รายงานวิจัยฉบับสมบูรณ์

โครงการ

“ฤทธิ์ของสารอนุพันธ์ capsaicinoids จากพริกต่อเซลล์เยื่อบุหลอดเลือดและหลอดเลือดของสมองมนุษย์”

โดย ดร. ภญ. พิมลรัตน์ เกตุสวัสดิ์สมคร

สิงหาคม 2565

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ดร. ภญ. พิมลรัตน์ เกตุสวัสดิ์สมคร หัวหน้าโครงการวิจัย

โรงเรียนแพทย์รามาธิบดี สถาบันการแพทย์จักรีนฤบดินทร์

คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานคณะกรรมการส่งเสริมวิทยาศาสตร์ วิจัยและนวัตกรรม (สกสว.)

รายงานฉบับสมบูรณ์

1. Executive summary

Cardiovascular disease (CVD) remains the leading cause of death worldwide (1). With a rapidly aging population and inadequate prevention, premature death from CVD has become epidemic. Vascular endothelial cells reside inside the blood vessel lumen and uniquely function to control various aspects of vascular homeostasis. Disruption of endothelium-mediated maintenance of vascular homeostasis leads to pro-inflammatory, pro-atherosclerotic, and pro-thrombotic phenotypes (2), resulting in excessive vasoconstriction, vascular leakage, thrombosis, and sustained immunological responses. A comprehensive systematic review and meta-analysis of human studies demonstrated that regular chili pepper intake is associated with significantly lower all-cause cardiovascular-related mortalities (3). These benefits are likely attributable to capsaicinoids, alkaloids substances responsible for the spiciness of chili peppers. While capsaicin has been extensively studied in improving cardio-metabolic profiles, pharmacological activities of other capsaicinoids remain elusive. The pungent capsaicinoids have restricted people usage and the on-going effort focuses on investigating the potential use of less irritating capsaicinoids or non-pungent capsiniods. Dr. Uthai Wichai and his team (4) have synthesized different non-pungent capsaicinoids derivatives from chili pepper. Although these compounds show great potential for future nutraceutical product development, their pharmacological activity is mostly unidentified. Our current studies investigated whether capsaicinoid analogues possess anti-inflammatory actions in endothelial cells.

Despite commonly use of a conventional cell culture (2D) technique in the laboratory, studies have shown that it inadequately emulates cellular and tissue microenvironment. Thereby, responses in standard cell cultures may not represent those in *in vivo*. With the current funding, our group has been developing physiologically relevant organ-on-a-chip (OOC) models as an alternative tool to better predict human outcomes. Thereby, the biological effects of capsaicinoids can be tested in both conventional cell cultures and 3D tissue engineered capillaries. Our results clearly showed that:

1. Dihydrocapsaicin (DHC) exerted robust anti-inflammatory actions in endothelial cells against TNFα. Other derivatives of DHC, namely nitro-DHC, DHC-sulfate and DHC-β-D-glucopyranoside did not affect TNFα-induced inflammatory responses in our studies.

2. Despite marked DHC's pharmacological activities in 2D endothelial cell cultures, findings in human capillary-on-a-chip appeared to differ. Although the effects of TNFα-induced endothelial dysfunction and

immunological responses could be replicated in 3D tissue engineered capillary, DHC did not impact TNFα responses in 3D model.

Even though discrepancies between 2D cell cultures and 3D human capillaries remain an important question at the moment and possibly require further investigation, findings of DHC eliciting cardiovascular protections are in accordance with previous *in vivo* reports.

During the past two years, we had experienced significant obstacles with the widespread of pandemic COVID-19 causing multiple lockdowns. Nevertheless, we were able to accomplish the overall goal. Experimental plans have been modified based on scientific merits to ensure that the project will be carried out successfully and the resource is well utilized. Not only the pharmacological action of various capsaicinoids was tested in our current studies, we have also positioned Thailand on the world map in developing/employing an organ-on-a-chip platform for future pre-clinical testing.

2. บทคัดย่อ

Capsaicin และ dihydrocapsaicin (DHC) เป็น capsaicinoids ที่สำคัญและพบได้มากในพริก จากการศึกษาที่ผ่านมาจำนวนมาก พบว่า capsaicin มีผลส่งเสริมสุขภาพและป้องกันการเกิดของโรคหัวใจและหลอดเลือด โดยไปเพิ่มการผลิตไนตริกออกไซด์ (NO) และลดการเกิดการอักเสบ ในขณะที่ capsaicin ได้รับการศึกษาอย่างกว้างขวาง แต่ว่าอนุพันธ์ capsaicinoids ชนิดอื่น ๆ ยังมีการศึกษาน้อยหรือแทบไม่มีเลย โดยเฉพาะกับระบบหัวใจและหลอดเลือด การศึกษาวิจัยในครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลโดยตรงของ capsaicinoid derivatives ชนิดต่างๆ ต่อการอักเสบของเยื่อบุผนังหลอดเลือดและผลต่อการหลั่ง NO โดยใช้แบบจำลองของเซลล์เยื่อบุหลอดเลือดที่แตกต่างกัน ทั้งแบบ เซลล์ปกติ 2D ทั่วไปและแบบสามมิติที่สร้างเป็นหลอดเลือดในไมโครชิพ ในการเลี้ยงเซลล์ปกติ เราพบว่าสารกระตุ้นการอักเสบ TNFα สามารถไปเพิ่ม Ser536 phosphorylation ของ p65 NFκB และการแสดงออกของ VCAM-1 และ ICAM-1 ซึ่งผลดังกล่าวของ TNFα ถูกทำให้ลดลงอย่างมาก ด้วย DHC เท่านั้น ในขณะที่สารอนุพันธ์ของ DHC ที่ทำการทดสอบ คือ nitro-DHC, DHC-sulfate และ DHC-β-D-glucopyranoside ไม่มีผล ทั้งนี้ผลทางเภสัชวิทยาของ DHC ยังสามารถไปลดฤทธิ์ของ TNFα ที่เหนี่ยวนำให้เกิดการยึดเกาะของโมโนไซต์กับเซลล์บุผนังหลอดเลือด นอกจากนี้ DHC ที่ความเข้มข้น 10 และ 50 ไมโครโมลาร์ ยังทำให้เพิ่มการหลั่ง NO อย่างมีนัยสำคัญ เมื่อทดสอบฤทธิ์ของ DHC ในโมเดลเส้นเลือดฝอยบนไมโครชิพแบบ 3 มิติ เราพบว่า DHC ไม่ได้มีผลต่อ การรั่วของหลอดเลือดอย่างมีนัยสำคัญ และยังไม่ช่วยลดปริมาณการเกาะติดของโมโนไซต์ในท่อหลอดเลือด ผลที่แตกต่างกันของ DHC ในโมเดลทั้งสองแบบนี้ต้องอาศัยการศึกษาเพิ่มเติม อย่างไรก็ตาม ฤทธิ์ต้านการอักเสบที่เด่นชัดของ DHC ในการเพาะเลี้ยงเนื้อเยื่อบุผนังหลอดเลือดอาจช่วยอธิบายกลไกการทำงานของ DHC ในระดับโมเลกุลของรายงานก่อนหน้านี้ที่แสดงให้เห็นถึงประโยชน์ของ DHC ในระบบหัวใจและหลอดเลือดในโมเดลสัตว์ทดลอง

Abstract

Capsaicin and dihydrocapsaicin (DHC) are major pungent capsaicinoids produced in red hot peppers. Capsaicin has been previously shown to promote vascular health by increasing nitric oxide (NO) production and reducing inflammatory responses. While capsaicin has been extensively studied, whether other capsainoid derivatives exerts cardiovascular benefits through similar mechanisms remains unclear. The current study aimed to investigate the direct effects of capsaicinoids on endothelial inflammation and NO release using different *in vitro* models. In conventional 2D endothelial cultures, we demonstrated that TNFα induces Ser536 phosphorylation of p65 NFκB and expressions of adhesion molecules, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1. These effects were robustly abrogated only by DHC, whereas its derivatives, namely nitro-DHC, DHC-sulfate and DHC-β-D-glucopyranoside did not appear to have impact on TNFα-induced responses. Consistently, DHC treatment led to a marked reduction in TNFα-mediated monocyte adhesion to endothelial cells. Additionally, NO production was significantly induced by DHC at 10 and 50 μM

compared to vehicle control. When tested in 3D human capillary-on-a-chip, the responses to TNFα-induced vascular leakage and monocyte adhesion were recapitulated. However, DHC did not appear to mitigate anti-inflammatory responses in 3D model. These discrepancies of results between the two models remain largely elusive. Nevertheless, the marked anti-inflammatory effects of DHC in endothelial cultures potentially help elucidate the molecular mechanisms of previous reports demonstrating the cardiovascular benefit of DHC in animal models.

3. เนื้อหางานวิจัย

Introduction

Cardiovascular disease (CVD) remains the leading cause of death worldwide (1). With a rapidly aging population and inadequate prevention, premature death from CVD has become epidemic. It is interesting to note that low and middle-income countries are now disproportionately affected, leading to high financial burdens (1). Vascular endothelial cells reside inside the blood vessel lumen and uniquely function to control various aspects of vascular homeostasis. By releasing different vasoactive agents (e.g., nitric oxide (NO), prostacyclin, and endothelin-1), endothelial cells are responsible for regulating vascular tone, oxidative stress, and local immune response (2). Endothelial dysfunction has been well recognized as a hallmark and independent predictor of cardiovascular events (5, 6) and often precedes apparent clinical cardiovascular symptoms (7). Moreover, evidence has increasingly suggested a significant positive correlation between endothelial dysfunction and mortality rate among COVID-19 patients (8) (9). Disruption of endothelium-mediated maintenance of vascular homeostasis leads to pro-inflammatory, pro-atherosclerotic, and pro-thrombotic phenotypes (2), resulting in excessive vasoconstriction, vascular leakage, thrombosis, and sustained immunological responses. Reduced NO bioavailability and increased oxidative stress are well documented as the underlying mechanisms of impaired endothelial functions (2).

The cardiovascular benefits of capsicum consumption have been well recognized (10). A comprehensive systematic review and meta-analysis of human studies demonstrated that regular chili pepper intake is associated with significantly lower all-cause cardiovascular-related mortalities (3). These benefits are likely attributable to capsaicinoids, alkaloids substances responsible for the spiciness of chili peppers. Capsaicin, a major capsaicinoid known to activate Transient Receptor Potential Vanilloid (TRPV)-1 channels has been well studied for its effects to relieve pain, combat obesity, and promote vasodilation (11). Mechanisms by which capsaicin mediates vasodilatation involve the release of calcitonin gene-related peptide (CGRP) by perivascular sensory neurons (12), as well as its direct stimulation of NO release in the vascular endothelium (13) (14). Treatment of endothelial cell cultures with capsaicin resulted in increased expression/activity of endothelial nitric oxide synthase (eNOS) and NO production (13). Similarly, capsaicin promotes vasodilation in a variety of isolated vessel beds including coronary (15), mesenteric (14), and basilar arteries (16) (17). Chronic treatment with dietary capsaicin reduced systemic blood pressure in genetically hypertensive rats (14) and prevented high-salt diet-induced nocturnal hypertension in mice (17). Moreover, capsaicin was shown to reduce lipid storage and atherosclerotic lesions in the mouse aorta, which was dependent on TRPV1 activation (18). These responses

correlate with reduced expression of inflammatory markers and increased eNOS activity in endothelial cell cultures following capsaicin exposure (19).

Despite the numerous studies demonstrating the pharmacological activities of capsaicin, its pungency has limited the wide usage in human. Currently, an ongoing effort focuses on investigating the potential use of less irritating capsaicinoids or non-pungent capsaicinoids. Dr. Uthai Wichai and his team have synthesized non-pungent capsaicinoid derivatives from chili pepper as well as isolated non-irritated capsaicin metabolites from fermented peppers. Although these compounds show great potential for future nutraceutical product development, their pharmacological activity is mostly unidentified. Our current studies aim to investigate whether these compounds possess any cardiovascular protective actions. In particular, we evaluated the potential pharmacological activities of different capsaicinoid derivatives on conventional human endothelial cultures and 3D tissue engineered capillaries.

The following data obtained during the support of this TRF funding are outlined below. As indicated, plans have been modified from original proposal based on findings and scientific justifications to maximize the chance of project success and usefulness of outputs generated. Although we originally proposed to test the overall hypothesis in human brain endothelial cell line (hCMEC/D3), there were many limitations critical for project progresses at which the data obtained from it would rather be uninformative. Nevertheless, evidences presented below include experiments conducted in hCMEC/D3, which had provided us rationale to make appropriate adjustment for the project.

Specific Aim 1: To investigate whether capsaicinoid derivatives protect human endothelial cell from an inflammatory mediator, TNFα

Experiment 1) To characterize hCMEC/D3 cells and determine the purity of our cell source

Rationale: Brain endothelial cells possess distinct characteristics including forming the restriction barrier and expressing various transporters to tightly regulate substances entering the brain. The difficulties of isolating brain endothelial cells and the loss of the specific expressions of certain proteins during standard culture have been well recognized as a major roadblock to study brain endothelium. Although various immortalized human brain endothelial cell lines have been developed to address these issues, each line differs significantly in term of barrier tightness (20). Based on previous reports, we chose to use hCMEC/D3 because they are stable, easily grown and have been widely used for brain circulation studies.

Methods: We have obtained these cells from Merck/Millipore. Cells were cultured according to the manufacture instruction and previous publications (21) using the medium supplemented with 5% fetal calf

serum (FCS), 0.5 ng/ml VEGF, 20 ng/ml IGF-1, 5ng/ml EGF, 10 ng/ml basic FGF, 0.2 ug/ml hydrocortisone, 1 ug/ml ascorbate, 1% penicillin/streptomycin. hCMEC/D3 cells were grown on rat tail collagen Type I coated flask and sub-cultured once they reached 85-95% confluency. Cells were expanded and properly stored in different aliquots at ECDD (Excellent Center for Drug Discovery, Mahidol). To perform immunofluorescence, hCMEC/D3 cells were plated in 24 well-plate until complete confluence was reached. After fixing with 4% formaldehyde, non-specific bindings were blocked using buffer containing goat serum for 60 minutes at room temperature. Samples were incubated with either 1:2000 CD31 or 1:150 ZO-1 antibodies overnight at 4°C. Appropriate fluorochrome-conjugated secondary antibody was incubated for 1 hour at room temperature in the dark followed by nuclei staining with DAPI for 5 minutes. No primary antibody negative controls were included to rule out the possibility of false positive.

