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Ex Vivo Human Aortic Punch Tissue: A New Approach for Investigating Angiogenesis in Cardiovascular Diseases

(Tisu Pukulan Aorta Manusia *Ex Vivo*: Pendekatan Baharu untuk Mengkaji Angiogenesis untuk Penyakit Kardiovaskular)

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ABSTRACT

Cardiovascular diseases (CVDs) remain the leading cause of mortality worldwide, with pathological angiogenesis contributing to disease progression. Conventional *ex vivo* angiogenesis assays often employ animal tissues, but physiological differences limit translational relevance. This study establishes a human *ex vivo* angiogenesis model using discarded aortic punch tissues from coronary artery bypass graft (CABG) surgeries, providing a clinically relevant platform for investigating angiogenic mechanisms. Two culture approaches of embedded and sandwich techniques were optimized using aortic punch tissues maintained in Matrigel for up to 27 days. Angiogenic sprouting was assessed microscopically, quantified with ImageJ, and analysed using repeated measure ANOVA with Bonferroni post hoc test. No sprouting was observed with the embedded method. In contrast, the sandwich method induced vessel sprouting, initiating at Day 7 with progressive increases in sprouting area and branching length through Day 27 ($p < 0.01$). Under hypoxic conditions (1% O_2), sprouting was significantly enhanced compared to normoxia (20% O_2), with greater sprouting area, branch number, and branching length. Hypoxia also elevated HIF-1α expression, particularly at Day 7 and peaked at Day 14 ($p < 0.05$) compare to normoxia, confirming hypoxia-driven angiogenic pathways. The sandwich culture method effectively supports angiogenesis in human aortic punch tissues, unlike the embedded approach. Hypoxia further amplifies angiogenic responses via HIF-1α signalling. This optimized human *ex vivo* assay offers a clinically relevant model bridging *in vitro* and animal-based systems, serving as a valuable tool for mechanistic studies and therapeutic exploration in cardiovascular disease.

Keywords: Angiogenesis; aortic ring assay; *ex vivo*; human aortic tissue

ABSTRAK

Penyakit kardiovaskular (CVD) kekal sebagai punca utama kematian di seluruh dunia, dengan angiogenesis patologi menyumbang kepada perkembangan penyakit tersebut. Ujian angiogenesis *ex vivo* konvensional lazimnya menggunakan tisu haiwan, namun perbezaan fisiologi antara spesies mengehadkan kerelevanan translasi. Kajian ini membangunkan satu model angiogenesis *ex vivo* manusia menggunakan tisu tebukan aorta yang tidak digunakan daripada pembedahan cantuman pintasan arteri koronari (CABG), yang menyediakan platform klinikal relevan untuk penyelidikan mekanisme angiogenik. Dua pendekatan kultur, iaitu teknik terbenam dan tertindih telah dioptimumkan menggunakan tisu tebukan aorta yang dikekalkan dalam Matrigel sehingga 27 hari. Percambahan angiogenik dinilai secara mikroskopik dianalisis secara kuantitatif menggunakan perisian ImageJ dan seterusnya dianalisis menggunakan ANOVA ukuran berulang dengan ujian post hoc Bonferroni. Tiada percambahan diperhatikan melalui kaedah terbenam. Sebaliknya, kaedah tertindih berjaya mendorong percambahan salur darah yang bermula pada hari ke-7 dengan peningkatan berterusan dalam keluasan percambahan dan panjang percabangan sehingga hari ke-27 ($p < 0.01$). Dalam keadaan hipoksia (1% O_2), percambahan didapati meningkat dengan ketara berbanding keadaan normoksia (20% O_2) dengan peningkatan keluasan percambahan, bilangan cabang, serta panjang percabangan. Hipoksia juga meningkatkan pengekspresan HIF-1α, khususnya pada hari ke-7 dan maksimum pada hari ke-14 ($p < 0.05$) berbanding dengan normoxia, sekali gus mengesahkan pengaktifan laluan angiogenik yang dipacu oleh hipoksia. Kaedah kultur tertindih terbukti berkesan dalam menyokong angiogenesis dalam tisu tebukan aorta manusia, tidak seperti kaedah terbenam. Keadaan hipoksia seterusnya memperkuat tindak balas angiogenik melalui pengisyaratan HIF-1α. Ujian *ex vivo* manusia yang dioptimumkan ini menawarkan model yang relevan dari segi klinikal, berfungsi sebagai jambatan antara sistem *in vitro* dan berasaskan haiwan, serta menjadi alat penting untuk kajian mekanisme dan penerokaan terapeutik dalam penyakit kardiovaskular.

Kata kunci: Angiogenesis; *ex vivo*; tisu aorta manusia; ujian cincin aorta

INTRODUCTION

Cardiovascular diseases (CVD) represent a critical research focus as these conditions continues to pose a significant global health economic burden, contributing to increasing mortality rates worldwide. According to the latest update from the World Health Organization (2021), heart attacks and strokes account for 85% of deaths associated with CVD. Notably, the development and progression of CVD are particularly driven by abnormal cellular biological function and inflammatory process that may lead to hardening the blood vessels, a condition known as atherosclerosis. This pathophysiology has been intricately linked with complex series of events including pathological angiogenesis (Zou et al. 2021).

