studies in the literature, although it has been known that the PCSEVs have cytoprotective effects, there are not any studies on the effects of these vesicles on cellular adhesion molecules induced in the CVI model [106]. According to our results, it was found that the protective effects of the PCSEVs on the cellular adhesion molecules of CVI-induced HBECs were consistent with both the gene expression results and the anti-inflammatory properties stated in the literature. In the results, it was observed that cellular adhesion proteins such as VCAM1 and ELAM1 in HBECs increased significantly under CVI induction. However, it was also observed that the increase of the protein levels in the CVI model significantly decreased in the treatment of SDEVs and HDEVs. Therefore, these declines in the nanovesicle groups indicate that the PCSEVs have the potential to regulate inflammation-induced cellular adhesion processes and limit leukocyte-endothelial interactions [284]. These findings indicate the ability of the PCSEVs to stabilize the inflammatory environment by protecting endothelial cell functions. Besides, changes in the expression of the ZO1 protein seem to be associated with the characteristic of endothelial cell barrier protection [285]. The decrease in protein expressions such as ZO1 and F-actin in the CVI group suggests that the inflammatory condition weakens the endothelial barrier by disrupting tight junction proteins [286]. However, a significant increase in the expression of ZO1 protein was observed when the cells were treated with the PCSEVs. The finding indicates that the SDEV and HDEV groups can protect tight junction proteins and rebuild endothelial barrier integrity. These results also support that the PCSEVs can limit the destructive effect of CVI induction on tight junction proteins. The decrease in ELAM1 protein expression and the increase in ZO1 protein levels indicate that the PCSEVs may not only limit the direct effects of inflammation but also balance the effects of the inflammatory environment by reorganizing the functions of the endothelial cells. Besides, the protective effects of the PCSEVs on PMA/Ionomycin-stimulated HBECs were also evaluated via the analysis of inflammatory cytokine profiles. The results demonstrated that the inflammatory condition caused a significant increase in IL8 cytokine expression, but had no significant effect on other cytokines. Nevertheless, no significant decrease in the expression of IL8 protein was detected when the PCSEVs were applied, indicating that the capacity of our nanovesicles to modulate inflammation-induced IL8 production was limited. Consequently, these findings demonstrate the potential of the PCSEVs to regulate inflammation-induced cellular adhesion and barrier functions in HBECs under the CVI model.

The results of the PCSEV treatment on the CVI-induced model revealed that these suspension culture-based nanovesicles primarily influenced cellular adhesion molecules in HBECs rather than inflammation-related markers associated with CVI pathology. In this respect, to investigate the cytoprotective effects of the PCSEVs, a T lymphocyte adhesion assay was performed on HBECs. Looking into the studies in literature, the activation effects of inflammation models induced by various stimuli on adhesion genes such as VCAM1 and ELAM1 in endothelial cells residing in different tissues have been reported [249,254]. It has been observed in many studies that various antioxidant molecules downregulate the activity of increased VCAM1 expression and prevent its upregulation. Although not all of these studies were followed up, these effects on the gene expressions discovered in a few studies have also been proven at the cellular level via adhesion assay [202,287]. Therefore, it was determined that anti-inflammatory or antioxidant molecules reduce the cellular adhesion capacity in inflammation-induced endothelial cells. According to our results, it was observed that the number of Jurkat cells adhering to HBECs significantly decreased as a result of the PCSEV treatment. These results demonstrate that the PCSEVs have the potential to protect endothelial cell functions by suppressing cellular adhesion processes triggered by CVI induction. The differences between the effects of PCSEVs reveal that SDEV has a higher efficacy compared to the HDEV treatment. These findings may be due to differences in biochemical composition between plant species or changes in the molecular components contained in vesicular structures. Therefore, these results suggest that the PCSEVs may regulate adhesion molecules in the HBECs during inflammation and thus alleviate the pathogenesis of vascular inflammation. Consequently, the potential of PCSEVs to modulate inflammation-induced leukocyte-endothelial interactions provides a promising basis for the use of the PCSEVs for therapeutic purposes.

