Étude de médicaments botaniques de la médecine traditionnelle chinoise pour la croissance des cellules endothéliales et l'angiogenèse

Thèse

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Résumé

Les médicaments botaniques, y compris ceux utilisés en médecine traditionnelle chinoise (MTC), traitent depuis longtemps les maladies cardiovasculaires où la dysfonction endothéliale est un facteur de risque bien établi. Dans la littérature, il existe de nombreux rapports sur les avantages dans la clinique et la santé des médicaments ou des préparations botaniques pour le système cardiovasculaire. Il a été indiqué que des médicaments botaniques, en particulier ceux ayant une capacité antioxydante puissante, protègent les cellules endothéliales (CE) en culture contre les radicaux libres et les oxydants. Cependant, il y a peu de recherches sur les médicaments botaniques dans le contexte de la cicatrisation et de la régénération tissulaire. Cette thèse a étudié quatre médicaments botaniques enregistrés dans la MTC pour explorer leurs effets sur la croissance des CE vasculaires et l'angiogenèse, deux événements activement impliqués dans la cicatrisation et la régénération tissulaire. Dans la première partie de la thèse, des cellules endothéliales ombilicales humaines (HUVEC) ont été cultivées en présence de différentes doses d'extrait d'astragale en forme de poudre, d'injection d'astragale, d'injection de puerarin et de proanthocyanidine. Parmi les quatre médicaments, la proanthocyanidine a montré un effet puissant sur la viabilité cellulaire et stimulé la croissance cellulaire d'une manière dépendante de la dose. En dehors de la gamme de doses efficaces, la proanthocyanidine était inefficace ou cytotoxique. Fait important, la proanthocyanidine testée était capable de maintenir une viabilité cellulaire comparable aux cellules supplémentées avec le milieu spécifique pour les CE avec un niveau bas ou normal de sérum, ce qui suggère le potentiel de la proanthocyanidine en tant que stimulateur de croissance et réactif angiogénique. Dans la seconde partie de la thèse, des études mécanistiques ont été réalisées en bloquant à la fois les récepteurs du facteur de croissance des cellules endothéliales (VEGFR) et les récepteurs du facteur de croissance des cellules épithéliales (EGFR). Cependant, les bloqueurs ont été inefficaces pour réduire l'effet stimulant de la proanthocyanidine sur les CE. En conséquence, il est conclu que la proanthocyanidine stimule probablement la croissance des CE à travers des récepteurs membranaires autres que VEGFR et EGFR. Dans la troisième partie de la thèse, il a été montré que la proanthocyanidine pouvait être chargée dans un cryogel d'alcool polyvinylique, puis libérée du gel à une concentration dans la gamme de la dose efficace. Ceci a démontré la faisabilité d'une libération lente de proanthocyanidine à

partir d'un support polymérique. Enfin, la propriété angiogénique de la proanthocyanidine a été testée sur un modèle de membrane chorioallantoïque d'embryon de poulet (CAM), montrant que ce médicament botanique était capable de stimuler le développement du système vasculaire.

Cette thèse a donc démontré pour la première fois que la proanthocyanidine est capable de moduler l'activité des CE humaines, particulièrement de réguler positivement l'activité et la croissance des CE en l'absence de facteurs de croissance, et que la proanthocyanidine peut être utilisée comme réactif angiogénique et libérée d'un support de médicament synthétique. En somme, cette thèse a démontré que les médicaments botaniques en médecine traditionnelle chinoise peuvent être utilisés comme substituts pour produits protéiques pour maintenir les cellules en culture et induire l'angiogenèse pour la cicatrisation et la régénération tissulaire.

Summary

Botanic drugs including those used in traditional Chinese medicine (TCM) have a long history of treating cardiovascular diseases, of which endothelial dysfunction is well established as a risk factor. In literature there exist extensive reports about the clinic and healthy benefits of botanic drugs or preparations to the cardiovascular system. Botanic drugs particularly those with potent antioxidative capacity have also been reported to protect endothelial cells (EC) in culture against free radicals and oxidants. However, there is little research about botanic drugs in the context of wound healing and tissue regeneration. This thesis studied four botanic drugs recorded in TCM to explore their effects on vascular EC growth and angiogenesis, two events actively involved in wound healing and tissue regeneration. In the first part of the thesis, human umbilical endothelial cells (HUVEC) were cultured in the presence of different doses of *astragalus* powder extract, *astragalus* injection, puerarin injection, and proanthocyanidin. Among the four drugs, proanthocyanidin showed a potent effect on cell viability and stimulated cell growth in a dose dependent manner. Outside the effective dose range proanthocyanidin was either ineffective or cytotoxic. Importantly, the proanthocyanidin under test was able to maintain a cell viability comparable with the cells supplemented with the commercially available EC growth medium at both low and normal serum conditions, which suggests the potential of proanthocyanidin as an EC growth stimulator and an angiogenic reagent. In the second part of the thesis, mechanistic studies were performed by blocking both endothelial cell growth factor receptors (VEGFR) and epithelial cell growth factor receptors (EGFR). However, the blockers were ineffective in reducing the stimulatory effect of proanthocyanidin on EC. Therefore it is concluded that proanthocyanidin stimulates EC growth through membrane receptors other than VEGFR and EGFR. In the third part of the thesis, it was shown that proanthocyanidin could be loaded into polyvinyl alcohol cryogel and then released from the gel at a concentration within the effective dose window. This demonstrated the feasibility of drug releasing of proanthocyanidin. Finally, the angiogenic property of proanthocyanidin was tested in chick embryo chorioallantoic membrane (CAM) model, showing that this botanic drug was capable of stimulating vasculature development. Preliminary data in rat subcutaneous model also support the angiogenic potential of proanthocyanidin.

This thesis therefore demonstrated for the first time that proanthocyanidin was capable of modulating the activity of human EC and in particular upregulating EC activity and growth in the absence of growth factors, and that proanthocyanidin may be used as an angiogenic reagent and released from a synthetic drug carrier. Consequently, this thesis has demonstrated that botanic drugs in traditional medicine may be used as substitutes of protein products to maintain cells in culture and to induce angiogenesis for wound healing and tissue regeneration.

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List of Abbreviations

ANG-1	Angiogenesis Prime-1
AMI	Acute myocardial ischemia
AKT/PKB	Protein kinase B
bFGF	Basic fibroblast growth factors
BCL-2	B-cell lymphoma 2
CAM	Chick chorioallantoic membrane
CVDs	Cardiovascular diseases
DAB	3, 3'-diaminobenzidine
DMSO	Dimethyl sulfoxide
ECGS	Endothelial cell growth supplement
ECM	Extracellular matrix
ECs	Endothelial cells
EDHF	Endothelium-derived hyperpolarizing factors
EDRF	Endothelium-derived relaxing factor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
EPCR	Endothelial protein C receptor
ERK	Extracellular regulated kinase
ET-1	Endothelin-1
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FnE	Fibrin fragment E
HIF-1α	Hypoxia induced factor
HSCs	Hematopoietic stem cells
HUVECs	Human umbilical vein endothelial cells
IL-8	Interleukin-8
LDL	Low-density lipoprotein
MAPK	Mitogen-activated protein kinase
MMPs	Matrix metalloproteinases

MTT	3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenylthiazolium
NO	Nitric oxide
NOSs	Nitric oxide synthases
OPC	Oligomeric proanthocyanidins
PAI-1	Plasminogen activator inhibitor-1
PAR1	Protease-activated receptor 1
PBS	Phosphate buffered saline
PD-ECGF	Platelet-derived endothelial cell growth factor
PDGF	Platelet-derived growth factor
PDGFR-α	Platelet-derived growth factor receptors alpha
PDGFR-β	Platelet-derived growth factor receptors beta
PGI2	Prostacyclin
PI	Phosphatidylinositol
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC-g1	Phospholipase C-g1
PAC	Proanthocyanidin powder
PVA	Polyvinyl alcohol
RTKs	Receptor tyrosine kinases
SH2	Src homology-2
TCM	Traditional Chinese medicine
TFPI	Tissue factor pathway inhibitor
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
tPA	Tissue plasminogen activator
VEGF	Vascular endothelial cell growth factor
VEGFR-1	Vascular endothelial cell growth factor receptor-1
VEGFR-2	Vascular endothelial cell growth factor receptor-2
VEGFR-3	Vascular endothelial cell growth factor receptor-3
VRAP	VEGFR-associated protein

VPF	Vascular permeability factor
vWF	von Willebrand Factor

Preface

This thesis aims to explore the potential angiogenic effect of some important botanic drugs used in traditional Chinese medicine (TCM). The first part of this work introduces the research background and reviews literature. Some of the herbs and remedies reported effective in TCM are reviewed. The second part of this thesis focuses on the effects of the selected Chinese medicines on endothelial cell viability and proliferation. The third part studies mechanisms by looking at if two important groups of membrane receptors are responsible for the stimulatory effect of proanthocyanidin on HUVEC proliferation. The fourth part tests the angiogenic effect of proanthocyanidin in vivo using a chick chorioallantoic membrane model. The fifth part investigates the feasibility of releasing proanthocyanidin from a polymeric drug carrier and the subcutaneous implantation of such drug loaded implants in rats. And the final part presents the general conclusions and perspectives.

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Chapter 1

Introduction

1.1 Background

Cardiovascular diseases (CVDs) are still among the leading causes of death in developed countries (1). They are reported as the top major killers during the past decade by World Health Organization in May 2014. Atherosclerosis, is the pathological narrowing of a blood conduit, which is commonly treated with endovascular stenting, by-passing with autograft, or the replacement of the diseased blood vessel with an artificial prosthesis. One of the critical issues following stenting and implantation of a vascular prosthesis is the absence of endothelialisation. The lack of endothelialisation in the lumen results in acute thrombosis and intima hyperplasia in long-term, which are the major causes of the failure of vascular prostheses. Re-endothelialization therefore has remained the most significant challenge in cardiovascular implants and has been the focus of intensive research activities in more than half a century.

Endothelialisation on an artificial surface depends on the homing, differentiation and proliferation of circulating progenitor cells and mature endothelial cells, a process closely regulated by various growth factors such as endothelial cell growth factor (VEGF). VEGFs are also potent growth factors involved in angiogenesis, which is the formation of new capillary blood vessels from existing vasculature. Angiogenesis is a critical process when treating ischemic vascular disease and for the survival of tissue-engineered scaffolds. New strategies to enhance endothelialisation and angiogenesis are therefore the central focus of cardiovascular therapies and tissue regeneration research.