Angiogenesis can be defined as the formation of new blood vessels from pre-existing vascular structures and plays a pivotal role in normal physiology processes such as embryonic development and tissue remodelling (Annex & Cooke 2021; Ye & Hines 2024). However, in the context of CVD, angiogenesis can contribute to diseases progression by facilitating neovascularization within atherosclerotic plaque which eventually led to plaque instability and increase the risk of adverse cardiovascular events (Annex & Cooke 2021). A comprehensive understanding of the mechanisms underlying pathological angiogenesis in CVD is essential for identifying and developing novel therapeutic targets.

Historically, a significant number of researchers have extensively relied on *in vitro* methods by utilizing animals or human cells to mimic angiogenesis events, such as HUVECs which have been widely used (Razmi et al. 2025). Additionally, *in vivo* and *ex vivo* approaches, involving various animal models especially mice have also become increasingly common (Klein & Hutmacher 2024; Mukherjee et al. 2022). In the context of *ex vivo* models, aortic ring assay is a widely used technique for angiogenesis studies with establishment of various organ-based models (Bellacen & Lewis 2009). Mice have predominantly been the primary source of samples for aortic ring assay in previous studies (Baker et al. 2011; Blot et al. 2021; Kapoor, Chen & Iozzo 2020). This assay involves culturing cross-sectional aortic rings from animals within a 3D matrix such as collagen or Matrigel, allowing endothelial cells to develop into sprouts and microvessel-like structure over (Baker et al. 2011). However, using mouse models has notable limitations due to species differences in genetic, physiological and immunological factors (Rydell-Törmänen & Johnson 2018). These differences might affect how the diseases manifest and respond to treatment which potentially cause the results might not directly translatable to human physiological pathological condition. Regardless of its potential in angiogenesis study, its application using adult human samples has yet to be widely applied and remains limited.

In this study, we aimed to develop a novel *ex vivo* model by utilizing discarded human aortic punch tissue surplus obtained from adult patients undergoing coronary artery bypass grafting (CABG) surgery. During CABG procedures, a small circular segment of the ascending aorta is routinely excised using an aortic punch device to create an opening for proximal graft anastomosis to enable the new graft vessel to be sutured onto the aorta, thereby bypassing the obstructed coronary artery. This excised tissue, which is typically discarded as surgical waste, was collected and repurposed to establish an *ex vivo* human aortic punch ring culture model for the investigation of angiogenesis.

This approach repurposes tissue that would otherwise be discarded, providing a unique and ethically sound opportunity to study angiogenesis directly within a context that is far more clinically relevant than traditional *in vitro* or animal *ex vivo* models. Establishment of this model provides a new platform for investigating the cellular and molecular mechanisms underlying pathological angiogenesis in the diseased adult human cardiovascular tissue, therefore potentially yielding new insights and therapeutic targets for CVDs.

MATERIALS AND METHODS

This study had been approved by Universiti Kebangsaan Malaysia Research Ethics Committee (Ethics Ref.no. UKM PPI/111/8/JEP-2024-698). Human aortic punch tissue was collected as detailed in the ethics protocol. This study was conducted in accordance with the ethical standard outlined in the Declaration of Helsinki and its later amendments.

SAMPLE COLLECTION

Aortic tissue discs were collected from consented adult patients (aged 20-80 years old) who underwent the CABG procedure in the operating theatre at Level 2, Hospital Cancelor Tuanku Mukhriz. The mean age of the patient donors was 58 ± 10.7 years. The diameter of the tissues collected were approximately 3 mm. The tissue was collected and placed in a sterile sample container containing media buffer and were kept in chiller until transported to the laboratory.

AORTIC PUNCH TISSUE PROCESSING AND CULTURING

The processing methods were optimized based on the protocols established by Blot et al. (2021), ensuring that all steps were conducted under sterile conditions. The aortic punch tissue (approximately 3 mm in diameter) was immediately transferred to a petri dish containing ice-cold sterile Phosphate-Buffered Saline (PBS, Sigma Adrich, USA), to prevent tissue desiccation. Adhering fat and connective tissue, specifically the adventitial layer, were carefully removed using fine surgical instruments. Tissue heterogeneity was minimized during the explant preparation process by ensuring that the following steps were standardized by size and thickness consistency. Pipette tips were pre-chilled overnight to prevent Matrigel (Corning®) polymerization in the tips. For the sandwich method, 30 µL of Matrigel was added to each well of a