Results: As expected, hCMEC/D3 cells were easy to culture and they exhibited regular brain endothelial cell morphology (bright field pictures shown in Figure 1). To ensure the proper protein expressions of brain endothelial cell markers, CD31 and brain endothelial cell tight junction proteins, ZO-1 were examined using a standard western blot analysis. As expected, the expressions of CD31 and ZO-1 were detected (Figure 2).

The subcellular localizations of CD31 appeared at cellular junction (Figure 3) as previously reported (21). ZO-1 localization was observed at cell-cell junction (Figure 3). Even though the current quality of image taken from a regular fluorescent microscope made it hard to appreciate ZO-1 localization. The signal in negative control was barely detected, indicating that the detected signals of CD31 and ZO-1 were specific. Together, we concluded that the culture condition of hCMEC/D3 cells and their characteristics in our laboratory were consistent with *prior* studies (21).

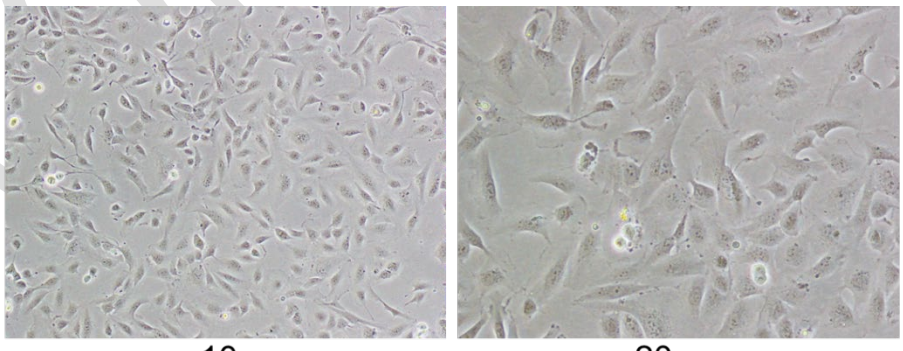


Figure 1: Typical human brain endothelial cells, hCMEC/D3 morphology. Bright field pictures are shown in magnification of 10x and 20x

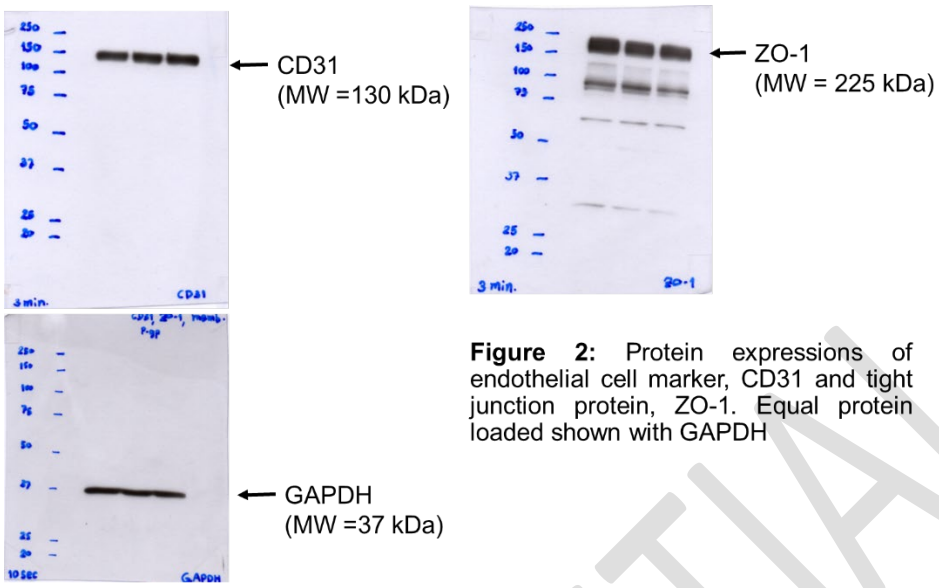


Figure 2: Protein expressions of endothelial cell marker, CD31 and tight junction protein, ZO-1. Equal protein loaded shown with GAPDH

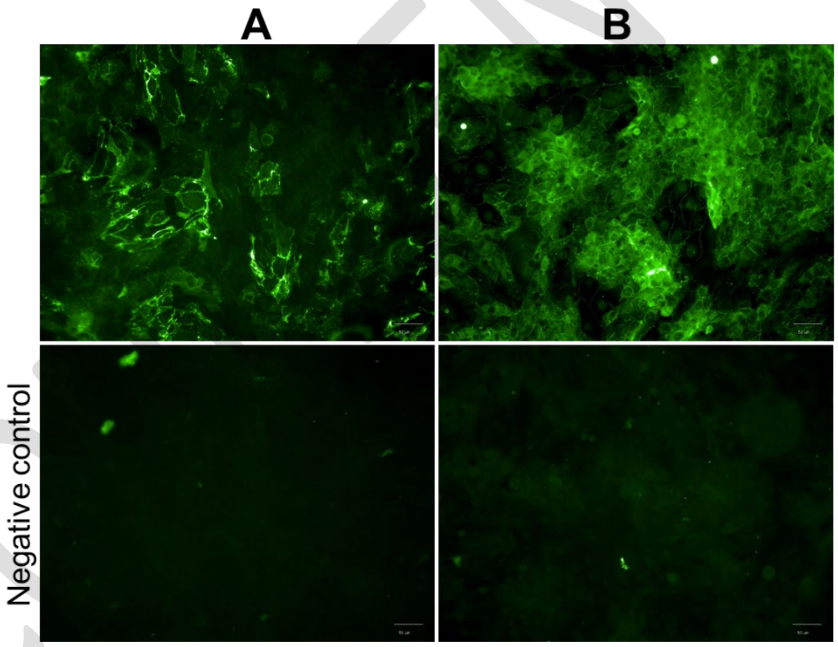


Figure 3: Representative immunofluorescence pictures demonstrating A) the endothelial cell marker, CD31 and B) tight junction protein, ZO-1. No primary antibody negative control showed minimum signal (n=3)

Conclusions/Discussions: From these experiments, our data provide a certain level of confidence that our typical characteristics of brain endothelial cells could be achieved as previously reported with our current culture conditions. We have continued to employ the same protocols and medium compositions as described above for subsequent experiments.

Experiment 2) To evaluate the appropriate concentration and time for capsaicinoid derivatives treatment in hCMEC/D3

Rationale: Despite benefits of capsaicin in cardiovascular diseases, its adverse effects have been well reported. For example, capsaicin can induce cell-cycle arrest or inhibit proliferation in cancer cells (22). In endothelial cells, high doses (>150 μM) of capsaicin can reduce cell viability significantly (23). Since cellular toxicity of these compounds are largely unknown in brain endothelial cells, we next tested the safety profile of capsaicinoid derivatives in comparison to capsaicin.

Methods: MTT assay was used to determine cell viability of hCMEC/D3 cells following capsaicinoid derivatives treatment. In brief, hCMEC/D3 cells were cultured in 96-well plates and treated with various concentrations of compounds at 0.5, 5, 10 and 50 μM for 24 hours. The media was then removed and MTT solution (a yellow tetrazole) at a final concentration of 0.5 mg/ml in serum-free media was added to each well. Following 3 hours of incubation at 37 ºC, the supernatant was carefully discarded and the formed formazan crystals were dissolved in DMSO. The absorbance was quantified using a Multiskan GO microplate reader (Thermo Fisher Scientific) at 570 nm. 4% Triton-X and Ellipicine (a known cytotoxic compound) were used as positive controls to validate the assay with maximum induction of cell death. In some experiments, capsaicinoid derivatives were treated in serum starved cells. Cell viability was calculated by percentage of control condition (untreated cells), which is defined by:

Cell viability = (Ab_treatment)/(Ab_control) ×100
Ab_treatment = the absorbance value of treatment condition
Ab_control = the absorbance value of untreated cells

Results: We received capsaicin and 4 capsaicinoid analogues from Dr. Uthai Wichai (Faculty of Science, Naresuan University). Their molecular weights and chemical structures are shown in Table 1.

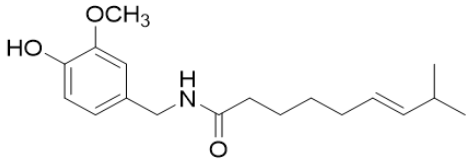
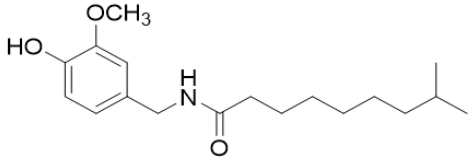
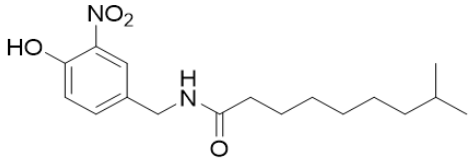
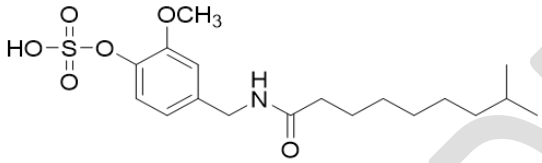
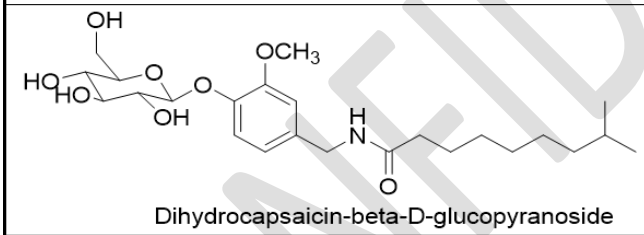
Compounds	Chemical Formula	MW.
 Capsaicin	$C_{18}H_{27}NO_3$	305.4180
 Dihydrocapsaicin	$C_{18}H_{29}NO_3$	307.4340
 Nitro-dihydrocapsaicin	$C_{17}H_{26}N_2O_4$	322.4050
 Dihydrocapsaicin Sulfate	$C_{18}H_{29}NO_6S$	387.4910
 Dihydrocapsaicin-beta-D-glucopyranoside	$C_{24}H_{39}NO_8$	469.5750

Table 1: Chemical structures and molecular weights of capsaicinoid derivatives tested in our studies

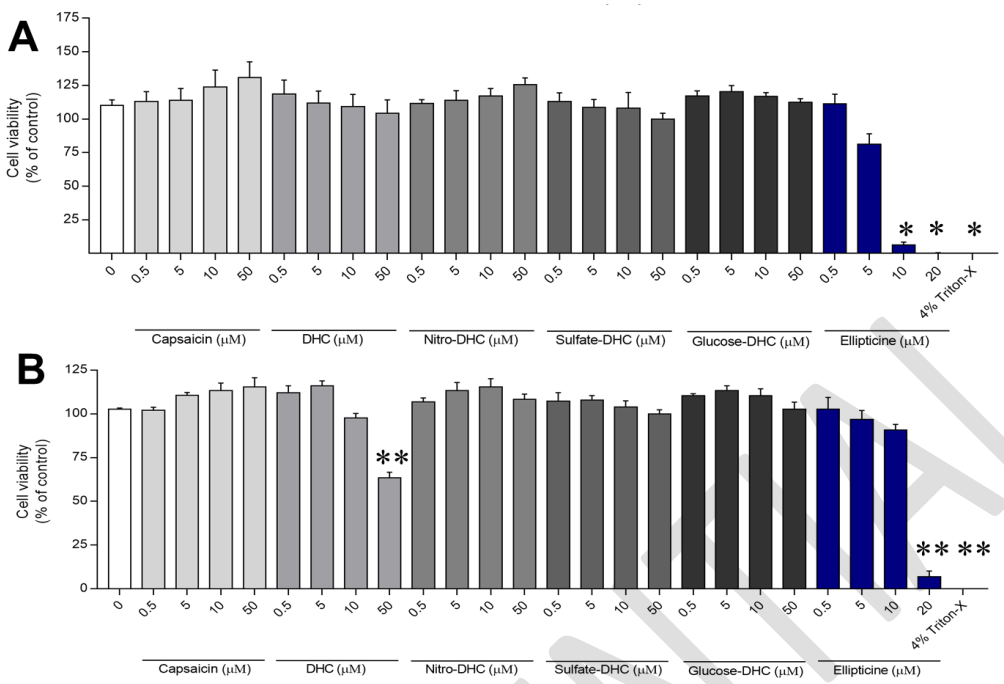


Figure 4: Cytotoxicity test with different concentrations of capsaicinoid derivatives. A) Complete medium with serum and growth factors and B) Serum and growth factor starved cells for 24 hours (n=3) *, ** $p<0.05$ vs DMSO control (0)

According to capsaicin and dihydrocapsaicin (DHC) chemical properties, they are lipophilic and readily dissolved in DMSO. We found that all other compounds are also soluble in DMSO; therefore, small aliquots of 50 mM of each derivative were made and kept in DMSO at -20 °C freezer until used. Next, we determined the viability of human brain endothelial cells following tested capsaiciniods treatment. Using MTT assay, only viable cells with active metabolism convert MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a yellow tetrazole compound to an insoluble formazan product with purple color, while dead cells are unable to produce the signal. The cell viability is determined by quantitatively measurement of absorbance at OD 590 nm. In our studies, hCMEC/D3 cells were incubated with indicated capsaicinoids at various concentrations from 0.5-50 μM for 24 hours. We chose this range of concentrations based on previous capsaicin reports. In some experiments, endothelial cells were starved prior to the capsaicinoids treatment. The stock solution of capsaicinoids was diluted with cultured medium. The final concentration of DMSO was controlled to 0.1%. We included 4% Triton-X and Ellipicine (a known cytotoxic compound) as positive controls. As expected, the signal was barely detected with 4% Triton-X treatment, indicating high cytotoxicity (Figure 4A and B). Ellipicine induced cell death in a concentration-dependent manner with the maximum effect occurred at 20 μM (Figure 4A and B). These positive controls validated the reliability of the assay. Capsaicin and its derivatives treatment at concentration of 0.5-50 μM did not affect endothelial cell viability compared to control (DMSO 0.1%) (Figure

4A). Similar findings were observed with endothelial cells deprived of serum and growth factors (Figure 4B), except DHC at 50 μM, which caused cytotoxicity compared to control (Figure 4B). Moreover, the cytotoxicity induced by Ellipicine only occurred at highest (20 μM) concentration with starved cells compared to complete media treated cells (Figure 4A and B).