In the study, it is understood that the PCSEVs demonstrate a protective effect on CVI-induced HBECs. Nevertheless, how these effects of the PCSEVs on endothelial cells occur is also quite informative point. Looking into the literature, some studies reported that SIRT1, PPARG and NRF2 pathways play an active role in the regulation of protective responses such as anti-inflammatory or antioxidant in CECs [288,289]. In the studies, it was proven that bioactive small molecules with protective properties have great potential owing to the activation of these pathways in endothelial cells. According to our results, we observe significant increases in the response of genes related to the NRF2 pathway in both treatments

with the PCSEVs. Accordingly, it is thought that the PCSEVs increase the capacity of HBECs to cope with inflammatory conditions and oxidative stress by activating the NRF2 signalling pathway. Furthermore, increases in the expression of NQO1 and HMOX1 genes indicate that they have the potential to alleviate the effects of proinflammatory stress in endothelial cells [290,291]. On the other hand, no significant change was observed in the expression of genes such as PPARG and SIRT1 in the SDEV treatment, while significant decreases were observed in these genes in the HDEV-treated cells. The decrease in the expression of the PPARG gene suggests that endothelial cell metabolism and lipid regulation may be modulated by these vesicles. Particularly, some studies emphasize the anti-inflammatory and lipid metabolism-regulating roles of PPARG [171,175]. However, the fact that HDEVs suppressed PPARG expression in our results suggests that this cell response may be an adaptation to rebalance energy metabolism. Besides, the decrease in the SIRT1 gene may indicate that NAD⁺-dependent deacetylase activities may decrease during inflammation [292,293]. The decreased expression of the SIRT1 gene suggests that the HDEVs tend to reduce cellular energy consumption while trying to balance cellular stress responses via the NRF2 pathway. On the other hand, the decreases in KI67 and AKT genes, which are associated with cell proliferation and survival, suggest that the PCSEVs may control cellular proliferation in inflammatory environments [294]. The decrease in AKT expression may also indicate that the metabolic activities and growth signals of cells are limited, which may be part of the strategy to prioritize antioxidant defense mechanisms [179,294]. In conclusion, it has been shown that the PCSEVs increase cellular antioxidant defense via the NRF2 pathway and may alleviate the negative effects of CVI pathology. These findings suggest that the PCSEVs primarily induce a protective response via the NRF2 signaling pathway and its downstream targets.

At this point of the study, it is seen that the PCSEVs have a critical potential to stimulate the protective effect on HBECs. However, dose- and time-dependent gene expression analyses are quite necessary in understanding how the endothelial cells respond to the treatment of these vesicles. Furthermore, dose dependency analysis was stated as an important study criterion for investigating the functional effects of the PCSEVs according to the MISEV2018 criteria [20]. In the context of our study, the effects of both SDEVs and HDEVs were examined in the expression of the HMOX1 gene in the HBECs. The results demonstrated that the HMOX1 expression significantly increased depending on dose and time factors. On

the other hand, an increase in the expression of the HMOX1 gene was also observed at low doses of SDEVs, while the inducing effect at the low doses of HDEVs disappeared more drastically. Upon examining the time-dependent analysis, it was determined in the studies that the protective effects of the PDEVs increased depending on time [295]. In our results, it was observed that the expression of the HMOX1 gene started to increase after 7 hours and reached a maximum level after 24 hours as a result of the HDEV treatment. It was also observed that the expression level stabilized after 48 hours. These results indicate that HMOX1 is an early responder gene to the PCSEV treatment and that the effect of these vesicles stabilizes over time. These findings suggest that the PCSEVs induce a protective response in HBECs and that this protective effect occurs through both dose- and time-dependent regulation.