Currently, all of the growth factors used to maintain endothelial cells in culture and to stimulate angiogenesis in a healing wound are proteins, with VEGFs representing the most potent and frequently used. Bioactive proteins are expensive, sensitive to preparation procedures and storage conditions, and quickly lose their activity in vivo. In traditional Chinese medicine (TCM), a number of well-known remedies and preparations, such as the injection and extract of *radix astragalus*, puerarin and proanthocyanidin, have been known to display potent angiogenic properties. Studies exploring the beneficial effects of botanical drugs in TCM on the cardiovascular system have been mainly carried out in Asian

countries, including China, Japan, and Korea. As for their usefulness in promoting endothelial cell proliferation and endothelialization, research in this regard is limited.

In this thesis, the roles of the four above-mentioned botanical drugs in regulating human vascular endothelial cell proliferation and angiogenesis were investigated. The outcome of this thesis may lead to the discovery of non-protein-based chemicals capable of promoting endothelial cell growth, as well as to the new strategies stimulating angiogenesis.

1.2 Literature review

1.2.1 Function and importance of endothelial cells

For any normal organ and tissue, it is impossible to cut off the blood supply for a long period of time. The primary function of blood vessels is to supply oxygen and nutrients to tissues and organs and to remove carbon dioxide and metabolic wastes. While the importance of blood vessels is self-evident, it is the endothelial cells that play a critical role in maintaining and regulating the normal physiological functions of blood vessels.

1.2.1.1 Discovery of endothelium

The primary function of endothelium, among other things, is the maintenance of blood flow (2). At macroscopic scale, the endothelium resembles a semi-transparent lining on the lumen of the entire circulatory system. While the circulation of blood was first described by William Harvey in 1628 (3,4), Malpighi was the first to report the vasculature as a network separating blood from the surrounding tissues (5). In the 1800s, von Recklinghausen discovered that blood vessels were actually lined by a layer of cells (6). Nevertheless, the function of this cellular layer was believed to be a static physical barrier at that time, based on the work of Starling in 1896 (7).

Only with the development of more sophisticated tools, such as electron microscopy, have the complex roles of endothelium been gradually revealed, including anti-thrombogenicity, secretion of growth factors and cytokines to interact with other cell populations such as smooth muscle cells and circulating cells, and the capacity to form new blood vessels in pathological conditions (8,9).

1.2.1.2 Endothelial cells

Endothelial cells (ECs) are the highly specialized cells (10) forming the inner layer of blood vessels and lining the entire circulatory system. From the heart to the smallest capillaries, ECs are the main regulator of vascular homeostasis. They not only interact with circulating cells but also with cells present in the vascular wall. In addition, as they constitute the interface between blood and tissue, they are mostly susceptible to changes in blood composition and blood flow (11). ECs are the main responder to all of these changes and play a central role in the mechanisms underlying the development of vascular disorders (11,12).

ECs cover the intimal surface and can spread out to a total area of up to 10 square metres (11). Vascular endothelial cells serve as a natural barrier between the blood vessel wall and blood flow and more importantly, they help the blood circulation to maintain the normal operation of human life. In addition, endothelial cells synthesize and secrete a variety of antithrombogenic chemicals to maintain blood flow and also messengers to crosstalk with smooth muscle cells. EC function is now recognized as being crucial to all aspects of vascular homeostasis (13). The active metabolism of these cells is thus necessary for the continuous adjustment of vascular tone (hence the control of blood pressure), the physiological regulation of leukocyte traffic from blood to tissues, and finally the maintenance of an antithrombotic and anticoagulant balance in the blood flow (14).

1.2.1.3 Barrier function

Endothelial cells lie on the luminal surface of blood vessels and form a monolayer called endothelium as a nonthrombogenic barrier. Because of its unique structure and metabolic properties, the endothelium is capable of selectively passing chemicals and cells through the vessel wall, meaning a selective permeability. When endothelial cells spall, the exposure of subendothelial collagen tissue leads to the adhesion and activation of platelets and the subsequent thrombosis involving fibrin formation and trapping of a variety of blood cells (15).

In an adult, the entire surface of endothelium comprises approximately 1 to 6×10^{13} cells that weigh approximately 1 kg and covers a surface area of 1 to 7 m^2 (16). The gate-keeping role of endothelium becomes effective through the presence of membrane-bound receptors for numerous molecules, including proteins (e.g., growth factors, coagulants and anticoagulant proteins), lipid-transporting particles (e.g., low-density lipoprotein [LDL]), metabolites (e.g., nitrous oxide and serotonin), and hormones (e.g., endothelin-1), and junctional proteins (9,17). Vascular endothelial cells regulate actin contractile function. At the same time, serotonin, histamine, and bradykinin stimulate actin contraction, thereby changing the cell gap width and tightness of cell junctions, in addition to influencing and regulating vascular permeability (18).

1.2.2 Blood flow regulation

The most important role of the endothelium is to maintain a continues blood flow that transports blood cells and nutrition to different locations and to collect metabolic products. This non-thrombogenic capacity of the endothelium is achieved through the small molecules and proteins secreted from ECs. However, this homeostat of endothelium can be modified because of localized inflammation and abnormal hemodynamics, creating a prothrombotic and antifibrinolytic microenvironment (19). Stimuli generated in such a microenvironment affect the secretion and uptake of vasoactive substances that constrict and dilate specific vascular beds (20, 21).

1.2.3 Vasoconstriction and vasodilation

ECs are responsible for the regulated synthesis and secretion of two potent. Short-lived vasodilators together provide minute-by-minute control of vascular tone and blood pressure. Namely, prostacyclin (PGI_2) synthesized from arachidonic acid released from membrane phospholipids and nitric oxide (NO) synthesized from arginine by nitric oxide synthases (NOSs). NO is an important vasodilator which has been found in virtually every vascular bed in many species, including humans. The lack of endothelial isoform of NOS in mice was shown to produce a phenotype with reduced endothelium-dependent vasodilatation and elevated systemic blood pressure (22). In some vascular beds, one or more other endothelium-derived vasodilators known as endothelium-derived

hyperpolarizing factors (EDHF) are also found to relax smooth muscle cells (23); however, the exact identity of EDHF remains uncertain. Failure of endothelium-dependent vasodilatation due to a lack of NO synthesis (and/or increased NO destruction by reactive oxygen species) is an early sign of hypercholesterolemia and atherogenesis, while a deliberate chronic inhibition of NOS exacerbates atherogenesis in animal models (24). Intriguingly, a deficit in endothelium-dependent relaxation can be reversed by adding extra arginine to the diet. The cause of the defect is not clear, although an increased production of endogenous inhibitors of NOS, such as asymmetric dimethylarginine, has recently been proposed (25).

Both NO and PGI_2 synthesis can be triggered rapidly and transiently by a variety of agents that cause increased cytoplasmic concentrations of Ca^{2+}, acting via G protein-coupled receptors, because both NO synthase and phospholipase A_2 (the initial rate-limiting enzyme in the PGI_2 synthetic pathway) are activated by Ca^{2+} (26). These agents are predominantly molecules generated during coagulation or platelet activation (e.g., bradykinin, thrombin, ATP), reflecting the other important role of NO and PGI_2 as platelet function inhibitors. However, there are dual controls on NO and PGI_2 synthesis, and each can also be activated by protein kinases. This is currently believed to be of particular relevance in the control of NO synthesis by what is arguably its most important physiological activator - shear stress. Increasing shear stress induces endothelial NO synthesis in the absence of detectable changes in cytoplasmic Ca^{2+}. This requires the activation of protein kinase B and is apparently dependent on the close physical association of NOS with the plasma membrane, particularly within its abundant invaginations known as caveolae, which are suggested to be microenvironments specialized in responding to external signals such as shear forces. Thus, an increased blood flow or pressure is sensed by the EC and leads to the feedback production of local vasodilators to maintain vascular homeostasis (27).

ECs are also the source of the potent vasoconstrictor peptide endothelin-1 (ET-1) (28). Its synthesis and secretion regulation remain less understood than are those of NO or PGI_2; however, there is evidence that ET-1 release can also be modulated by physical

forces such as shear or stretch in vitro. ET-1 is more likely to be a tonic modulator of vascular tone due to its long-lasting action. There is evidence of increased circulating ET-1 levels in various forms of human hypertension; in this regard, ET receptor antagonists show definite clinical potential in the treatment of hypertension and heart failure (14,28).

Vascular endothelial cells have a complex enzyme system that synthesizes and secretes a variety of biologically active substances. Vascular endothelial cells can degrade serotonin, histamine, and norepinephrine (29). Endothelial cell dysfunction is closely related to a variety of cardiovascular diseases, including hypertension, coronary heart disease, peripheral vascular disease, diabetes, chronic renal failure, and autoimmune diseases.

1.2.4 Regulation of thrombosis and fibrinolysis

ECs are the major source of circulating von Willebrand Factors (vWF) that are important in regulating blood coagulation. This highly multimeric glycoprotein has two separate biological functions, serving as the carrier for coagulation Factor VIII and as the cofactor required by platelets to bind to exposed collagen when the vessel wall is damaged, thus initiating the formation of a hemostatic plug (30). vWF is secreted by two pathways: a constitutive one and a one that is triggered by exocytosis from storage granules (Weibel-Palade bodies) that contain high molecular weight multimers (with greater biological activity). Secretion from the granules is rapid and is initiated by a fairly small range of agonists, of which thrombin and histamine are the best described. The granule membrane contains P-selectin, and thus vWF secretion is accompanied by the transient expression of P-selectin at the EC surface, as granule and plasma membranes fuse for exocytosis. The intracellular transduction pathways leading to granular secretion include an elevation of cytoplasmic Ca^{2+}, along with other signals that are poorly understood. Plasma vWF levels are quite stable in healthy individuals, yet are transiently elevated several folds during infection, similar to other acute phase reactants, which is likely to contribute to an increased prothrombotic risk. The mechanism causing this elevation is unclear; pro-inflammatory cytokines do not directly induce vWF release, though they can enhance thrombin-induced secretion. In disease states in which there is vascular involvement (autoimmune vasculitic

diseases, atherosclerosis, etc.), plasma level of vWF is chronically elevated, presumably reflecting ongoing endothelial activation or damage. Again, the underlying mechanisms have not yet been elucidated (31).