Conclusions/Discussions: Capsaicinoid derivatives at concentrations tested, 0.5-50 μM did not cause a significant damage to hCMEC/D3 cells, at least, with completed media. Thus, concentrations of these compounds at this range will be used in subsequent experiments. If greater than 50 μM is required, MTT will be used to test cell viability. Cautions should be taken when exposing starved cells with concentration of DHC greater than 50 μM.

Experiment 3) To examine the protective effects of tested capsaicinoids against TNFα-induced inflammatory responses

Rationale: Accumulating studies have demonstrated the benefit of capsaicin in inflammation (11) occurs *via* TRPV1 dependent mechanism. Genetic ablation of TRPV1 aggravated neutrophil infiltration, pro-inflammatory cytokine production, and adhesion molecule expression in mouse model of endotoxic shock (24). Conversely, activation of TRPV1 by capsaicin inhibited lipopolysaccharides (LPS)-induced inflammatory responses in endothelial cells through PI3K/Akt/eNOS/NO dependent mechanism (25). However, most of previous studies focused on anti-inflammatory effects of capsaicin in endothelium from conduit arteries. Particularly, little is known about the roles of capsaicin in brain endothelium. Here, we asked whether capsaicinoids could exert the protective effects against TNFα- induced inflammatory responses in human brain endothelial cells.

Methods: We had performed the following experiments:

Experiment 3.1) We examined TRPV1 expression in brain endothelial cells in the presence or absence of inflammatory stimulus using qRT-PCR. hCMEC/D3 cells were cultured as described above and total RNA was purified using Monarch® Total RNA Miniprep Kit (New England Biolab, USA) following the manufacturer instruction. Subsequently, the concentration of RNA was measured using Nanodrop. The purity of RNA was determined by evaluating 260/230 and 260/280 ratio. Poor quality RNA with any ratio less than 1.8 was discarded. 1000 ng of RNA was transcriptionally reversed to cDNA using iScript Reverse Transcription Supermix (Bio-Rad, USA). Human TRPV1 transcript was quantitatively detected using TaqMan Real-time PCR Assays (Thermofisher Scientific). GAPDH mRNA was used as an internal control.

Experiment 3.2) We investigated responses of hCMEC/D3 cells to TNFα-induced inflammation with or without capsaicinoids pre-treatment. In this experiment, brain endothelial cells were incubated with different concentrations of TNFα for 24 hours and iNOS transcript was measured using TaqMan Real-time PCR Assays as described above. We also examined VCAM (vascular cell adhesion molecule) -1 protein expression, which is another inflammatory marker commonly induced by TNFα using a standard western blot analysis. In brief, hCMEC/D3 cells were treated with different concentrations (0, 0.1, 1, 5, and 10 ng/ml) of TNFα diluted in serum free media for 6 hours. In some experiments, cells were either pre-treated with 50 μM capsaicinoids or 0.1% DMSO vehicle control for either 30 minutes or 17 hours followed by 6 hours incubation of 10 ng/ml TNFα. Cells were washed with chilled 1xPBS twice and lysed with RIPA lysis buffer containing proteases and phosphatases inhibitors. Cell lyses was centrifuged and the supernatant was collected. Protein samples were loaded and separated in SDS-page gel followed by electrophoretically transferred to a nitrocellulose membrane. Non-specific proteins were blocked by 5% non-fat milk and the membrane was incubated with 1:1000 VCAM-1 antibody (Cell Signaling, USA) overnight at 4^{O}C. Diluted anti-rabbit IgG was incubated with the membrane for 1 hour at room temperature followed by washing steps. Specific VCAM-1 protein was detected using chemiluminescent HRP substrate and visualized in Chemidoc system (Bio-Rad, USA).

Results:

Experiment 3.1) Previous studies have shown that TRPV1 is ubiquitously expressed, including in endothelial cells (26). The benefits of TRPV1 activation by capsaicin on brain circulatory system with promoting vascular relaxation and preventing stroke has been well reported (17), alluding vascular TRPV1 is present in the brain. However, those prior studies rely heavily on the use of antibodies, which have been proven later to be non-specific (27). hCMEC/D3 cells were commonly used in blood brain barrier research but the expression of TRPV1 has never been investigated. At baseline, TRPV1 transcript was barely detectable with Cq over 35 cycles, which is considered very low expression. TNFα has been shown to induce TRPV1 gene expression in other cell types (28). Thus, we asked whether TRPV1 could be upregulated during inflammatory condition. hCMEC/D3 cells were incubated with different concentrations of TNFα for 24 hours and TRPV1 mRNA was examined. We observed a slight induction of TRPV1 mRNA following TNFα treatment in hCMEC/D3 cells (about 1.35 folds compared to DMSO vehicle control, n=2-4) but the overall expression after treatment was still difficult to detect.

Experiment 3.2) Although we found no evidence of TRPV1 expression in hCMEC/D3 cells, it is entirely possible that capsaicinoids may exhibit pharmacological activities *via* TRPV1 independent mechanism. An inflammatory

marker, iNOS in brain endothelial cell line in response to TNFα was examined. Surprisingly, iNOS mRNA was hardly detected in hCMEC/D3 cells and TNFα did not appear to induce iNOS expression in this cell (Not detectable, n=3). Next, we tested if TNFα could induce VCAM-1 protein expression in brain endothelial cells. VCAM (Vascular Cell Adhesion Molecule)-1 is an immunoglobulin-like adhesion molecule that is predominantly expressed on the surface of endothelial cells (29). Even though its expression is barely detectable at baseline condition, VCAM-1 is rapidly induced by pro-inflammatory cytokines, including TNFα (29). Here, we showed that treatment with TNFα robustly increased VCAM-1 in concentration dependent manner both at 4 or 6 hours incubation (Figure 5).

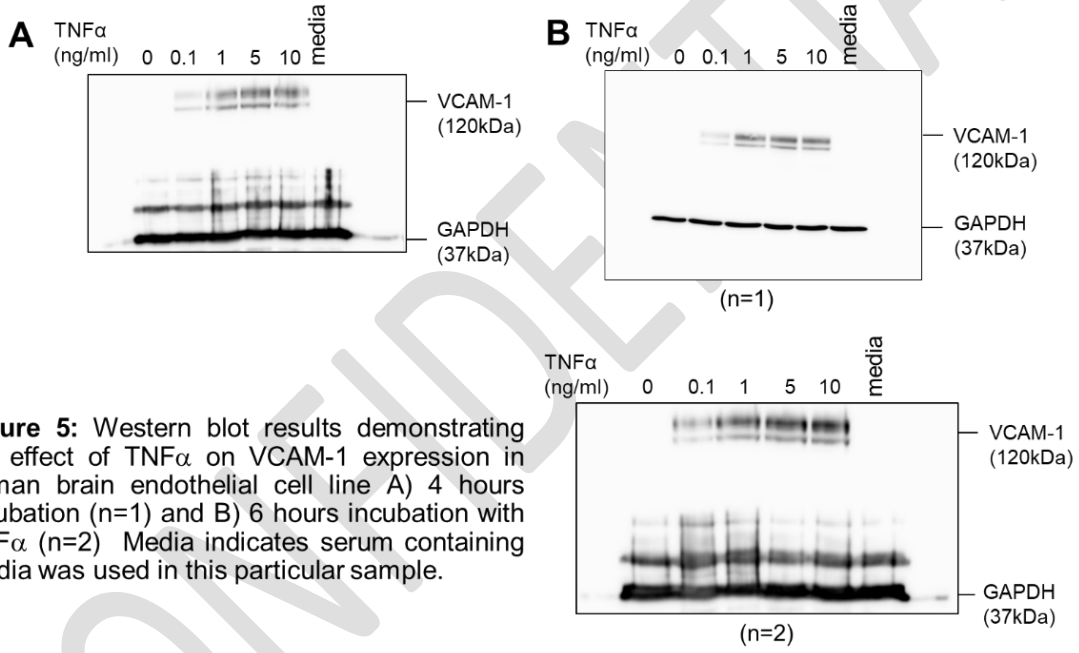


Figure 5: Western blot results demonstrating the effect of TNFα on VCAM-1 expression in human brain endothelial cell line A) 4 hours incubation (n=1) and B) 6 hours incubation with TNFα (n=2) Media indicates serum containing media was used in this particular sample.

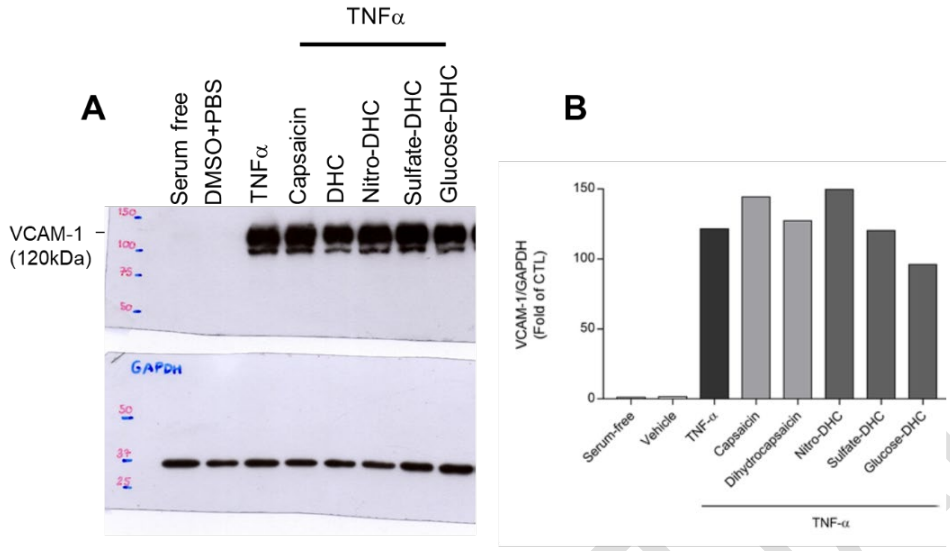


Figure 6: VCAM-1 expression after 30 min pre-incubation of capsaicinoids (50 µM each) following by TNFα (10ng/ml) treatment for 6 hr A) Representative blot of different capsaicinoids responses to TNFα treatment B) Quantitative of western blots results (n=2)

As expected, VCAM-1 was not detected at baseline condition with regular media and overnight starvation did not appear to induce VCAM-1 (Figure 5). Next, we asked whether each capsaicinoid derivative could mitigate the induction of VCAM-1 by TNFα. hCMEC/D3 cells were treated with 50 µM of different capsaicinoids for 30 min and followed by 10 ng/ml TNFα incubation for 6 hours. There was a marked increase in VCAM-1 expression with TNFα alone. However, treating brain endothelial cells with specific capsaicinoid derivatives did not result in an altered VCAM-1 expression (Figure 6). Similar findings were observed with longer time of capsaicinoids incubation to 17 hours (Figure 7). We conclude that capsaicin and tested capsaicinoid derivatives minimally counteracted the effects of TNFα-induced VCAM-1 in hCMEC/D3 cells.

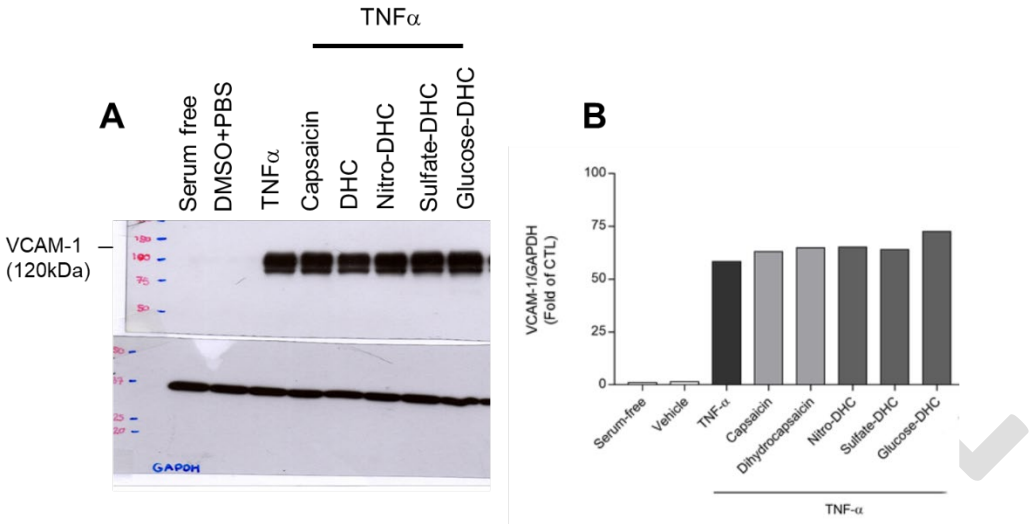


Figure 7: VCAM-1 expression after 17 hours pre-incubation of capsaicinoids (50 µM each) following by TNFα (10ng/ml) treatment for 6 hr A) Representative blot of different capsaicinoids responses to TNFα treatment B) Quantitative of western blots results (n=2)

Conclusions/Discussions: Although our results here with 2D cultures appear unexpected, it is entirely possible that hCMEC/D3 cells, at least, in static condition (traditional 2D cultures) failed to express TRPV1. It is well documented that the expression profiling of cells grown in 2D cultures often differs from their native counterpart (30). Moreover, hCMEC/D3 is an immortalized endothelial cell line, commonly used in blood brain barrier research (31) has possibly lost expressions of other key molecules. Our current findings are consistent with prior studies demonstrating an upregulation of VCAM-1 in response to inflammatory mediators (31) in hCMEC/D3. However, incubation of capsaicinoids did not affect the response of TNFα. This is possibly attributable to the lack of TRPV1 expression in this cultured condition with hCMEC/D3. To date, there is no evidence in the literature demonstrated the expression pattern of TRPV1 in hCMEC/D3. Nevertheless, there was only one study investigated the effects of capsaicin on blood brain barrier tightness using another human brain endothelial cell line (23) but whether or not TRPV1 was involved has not been investigated.