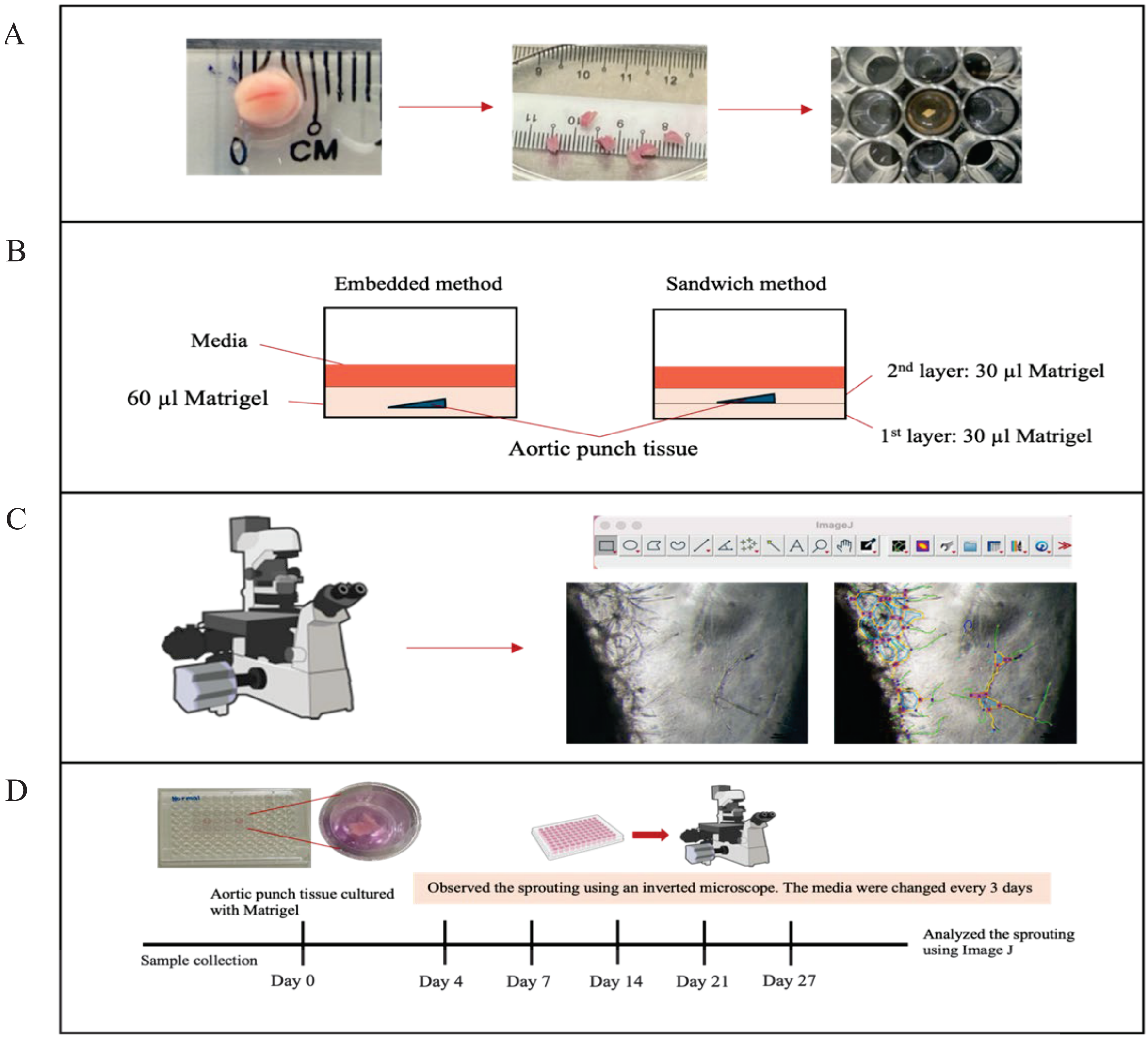


FIGURE 1. Overview of aortic punch processing using the sandwich and embedded methods. (A) Tissue preparation: the aortic punch was rinsed with sterile, cold PBS, cut into small segments, and cultured in Matrigel under 5% CO_2, with medium changes every three days. (B) Culture methods: processed tissues were seeded in a 96-well plate using either the embedded or sandwich method. For the embedded method, a single 60 µL layer of Matrigel was added to each well, and tissue segments were placed immediately before gel polymerisation. For the sandwich method, 30 µL of Matrigel was first added to form the bottom layer and allowed to solidify at 37 °C for 1 h. Tissue segments were washed with cold PBS, gently dried, and placed on the solidified layer, followed by an additional 30 µL of Matrigel to form the top covering layer. (C) Observation: cultured tissues were monitored using an inverted microscope and analysed for angiogenic outgrowth with 4× magnification (scale bar: 100 µm). (D) Timeline: tissue cultures in Matrigel were maintained and observed for up to 27 days

96-well plate, which was then incubated at 37 °C for 1 h to allow complete solidification. Next, the tissue was carefully cut into small segments (approximately 1 mm × 1 mm). As the gel polymerized, the tissue segment was washed with cold PBS and then dried with absorbent paper. The tissue was then placed on Matrigel before adding an additional 30 µL of Matrigel to cover the tissue.

In contrast, for the embedded technique, 60 µL of Matrigel was added to the well of a 96-well plate, and the tissue was immediately placed into the well before the gel polymerized. The gel was then allowed to polymerize for 1 h at 37 °C. While the gel polymerised, the complete endothelial cell media (ECM, ScienCell, USA), with 10% foetal bovine serum (FBS, Thermo Fisher Scientific, USA), 2% endothelial cell growth serum (ECGS ScienCell, USA) and 1% Penicillin-streptomycin (Thermo Fisher Scientific, USA) was freshly prepared. A total of 150 µL of complete media was added to the well with tissue and 200 µL of PBS was added to empty wells surrounding the tissue to avoid evaporation. The tissue culture was incubated at 37 °C with 5% CO_2 for sprouting formation to occur. The media were changed every 3 days. Figure 1 illustrates the workflow of the methods used in this study to optimize the aortic punch culture. For the sprouting assay, a total of N=3 independent biological replicates (representing three different human donors) were used.