Thrombin, converted from prothrombin during the clotting process, is a major physiological modulator of EC behaviour (32). It triggers the secretion of NO and PGI_2, that not only affect vascular tone but also are powerful platelet activation inhibitors. The secretory responses are predominantly a consequence of the interaction between thrombin and its specific G protein-coupled receptor (protease-activated receptor 1, PAR1), one of a growing family of receptors that are activated by the protease clipping of an N terminal extracellular peptide from the receptor itself, thus exposing a sequence that then binds as a "tethered ligand" to another region of the receptor and self-activates it.

Secretion of vWF, thrombin triggered NO and PGI2 forming the main physiological homeostatic systems ensure that the effects of locally generated thrombin are spatially confined, thus preventing unwanted systemic procoagulant effects (14). The rest of these are captured and inhibited by antithrombin. Although antithrombin is synthesized primarily in the liver, it avidly binds to the surface glycosaminoglycans of endothelium. When it binds thrombin, the inactive complex is released back into the circulation and is rapidly cleared by the liver. Thrombomodulin is an endothelial transmembrane protein. When thrombin binds to thrombomodulin, its conformation changes, thereby rendering it far less efficient at cleaving fibrinogen, yet far more efficient at cleaving and activating circulating protein C. Activated protein C is an anticoagulant that deactivates coagulant factors Va and VIIIa. Recently, a specific endothelial transmembrane protein was characterized to bind protein C which may enhance its ability to interact with thrombomodulin-bound thrombin, known as endothelial protein C receptor (EPCR) (13). Soluble forms of thrombomodulin are found in the circulation, and their levels, similar to those of vWF, may reflect aspects of EC activation or damage (33).

As aforementioned, healthy ECs contribute to the maintenance of normal blood fluidity through their anticoagulant and antiplatelet effects; however, when activated by

exposure to the inflammatory cytokines, endotoxin or thrombin, EC phenotype becomes less anticoagulant, in addition to modulating leukocyte adhesion and migration. The two main elements controlling fibrinolysis, namely, tissue plasminogen activator (tPA) and its physiological inhibitor, plasminogen activator inhibitor-1 (PAI-1), are EC secretory products that are both continuously released into the bloodstream, with PAI-1 normally in excess. tPA can also be acutely released from small granular stores, notably in response to thrombin (34). Following endothelial activation by inflammatory cytokines or endotoxin, the rate of PAI-1 secretion is enhanced, whereas constitutive tPA secretion is either unaltered or diminishes. It has also been shown that PAI-1 secretion can be enhanced by treating the endothelium with glycated or oxidized lipoproteins, which suggests one mechanism explaining the increased procoagulant risk found in diabetes and atherosclerosis (35).

All extravascular cells constitutively express transmembrane protein tissue factor (known as thromboplastin when combined with cofactor membrane phospholipids) responsible for binding and activating Factor VIIa. Although this “extrinsic” coagulation pathway for the activation of Factor X (and hence the conversion of prothrombin to thrombin) was for many years regarded as subordinate to the first described “intrinsic” pathway, it is now clear that tissue factor is the primary physiological initiator of coagulation (36). In contrast, and as required to prevent intravascular coagulation, blood cells and endothelium do not normally express tissue factor. In vitro, both monocytes and ECs can be induced to express tissue factor by exposure to inflammatory cytokines or endotoxin; and there is good evidence showing that monocyte expression of tissue factor occurs in vivo and contributes to intravascular coagulation, seen for example in bacteremia (37). However, EC expression of tissue factor in vivo has been much less convincingly demonstrated and appears to be under tighter control than that in monocytes. ECs are rather the source of the most important physiological inhibitor of the extrinsic pathway, namely, tissue factor pathway inhibitor (TFPI, previously referred to as EPI or LACI). This protein is secreted, circulates in plasma, and is also found bound at the EC surface.

Healthy ECs are vital for vascular homeostasis. Through the secretion or surface expression of a series of specific molecules, these cells ensure that under normal conditions blood flow is appropriately regulated and that intravascular platelet activation and blood coagulation are avoided. In response to pathophysiological mediators, EC properties are dynamically modulated, both to support/repair vessel growth and to guide the resolution of an inflammatory or infectious process. In many instances, this temporary alteration of EC phenotype contributes to the successful restoration of vascular homeostasis (14).

1.2.5 Angiogenesis

Angiogenesis refers to a physiological process through which new blood vessels are formed from existing ones to provide a new blood supply. After the development of the first embryonic vessels, angiogenesis is the second most vital process during development and disease.

There are two types of angiogenesis: sprouting angiogenesis and intussusceptive angiogenesis. The sprouting of blood vessels is formed by endothelial cells in the direction of angiogenic stimuli such as VEGF-A. The basic process of sprouting angiogenesis is composed of capillary basement membrane enzyme degradation, endothelial cell proliferation and migration, endothelial cell tube formation, vascular fusion, vessel trimming, and adventitial cell stabilizing (38). A key component in sprouting angiogenesis is a cell-cell signaling system known as Delta-Notch signaling. Although the aspects of the Delta-Notch signaling pathway are not fully understood, many studies have shown that the generation of normal blood vessels is dependent on the concentration of vascular endothelial growth factor (VEGF-A) present in the surrounding tissue. Intussusceptive angiogenesis, on the other hand, involves a process of vascular lumen splitting, also called splitting angiogenesis. Intramural vascular interstitial tissue intrudes into existing vessels and forms a pillar throughout the vascular tissue, thereby forming a plurality of blood vessels. A single vessel splits into two vessels by extending the vessel wall into the lumen. Compared to sprouting angiogenesis, splitting angiogenesis is more efficient because it takes place through a reorganization of existing endothelial cells that does not require endothelial proliferation and migration.

1.2.5.1 Angiogenesis in wound healing

Angiogenesis is a vital physiological process that is essential for normal wound healing. Many factors regulate wound angiogenesis, including hypoxia, inflammation, and growth factors. Immediately following injury, angiogenesis is triggered by a variety of molecular signals, including cytokines, growth factors, and cell-matrix interactions (39). Clinically, 3 to 5 days after injury, newly formed capillaries are first visible in the wound, and their appearance is synonymous with granulation, creating a temporary matrix consisting of proliferating vessels, migrating fibroblasts, and new collagen synthesis. New capillaries proliferate through a series of biological events to form granulation tissue on the wound bed. In the terminal stage of healing, inflammation subsides, angiogenesis regresses, and the stable tissue matrix forms. Damaged granulation is a sign of chronic diabetic wounds, as well as vein and arterial deficiency. Angiogenesis has thus become the main focus of wound biologists and surgeons.

The field of angiogenesis research began in the 1960s with the discussions of how new blood vessels support solid tumor growth (40), despite the fact that physiologists had long recognized that neovascularization takes place during normal regeneration. Hyperplastic capillaries bring oxygen and micronutrients to the growing tissue and remove metabolic wastes. The secretion of vasculature-secreted paracrine factors prevents apoptosis or programmed cell death, promoting the survival of adjacent cells. Because wound healing requires angiogenesis, its induction is beneficial in many clinical situations to achieve wound closure.

Wound healing occurs in three major overlapping stages: 1) hemostasis and inflammation; 2) proliferation; and 3) remodeling (41). First, tissue damage leads to the release of various pro-inflammatory mediators and growth factors such as basic fibroblast growth factors (bFGF) that are usually stored in intact cells and extracellular matrix. During hemostasis, thrombin is among the first coagulation factors present in the wound that upregulates VEGF receptor and enhances the role of the growth factor (42). Cells exposed to thrombin also release gelatinase A, thereby promoting the localized dissolution of the

basement membrane, which is a necessary early step in angiogenesis. Platelets are among the first cells that are activated in acute phase of wound healing. Platelet-derived growth factor (PDGF), VEGF, transforming growth factor (TGF-α, TGF-β), bFGF, platelet-derived endothelial cell growth factor (PD-ECGF) and angiopoietin 1 (Ang-1) all stimulate endothelial proliferation, migration, and tube formation (43). Wound angiogenesis is then amplified by inflammation (42). Macrophages and monocytes release numerous angiogenic factors, such as PDGF, VEGF, Ang-1, TGF-α, bFGF, interleukin-8 (IL-8), and tumor necrosis factor alpha (TNF-α) (44). Several growth factors (PDGF, VEGF, and bFGF) play a synergistic role in the vascularisation process. Decomposition of the damaged tissues further releases matrix-bound angiogenic stimuli. Fibrin cleavage produces fibrin fragment E (FnE) that directly stimulates angiogenesis and enhances the effects of VEGF and bFGF. The expression of the inducible COX-2 enzyme during the inflammatory phase also leads to VEGF production and other angiogenesis promoters (45).

Hypoxia is also an important driver of wound angiogenesis (46). Acute hypoxia causes hypoxia induced factor (HIF-1α) to trigger VEGF production. One property of VEGF is its capacity to induce edema through elevated permeability; its alternative name is therefore vascular permeability factor (VPF). Hypoxia also results in the production of NO by endothelial cells (46). NO promotes vasodilation and angiogenesis to improve local blood flow. Some of the newly formed blood vessels will become mature and stable (47). Vascular stability is controlled by Ang-1 and its receptor Tie2, as well as smooth muscle cells and pericytes. The binding of Ang-1 to Tie2 on activated endothelial cells leads to the production of PDGF and the recruitment of smooth muscle cells and pericytes for the newly formed vasculature. PDGF deficiency leads to abnormalities, resulting in the formation of immature vessels (48). At the end of healing, angiogenesis is inhibited and the level of growth factors decreases with tissue hypoxia recovery and subsided inflammation. Endogenous angiogenesis inhibitors thus become the main force, with a stable depletion of ECs inhibiting TGF-β and vascular proliferation. The interferon-β epidermal production also inhibits angiogenesis (49). Endostatin is the cleavage product of collagen XVIII, which surrounds the vascular basement membrane and inhibits wound blood vessels, as does another molecule called angiostatin.

1.2.5.2 Angiogenesis in tumor growth

Physiological angiogenesis is only present in the reproductive system and wound healing process, which is both transient and strictly controlled. Uncontrolled angiogenesis leads to a variety of diseases, including diabetic retinopathy and rheumatoid arthritis (50). Tumor growth and metastasis are vascular-dependent (51). Nearly two decades of research prove that angiogenesis plays an important role in tumor growth.