Experiment 4) To investigate whether tested capsaiciniods increase activation of eNOS and NO production

Rationale: Prior evidence has shown that capsaicin increased eNOS expression and promoted NO production in endothelial cells through TRPV1 receptor (32, 33). This was shown as a major mechanism by which capsaicin exerts cardiovascular benefits. Although various types of endothelial cells such as those derived from human umbilical vein (HUVEC) (25), bovine aorta (33) and mouse mesenteric arteries (14) have been used to test the effects of capsaicin, activation of TRPV1 in human brain endothelial cells remains largely unexplored.

Methods: We measured cellular and released NO following capsaicin treatment in hCMEC/D3 using chemiluminescence method. Human brain endothelial cells were cultured in 6 well-plate until reaching 90% confluence and were switched to serum and growth factors free media overnight. Subsequently, cells were treated with either 10 µM, 50 µM capsaicin or 0.1% DMSO for 5 minutes and cultured media was collected before harvesting the cells. Cells were washed with chilled nitrite free PBS twice and 50 µl of PBS was added before scraping cells from the plate. Cultured media and cell lysates were injected directly into a tri-iodide contained solution purge vessel to measure the amount of nitrite, that was reduced to NO gas. Total protein amount in each sample was measured by Bradford assay. Different volume of 1 µM standard sodium nitrite solution between 10-100 µL were injected into a purge vessel to create a standard curve. The nitrite levels were calculated from the slope of a graph plotted between standard solution and the area under the curve of the chemiluminescence detector signals. Acetylcholine was included as our positive control.

Results: In this experiment, we used acetylcholine as our positive control and observed a maximum increase in NO production in a range of 1.4-2.1 folds compared to vehicle control. Similarly, 10 µM capsaicin caused an induction of NO between 1.3-1.5 folds of those in control (Representative data shown in Figure 8). Despite this induction, the variability was quite large between experiments (n=3). Even with positive control groups, acetylcholine did not consistently increased NO. Capsaicin-induced NO production also occurred in similar manner to acetylcholine but with less magnitude.

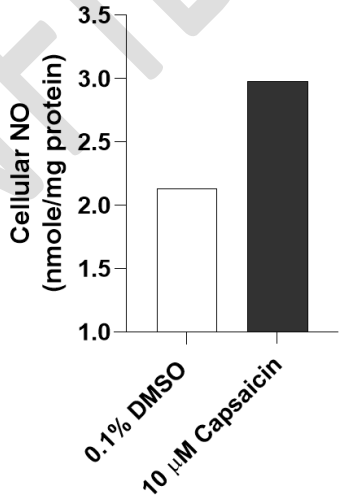


Figure 8: Capsaicin-induced cellular NO production in brain endothelial cells measured by chemiluminescence method (*preliminary data*).

Conclusions/Discussions: It is well recognized that NO is a key marker for endothelial function, we measured NO in hCMEC/D3 with chemiluminescence method, which is highly sensitive with detection in pmole range (34). The inconsistent findings with NO generation in hCMEC/D3 cells from our studies could be potentially explained by the low expression of eNOS. eNOS protein level detected by Western blotting was much lower in hCMEC/D3 compared to another endothelial cell type (Human Umbilical Vein Endothelial Cells; HUVEC). Even with the stimulation with positive control, acetylcholine, the induction of NO was highly variable with modest amount of NO detected. Similar results were observed with capsaicin treatment. We speculate that the inconsistent expression of eNOS in hCMEC/D3 cell line contributes to large variations between experiments and possibly NO generated hardly exceeds the detection threshold.

Discussions

Due to difficulties encountered in above experiments, I decided to modify proposed studies in 2D by using the primary endothelial cells, instead of immortalized cell line. Utilizing hCMEC/D3 posts many limitations with inconclusive results, which could be attributable to limitations of immortalized cells lacking critical downstream mediators necessary for capsaicinoid functions. Primary cultures will allow us to test the central hypothesis in conditions closer to *in vivo* states than those with cell lines. Unfortunately, the quality of primary human brain endothelial cells from commercial sources has been widely questioned in the literature. The inconsistency between batches and rapid phenotypic changes with regular cultures have been well described (35) Overall, the aim of this project is to evaluate pharmacological activities of novel capsaicinoid compounds in endothelial cells to support the use of chili pepper. A large number of studies utilize HUVEC (Human umbilical vein endothelial cell) to evaluate the cardiovascular effects of capsaicin. Generally, HUVEC is a primary endothelial cell source that has been widely used since 1970 for endothelial biology. HUVEC can be obtained commercially and they are easily grown in a standard cell culture laboratory. Our initial screening of HUVEC detected key molecules expressions necessary to test our hypothesis. For example, CD31 (an endothelial marker) and endothelial nitric oxide synthase (eNOS) were readily detected in our HUVEC cultures (*data not shown*). Another advantage of using HUVEC in our studies is that we can compare our findings to prior studies in the literatures. Thereby, it provides another level of confidence regarding validity and accuracy of our results.

Modifications of plans to move project forward

To ensure that 1) our project will be carried out successfully and 2) the effects of capsaicinoid derivatives could be investigated in appropriate endothelial models, we decided to test our hypothesis using a well-established endothelial cell source, HUVEC (Human umbilical vein endothelial cell). Adjustment of plans was made based

on above findings in brain endothelial cell lines. Unexpected roadblocks with loss of key mediator expressions necessary to test our hypothesis in hCMEC/D3 cells. Moreover, the inconsistency of results obtained from hCMEC/D3 made it difficult to interpret our findings. Although we recognized that the source of HUVEC does not originate from brain, these cells have been shown to retain protein expression profiling closer to *in vivo* (36), thereby meaningful scientific evidence could be acquired. With unresolved issues in the field regarding primary human brain endothelial cell source, testing pharmacological activities of capsaicinoid analogues probably cannot be achieved with standard 2D cultures.

We employed HUVEC to perform experiment 3 and 4 and data are presented below:

Experiment 3) To examine the protective effects of tested capsaicinoids against TNFα-induced inflammatory responses

Methods: To investigate whether capsaicinoid derivatives protect against TNFα-induced inflammation in human endothelial cells. Primary Human umbilical vein endothelial cells (HUVEC; PromoCell) were cultured in endothelial cell growth medium containing 2% fetal calf serum, 5 ng/ml EGF, 10 ng/ml bFGF, 20 ng/ml IGF-1, 0.5 ng/ml VEGF, 1 µg/ml ascorbic acid, 22.5 µg/ml heparin and 0.2 µg/ml hydrocortisone. Cells were cultured using a standard protocol. Typical morphology of HUVEC and an expression of a specific endothelial cell marker, CD31 were shown in Figure 9. As expected, the expressions of these markers were readily detected, indicating proper culture of endothelial cells.

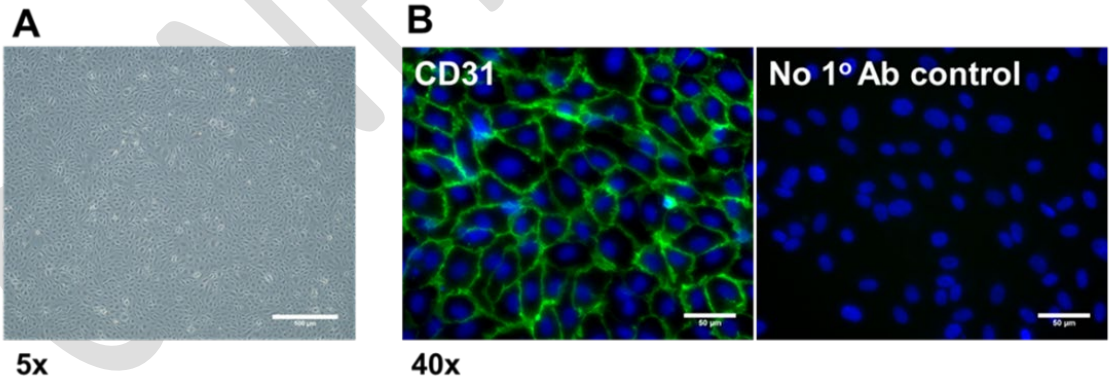


Figure 9: Representative images of typical morphology of Human Umbilical Vein Endothelial Cells (HUVECs) (A) and expression of an endothelial cell marker, CD31 (B) (n=4)

In our experiments, only HUVEC from passage 3-6 was utilized because primary cell is notoriously known for de-differentiation once sub-cultured multiple times. To access an anti-inflammatory action of capsaicinoids, endothelial cells ($2x10^5$ cells per well) were grown in 6- well plate and subsequently maintained in complete media. Once reached 90-95% confluence, HUVEC were pre-treated with different capsaicinoid derivatives at 50 μM for 30 minutes followed by co-incubation with specific capsaicinoids and 1 ng/ml TNFα for 4 hours. Prior to capsaicinoids experiments, cells were treated with TNFα at different concentrations to determine the appropriate range of concentrations that led to inflammatory responses. All drugs and vehicle were diluted in serum-free media. Matched concentration of vehicle treated group was included as a negative control. Cells were washed with chilled 1xPBS twice and lysed with protein lysis buffer containing proteases and phosphatases inhibitors. Cell lyses was centrifuged and the supernatant was collected. Standard Western blot analysis was performed to detect VCAM-1, ICAM-1, Phospho-NF-κB p65 (Ser536) and NF-κB p65 (Cell Signaling). Proteins were detected using chemiluminescent HRP substrate and visualized in Chemidoc system (Bio-Rad, USA). GAPDH was used as a loading control in all experiments.

Additionally, we asked if capsaicinoids could affect an immune response, monocyte adhesion to endothelial surface in response to TNFα treatment. HUVECs were seeded in a 24 well-plate until reaching 95% confluency. Endothelial cells were stimulated with 1 ng/ml of TNFα for 4 hours to induce inflammatory responses. To visualize the monocyte adhesion, U-937 cells were labeled with 10 μM of Green CMFDA Dye (ab145459, Abcam). Monocytes were then added to the stimulated endothelial cells and incubated at 37 °C with 5% CO_2 for 30 minutes. Non-adherent cells were washed gently with 1xPBS three times. Cells were fixed with 4% PFA at room temperature for 15 minutes. Nuclei were counter-stained using Vectashield® (Vector laboratories). Total fluorescent intensity of monocyte adhesion was measured using a microplate reader (BioTek, Synergy Neo 2) at an excitation/emission of 492/517 nm. Images of monocyte adhesion were captured using a Nikon Eclipse TS2. All experiments were done in triplicates.

Results:

3.1. Anti-inflammatory activity of capsaicinoid derivatives in TNFα-treated endothelial cells

TNFα is a pleiotropic cytokine known to induce inflammatory responses in different cell types (37), including endothelial cells. Incubation of HUVECs with TNFα at concentrations of 0.1, 1, 5, or 10 ng/ml for 4 hours resulted in an upregulation of adhesion molecules, vascular cell adhesion protein (VCAM)-1, and intercellular adhesion molecule (ICAM)-1 in a concentration-dependent manner (Figure 10).

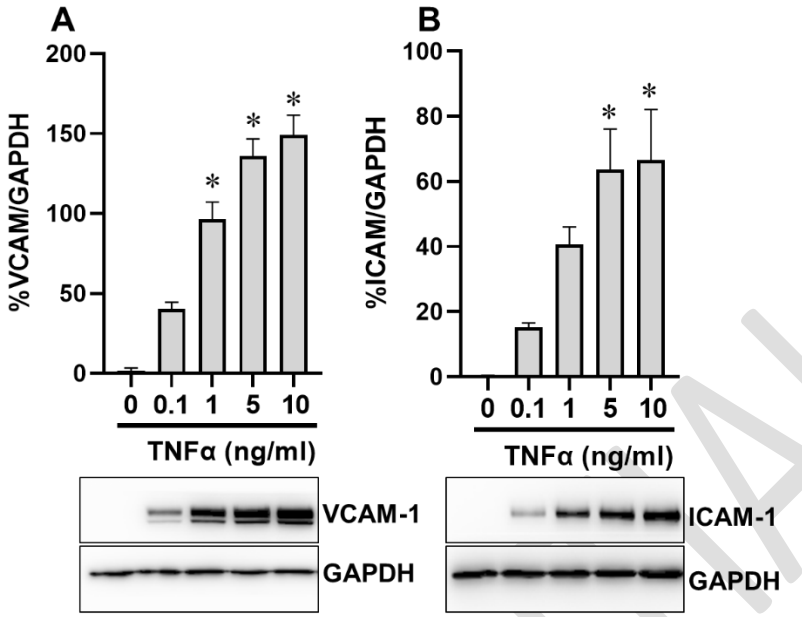


Figure 10: TNFα increased VCAM-1 and ICAM-1 expressions in HUVEC. Significant inductions of VCAM-1 (A) and ICAM-1 (B) were apparent following 4 hours of TNFα treatment compared to vehicle control. (n=3); * $p<0.05$ *vs* vehicle control

To examine the potential cytotoxicity of capsaicinoids against endothelial cells, we quantified HUVEC viability in response to treatment with various concentrations (0-500 μM) of capsaicin and DHC using resazurin cell viability assay. As expected, 4% of Triton X markedly reduced cell viability to 3.11% of control (n=3). Exposure of endothelial cells to capsaicin from 0.5-100μM did not cause significant cytotoxicity (Figure 11A). DHC between 0.5-50 μM did not affect HUVEC viability but exposure of DHC at 100μM and 500μM resulted in increased endothelial cytotoxicity (Figure 11B). In subsequent experiments, capsaicin and DHC at 50 μM were used to ensure that the results obtained were not confounded by reduced endothelial cell viability.