POSITION OF AORTIC TISSUE CULTURE

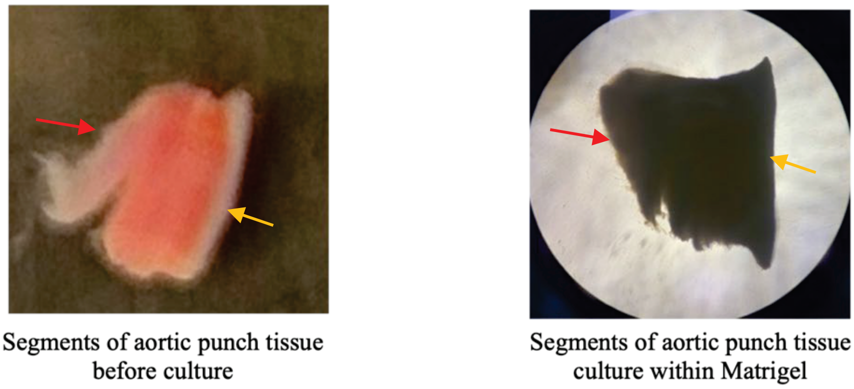
Human aorta consists of the 3-wall layer (adventitia, media and intima). Endothelial cells which line the intima layer where the angiogenesis will be formed. There for the aortic punch that are collected will be dissect and positioned into the well as shown in Figure 2.

TISSUE CULTURE ENVIRONMENT

The *ex vivo* aortic ring assay is a most technique used for examining sprouting angiogenesis (Mehta & Mahmoud 2022). This method is highly adaptable and can be employed to explore various growth factors influence vascular sprouting in a setting that closely mimics physiological conditions. In this study, we focused on optimise protocol for studying vascular sprouting using human aortic punch tissue exposed to different environments: Normal conditions (normoxia) and hypoxic conditions (hypoxia). For the normal condition, the tissue was incubated in a standard incubator set to 20% O_2 and 5% CO_2, with the remaining 75% being N_2. In the hypoxic treatment group, the samples underwent similar processing in a hypoxic environment (1% O_2, 5% CO_2, 94% N_2) to encourage angiogenic sprouting. Prior to hypoxia incubation, the tissues were incubated under normal conditions for 24 h to prevent stress-induced and abrupt changes in the tissue. The sprouting formation was observed using an inverted microscope and analyzed with ImageJ with comparisons made between normoxia and hypoxia at designated time points.

TISSUE PROTEIN EXTRACTION

Aortic punch tissues were rinsed with pre-cooled PBS, weighed, and homogenized in RIPA lysis buffer (1 mL per 0.3 g tissue; Elabscience, China) using a tissue homogenizer. Homogenates were incubated on ice for 1 h and vortex every 20 min (10 s). Samples were centrifuged at 12,000 rpm for 20 min at 4 °C, and the supernatants containing proteins were collected and stored at –80 °C until further use.



Segments of aortic punch tissue before culture

Segments of aortic punch tissue culture within Matrigel

FIGURE 2. Cross-section of aortic punch tissues and position of the aortic punch section in Matrigel culture under a microscope. The red arrow indicates the intima, which is composed of endothelial cells and appears pinkish upon gross inspection. The yellow arrow indicates the adventitia, which consists primarily of smooth muscle cells and fibroblasts and appears slightly yellowish. Vessel sprouting is observed from the endothelial (intimal) side

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR HIF-1α

A sandwich ELISA was performed using a commercial kit (Elabscience, China) according to the manufacturer's protocol. Briefly, 100 µL of standard, blank, or sample was added to each well and incubated at 37 °C for 90 min. After incubation, the wells were decanted and 100 µL of Biotinylated Detection Antibody working solution was added, followed by a 60 min incubation at 37 °C. Wells were then washed three times, and 100 µL of HRP-conjugated working solution was added, with incubation for 30 min at 37 °C. Subsequently, 90 µL of substrate reagent was added and incubated for 15 min at 37 °C, protected from light. The reaction was stopped by adding 50 µL of stop solution to each well, and absorbance was measured at 450 nm using a microplate reader.

STATISTICAL ANALYSIS

The sprouting was observed at days 0, 4, 7, 14, and 21 days. The sprouting area and branching were then calculated using Image J software. Longitudinal changes across the culture period were analysed using repeated measures ANOVA in IBM SPSS Statistics version 26. Mauchly's test was used to assess the assumption of sphericity, and Greenhouse–Geisser correction was applied when necessary. Post-hoc pairwise comparisons were performed using Bonferroni adjustment. Independent T-test was used to compare between normoxia and hypoxia group across the time point. A p-value < 0.05 was considered statistically significant.