Tumor angiogenesis is a complex process consisting of five basic steps: 1) an imbalance of tumor angiogenesis factors, angiogenesis inhibitory factors, and angiogenesis phenotype; 2) a degradation of the vascular endothelial basilar membrane; 3) endothelial cell migration and proliferation into the tumor tissue; 4) the channeling and branching of endothelial cells to form vascular rings; and 5) the formation of a new basilar membrane (52). As is evidenced in this process, tumor metastasis and angiogenesis are similar in terms of molecular mechanisms, as both need to regulate cell adhesion, matrix degradation, and cell movement in order to achieve tumor cell metastasis and angiogenesis.

1.2.5.3 Angiogenesis in tissue engineering

Sufficient blood supply is the decisive factor in ensuring the survival of engineered tissues. It is well-known that cells can only survive in distance of 150-200 μm away from blood supply. Cells that are more than 200 μm from the capillaries are unable to survive due to the lack of nutrients and oxygen. If any cell mass is greater than 1 mm^3 with no blood vessel growth, it will die (53).

The inward growth of newly formed blood vessels in an implanted tissue construct is a highly dynamic process. The first step of this process occurs via angiogenic growth factors, such as VEGF and bFGF (54). These factors may be produced by host tissue cells due to tissue damage during the implantation process or to inflammation of the implant, as described in section 2.5.1. On the other hand, tissue constructs can be produced using artificial protein delivery systems or different cell types capable of releasing angiogenic growth factors (55).

When angiogenesis is activated, host microvascular endothelial cells begin to produce matrix metalloproteinases (MMPs), resulting in the degradation of the basement membrane in vasculature surrounding the implant. Ideally, the sequential events of angiogenesis would eventually lead to the growth of capillaries into the implanted tissue structure, and connect with each other to form new blood perfusion microvascular networks (56). The walls of these networks are ultimately stabilized by the production of extracellular matrix compounds and the recruitment of smooth muscle cells and pericytes (57). Thus, the successful vascularization of tissue constructs through angiogenesis depends on the coordination of various cytokines/growth factors and cellular mechanisms, particularly the close interactions between the host tissue and the implant. This multi-step angiogenesis vascularization process provides various possibilities to stimulate and accelerate the formation of vascular networks in tissue constructs.

1.2.6 Survival of endothelial cells with VEGF

VEGF is a key regulator of physiological angiogenesis during embryogenesis, skeletal growth, and reproductive functions. VEGF has also been implicated in pathological angiogenesis associated with tumors, intraocular neovascular disorders, and other conditions. The biological effects of VEGF are mediated by two receptor tyrosine kinases (RTKs) vascular endothelial cell growth factor receptor-1 (VEGFR-1) and vascular endothelial cell growth factor receptor-2 (VEGRR-2), which differ considerably in terms of their signaling properties. Non-signaling co-receptors also modulate VEGF RTK signaling.

1.2.6.1 VEGF is required for the survival of cells

A well-documented in vitro activity of VEGF is how it promotes the growth of ECs derived from arteries, veins, and lymphatics. Indeed, VEGF induces a potent angiogenic response in a variety of in vivo models. VEGF delivery has also been shown to induce lymphangiogenesis in mice (58). Although ECs are the primary target of VEGF, several studies have reported mitogenic effects on certain non-EC types (59). Recent studies have also shown that VEGF stimulates surfactant production by alveolar type II cells (60).

VEGF is a survival factor for ECs, both in vitro and in vivo. In vitro, VEGF prevents apoptosis induced by serum starvation. Gerber et al. demonstrated that this activity is mediated by the phosphatidylinositol (PI)-3 kinase-Akt pathway (61). VEGF also induces the expression of anti-apoptotic proteins Bcl-2 and A2 in endothelial cells (62,63). In vivo, the pro-survival effects of VEGF are developmentally regulated. VEGF inhibition results in extensive apoptotic changes in the vasculature of newborn but not adult mice (64). Furthermore, a marked VEGF dependence has been found in ECs of newly formed but not of established vessels within tumors. Coverage by pericytes is suggested as one of the key events resulting in the loss of VEGF dependence (65).

VEGF also affects bone marrow-derived cells by promoting monocyte chemotaxis and inducing colony formation by mature subsets of granulocyte-macrophage progenitor cells. VEGF delivery in adult mice has been shown to inhibit dendritic cell development, increase the production of B cells, and entail the generation of immature myeloid cells (66). Conditional gene knock-out technology has been used to achieve selective VEGF gene ablation in bone-marrow cell isolates and hematopoietic stem cells (HSCs) (67). VEGF-deficient HSCs and bone marrow mononuclear cells were shown to not repopulate lethally irradiated hosts, despite the excessive coadministration of wild-type cells (68). These studies also point to an internal autocrine loop, unblocked by extracellular inhibitors such as antibodies, by which VEGF controls HSC survival during hematopoietic repopulation.

1.2.6.2 Types of VEGFs

The VEGF family consists of five members: VEGF-A, placental growth factor (PGF), VEGF-B, VEGF-C, and VEGF-D. A number of VEGF-related proteins encoded by viruses (VEGF-E) and found in the venom of some snakes (VEGF-F) have also been discovered.

The main functions of VEGF-A are to promote angiogenesis by upregulating EC migration and proliferation, enhance $\alpha v\beta 3$ activity that promotes the creation of blood vessel lumen, and create fenestrations leading to EC chemotaxis to macrophages and granulocytes (69). Furthermore, it may promote vasodilation by indirectly releasing NO.

The main function of VEGF-B is to promote embryonic angiogenesis, while that of VEGF-C is related to lymphangiogenesis (70). As for VEGF-D, it is required for the development of the lymphatic vasculature in the lung. PGF is crucial to vasculogenesis and is also necessary for angiogenesis during various pathologies such as ischemia, inflammation, wound healing, and cancer (71). In addition to endothelial cells, VEGF-A also affects a number of other cell types, e.g., monocyte/macrophage, neurons, cancer cells, and kidney epithelial cells.

VEGF-A is generated by the alternative splicing of a single pre-mRNA species which in humans exists in 121, 145, 165, 189, and 206 amino acids five different isoforms (72). The region encoding VEGF-A spans 14 kb and contains eight exons. The isoforms differ in how they bind to heparan sulfate and extracellular matrix (ECM). VEGF-A_{121}, which lacks the region encoded by exons 6 and 7, does not bind to heparan sulfate and is freely diffusible, whereas the other isoforms bind to heparan sulfate and ECM. VEGF-A_{121} is not a substitute for other isoforms, as mice expressing only VEGF-A_{120} (mouse VEGF is one amino acid shorter) were found to die early post-natally and display signs of cardiomyopathy (73). VEGF-A was originally identified as vascular permeability factor (VPF) - a designation reflecting its important and unique in vivo function (74). Another important feature of VEGF-A is its increased expression during conditions of hypoxia, which is regulated by a hypoxia-responsive element in its promoter (75). Thus, VEGF is crucial for vascular development, as well as for physiological and pathological angiogenesis. There is also evidence that VEGF-A may stimulate lymphangiogenesis (76). Several mammalian VEGF-related proteins [VEGF-B, -C, -D, and PGF] have been described. VEGF-B and PGF bind only to VEGFR-1, while VEGF-C and VEGF-D are expressed as propeptides that primarily bind to vascular endothelial cell growth factor receptor-3 (VEGFR-3). Processed mature ligands bind to VEGFR-3 with greater affinity and can also bind and activate VEGFR-2 (77). Aside from these mammalian VEGF-family proteins, a sheep parapoxvirus open-reading frame encoding a VEGF-related protein was shown to denote VEGF-E binding only to VEGFR-2 (72,78).

1.2.6.3 The receptors

a) VEGFR-1

VEGFR-1 is a receptor for VEGF-A, VEGF-B and PGF with high-affinity. It is also known as Flt-1 and expressed not only in vascular endothelial cells but also in a range of non-endothelial cells such as macrophages, haematopoietic stem cells and monocytes (72). Vegfr-1knockout mouse embryos die at day 8.5-9.0 because of obstruction of vessels by an overgrowth of endothelial cells (79). The increase in the number of endothelial progenitors in the absence of VEGFR-1 implies a negative regulatory role during vascular development that appears to be exerted by the soluble form of VEGFR-1 (sVEGFR-1), which lacks the transmembrane and intracellular part of the receptor (80). sVEGFR-1 is found expressed in a large amount in the placenta during pregnancy. With high affinity, it binds VEGF-A by preventing the binding of growth factor to the functional VEGFRs. Deletion of the intracellular domain of VEGFR-1 (Vegfr-1 tk-/-) is compatible with normal vascular development, which strongly suggests that the physiological role of VEGFR-1 in the development may be to sequester excess VEGF (81). Hematopoietic stem cells, as well as circulating CD97 monocytes and macrophages express VEGFR-1 which plays a key role in these cells migration. Indeed, macrophages from Vegfr-1 tk-/- mice cannot migrate towards VEGF-A or PGF (82). Accumulating hematopoietic cells in tumor tissue have recently been shown that can promote tumor vascularization. The growth of tumors expressing PGF is impaired in Vegfr-1 tk-/- mice, which suggested that the VEGFR-1 intracellular domain may regulate tumor angiogenesis (72,83). Furthermore, more researches and data have shown that VEGFR-1 function inhibited by neutralizing antibodies leads to tumor growth reducing and a number of perivascular hematopoietic cells decreasing in the tumor (72,84). Other non-endothelial cell types including spermatogenic cells, Leydig cells and osteoclasts also express VEGFR-1.

VEGF-A is bound by VEGFR-1 with high affinity. However, ligand binding results in a maximal twofold in kinase activity increasing (85). Only when in cells engineered to overexpress the receptor, the activation of VEGFR-1 can be discerned. Phosphorylation sites of VEGFR-1 have been labeled in these models. Remarkably, VEGFR-1 appears not to be phosphorylated on consensus positive regulatory tyrosine residues, which may

explain the reason why it has a low level of kinase activity. VEGFR-1 signal transduction and activation are correctly revealed possibly only when analyzed in the receptor expressing of primary endothelial cells or monocytes/macrophages endogenously (86).