In the last part of the study, it was investigated which pathways the PCSEVs interacted with to generate these protective responses in HBECs. In the literature, it has been known that some therapeutic approaches can induce anti-inflammatory and antioxidant effects on endothelial cells via the NRF2 pathway [296,297]. Besides, studies on the effects of PDEVs have shown that the edible nanovesicles can also activate the NRF2 pathway in various cells [298]. Upon examining our results, it was seen that the PCSEVs activate the NRF2 pathway and thus generate an antioxidant response. In studies on the expression of NRF2 protein in the literature, its various isoforms were examined by western blot analysis. In this respect, it was stated that the active form of NRF2 protein was the 100 kDa band and the 68 kDa band was the inactive form [268]. Accordingly, it was understood that the SDEVs not only activated the NRF2 pathway but also significantly increased its expression. Furthermore, it was also found that the expression of the NQO1 enzyme increased in both PCSEV treatments. According to these findings, the results indicate that the NRF2 pathway has an important role in the protective effects revealed by the PCSEVs. On the other hand, it has been observed that the PCSEVs also affect the phosphorylation status of the upstream pathways. In the literature, there are no studies on the phosphorylation levels or activation of these pathways in endothelial cells by these suspension-derived nanovesicles. In our results, it was found that the PCSEVs also regulate the phosphorylation of upstream signaling pathways such as ERK 1/2, AKT and P38. The increase in ERK 1/2 phosphorylation is evaluated as a protective response that may contribute to the activation of the NRF2 pathway. In the literature, ERK 1/2 was defined as a pathway that increases the

nuclear translocation and transcriptional activity of NRF2 protein [299]. Thus, it is understood that the PCSEV treatment facilitates coping with cellular stress by supporting the NRF2 pathway via ERK 1/2. On the other hand, it was observed that AKT and P38 phosphorylations were suppressed by the treatment of both SDEVs and HDEVs. While AKT is generally associated with cellular growth and metabolism, P38 is more commonly known as a pathway that regulates inflammatory responses [300]. Suppression of AKT and P38 indicates that inflammation-related signaling pathways are reduced and that the PCSEVs stimulate the pathways to control inflammation and maintain cellular homeostasis [179,294]. This suggests that these nanovesicular structures struggle with oxidative stress by both reducing inflammatory responses and activating the NRF2 pathway. In conclusion, our study demonstrates that the PCSEV treatment activates the NRF2 pathway to generate an antioxidant response, while also activating cellular protective mechanisms by suppressing inflammatory signaling pathways. Detailed analysis of the effects of PCSEVs on different pathways increases the potential of such therapeutic approaches in the treatment of diseases associated with oxidative stress and inflammation.

5. CONCLUSION

This study, aims to investigate the characteristic features of suspension culture media-derived nanovesicles as the PCSEVs, their potential effects on the CVI model, and the mechanisms underlying these effects. Accordingly, the study consisted of three main parts; isolation and characterization of the PCSEVs, modelling of CVI pathology, and investigation of potential anti-inflammatory effects of these nanovesicular structures. In the first stage of the study, the PCSEVs were successfully purified and characterized. It was understood that both the biophysical and molecular properties of the PCSEVs were compatible with the biological properties of PDEVs. In the second stage, *in vitro* CVI pathology was modelled in the HBECs upon an investigation of the effects of the PCSEVs. According to the characteristics of the CVI model, it exhibited critical features of pathologies such as endothelial inflammation and dysfunction, increased adhesion molecule expression, and the disruption of tight junction proteins, and it was understood that the CVI model was compatible with its biological counterpart like cerebrovascular tissue. In the last stage, it was observed that the PCSEVs led to a protective and therapeutic response against the CVI induction, and these effects mainly occurred by modulating adhesion molecule expression. In the results, the PCSEVs demonstrated the potential to alleviate inflammation by reducing excessive T lymphocyte accumulation in HBECs. Further analysis revealed that the therapeutic effects of the PCSEVs were mediated through key signaling pathways. In particular, the PCSEVs were shown to suppress the p-AKT and p-P38 pathways associated with inflammatory response, while activating the ERK1/2-NRF2 pathway associated with antioxidant and cellular protection. These findings highlight the PCSEVs as potent therapeutic agents with the potential to alleviate CVI pathology. However, further studies are needed to transform these *in vitro* results into clinical applications. Future studies should include *in vivo* experiments in animal models to confirm the efficacy and safety of the PCSEVs. Furthermore, further investigation of the molecular mechanisms underlying the protective effects of the PCSEVs is critical to optimize its therapeutic potential.

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