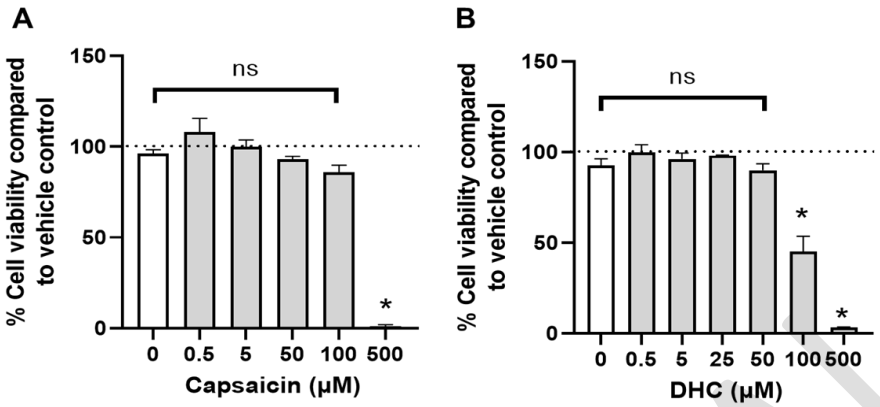


Figure 11: The cytotoxicity profiles of capsaicin and DHC. Endothelial cells were treated with different concentrations of capsaicin (A) or DHC (B) for 5 hours. Matched vehicle controls (DMSO) were also included to rule out the possibility that the toxicity was from vehicle. Cell viability was determined using resazurin cell viability assay. The data were expressed as percent cell viability compared to their corresponding vehicle controls. (n=3-6); *p<0.05 *vs* control (0 μM). ns = not significant

Despite extensive studies with capsaicin, it remains largely unknown whether other capsaicinoid derivatives possess the same cardiovascular benefits and if this occurs through similar mechanisms as those by capsaicin. Therefore, we next tested the pharmacological activities of capsaicin and other capsaicinoid derivatives, dihydrocapsaicin (DHC), nitro-DHC, DHC-sulfate and DHC-β-D-glucopyranoside on TNFα-induced endothelial inflammation. Consistently, 4 hours exposure of 1 ng/ml TNFα to endothelial cells robustly increased expressions of VCAM-1 and ICAM-1 (Figure 12A and B). Capsaicin trended to mitigate the induction of ICAM-1 induced by TNFα in HUVEC (Figure 12A and B). Strikingly, incubation of DHC dramatically decreased TNFα -induced VCAM-1 and ICAM-1 expressions (Figure 12A and B).

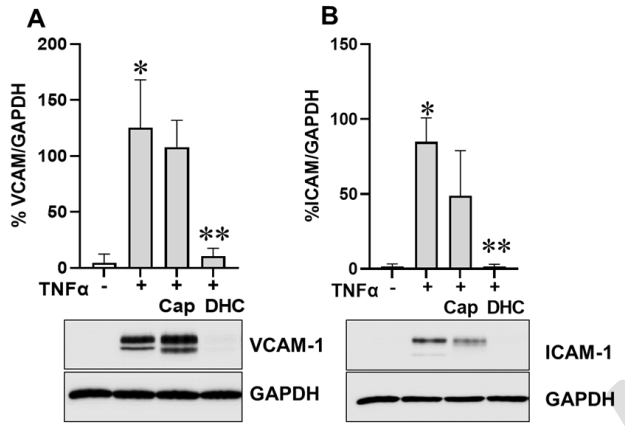


Figure 12: DHC counteracted TNFα-induced inflammatory responses in HUVECs. Expressions of VCAM-1 (A) and ICAM-1 (B) in HUVECs following 50 μM of Cap (capsaicin) or DHC treatment in the presence of TNFα for 4 hours. (n=3) ;* p<0.05 *vs* vehicle control; ** p<0.05 *vs* TNFα alone

Next, we asked if other concentrations of DHC could elicit inhibitory responses against TNFα in endothelial cells. As shown in Figure 13, only DHC at 50 μM significantly decreased TNFα-induced expression of adhesion molecules, VCAM-1 and ICAM-1.

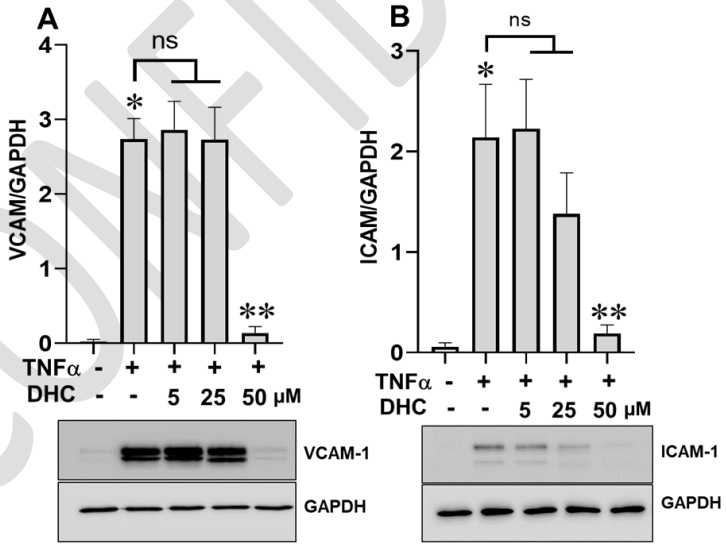


Figure 13: DHC at 50 μM decreased TNFα-induced adhesion molecule expressions. Different concentrations of DHC were co-incubated with 1 ng/ml TNFα in HUVECs for 4 hours and expressions of VCAM-1 (A) and ICAM-1 (B) were examined. (n=3-4); * p<0.05 *vs* vehicle control; ** p<0.05 *vs* TNFα alone; ns = not significant

In addition, there was no effect of nitro-DHC, DHC-sulfate or DHC-β-D-glucopyranoside against TNFα-induced inflammation (Figure 14).

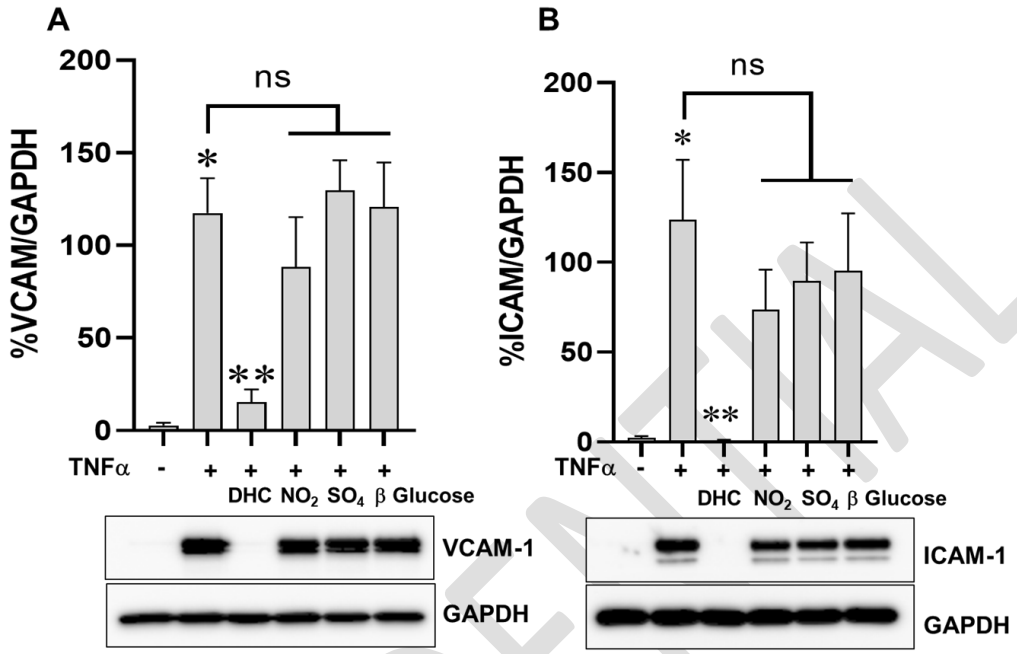


Figure 14: The effects of other capsaicinoids on TNFα-induced expressions of VCAM-1 (A) and ICAM-1 (B) in HUVEC. Representative pictures of n=3. ;* p<0.05 *vs* vehicle control; ** p<0.05 *vs* TNFα alone

3.2. DHC ameliorated TNFα-induced NFκB activity in endothelial cells

Given that the expression of VCAM-1 and ICAM-1 induced by TNFα requires activation of NFκB (38), we next examined the effects of DHC on the phosphorylation of the NFκB p65 subunit, at Ser536, which is sensitive to inflammatory stimuli (39). Treatment of TNFα increased NFκB activity by about 5-fold compared to vehicle control. In the presence of DHC at 50 μM, the phosphorylation of p65 was significantly diminished compared to TNFα alone (Figure 15). There was no change in total p65 expression. Unlike DHC, capsaicin did not affect NFκB p65 phosphorylation induced by TNFα (Figure 15A).

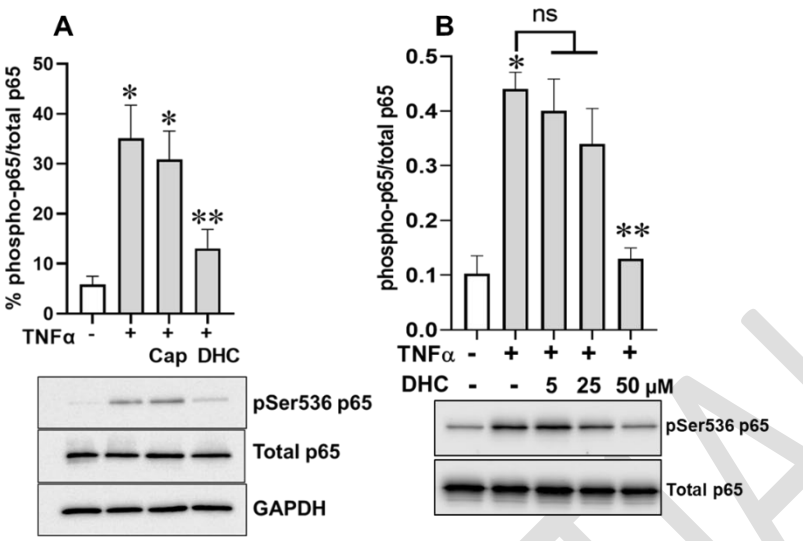


Figure 15: DHC inhibited Ser536 phosphorylation of p65 NFκB induced by TNFα. The effects of 50 µM of Cap (capsaicin), 50 µM DHC (A), or DHC at different concentrations (B) were tested in HUVECs (n=3-4);* p<0.05 *vs* vehicle control; ** p<0.05 *vs* TNFα alone, ns = not significant

Neither nitro-DHC, DHC-sulfate or DHC-β-D-glucopyranoside altered activation of NFκB (Figure 16).

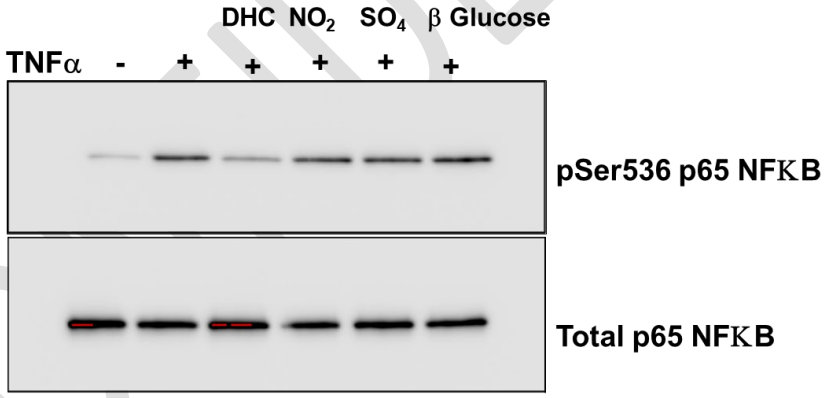


Figure 16: The effects of other capsaicinoids on TNFα-induced phosphorylation Ser 536 p65 of NFκB and total p65 of NFκB in HUVEC. Representative pictures of n=3.

3.3. Decreased TNFα-induced monocyte adhesion to endothelial cell by DHC

Upregulation of adhesion molecules, such as VCAM-1 and ICAM-1, has been proposed as a prerequisite for facilitating luminal leukocyte-endothelial interactions in response to inflammation (40). Thus, we determined whether DHC could mitigate TNFα-induced monocyte adhesion in HUVEC cultures. Fluorescently labeled human monocytes (U-397) were co-cultured with HUVECs that were pre-stimulated with either TNFα or TNFα + DHC to quantitate the extent of monocyte-HUVEC adhesion. As expected, monocyte-HUVEC adhesion was robustly increased with TNFα treatment. However, this monocyte-endothelial cell interaction was significantly diminished in the presence of DHC (Figure 17). Taken together, we concluded that DHC alleviated TNFα-induced vascular inflammatory responses.

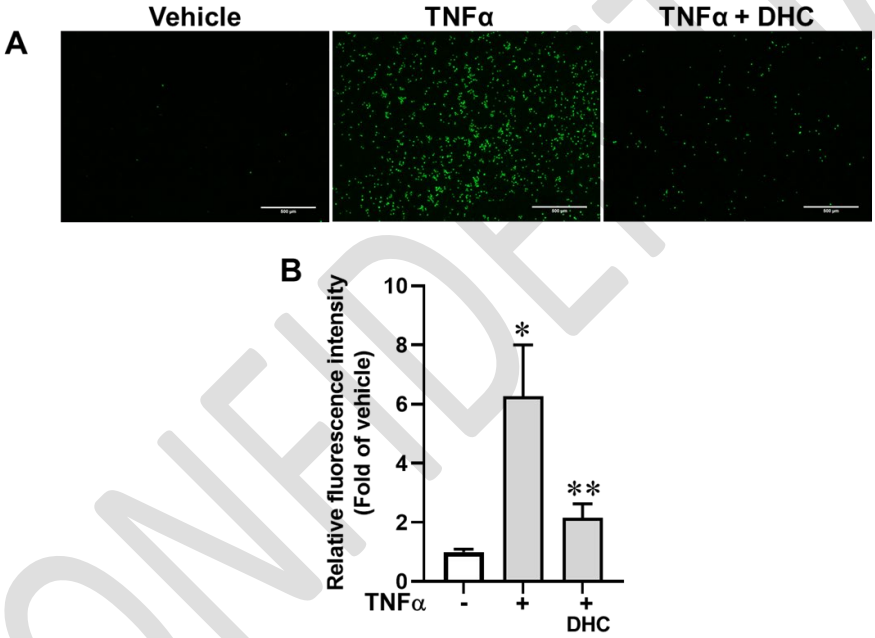


Figure 17: DHC decreased monocyte adhesion to endothelial cell surface following TNFα treatment. (A) Representative images of labelled monocytes adherence to HUVEC after stimulation with TNFα for 4 hours and (B) Quantitative data of florescence intensity from monocytes that adhered to endothelial cells were presented as relative fold of vehicle control. Experiments were repeated five times. (n=5); * $p<0.05$ vs. vehicle control; ** $p<0.05$ *vs* TNFα alone

Experiment 4) To investigate whether tested capsaiciniods increase activation of eNOS and nitric oxide (NO) production in human endothelial cells

As our results so far highlighted the significance of DHC, we then evaluated whether DHC mediates NO production in endothelial cells.