VEGFR-1 has been shown to negatively influence VEGFR-2 function in cells expressing chimaeric VEGFR proteins (87). Antagonizing signals from VEGFR-1 overriding mitogenic signals from VEGFR-2 has been shown to depend on phosphoinositide 3-kinase (PI3K), although the exact mechanisms have not been identified yet. Of interest is that receptor heterodimerization is not a prerequisite for the negative influence of VEGFR-1. Thus, the signaling capacity of VEGFR-1 and -2 heterodimers is similar to that of VEGFR-2 homodimers, at least with regard to such signal transducers as phospholipase C-g1 (PLC-g1).

b) VEGFR-2

VEGFR-2, also known as KDR or Flk-1, is a 200-230-kDa high affinity receptor for VEGF-A, VEGF-C and -D and VEGF-E. It is expressed in both vascular endothelial and lymphatic endothelial cells as well as in several other cell types, such as megakaryocytes and haematopoietic stem cells. It was indicated that the receptor is crucial to vascular development as proved Vegfr-2 -/- embryos die by embryonic 8.5-9.5 days, exhibiting defects in the development of endothelial and haematopoietic precursors (88).

VEGF-A binds to the second and third extracellular IgG-loop of VEGFR-2 with a Kd of 75-125 pM, which is lower than the affinity of VEGF-A for VEGFR-1. There are at least 6 autophosphorylation sites identified for VEGFR-2, which are Tyr951 and Tyr996 in the kinase-insert domain, Tyr1054 and Tyr1059 in the kinase domain, and Tyr1175 and Tyr1214 in the C-terminal tail (89). Several proteins have been found to associate, via their Src homology-2 (SH2) domain, with specific autophosphorylated tyrosine residues. Tyr951 creates a binding site for the VEGFR-associated protein (VRAP), while Tyr1175 creates a binding site for Sck and PLC-g1 (90).

VEGFR-2 is the major mediator of several physiological and pathological effects of VEGF-A on endothelial cells, including migration, proliferation and survival and permeability (91). VEGFR-2, like many other receptors, induces proliferation by activating the classical extracellular signal-regulated kinase (Erk) pathway (p42/44 mitogen-activated protein kinase), leading to gene transcription. In the case of VEGFR-2, it was originally reported that Raf was directly activated by protein kinase C (PKC) in a Ras-independent manner, however recent data has shown that VEGF-A is capable of stimulating Ras through a pathway that requires PKC and sphingosine kinase (92). PI3K is also activated by VEGFR-2, which leads an increase in the lipid phosphatidylinositol (3,4,5) P3. At the same time, several important intracellular molecules are activated, including Akt (also denoted as protein kinase B or Akt/PKB) and the small GTP-binding protein Rac (72). The Akt/PKB pathway regulates cellular survival by inhibiting pro-apoptotic pathways such as B-cell lymphoma 2 (Bcl-2)-associated death promoter homologue (BAD) and Caspase 9. This pathway also activates endothelial nitric oxide synthase (eNOS), which generates NO, involved in the increase in vascular permeability and cellular migration observed with VEGF-A. The small GTP-binding protein Rac has also been implicated in the regulation of vascular permeability and cellular migration. Other components involved in VEGFR-2-dependent cytoskeletal regulation and cell migration include p38 mitogen-activated protein kinase (MAPK) and focal adhesion kinase (FAK) and its substrate paxillin (93). VEGFR-2 also activates several other important intracellular signaling molecules, particularly Src. While it is not yet clear exactly how VEGFR-2 interacts with Src or its downstream role, mice lacking the Src family members Src and Yes have been shown to display impaired VEGF-A-induced vascular permeability (94).

c) VEGFR-3

VEGFR-3, also known as Flt-4, is a 195-kDa high affinity receptor for VEGF-C and VEGF-D. VEGFR-3 has different distinct features including cleavage during synthesis within the fifth extracellular immunoglobulin loop, while by a disulfide bridge the two regulating polypeptides kept together (95). In humans, there are one short and one long VEGFR-3 splice variants, with the latter exhibiting a C-terminal extension of 65 amino acids (96,97). It is created by retroviral insertion (98). There are researches showing that

embryos lacking VEGFR-3 expression was found dead at 9.5 days because of deficient vessel remodeling (99). Larger vessels became disorganized, resulting in fluid accumulation and cardiovascular failure. These effects may potentially be caused by VEGFR-3 losing directly, or by an indirect effect caused by a VEGF-C and -D availability increasing to activate VEGFR-2. VEGFR-3 expression is detected primarily in lymphatic endothelial cells which are physiologically distinct from blood vascular endothelial cells. Homeodomain transcription factor Prox-1 has been shown that can regulate the lymphatic endothelial cell phenotype (100) and lead to chronic lymphedema by inactivating point mutations in it (101). It was found that blocking VEGFR-3 function can lead to a regression of lymphatic vessels and features of lymphedema in the skin of transgenic mice by using overexpression of a soluble recombinant VEGFR-3, while no apparent effect was found on the blood vasculature (102). Tumor lymphangiogenesis can also suppress by soluble VEGFR-3 with metastasis to regional lymph nodes decreasing. VEGFR-3 function enhanced through the tumor-specific VEGF-C overexpression in breast cancer, fibrosarcoma and melanoma models and that of VEGF-D in a human embryonic kidney (293EBNA) tumor model have been proved to increase lymphangiogenesis and promote the spread of metastasis (103).

1.2.7 Traditional Chinese medicine and angiogenesis

Arterial occlusive diseases such as coronary heart disease and lower extremity arterial occlusion are serious hazards to human health. Medications as well as interventional and conventional surgeries remain the primary treatment methods employed. However, for patients with multiple surgical procedures and a lack of autologous grafts, the problems are still unable to undergo revascularization, which is why, in recent years, research on pro-angiogenesis therapy has gained interest. Angiogenesis occurs throughout life, involving embryonic maturation, individual growth and development, wound healing, cancers, and other physiological and pathological processes.

In the long history of TCM, various natural products have been used to treat CVD. Some of them have reportedly performed well in promoting angiogenesis, as described below.

1.2.7.1 Dang-gui-bu-xue-tang (当归补血汤)

Literally, this name means a blood enhancing remedy (bu-xue-tang) based on Chinese angelica root (Dang-gui). In TCM it is often used to treat patients who are weak because of losing blood and anemia. Lei Yan et al. investigated the effect of astragalus and angelica in various combinations on angiogenesis using a chick chorioallantoic membrane (CAM) model (104). The results of astragalus and angelica 5:1 with regard to performance in angiogenesis and vascular counting were found to be significantly better than those of the control group and other combinations. They concluded that Dang-gui-bu-xue-tang had promoted CAM angiogenesis and that the 5:1 ratio recommended by the original remedy is the ideal combination.

Endothelial cells are the target cells during angiogenesis, while their migration and proliferation determine the angiogenic process. Lei Yan et al. explored the influence of astragalus and angelica on human umbilical vein endothelial cell (HUVEC) proliferation and cell cycles by means of an MTT assay for cell proliferation, cell cycle analysis by flow cytometry, and the SABC method to detect the expression of vascular endothelial growth factors through the establishment of an in vitro cultured HUVECs model (105). Results showed that astragalus and angelica were not only capable of promoting vascular endothelial cell proliferation and DNA synthesis but also of having a synergistic effect when the ratio of astragalus and angelica was 5:1. These drugs may thus have an effect on the development of new blood vessels in ischemic myocardium they concluded.

1.2.7.2 Shuang-long-wan (双龙丸)

Shuang-long-wan, literally double dragon bill, is a remedy made of centipede, scorpion, and earthworm, which is used to improve circulation, in addition to other effects. Yang Zufu et al. studied the effect of Shuang-long-wan on ischemic myocardium angiogenesis and the molecular mechanisms using the acute myocardial ischemia (AMI) model in rats (106). The expression of VEGF-mRNA and bFGF-mRNA was detected with RT-PCR; and the production of VEGF and bFGF was measured with immunohistochemistry. Their findings showed that angiogenesis in the ischemic myocardium in terms of the number of vessels in both high- and low-dose groups increased

compared to that observed in the control group. This shows that Shuang-long-wan has an angiogenic effect in ischemic myocardium and that an elevated dose (6.72 g/kg) significantly upregulated the expression and secretion of VEGF and bFGF.

1.2.7.3 She-xiang-bao-xin-wan (麝香保心丸)

She-xiang-bao-xin-wan is made of musk, ginseng extract, bezoar, cinnamon, oriental sweetgum, toad, and borneol. It is used to treat ischemic angina, chest tightness and myocardial infarction. Wang Shanshan et al. investigated the effect of She-xiang-bao-xin-wan on myocardial infarction, coronary collateral angiogenesis, and the underlining mechanism in rat acute myocardial infarction model (107). The authors found that the infarct area in the treatment group was significantly smaller than that observed in the control group. The expression of VEGF, bFGF and vWF factors, and the density of vasculature in the marginal zone of infarct myocardium increased significantly, compared to the results of the control group. It was thus proposed that She-xiang-bao-xin-wan could promote coronary collateral perfusion to ischemic tissues, with a protective effect on the ischemic myocardium. The mechanism may be related to the increased expression of VEGF and bFGF.

1.2.7.4 Xin-mai-tong (心脉通)

Xin-mai-tong capsule primarily consists of Jilin red ginseng, panax, rhubarb root, dry earthworm, *Citrus aurantium*, etc., reportedly to improve circulation. Meng Jun et al. studied the effect of Xin-mai-tong capsule on VEGF expression in 46 randomly treated patients with acute myocardial infarction (108). Within a week, in test and control groups, the VEGF levels in patients' serum differed significantly. The authors suggested that Xin-mai-tong might have induced angiogenesis, increased the formation of collateral vessels, and improved blood supply to the infarct zone. Various mechanisms were proposed, including to regulate vasoactive factor metabolism, promote VEGF expression and the subsquant myocardial blood supply and prevent the damage of oxygen free radicals.

1.2.7.5 Tong-xin-luo (通心络)

Tong-xin-luo, meaning dredge heart circulation, is composed of ginseng, leech, scorpion, centipede, root of common peony, etc. It is used to treat symptoms caused by ischemic heart diseases. Wang Wenjian et al. studied the effect of Tong-xin-luo on angiogenesis using CAM model (109). The experiment was designed as four randomly selected groups: blank serum group, bFGF group, and Tong-xin-luo high- and low-dose groups. Vasculature development was found to be significantly higher in the high-dose group than in the blank serum group and was not significantly different from that of the bFGF group. There were no significant differences between the low-dose group and the blank serum group. It was concluded that Tong-xin-luo promoted CAM angiogenesis and therefore may have angiogenesis-promoting effects.