RESULTS

First, the efficacy of embedded and sandwich culture techniques was evaluated using human aortic punch tissue over a 27-day incubation period. A clear disparity in angiogenic potential was observed between the two methods. In the sandwich technique, microvessel sprouting initiated by Day 7 and exhibited progressive expansion throughout the culture duration (Figure 3(B)). Conversely, the embedded technique yielded no observable sprouting under microscopic evaluation at any point during the study (Figure 3(A)).

Quantitative assessment via the Image J Angiogenesis Analyzer conformed the disparity between the methods. Within the sandwich model, the sprouting area (Figure 3(C)) showed significantly increase over time ($F(3,42) = 10.55$, $p < 0.001$). Bonferroni post-hoc analysis showed that sprouting at Day 21 (2410138.4 ± 362591 pixel^2) and Day 27 (3562992.4 ± 304283 pixel^2) were significantly higher than Day 7 (159308.10 ± 45845 pixel^2) and Day 14 (931994.1 ± 260911 pixel^2). Similarly, total branching length were also significantly elevated by Day 21 (6655.67 ± 429.04 pixel) and 27 (7039.00 ± 278.83 pixel) compared to the Day 7 (3762.73 ± 528.773 pixel) as shown in Figure 3(D) ($p<0.01$). In contrast, all quantitative parameters of sprouting area and total length branching for the embedded method remained at zero throughout the 27-day period, establishing the sandwich technique as the superior model for human aortic punch sprouting assay.

Following the validation of the sandwich model, the influence of oxygen on sprouting kinetics was investigated by comparing normoxic (20% O_2) and hypoxic (1% O_2) environments. Observations were performed at designated time points from Day 4 to Day 21. Although the previous culture period was initially extended to Day 27, quantitative analysis demonstrated that key sprouting parameters, including total branch number and sprouting area, had already increased significantly by Day 21 when compared with Day 7. This indicates that Day 21 represents a sufficient time point for assessing angiogenic sprouting in this model.

As shown in Figure 4(A)-4(B), vessel outgrowth was clearly observed under an inverted microscope, with red arrows indicating sprouting regions between normoxia and hypoxia. Quantification of branch formation (Figure 4(C)-4(E)) showed sprouting vessel are higher in hypoxia group tissue culture compare to normoxia group across all time points ($p < 0.05$). Within the hypoxic group, a significant time-dependent increase was observed, with the branching area, total branching length and number of branches on Day 21 being significantly greater than on Day 7 ($p < 0.05$).

Protein expression of HIF-1α was also evaluated to assess the cellular response to hypoxic conditions on aortic punch tissue culture. HIF-1α is a key transcription factor that regulates cellular adaptation to low oxygen levels by activating genes involved in angiogenesis, metabolic adaptation, and cell survival (Casillas et al. 2021). In the present study, HIF-1α protein levels were consistently higher under hypoxic conditions compared with normoxic conditions across the culture period (Figure 5). A significant increase in HIF-1α expression was observed on Day 7 and Day 14 in the hypoxia group compared with the normoxia group ($p < 0.05$), with the highest level detected on Day 14. These findings indicate activation of hypoxia-responsive pathways, potentially promoting angiogenic signalling through factors such as VEGF (Magar et al. 2024).

DISCUSSION

The aortic ring assay is a well-established *ex vivo* model for studying angiogenesis, allowing vascular sprouting to be observed from intact tissue under controlled conditions. This model preserves the interaction between endothelial cells and surrounding vascular components, providing a valuable bridge between *in vitro* and *in vivo* systems (Aplin & Nicosia 2019; Kapoor, Chen & Iozzo 2020).

Previous studies have demonstrated the feasibility of using human vascular explants for angiogenesis research. For example, Seano et al. (2013) successfully utilized arterial explants from human umbilical cords in an *ex vivo* assay to model tumour angiogenesis. In contrast, in current study we developed a modified aortic ring assay using