Methods: We quantified cellular NO following either DMSO, capsaicin or DHC treatment in HUVECs using a chemiluminescence method as previously described (41). Briefly, cells were cultured in 6 well-plates until reaching 90% confluence and treated with vehicle, capsaicin, or DHC for 30 minutes. Conditioned media was then collected before harvesting the cells. Cells were washed twice with chilled nitrite-free PBS. and 50 µl of PBS was added to the cells prior to plate scraping. Cultured media and cell lysates were injected directly into a tri-iodide contained solution purge vessel to measure the amount of nitrite and NO by chemiluminescence. The amount of total protein in each sample was measured using a Bradford assay. Different volumes of 1 µM standard sodium nitrite solution between 10-100 µL were injected into a purge vessel to create a standard curve. The nitrite levels were calculated from the slope of a graph plotted between the standard solution and the area under the curve of the chemiluminescence detector signals.

Results: Prior studies have shown that capsaicin promotes vasodilation in association with increased NO release in various vascular beds (14-17). Consistent with this, we observed an increase in cellular NO production by 50 µM of capsaicin compared to vehicle control (Figure 14). Incubation of HUVEC with DHC for 30 min led to an increase in cellular NO in a concentration-dependent manner, with significant effects at 10 and 50 µM, compared to control (Figure 18).

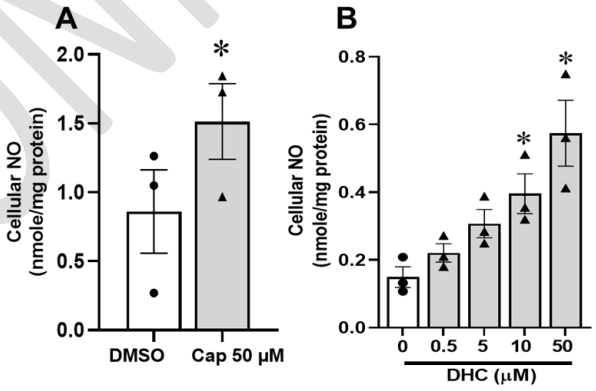


Figure 18: Capsaicin and DHC mediated NO production. Cellular NO was measured following 30 minutes incubation of 0.1% DMSO, cap (capsaicin 50µM) (A), or different concentrations of DHC (B) in HUVEC. (n=3); *p<0.05 *vs* vehicle control (DMSO)

Conclusions/Discussions: We have demonstrated the following: 1) DHC mitigated the TNFα-mediated activation of NFκB and its molecular targets more robustly than capsaicin; 2) pretreatment with DHC significantly blunted monocyte adhesion to the endothelial cell surface; 3) NO production was induced by DHC. Our data provide additional scientific evidence to support the notion that chili pepper consumption is beneficial to cardiovascular health.

Specific Aim 2: To examine the biological activities of capsaicinoids derivatives on 3D human capillary-on-a-chip

Experiment 5) To establish and optimize the protocol for a 3D human capillary model

Rationale: Emerging evidence has currently highlighted the significance of utilizing an organ-on-a-chip model in drug development. Specifically, this sophisticated *in vitro* technology is designed to create a three-dimensional (3D) tissue to capture organ-level functions. This can be achieved through advanced cell compartmentalization and microfluidic development. The high fidelity of an organ-on-a-chip provides valuable *in vitro* tool that potentially generates reliable predictions of compound safety and efficacy in human. Our original project proposed to establish the 3D human brain capillary model using a standardized microfluidic device. However, we also have been concurrently developing human capillary using HUVEC. With limitations of microfluidic field regarding low reproducibility, we carefully chose a platform that serves our current needs. Priorities for considerations include low variability between experiments but high compatibility with common lab equipment.

Methods:

3D Cell culture: We used a commercially available microfluidic platform to establish 3D human capillary. This particular chip design enables a perfused tubular tissue to be formed in adjacent to collagen gel. To culture cells in a microfluidic device, collagen type I was casted as extracellular matrix for cells to adhere. Neutralized collagen was added in the gel inlet and allowed polymerization at 37°C. Depending on experiments, HUVECs (conduit blood vessel origin) or hCMEC/D3 (brain capillary endothelial cell line) suspension at a concentration of $2x10^7$ cells/ml was seeded into the chip. 50 µl of complete cell culture media was then added in the perfusion inlet before placing the plate to the incubator at 37°C. After three hours of incubation, 50 µl of media was added to the media outlet. Plate was placed on an interval rocker for continuous perfusion. Pictures of capillary were taken daily and culture media was refreshed every couple of days**.**

Viability assay: After 5-6 days, the viability of endothelial cell tube was assessed using LIVE/DEAD Viability/Cytotoxicity kit for mammalian cells (ThermoFisher Scientific). Following the manufacturer protocol,

live cells were stained with green-fluorescent calcein-AM, whereas red-fluorescent ethidium homodimer-1 indicates dead cells.

Immunofluorescence: Endothelial cell tube was fixed with 4% formaldehyde for 15 min. The capillary was then permeabilized with 0.3% Triton X-100 for 10 minutes before incubating with blocking solution containing goat serum. CD31 primary antibody (Cell Signaling) was incubated with the capillary overnight at 4°C on interval rocker. The blood vessel was then washed and incubated with Alexa Fluor 488 anti-mouse antibody (Cell signaling) for 30 minutes. In some experiments, DAPI or phalloidin-rhodamine conjugate antibody was stained to visualize DNA and F-actin, respectively. Cells were washed and proceeded to fluorescence microscopy. Composite images of 3D structure were obtained from confocal microscopy.

Results:

Both HUVEC or hCMEC/D3 endothelial cells typically formed 3D structure quite consistently after seeding cells for a few days (Figure 19, 20 and 21).

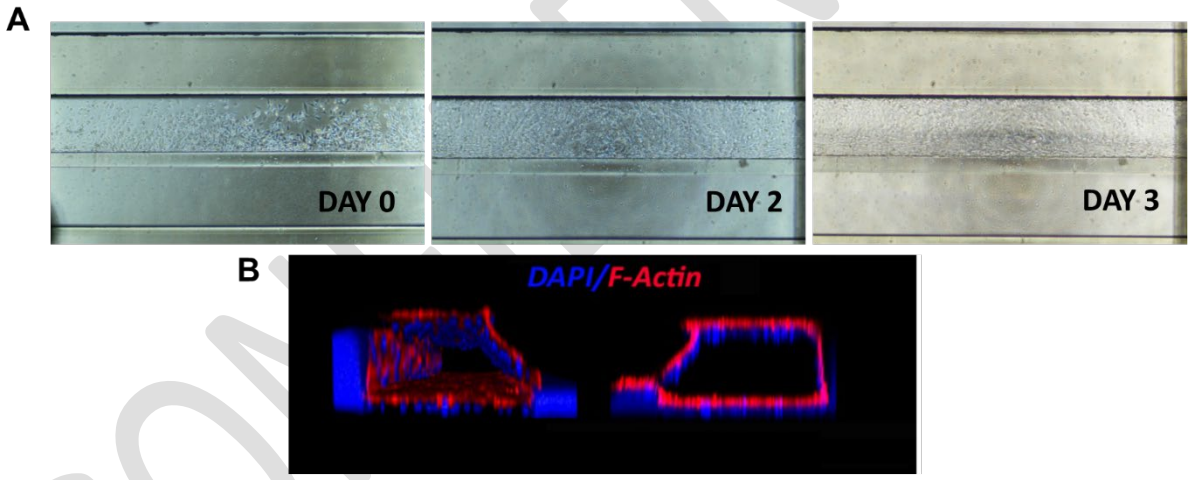


Figure 19: Typical endothelial cells in microfluidic device following cells seeding from A) Day 0 onwards. B) Composite confocal microscopy image reconstructions showed a hollow tubular structure.

Composite confocal microscopy image reconstructions showed a hollow tubular structure adjacent to collagen matrix (Figure 19B) as expected. We observed that the quality of collagen and the number of cells seeded as well as technical handing were key determinants of confluent tubular structure. Endothelial microvessels were stable for at least 7 days in a standard cell culture incubator. We were able to reproducibly generate the 3D capillary with this method.

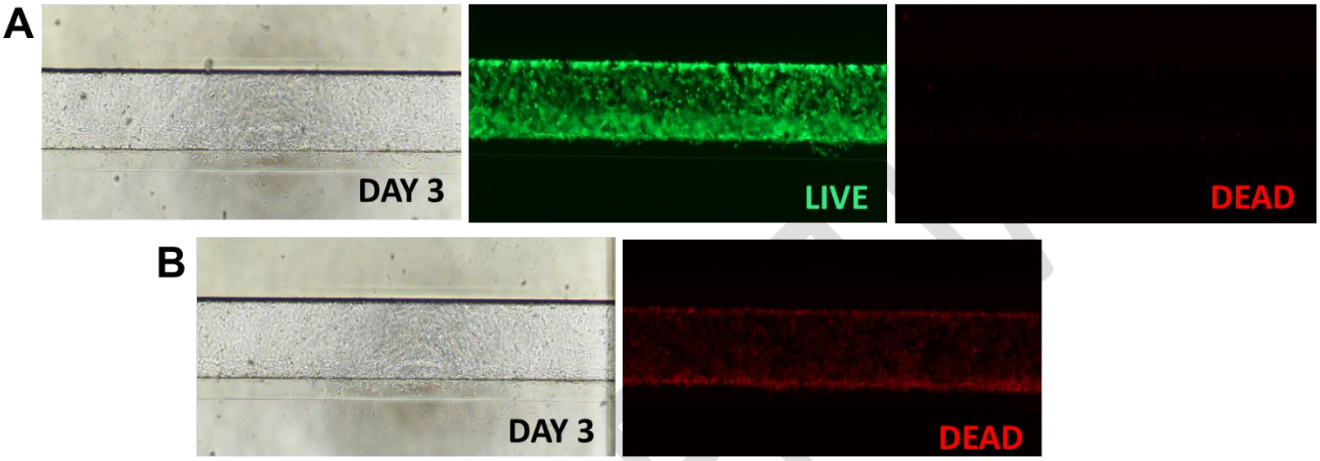


Figure 20: Live/Dead staining of human brain endothelial cell tube at Day 3. Green indicates Live cells, whereas Red indicates Dead cells. A) Representative images of 3D microvessel following Live/Dead staining (n=3) and B) Endothelial cell tube treated with 100% Ethanol showing dead cells with red color (n=3)

The capillary's viability was then assessed using Live/Dead assay. In live cells, non-fluorescent calcein AM is converted to a green-fluorescent calcein AM by intracellular esterases. The dead cells were detected by red-fluorescent ethidium homodimer-1, a cell membrane impermeable dye that emits red fluorescence once bound to DNA. The death stain control was included in our studies with a microvessel treated with 100% ethanol for 5 minutes (Figure 20B and 21B). We observed that both HUVEC and hCMEC/D3 cells in 3D capillary structure were robustly viable with minimal cell death (Figure 20A and 21A).

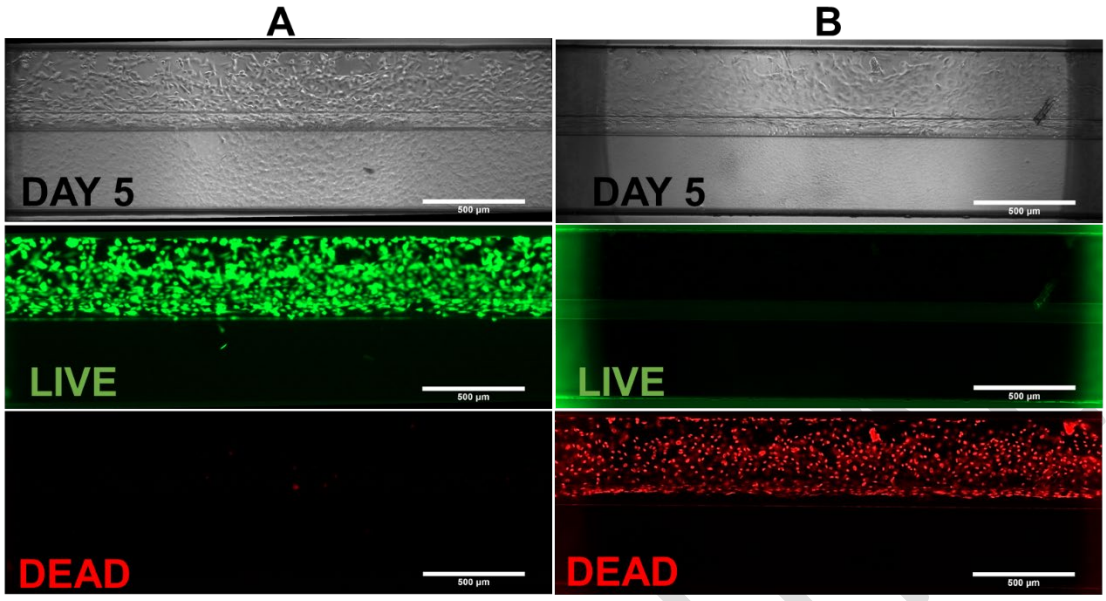


Figure 21: Live/Dead staining of HUVEC at Day 5. Green indicates Live cells, whereas Red indicates Dead cells. A) Representative images of 3D microvessel following Live/Dead staining (n>10) and B) Endothelial cell tube treated with 100% Ethanol showing dead cells with red color (n>3)

Lastly, we stained for endothelial marker, CD31 in HUVEC capillary tube. CD31 was readily detected in our 3D cell culture (Figure 22), consistent to prior reports.