1.2.7.6 Tonifying kidney medicine

Tonifying kidney medicine includes a variety of preparations from a wide spectrum of natural products such as placenta, *ligustrum lucidum*, *morinda citrifolia*, dodder, *wulingzhi*, etc. Using elderly female golden hamsters as the experimental model mimicking the physiological symptoms of kidney deficiency in the elderly, Zhang Shucheng et al. studied the pharmacological effect of tonifying kidney medicines (110). The authors found that the angiogenic effect on uterine tissue significantly increased, which suggests that these specific ingredients have angiogenic potential. The authors also used self-control methods to compare the growth factor expression in endometrial tissues before and after treatment. VEGF, VEGFR, bFGF/FGF, platelet-derived growth factor receptors alpha (PDGFR-α) and epidermal growth factor receptor (EGFR) were investigated by a comparative immunohistochemistry analysis, which revealed a significant increase in the number of cells showing intensive stains of five types of the factors in the group taking the medicine, suggesting that the tonifying remedy had an effect in promoting angiogenesis. Using immunohistochemical methods, Wang Lei et al. observed similar effects on bFGF and VEGF expression in the elderly golden hamster uterus, supporting that these drugs could promote local angiogenesis (111).

1.2.7.7 Nv-zhen-yun-yu-tang (女贞孕育汤)

This formula contains dodder, ligustrum lucidum, medlar, angelica, and salvia, as well as 14 other Chinese herbal ingredients. Xia Yuwei et al. observed 45 anovulatory infertility cases who took three periods of Nv-zhen-yun-yu-tang decoction treatment (oral administration), while the patients took clomiphene were settled as control (112). Blood flow parameters, resistance index and pulsatility index of ovarian and uterine were determined. CAM model was used to detect the angiogenesis effect of patients serum who took the medicine. Results showed pregnancy rate of the treatment and control group was 55.55% and 52.17% respectively. The blood supply of ovaries was improved in ovulation side obviously after treatment than that of non-ovulation side, which accelerates the angiogenesis, growth and development of endometrium. In CAM model, angiogenesis performed both in treatment group and clomiphene group, and was better than blank control.

1.2.7.8 Salvia miltiorrhiza (丹参)

Liu Qigong et al. studied the effect of *Salvia* injections on the coronary collateral vessels in a canine myocardial infarction model (113). Coronary angiography and myocardial tissue pathological analysis revealed that the myocardial capillary and infarct zone vascular density increased significantly in the treatment group. Xu Jie et al. investigated the mechanisms of angiogenesis of salvianolate, the water-soluble component of *Salvia*, by observing the effects of the *Salvia* polyphenol salt on monocyte-induced endothelial cell migration (114). The authors were able to show that salvianolate could promote monocyte-induced endothelial cell migration, thus promoting the synthesis and secretion of VEGF and bFGF in monocytes. It was concluded that salvianolate played an important role in promoting endothelial cell migration.

1.2.7.9 Panax notoginseng (PNS) (三七)

Yan Yanfang et al. confirmed that the main ingredients of PNS, e.g., Rb1, Rg1 and Re, have a significant protective effect on hypoxia-induced injury to vascular endothelial cells, thereby indirectly suggesting that PNS may promote angiogenesis (115). Coronary angiography also showed that PNS promoted collateral circulation in experimental myocardial infarction, supporting the angiogenic property of PNS (116).

1.2.7.10 Puerarin

Puerarin is an isoflavone derivative isolated from *pueraria lobata* or pueraria *thomsonii* Benth, which are popular botanic drugs in TCM known to reduce fever, headache, diarrhea, and hypertension, among other things. Because it can increase coronary blood flow, puerarin has been used to treat coronary heart disease in China. Zhang et al. studied the effect of puerarin on angiogenesis in myocardium of rat with myocardial infarction and their results demonstrated that puerarin may induce therapeutic angiogenesis in myocardium of myocardial infarction rats by inducing VEGF and eNOS expression (117). In another study, Wu et al. investigated the cardio-protective effect of a complex preparation made of puerarin and *Salvia* extract on acute ischemic myocardial injury in rats (118). The results indicated that puerarin and *Salvia* extract complex exerted significant cardio-protective effect against acute ischemic myocardial injury.

1.2.7.11 Proanthocyanidin

Proanthocyanidins (Figure 1.1 A) are a class of oligomers of flavan-3-ols such as epicatechin (Figure 1.1 B), and are also the precursors of plant pigments known as anthocyanidins and anthocyanins that are widely found in a variety of plants. Chemically, most proanthocyanidins are composed by catechin and epicatechin in different ratios. They were discovered in 1947 by Jacques Masquelier and reported in his Ph.D. thesis as a colorless fraction from the red-brown skin of peanuts (119). Nowadays, according to the polymerization degree, these polyphenolic compounds are commonly classified as oligomers and even polymers. Oligomeric proanthocyanidins are well-known by the abbreviation “OPCs” which are often referred as nutritional supplements.

Proanthocyanidins can be found in most plants especially in the skin, seeds and seed coats of purple or red pigmented fruits such as in grape skin and seeds, blueberries, cranberries, aronia, hawthorn, rosehip and sea buckthorn (120). Since such fruits are commonly consumed, proanthocyanidins are common in our daily diet. Intake of antioxidants such as proanthocyanidins was used to explain the so-called French Paradox; however there has been no solid evidence to support the claimed benefits to coronary heart

diseases. Nevertheless, diets such as red wines rich in polyphenols and proanthocyanidins continue to attract attention as potential cardiac-protective supplements (121).

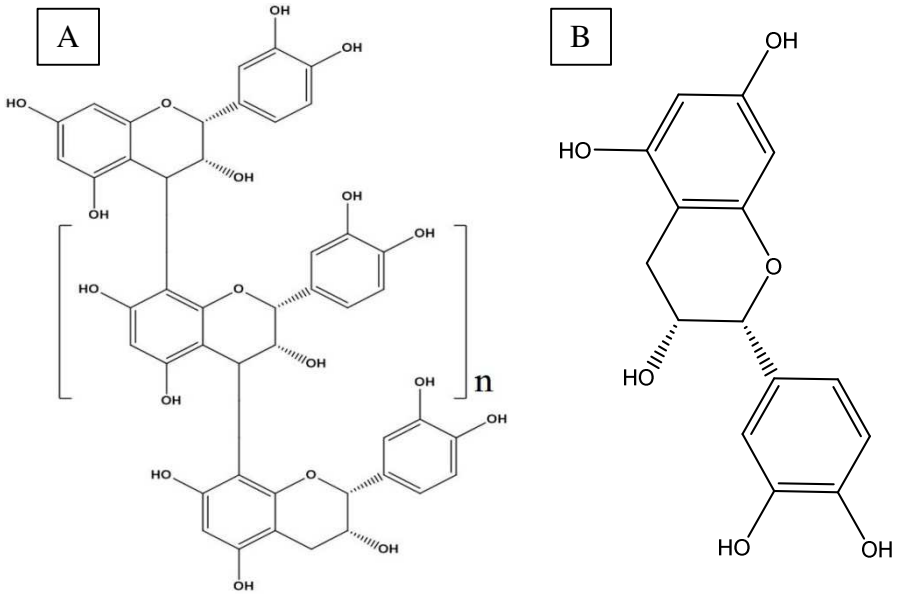


Figure 1.1 Chemical structures of proanthocyanidins (A) and epicatechin (B)

Proanthocyanidins have been reported to exhibit a protective effect against cardiovascular disease. Zhang et al. used high-carbohydrate/high-fat diet and streptozotocin to induce diabete in rats and treated them with different concentration of grape seed proanthocyanidin extracts (GSPE) for 24 weeks. The results showed that inflammatory response and intima-media thickness in the diabetic rats were reversed by GSPE ($p < 0.05$), and glycation end products and receptor of advanced glycation end products expression of aortic root were effectively lowered by the high concentration of GSPE ($p < 0.05$). This study evidenced that GSPE may be an effective agent to protect vasculature from diabetes-caused inflammation and endothelial dysfunction (122). Liang et al. studied the effect of GSPE on arterial remodeling by treating the spontaneously hypertensive rats with GSPE daily. The result showed that the administration of GSPE alleviated hypertension significantly by reducing endothelin‐1production, increasing NO production and ameliorating oxidative stress by improving superoxide dismutase and catalase and reducing malondialdehyde formation. This indicates that GSPE may attenuate arterial remodeling induced hypertension by repressing oxidative stress (123). Xu et al. researched the effect of

GSPE on liver ischemia/reperfusion injury and alleviation of endoplasmic reticulum stress. The result showed that serum aminotransferase, apoptotic cells, Suzuki scores, methane dicarboxylic aldehyde level were decreased in the GSPE group. Pro-inflammatory factors were downregulated while anti-inflammatory factors upregulated by GSPE. It was concluded that GSPE possesses antioxidative, anti-inflammatory and antiapoptotic effects by relieving endoplasmic reticulum stress to protect the liver against ischemia/reperfusion injury (124).

While many TCM remedies are claimed to be clinically effective in treating CVD, there lacks systematic research to substantiate the efficacy and mechanisms of the many widely claimed remedies. In addition, although some of these herbal medicines are reported to be effective in proliferating endothelial cells, clear evidence is limited and is as yet unsupported by other research, particularly outside of China. Therefore carefully designed studies are necessary to verify the efficacy of these remedies or drugs.

To avoid the complexity of multiple component remedies and considering the availability of the reportedly angiogenic drugs in the market, this thesis selected four preparations from TMC, which are *Astragalus* powder extract, *Astragalus* injection, puerarin injection, and proanthocyanidin. The selection of the drugs was based on TMC literature and candidate's previous work.

Table 1.1 The four botanic drugs selected in this study

Name of drug	Form	Reported clinic benefit	References
Astragalus powder extract	Powder extract	To enhance immune function To relieve allergy symptoms To improve the health of heart To ameliorate circulation and speed up wound healing To protect liver	Lei Y, et al. Zhongguo Zhong Yao Za Zhi. 2003. Chinese.
Astragalus injection	Injection	To ameliorate circulation To remove stasis To treat viral myocarditis	Shuo Zhang, et al. World J Cardiovasc Dis. 2013.
Puerarin injection	Injection	To treat coronary heart disease To treat angina pectoris To treat myocardial infarction To treat ischemic stroke To treat sudden deafness	Zhang S, et al. Biol Pharma Bull. 2006. Wu L, et al. Phytomedicine. 2007.
Proanthocyanidin	Standard reagent, powder	To treat diabetes To protect heart To treat hypertension To relieve oxidative stress To treat cancer	Zhang Z, et al. Int J Food Sci Nutr. 2015. Liang Y, et al. Mol Med Rep. 2016.