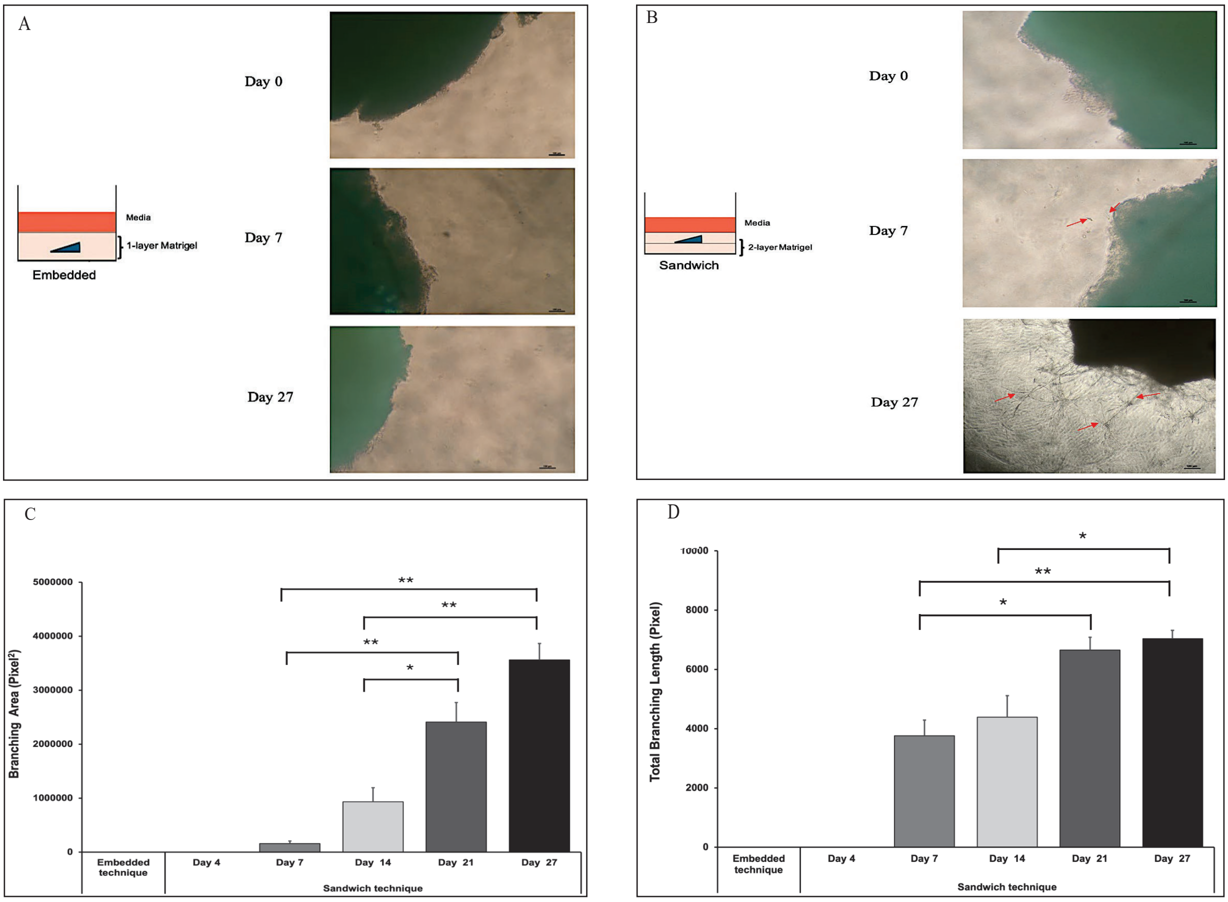


FIGURE 3. Comparison of sprout formation between the embedded and sandwich techniques in aortic punch tissue culture. Representative inverted microscope images of aortic punch tissue cultured with Matrigel using the embedded technique (A) and sandwich technique (B) at Day 0, Day 7, and Day 27 (scale bar: 100 µm). Sprout formation was observed only in the sandwich technique (red arrows). (C) Quantification of sprouting branching area showed a progressive increase over time, with significant differences observed between Day 7 and Days 21 and 27. (D) Total branching length also increased over time, with significantly higher values at Days 21 and 27 compared to Day 7. Data are presented as mean ± SEM. N= 3 (biological); * $p < 0.05$; ** $p < 0.01$

human aortic punch tissue obtained as surgical waste from CABG procedures. This approach provides a translationally relevant human model for investigating angiogenesis in the context of cardiovascular disease. During CABG surgery, a circular section of the ascending aorta is removed using a punch tool to create an opening for graft anastomosis. These discarded tissues contain viable vascular cells, including smooth muscle and endothelial cells, and typically retain normal histological characteristics suitable for culture.

A key methodological finding of this study was that angiogenic sprouting occurred only when the sandwich culture technique was used, while the embedded technique failed to induce any sprouting. In the embedded approach, the tissue was surrounded by a single layer of Matrigel, which may have limited adhesion between the tissue and the matrix, causing the explants to settle at the bottom of the well and reducing structural support. In contrast, the sandwich method, where the tissue is placed between two Matrigel layers, provided improved matrix support and nutrient diffusion, thereby facilitating sprouting. The sandwich technique has been extensively utilized in prior research concerning animal aortic ring assays (Bellacen & Lewis 2009; Ernens et al. 2015; Iqbal et al. 2017; Kapoor, Chen & Iozzo 2020). Current study results corroborate that the sandwich technique offers enhanced tissue support and nutrient availability, elucidating its efficacy in comparison to the embedded method.

Using the optimized sandwich culture method, vascular sprouting was first detected on Day 7 and progressively increased until Day 27 with significant increases in sprouting area and total branch length over time. Compared with animal models, the slower angiogenic response observed in human tissues may reflect species-specific differences in angiogenic signalling and cellular interactions (Simons et