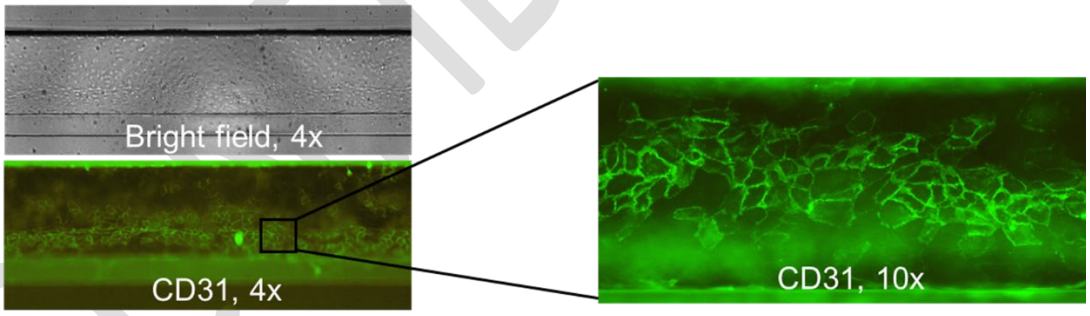


Figure 22: Expression of an endothelial marker, CD31 in 3D human capillary tube. (n>5)

Conclusions/Discussions: Although some pilot studies with brain capillary-on-a-chip protocol were generated as shown above, we did experience some inconsistencies with cell growth when seeded in microfluidic devices during the last phase of our experiments. A number of studies were carried out to improve tubular formation quality but there was a significant retard of hCMEC/D3 growth in the chip. *Due to the time constraint to overcome the obstacles encountered with brain endothelial cell line, we decided to move the project along with HUVEC.* These primary endothelial cells have been frequently deployed in creating microvessels in various

microfluidic devices. Similarly, we were able to consistently tissue engineer 3D human capillary with HUVEC without issues.

Experiment 6) To investigate the inflammatory responses induced by TNFα in endothelial cell tube and whether treatment with specific capsaicinoid mitigates the effects of TNFα

Rationale: Endothelium forms a semi-permeable barrier and separates blood from tissue (42). Selective entry of different molecules is attributable to transcellular and paracellular transport. Drugs or different pathological conditions leading to increased endothelial permeability may cause interstitial edema, decreased microcirculatory perfusion, and eventually to organ dysfunction (43).

Methods: We next investigated whether our 3D human capillary exhibited functional barrier. Using live cell time-lapse imaging, the permeability to 70 KDa of FITC-Dextran was subsequently evaluated in the period of 30 minutes. An increase in fluorescence intensity in the adjacent collagen channel as dextran diffused out of the lumen was quantified every 5 minutes. The leakage of endothelial cell tubes was expressed as a ratio of fluorescence signal in the collagen and lumen. We included cell-free tubes as an additional control to observe the maximal leakage. To test the integrity of endothelial cell tubes, some capillaries (5 days post cell seeding) were luminally exposed to an inflammatory agent, 1 ng/ml TNFα for 24 hours. In some experiments, the leakage of 3D capillary following DHC incubation with or without TNFα treatment was also determined.

Results: Under *in vivo* conditions, the macromolecules with molecular weight larger than albumin (>66.5 kDa) is usually retained in the circulation (44). To recapitulate restrictive barrier of endothelium, 70KDa of FITC dextran was luminally perfused in the 3D human capillary and monitored the leakage of green fluorescence to the adjacent collagen channel. There was a robust leakage of dextran from luminal side to basolateral compartment overtime in a cell free tube. Remarkably, the fluorescent signal was mostly retained within the lumen of 3D microvessel (Figure 23), indicating restriction barrier of endothelial cell tube to 70KDa dextran.

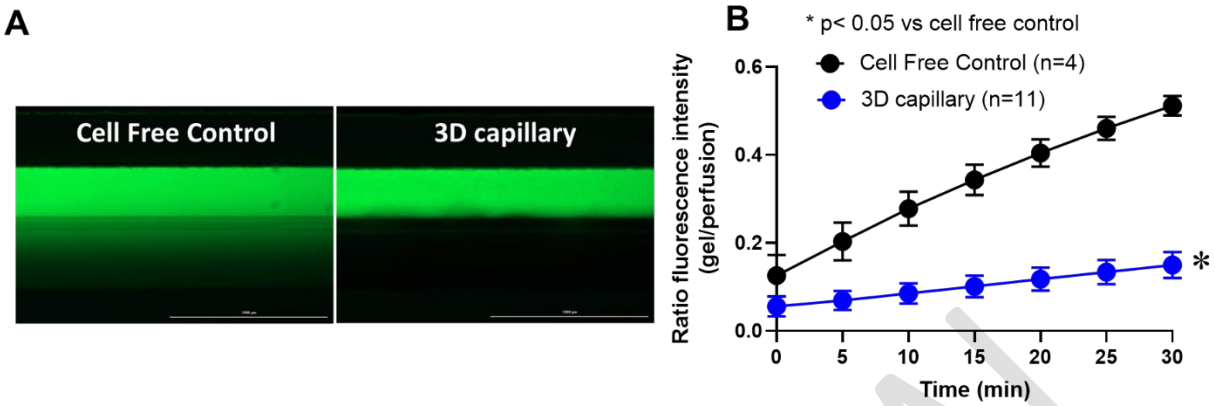


Figure 23: Permeability assay with 70KDa Dextran in human capillary-on-a-chip compared to cell free control. A) Time-lapse studies to monitor fluorescently labelled dextran leakage to adjacent collagen channel over a period of 30 minutes. B) Quantitative of fluorescent intensity from in gel normalized by in lumen (n=4-11)

Prior studies have shown that a pro-inflammatory cytokine, TNFα triggers endothelial barrier dysfunction resulting in edema formation (45). To qualify our 3D model, we asked whether the barrier integrity of endothelium could be modulated by TNFα. There was a minimal leakage of fluorescence signal from luminal side to basolateral compartment in vehicle treatment group compared to cell free tubes (Figure 24). Exposure of the 3D microvessel to TNFα for 22 hours resulted in a significant increase in permeability to FITC dextran (Figure 24). These findings led us to conclude that the response to a pro-inflammatory cytokine, TNFα-induced disruption of endothelial barrier function could be simulated in our 3D human capillary.

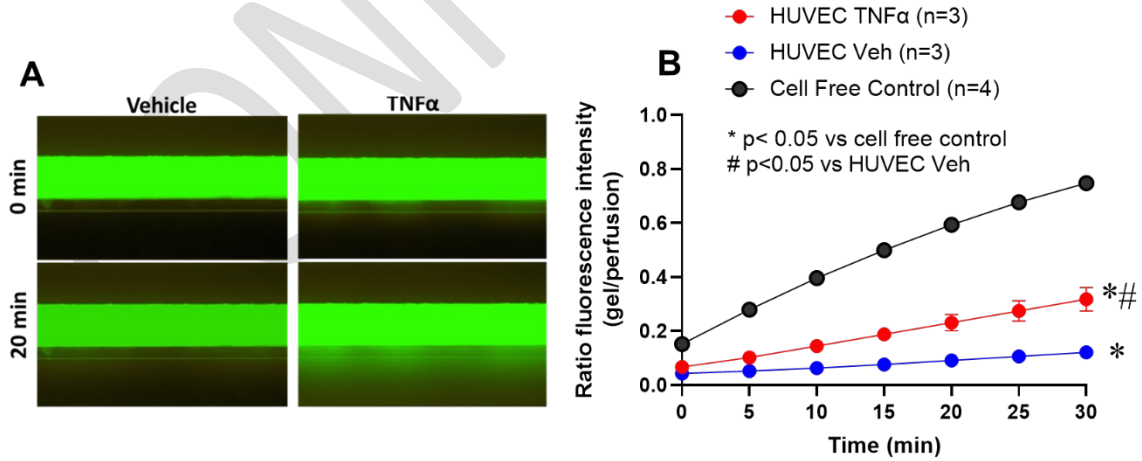


Figure 24: Impaired vascular integrity induced by TNFα. Permeability assay with 70KDa Dextran in human capillary-on-a-chip with or without 1 ng/ml TNFα treatment for 22 hours. A) Time-lapse studies to monitor fluorescently labelled dextran leakage to adjacent collagen channel over a period of 30 minutes. B) Quantitative of fluorescent intensity from in gel normalized by in lumen

Next, we asked whether a capsaicinoid analogue would mitigate the effects of TNFα-induced vascular leakage in 3D capillary. Since DHC exerted the most robust anti-inflammatory effects in 2D conventional endothelial cultures, we next tested if DHC could counteract increased permeability induced by TNFα in 3D capillary tube. Similar to above studies, there was a significant increase in vascular leakage following TNFα treatment compared to vehicle group (Figure 25A). Unexpectedly, co-incubation of 50 μM DHC and TNFα did not restore vascular permeability following TNFα treatment (Figure 25A). Neither DHC at 10 nor 50 μM alone altered endothelial barrier to 70KDa Dextran (Figure 25B).

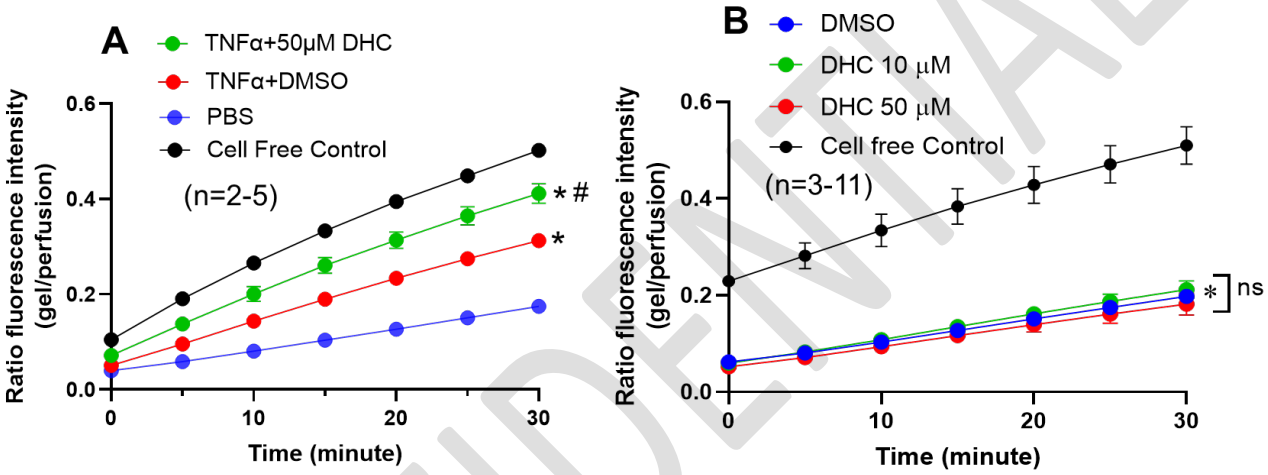


Figure 25: Effects of DHC on vascular leakage. Permeability assay with 70KDa Dextran in human capillary-on-a-chip. Time-lapse studies to monitor fluorescently labelled dextran leakage to adjacent collagen channel over a period of 30 minutes. A) DHC pre-treatment of 3D endothelial cell tubes for 30 minutes prior to co-incubation with TNFα for 22 hours and B) DHC alone exposure to HUVEC tubes for 22 hours. *p<0.05 vs. cell-free control, $^{\#p}$<0.05 vs. TNFα+DMSO; ns= not significant

Conclusions/Discussions: Our current studies utilized 3D human tissue engineered microvessel to interrogate the potential modulator of endothelial integrity. Not only that we demonstrated a significant restriction of our tissue engineered capillary, but we also observed endothelial barrier disruption following TNFα treatment. We have been conducting permeability assay in 3D capillary repeatedly with high reproducibility (total biological replicates >50). When compared to previous reports, our data appear comparable to others(44) (46). Despite robust anti-inflammatory responses observed with DHC in 2D cell cultures, the leakage of 3D microvessel induced by TNFα was not restored by DHC. Indeed, incubation of DHC exacerbated disruption of endothelial barrier. Apparently, this was not expected. Data available in the literature remains controversial. Capsiate and

dihydrocapsiate, non-pungent derivatives have been shown to reduce VEGF-induced endothelial permeability *in vitro* (47). Pre-treatment of capsaicin to mouse endothelial cells alleviated barrier leakage induced by high glucose (48). Despite these findings, other groups have reported an increase in permeability by opening tight junctions of endothelial cells following 100 μM of capsaicin treatment (23). Possibly, an additive effect of DHC and TNFα on rearrangement of tight junction protein and cytoskeleton contributes to unexpectedly increase in capillary leakage in our 3D model.

Experiment 7) To examine whether tested capsaiciniods treatment will affect TNFα-induced monocyte adhesion in 3D human capillary tube

Rationale: Although measuring NO in 3D capillary would be an important marker to assess endothelial function, quantification of NO in microfluidic device was not simple. Indeed, other readouts that would be more relevant to our overall hypothesis include evaluation of monocyte adhesion and quantification of cytokine release. These assays will allow us to examine the effects of capsaicinoids on TNFα-induced endothelial inflammation in 3D human capillary. In this particular experiment, we examined whether DHC could mitigate monocyte adhesion-induced by TNFα in 3D capillary.