1.3 Hypothesis

The four botanic drugs can regulate and promote endothelial cell growth and angiogenesis.

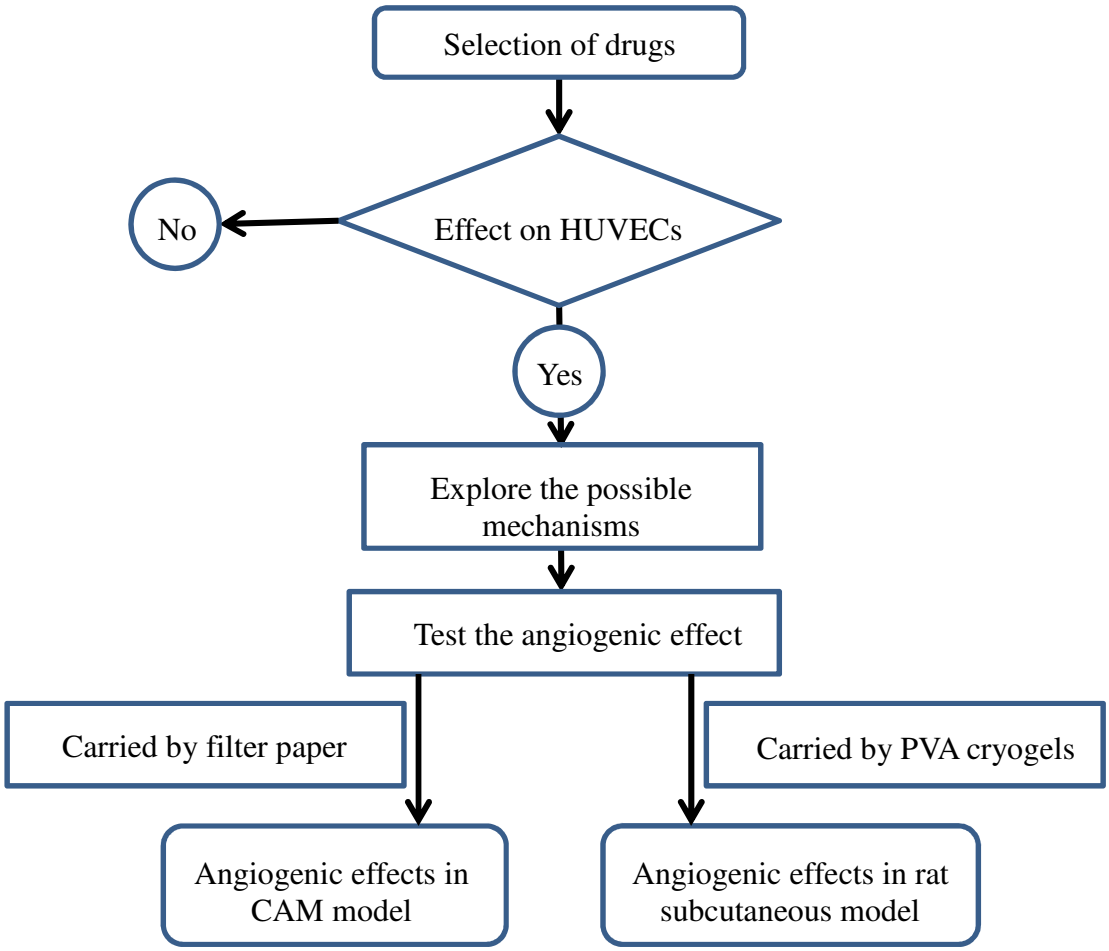
1.4 Objectives

1. To study the effect of the selected botanic drugs on endothelial cell activity and proliferation in vitro.

2. To explore the possibility of releasing the botanic drugs from a polymeric carrier.

3. To investigate the angiogenic effect of the botanic drugs.

1.5 Research plan



Chapter 2

Effects of four Chinese medicines on endothelial cell viability and proliferation

2.1 Introduction

Blood vessels are distributed throughout the human body to transport blood in and out of tissues and organs to keep them alive. The entire lumen of blood vessels is covered by a single layer of flat cells named endothelial cells (ECs), which play a critical role in regulating the normal physiological function of the circulatory system. Many cardiovascular diseases are related to the dysfunction and abnormal pathophysiological changes of the endothelium. Because of this, regulation of EC activities has been the center of many research areas such as revascularization of infarcted myocardium, reendothelialization of vascular implants, and perfusion of tissue engineered products.

New strategies to enhance the viability and proliferation of ECs therefore will have a huge impact in medicine and biomedical engineering. Modern angiogenic approaches use angiogenic growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblasts growth factors (bFGF) (125). Growth factors are bioactive proteins that are expensive, sensitive to preparation procedures (easily be denatured), and lose their activity quickly in vivo.

In traditional Chinese medicine (TCM) there are well-acknowledged botanic drugs that reportedly promote angiogenesis. However, studies to investigate the angiogenic property of remedies in TCM have been mainly limited in Asian countries. Research on their usefulness in promoting endothelialisation has been very limited. Study of such in the context of wound healing and tissue engineering has not been reported.

Astragalus is an herb and its root is used to make medicine in TCM. Lei Yan et al. investigated *Astragalus* and *Angelica* for their angiogenic effect in chick chorioallantoic membrane (CAM) model (104). The results showed that *Astragalus* preparation enhanced angiogenesis by stimulating endothelial cell migration and proliferation. The authors also explored the influence of *Astragalus* and *Angelica* on human umbilical vein endothelial cell (HUVECs) proliferation and cell cycles (105). The results showed that *Astragalus* and *Angelica* could promote HUVEC proliferation and DNA synthesis. It was suggested that these drugs may benefit the development of new blood vessels in the ischemic myocardium.

Puerarin belongs to isoflavones and can be found in a number of plants and herbs, such as the root of *Pueraria* (*Radix puerariae*) (126). In TCM, puerarin is known for its functions of protecting tissues from ischemic injury by improving blood supply, reducing hypertension, relieving angina pectoris, scavenging free radicals, lowering cholesterol and improving platelet function. This drug therefore has been widely used to treat ischemic heart and brain vascular diseases and diabetic complications in China (127). Wang Chunling et al. reported that puerarin had a protective effect against the oxidative damage to the DNA of HUVECs (128). The results showed that the breaks of DNA strands caused by hydrogen peroxide significantly decreased with a pre-treatment of puerarin. This suggests that puerarin can function as an antioxidant and protect against the oxidation induced DNA damage. There are also similar studies reported that a pre-treatment of endothelial cells with procyanidins had a protective effect on the cells against hydrogen peroxide. The mechanisms were thought to be mediated by anti-lipid peroxidation, oxygen free radical scavenging, and anti-apoptosis (129).

In this chapter, according to the literature review, four types of botanic drugs were selected to test the feasibility of using them to regulate endothelial cell viability and proliferation.

2.2 Materials and Methods

2.2.1 Materials

Human primary umbilical vein endothelial cells line (PCS-100-013) was purchased from ATCC (ATCC, Manassas, VA, USA). Medium 199, endothelial cell growth supplements (ECGS), heparin, 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenylthiazolium (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Fetal bovine serum (FBS) and trypsin was from Fisher Scientifique Limitee Company (Ottawa, ON, Canada). Penicillin-Streptomycin and L-Glutamine were from Gibco (Burlingtoqiun, ON, Canada). Ordinary invert microscope if from Olympus (Japan) while Quant Universal Microplate Spectrophotometer is from Bio-Tek Company (USA), and phase hemacytometer was purchased from VWR (Canada).

Astragalus Extract was prepared from the roots of Astragalus membranaceus Bge. var. mongholicus Hsiao in form of brown powder. It was purchased from Shanxi Herbsoul Natural Products (Xi'an, China). *Astragalus* injection was in form of pale yellow transparent liquid and purchased from Chengdu Diao Pharmaceutical Group (Chengdu, China). Puerarin injection was in form of transparent liquid and purchased from Harbin Sanlian Pharmaceutical Industry (Harbin, China). Every 1 ml *Astragalus* injection contained 2 g crude *Astragalus*, and every 1 ml puerarin injection contained 0.05 g crude puerarin. Proanthocyanidin was purchased in form of brown powder with a purity of 99.37% purchased from Tianjin Jianfeng Natural Product R & D (Tianjin, China).

2.2.2 Methods

2.2.2.1 Culture of HUVECs

A complete medium was prepared by adding 15% heat inactivated FBS, 20 μg/ml ECGS, 0.9 g/l heparin and 292 g/l L-Glutamine into the M199 cell culture medium. Bovine gelatine (type B) was dissolved in hot H_2O at the ratio of 1% (w/v) and filtered when it was still hot with a 0.22 μm filter.

To expand HUVECs, a T-75 canted neck flask was prepared by adding 5ml pre-warmed 1% gelatin to cover the seeding surface area. In 5 min the gelatin solution was removed and then the flask was firstly rinsed twice with deionized water and then with the complete growth medium once. Then the flask was placed in a humidified incubator at 37 °C and in 5% CO_2, allowing the medium to pre-equilibrate under the designed temperature and pH before adding cells. Thus, the flask was prepared and ready to use.

A vial of cryopreserved HUVECs containing 2 x 10^5 cells was thawed by a gentle agitation in a 37 °C water bath for approximately 2 minutes before being removed from the water bath and disinfected by spraying with 70% ethanol. The cells were then transferred from the cryovial to a conical tube containing 30 mL of complete growth medium by using a sterile pipette. After being pipetted gently to homogenize the suspension, the cells of equal number were transferred to the previously prepared flasks. The flasks were gently

rocked to achieve even cell distribution and then placed in the cell culture incubator at 37 °C in a 5% CO_2 atmosphere. The cells were incubated with culture medium refreshed every 48 hours.

Cells were passaged when cultures had reached approximately 80% confluence. Trypsin-EDTA and the complete growth medium were warmed to 37 °C prior to use. To detach cells, the flask was aspirated carefully to remove culture medium, and then rinsed two times with 5 mL phosphate buffered saline (PBS) to wash away trace of serum. After that, 3 ml pre-warmed trypsin-EDTA was added. The flask was gently rocked to ensure the complete coverage of trypsin over the cells and then aspirated to remove the excess fluid. Cells were then observed under a microscope. Typically within 5 minutes, cells pulled away from each other and rounded up. By gently tapping the sides of the flask the cells became detached from the flask surface. When majority of the cells appeared detached, 10 mL of the pre-warmed complete growth medium was added to the flask. The cell suspension was gently pipetted to dissociate all cells and then transferred to a sterile centrifuge tube. Another 10 mL complete growth medium was added to the flask to collect any additional cells. Finally, the cells were counted using a hemacytometer and seeded to new flasks or plates. Cells of passage 4 to 7 were used for the following experiments.