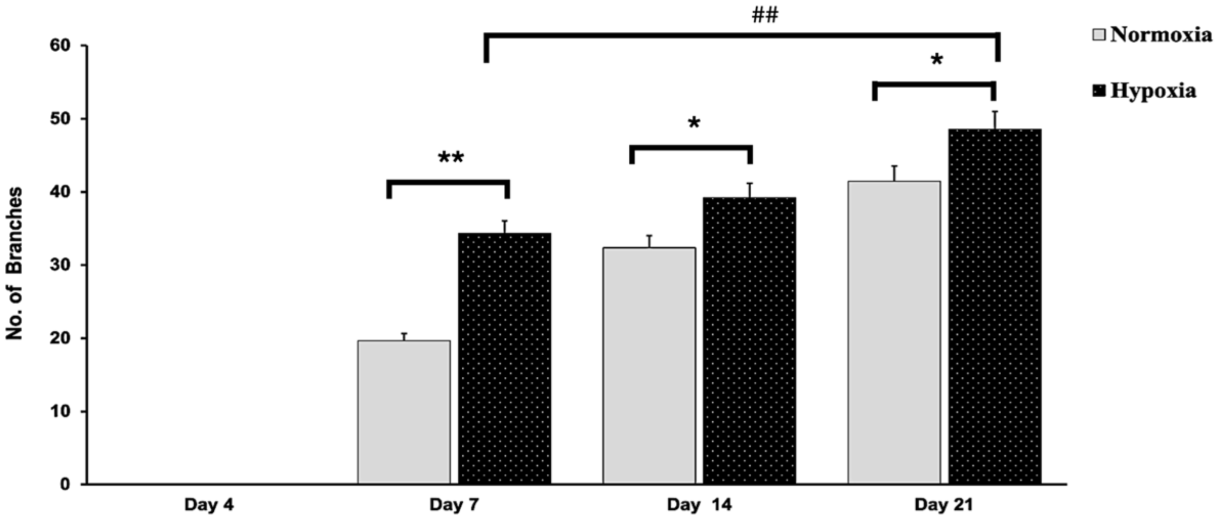


FIGURE 4. A: Sprouting vessels between normoxia and hypoxia condition (1% O_2). Red arrows represent sprouting vessel observe under inverted microscope with 4x magnification. Scale bar = 100 µm. B: Branching area observed between normoxia and hypoxia across the time point. The area of hypoxia group is significantly higher compared to normoxia on Day 7 and Day 14. C-D: Branching area and total branching length also increased over time. Significant differences were observed between normoxia and hypoxia at Day 7 and Day 14. Under hypoxic conditions (1% O_2), the branching area and total branching length on Day 21 was significantly elevated compared to Day 7. E: The number of branches and significant differences were observed between normoxia and hypoxia at each time point. Under hypoxic conditions, the number of branches on Day 21 was significantly higher than Day 7. * ($p < 0.05$), ** ($p < 0.01$) indicates the significant between hypoxia and normoxia. # ($p< 0.05$), ## ($p < 0.01$) indicates significant between hypoxia groups. N = 3. Data are presented as mean ± SEM

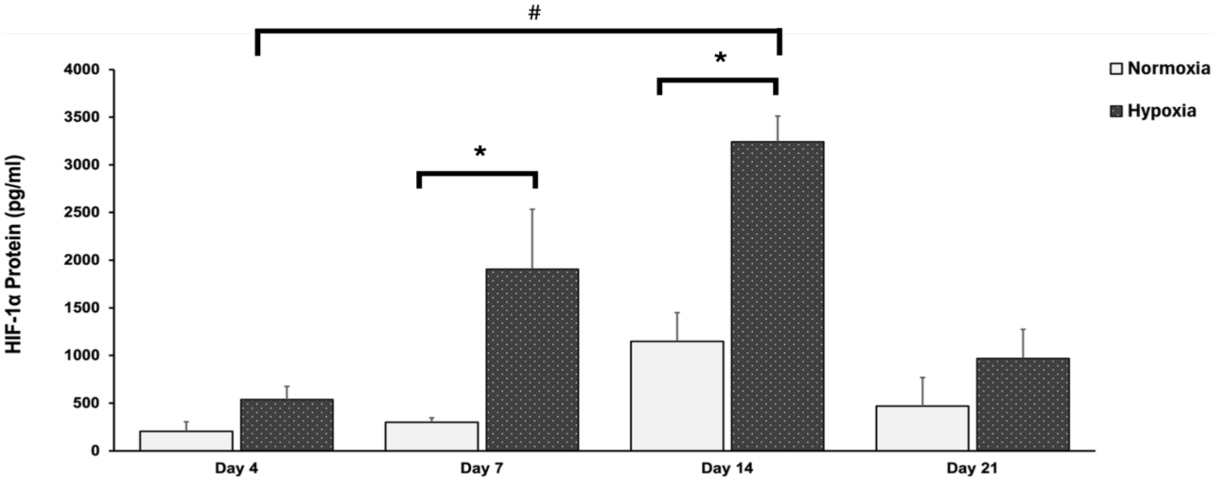


FIGURE 5. HIF-1α protein expression in aortic punch tissue under normoxic (20% O_2) and hypoxic (1% O_2) conditions. HIF-1α concentrations (pg/mL) were measured on Days 4, 7, 14, and 21 showing a significant increase under hypoxia, especially at Day 7 and Day 14. * ($p < 0.05$), ** ($p < 0.01$) indicates the significant between hypoxia and normoxia. # ($p< 0.05$), ## ($p < 0.01$) indicates significant between hypoxia groups. N = 3. Data are presented as mean ± SEM