Methods: Human monocyte cells, U-937 were harvested and suspended in RPMI medium. They were labelled with 10 μM Green CMFDA Dye (ab145459, Abcam) and centrifuged at 200 × g for 5 minutes. Monocytes were re-suspended in MV2 endothelial cell medium. Concurrently, the 3D endothelial microvessels were stimulated with TNFα (0, 1, 10 ng/ml) for 6 hours. The nuclei of endothelial microvessels were stained with 5 μg/ml Hoechst (H3570, Thermo Fisher Scientific) for 20 minutes at 37°C. After washing endothelial tube with MV2 medium for 5 minutes, the labeled monocyte in the suspension will be added to luminal side of the HUVEC microvessel. Following incubation for 15 minutes at 37°C on a rocking platform, the microvessels were gently washed with HBSS for 5 minutes twice. Images of luminal side of endothelial tube were taken using fluorescent microscope (Zeiss). If necessary, confocal microscopy was used to create Z-stack of monocyte binding to endothelial surface. The numbers of monocyte adhesion to endothelium were counted using ImageJ. I some studies, DHC was pre-treated for 30 minutes prior to co-incubation with TNFα for 6 hours before performing monocyte adhesion assay.

Results: Adhesion of monocytes to endothelial surface is an early step of an atherosclerotic plaque initiation. Various cytokines including TNFα have been shown to mediate monocyte adhesion through upregulations of adhesion molecules such as VCAM-1 and ICAM-1 (49). Based on our 2D endothelial cell results, DHC significantly

reduced TNFα-induced monocyte adhesion. Next, we hypothesized that monocyte adhesion to endothelial surface induced by TNFα would also be decreased following DHC exposure in 3D human capillary. We first established an assay to demonstrate that TNFα increased monocyte adhesion to the luminal surface of endothelial cell tube. As shown in Figure 26, there was minimal monocyte-endothelial interaction in vehicle

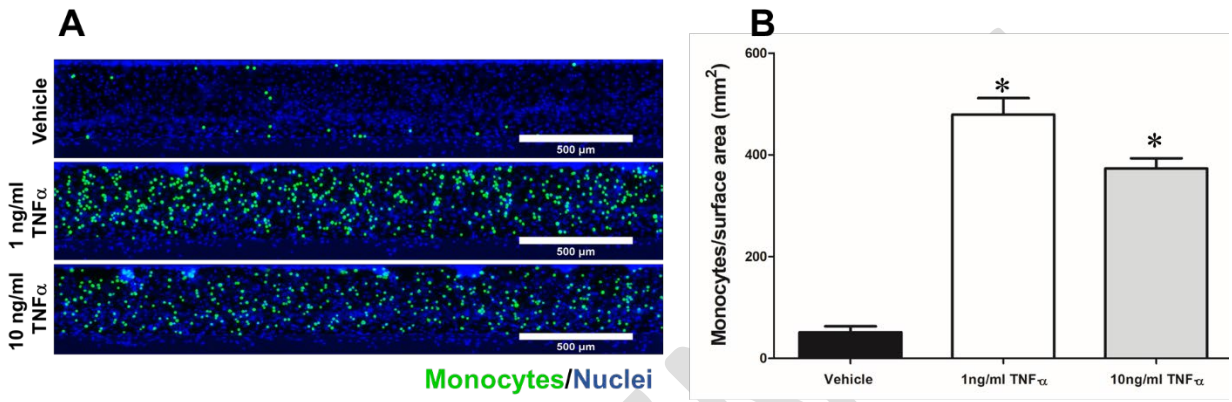


Figure 26: Monocyte adhesion studies in 3D human capillary-on-a-chip at baseline and after TNFα treatment. Monocytes labelled with Green CMFDA Dye was perfused luminally to the microvessel. A) Images of luminal side of endothelial tube were taken using fluorescent microscope. B) Quantification of monocyte adhesion normalized by surface area. *p < 0.05 vs vehicle (n>3)

control, similar to *in vivo* conditions. TNFα stimulation for 6 hours led to a marked induction of monocyte adhesion, consistent with increased adhesion molecules expressions (Figure 26). Next, we tested whether DHC could diminish monocyte adhesion to apical surface of 3D endothelial cell tube. Again, there was a significant

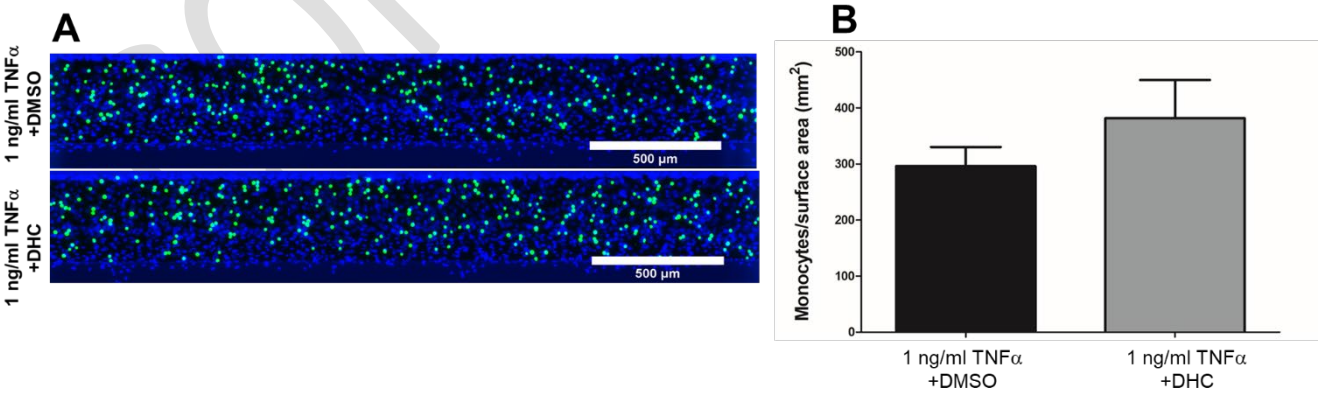


Figure 27: The effects of DHC on monocyte adhesion studies in 3D human capillary-on-a-chip following TNFα treatment. Monocytes labelled with Green CMFDA Dye was perfused luminally to the microvessel. Images of luminal side of endothelial tube were taken using fluorescent microscope. (n≥3)

increase in adherence of monocyte to endothelium following TNFα treatment. However, exposure of capillary with DHC did not decrease TNFα-induced immune responses (Figure 27).

Conclusions/Discussions: We have successfully developed an assay and workflow to monitor an immune response in 3D human capillary. This model recapitulates monocyte adhesion to endothelial surface in more physiologically relevant than those in conventional culture. In normal condition, monocyte adherence to luminal wall minimally occurs in *in vivo*, similar to what we observed here. Upon stimulation with TNFα, a robust increase in monocyte adhesion was detected, providing a useful tool to screen drugs or compounds that possibly modulate immunological responses. Although we have provided a strong evidence demonstrating the impact of DHC in alleviating TNFα-induced monocyte adhesion in 2D cultures, the results differed in 3D capillary. The reasons for these discrepancies remain unclear and probably require further investigation. Perhaps, expressions of mediators necessary for DHC functions observed in 2D were not present when endothelial cells were exposed to flow or grown in 3D context. Alternatively, DHC might be degraded more rapidly with 3D cultures leading to loss of its functions.

Discussions

We believe that we have demonstrated significant progress to advance scientific discoveries supported by funding from TRF in these past two years Despite difficulties during COVID-19 causing multiple rounds of lockdown, we were able to provide additional pharmacological evidence to support the use of chili pepper. Given the potent effects of DHC on the endothelium shown here in 2D cultures, we postulate that the continuation of capsaicinoid intake will result in protection against endothelial dysfunction. This notion is in line with a previous meta-analysis of human studies that demonstrated an association between regular chili pepper intake and lower all-cause cardiovascular-related mortalities (3). Interestingly, more recent evidence has increasingly suggested a significant positive correlation between endothelial dysfunction and mortality rate among COVID-19 patients (8) (9). Given our data here together with other reports, we hypothesize that *capsicum* consumption as part of our food routine may help attenuate the severity of cardiovascular symptoms associated with COVID-19. Although limitations of capsaicin and DHC-induced irritation has been well recognized, developing alternative, non-pungent capsaicinoid analogs will provide possible therapeutic options with better safety profiles for clinical use. In our studies, we tested the effects of capsaicinoid derivatives only with endothelial cells. It remains largely unknown whether these capsaicinoid analogues exert their cardiovascular protective activities involving other cell types. Multiple lines of evidences have strongly demonstrated that immune system and inflammation play a critical role in hypertension and related cardiovascular diseases (50).

Investigating whether these non-pungent capsaicinoids derivatives exhibit their biological activities in immune cells will be significant.

With this funding from TRF, we also successfully developed tissue engineered human capillary in a microfluidic device. Although this technology is relatively new in Thailand, interest in the area of organ-on-a-chip has been exponentially increasing in other countries. Recent findings from multiple laboratories have suggested that data obtained from 3D organ models are more translational to human and this novel technology platform will eventually decrease the animal use for drug testing (51). Although an organ-on-a-chip model was firstly established to resolve high cost of drug development, applications of this technology currently extends far beyond preclinical screening of compounds. It provides a useful tool to imitate human diseases or organ level functions of those that are difficult to model in animals. Moreover, toxins or infectious substances can be examined in an organ-on-a-chip to investigate their impact on human health *in vitro.* For example, Tissue Chips in Space initiative enables the study of microgravity environment on the human body using various organ-on-a-chip models (52). A European Union Horizon has been developing placenta model to investigate molecular interaction during normal and pregnancy-related diseases conditions (53). Currently, major limitations of an organ-on-a-chip including low reproducibility and difficult to use issues have restricted regular users to widely adopted this emerging technology (54). With my past experience working with different platforms of microfluidic devices, ease of use and high reproducibility have been my focus to choose the appropriate chip design for our current purposes. 3D human organ-on-a-chip model in our laboratory recapitulates key functions of endothelium and it is highly reproducible. This offers tremendous opportunities for compound testing and human organ function modelling in Thailand.

4. ภาคผนวก

Despite significant progress made with this project, we have modified and conducted our studies based on progressive scientific findings to maximize outputs and usefulness of outcomes. The following table provides details of how project differs from original plans.

Original plans	Actual studies
1) To characterize hCMEC/D3 cells and determine the purity of our cell source	hCMEC/D3 and HUVEC cells markers were characterized to ensure the identity and purity of cell sources.
2) To evaluate the appropriate concentration and time for capsaiciniods derivatives treatment in hCMEC/D3	Cytotoxicity assay has been conducted to determine appropriate concentration of capsaicinoids for subsequent studies in endothelial cells.
3) To examine the protective effects of tested capsaicinoids against TNFα-induced inflammatory responses	-The results with hCMEC/D3 cells were rather inconsistent when testing capsaicinoids effects on anti-inflammation and production of NO. This is probably due to lack of key mediator expressions such as TRPV1 receptor and eNOS, respectively. With this limitation, we decided to conduct our subsequent experiments with HUVECs to ensure the success of the project. Data were then generated from HUVEC to test the overall hypothesis.
4) To investigate whether tested capsaiciniods increase activation of eNOS and NO production in the presence or absence of TNFα treatment	
5) To establish and optimize the protocol for a 3D human brain capillary model	-Protocols and workflows for 3D human capillary were established with hCMEC/D3 (brain specific) and HUVEC (conduit vessel origin) cells. Specifically, viability and 3D tubular structure were shown. Moreover, the barrier integrity of endothelium was apparent in 3D capillary compared to cell-free control.
6) To investigate the inflammatory responses induced by TNFα in EC tube and whether treatment with specific capsaicinoids mitigates the effects of TNFα	-The inflammatory responses induced by TNFα in 3D capillary tube were investigated. There was an increase in permeability following 22 hours of TNFα treatment. However, DHC, a capsaicinoid analogue did not restore TNFα-induced vascular leakage.

7) To examine whether tested capsaiciniods treatment will increase total nitric oxide production in human capillary tube following TNFα incubation	-To cohesively investigate inflammatory partway, immunologic response assay was established instead of measurement of NO. Specifically, we developed an assay which we can visualize monocyte adhesion to luminal surface of 3D capillary. As expected, TNFα markedly increased monocyte adhesion, a normal immunologic response following cytokine treatment. However, exposure of DHC did not lessen the response of TNFα.

ผลที่ได้รับเมื่อสิ้นสุดการวิจัย

1. Outputs

Different capsaicinoids derivatives were tested for their potential pharmacological activities against TNFα-induced endothelial inflammation. Based on our current data, DHC appears to exert strong effects to mitigate inflammation, monocyte adhesion and increase NO release, at least, in conventional cultures. We submitted the results for publication which is currently under revision in Q1 journal.

Notably, 3D human capillary-on-a-chip has been developed successfully with high fidelity and resemblance of *in vivo* endothelial functions in our current studies. Additional scientific articles relating to utilization of this platform have been under preparation. Lastly, we have been training our graduate students and research scientists to work with 3D tissue engineering platforms. This opportunity enables our personnel to be skillful with the new technology, which had not been available elsewhere in Thailand. Due to this funding, we are able to train a Master degree student who will defend her thesis at the end of this year. She has been instrumental in developing human capillary-on-a-chip and its associated assays downstream.

2. Outcomes

The pharmacological activities of previously under-appreciated capsaicinoid analogue, DHC have been described in our current studies. Our present study highlights the benefits of DHC treatment on human endothelial cells and provides evidence to support cardiovascular benefits from capsicum consumption.

In addition, we are able to successfully develop 3D capillary-on-a-chip with high reproducibility. To our knowledge, this technology is not available elsewhere in Thailand. Having successful setup of an organ-on-a-

chip model in our laboratory will put forward the possibilities of testing compounds of interests in a more resemblance of human organ functions than those from conventional cultures.

3. Impact

Cardiovascular disease (CVD) remains the leading cause of death worldwide (1). With a rapidly aging population and inadequate prevention, premature death from CVD has become epidemic. It is interesting to note that low and middle-income countries are now disproportionately affected, leading to high financial burdens (1). Our studies provided scientific evidence and added new public knowledge of potential cardiovascular benefits of capsicum consumption. Accumulating of scientific evidence ultimately will lead to development of high value nutraceuticals of capsicum products and helps stimulate the agriculture of hot peppers. Possibly, this will create high financial return to farmers and local businesses. Additional impact from data generated from this project also includes the availability of 3D human capillary-on-a-chip that can be employed to test safety and efficacy of other compounds of interest in the future. This could eventually help generate safer and more efficacious products.

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