al. 2015; Staton et al. 2004). Previous studies using human vascular explants such as saphenous vein or umbilical cord arteries have similarly reported sprouting occurring between Day 10 and Day 15 which supporting the relatively slower kinetics observed in human *ex vivo* models (Barton et al. 2024; Seano et al. 2013). However, the angiogenic potential of these tissues may be also influenced by the underlying vascular pathology of CABG patients such as atherosclerosis, hypertension or diabetes also need to consider in future investigation (Denny et al. 2014). These conditions may induce endothelial dysfunction or cellular senescence, potentially requiring stronger stimuli to initiate angiogenesis compared with healthy animal tissues. This may explain the delayed sprouting observed in the present study which began on Day 7, whereas rodent aortic ring assays often exhibit sprouting within 3-5 days (Bellacen & Lewis 2009; Kapoor, Chen & Iozzo 2020).

The study further evaluated angiogenic responses under normoxia (21% O_2) and hypoxia (1% O_2), demonstrated significantly increased sprouting under hypoxia. Hypoxic environments are known to alter cellular gene expression and promote angiogenesis through activation of hypoxia-responsive signalling pathways (Acharya et al. 2023; Pavlacky & Polak 2020). Notably, HIF-1α protein expression demonstrated that human aortic punch tissue was elevated from Day 4 until Day 14 and then started to decline. Previous research reported that HIF-1α protein levels typically increase during the early to mid-phases of angiogenesis, corresponding to active endothelial proliferation, migration and capillary sprouting. After that, HIF-1α will decrease as tissue oxygenation and vessels maturation improves (Hashimoto & Shibasaki 2015; Walton et al. 2013). HIF-1α protein often peaks early and rapidly stabilized under acute hypoxia which then declines despite low oxygen conditions and replaced by HIF-2α. This is because, HIF-1α is tightly regulated by oxygen-dependent prolyl hydroxylases that mark it for degradation when oxygen is available (Magar et al. 2024). Early hypoxia induces strong expression of HIF-1α to trigger angiogenic factors like VEGF and angiopoietins which can promote sprouting. As the branching progressively increases over time, this suggests that the tissues commence to respond to hypoxic stress, potentially enhancing cellular adaptations. Prior investigations have indicated that extensive exposure to hypoxia can induce significant morphological alterations in cultured cells, including increased branching and improved survival rates due to metabolic adaptations (Teixeira et al. 2015). Consistent with Lee at al. (2011), they also reported hypoxic exposure led to a three-fold augmentation in vessel sprouting at the severed edges of the mouse aortic ring assay, emphasizing the role of hypoxic environments in stimulating angiogenesis.

Despite these promising findings, several limitations should be considered when interpreting the results. The study was conducted using a relatively small number of biological donors (N = 3) due to the limited availability of discarded aortic punch tissue obtained from coronary CABG procedures. This constraint may limit the statistical power of the analysis and the generalisability of the findings, particularly given the potential variability in vascular characteristics among patients with underlying cardiovascular disease. In the current study, angiogenic sprouting was primarily assessed through morphological observation and quantitative analysis using ImageJ. Although this approach provides useful measurements of sprouting area and branching patterns, endothelial-specific validation was not performed. The absence of markers such as CD31, VE-cadherin, or von Willebrand factor limits definitive confirmation that the observed sprouts originated from endothelial cells. Incorporation of immunohistochemical or molecular analyses in future studies would strengthen the identification of endothelial-derived vascular structures. In addition, imaging of sprouting structures within a three-dimensional matrix may be constrained by limited depth of field, potentially obscuring vessels located at different focal planes (Bellacen & Lewis 2009; Nicosia 2009). Variability between patient-derived tissues and differences in tissue handling may also contribute to heterogeneity in angiogenic responses which should considered in further study.

Furthermore, the current investigation mainly focused on sprouting behaviour and HIF-1α expression under hypoxic conditions. While HIF-1α is a key regulator of hypoxia-induced angiogenesis, additional angiogenic mediators, including VEGF and angiopoietins were not evaluated. Assessing these downstream signalling molecules in future studies would provide a more comprehensive understanding of the molecular mechanisms driving angiogenesis in this model. Nevertheless, despite these limitations, human aortic punch explant model might provide a promising platform for translational angiogenesis research especially in cardiovascular diseases. By utilising clinically derived human vascular tissue, this model offers a physiologically relevant system for investigating mechanisms of vascular remodelling in human and may serve as a useful tool for evaluating potential pro-angiogenic or anti-angiogenic in cardiovascular therapeutic strategies.

CONCLUSION

The aortic ring assay is one of the important *ex vivo* model for studying angiogenesis, providing insights into the interactions between endothelial cells and vascular tissue. The use of human aortic punch tissue from coronary artery bypass graft procedures represents a significant advancement, offering a clinically relevant platform for investigating cardiovascular angiogenesis. The sandwich culture technique effectively supported vascular sprouting and elevated in HIF-1α expression under hypoxia coincided with sprout initiation, highlighting its role as a key molecular regulator. This optimized human aortic punch model may provide a translational tool for future mechanistic studies and for screening pro- or anti-angiogenic therapies in cardiovascular research.

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