2.2.2.2 Drug preparation

Astragalus and puerarin injections were used as received. *Astragalus* extract and proanthocyanidin were dissolved in M199 without serum and then filtered by 0.22 micron filter for the sterilization. The final concentration of *Astragalus* extract was 10mg/ml and the final concentration of proanthocyanidin was 1 mg/ml. All the drugs were opened or prepared freshly before use.

2.2.2.3 Cell growth

HUVECs were diluted to 1×10^4 cells/ml in complete medium containing 15% FBS and 25 μg/ml ECGS. Five hundred microliters (500 μl) of the cell suspension was added into each well of a 48-well plate. After 24 hours the complete medium was aspirated and replaced with the basic medium (without serum and growth factors). The culture was continued for overnight before adding test or control media. To prepare test media, a serial

dilution of each drug was prepared by mixing a treatment medium containing 5% FBS or 15% FBS but without ECGS, and 1.0 ml of the medium containing different concentrations of *Astragalus* injection, puerarin injection, *Astragalus* extract and proanthocyanidin. The test media therefore contain different concentrations of drugs in a medium of 5% or 15% FBS without ECGS. Medium without FBS and ECGS was used as blank control. Medium with 25 μg/ml ECGS and 5% or 15% FBS was used as positive controls. The compositions of all the media are listed in Table 2.1. The cultures were carried out for 24 h, 48 h and 72 h in triplicate for each condition.

Table 2.1 Culture media and their compositions

Culture media	**Compositions**
Basic medium	M199 1% penicillin/streptomycin 0.9 g/L Heparin 292 g/L L-Glutamine
Treatment medium (Blank control)	Basic medium 5% FBS or 15% FBS
Complete growth medium (Positive control)	Basic medium 15% FBS 25μg/ml ECGS
Test medium	Treatment medium Drugs of various concentrations

2.2.2.4 MTT test

After every 24 h, the cultures were washed and medium replaced with 500 μl fresh medium containing 5% FBS, to avoid any possible interaction between the drugs under test and staining reagents. Then, 50 μl of MTT reagent of 5 mg/ml in concentration was added to each well. After an additional incubation for 4 h at 37 ℃, the medium was carefully aspirated and 500 μl DMSO was added to dissolve formazan crystals. The dissolved

formazan solutions (100 μl) were transferred to a 96-well plate and their optical absorptions at 570 nm were recorded with a plate (μQuant Bio-Tek Company, USA).

2.2.2.5 Cell count

HUVECs were seeded to 6-well plates at a density of 2,500 cells per cm^2 and treated with the drugs as described in previous section Cell growth. After 72 h, culture media were aspirated and cells were rinsed twice with PBS. Then, 0.25% trypsin-EDTA was added to each well to detach cells. The detached cells were collected in a centrifuge tube and centrifuged at 150×g for 5 min. After aspirating the supernatant, 100 μl medium was added to resuspend the cells by gently pipetting the cell suspension. Trypan blue was added and live cells were counted at least 4 times using a phase hemacytometer.

2.2.2.6 Hoechst staining

For Hoechst staining, sterilized round cover glasses were placed into culture plate and coated with a solution of 1% of gelatin before cell seeding. HUVECs were seeded onto the gelatine coated cover glasses at a density of 3.2×10^4 cells per surface area and cultured with the selected dose of drugs, in comparison with positive and blank controls. At 72 h of culture, the culture medium was removed and 2 ml Hoechst fixative (alcohol ： glacial acetic acid, 3：1) was added to keep the cells completely immersed for 2 min. Then, the fixative was refreshed and the fixation was continued for another 5 min. After, the fixed cells were washed 3 times with pyrogen-free water and 5 ml of Hoechst working solution diluted to 1% was added. The Petri-dishes were covered with aluminum foil and kept at room temperature for 15 min. Finally, Hoechst solution was removed and the cells were washed 3 times with pyrogen-free water before being observed with a fluorescence microscopy and photographed.

2.2.2.7 Immunocytochemical staining

Immunocytochemical staining for von Willebrand factors (vWF) was performed at the end of 72 h of culture on cover glass. To do this, cells were rinsed with PBS and then fixed with a PBS solution of 95% methanol and 1% acetic acid. After being rinsed with 80% methanol in PBS, 3% H2O2 was added as endogenous peroxidase blocker and incubated

for 10 min before being rinsed again with PBS for 3 x 5 min. Normal chicken serum diluted to 1:200 in PBS was added as blocking serum and incubated for 20 min before adding primary antibodies. Monoclonal mouse anti-human vWF antibodies were added at a 1:500 dilution and the plates were kept at 4 ℃ overnight. Then the cells were warmed up to room temperature and rinsed in PBS for 3 x 5 min. Chicken anti-mouse biotinylated antibodies were added as the secondary antibody at a 1:500 dilution and kept at room temperature for 40 min. Then the cells were incubated in avidin-peroxidase for 40 min at room temperature. To visualize the staining, cells were treated with the peroxidase substrate 3,3'-diaminobenzidine (DAB) for 5 min at room temperature, rinsed with deionized water, and counterstained with hematoxylin for 30 sec.

2.2.2.8 Statistical analysis

Data were expressed as mean ± S.D. Student t-test was used to determine statistical significance between control and test groups. $P<0.05$ was considered statistically significant and $P<0.01$ was considered statistically highly significant.

2.3 Results

2.3.1 Effect of Astragalus injection on cell growth

Figure 2.1 shows the metabolic activity of HUVECs at different concentrations of astragalus injection in 5% of FBS for 24, 48 and 72 h, in comparison with the blank (5% FBS alone) and positive (5% FBS + ECGS) controls. At 24 h there was already a significant difference between controls and some drug groups. At 48 and 72 h, except the group of 500 mg/ml of drug showing a stable cell activity similar to that of the blank control, all other drug groups showed a significant decrease in cellular activity with respect to blank control. Nevertheless, in a wide range of drug concentrations ranging from 100 μg/ml to 250 mg/ml, HUVECs showed a tolerance to the astragalus injection. High cytotoxicity appeared only at the highest concentration tested, i.e., 1.0 g/ml ($p<0.05$). Positive control showed normal cell growth over three days.

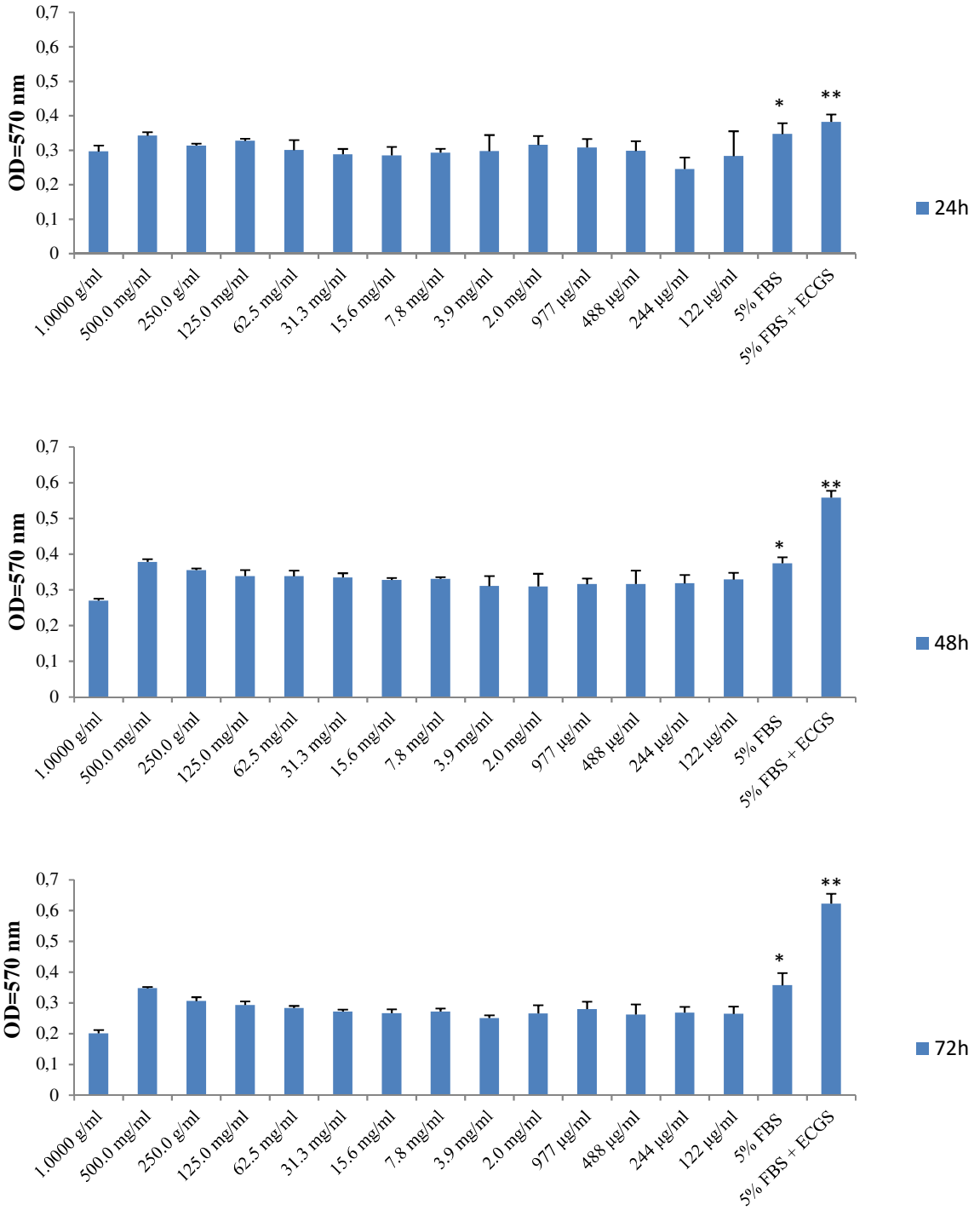
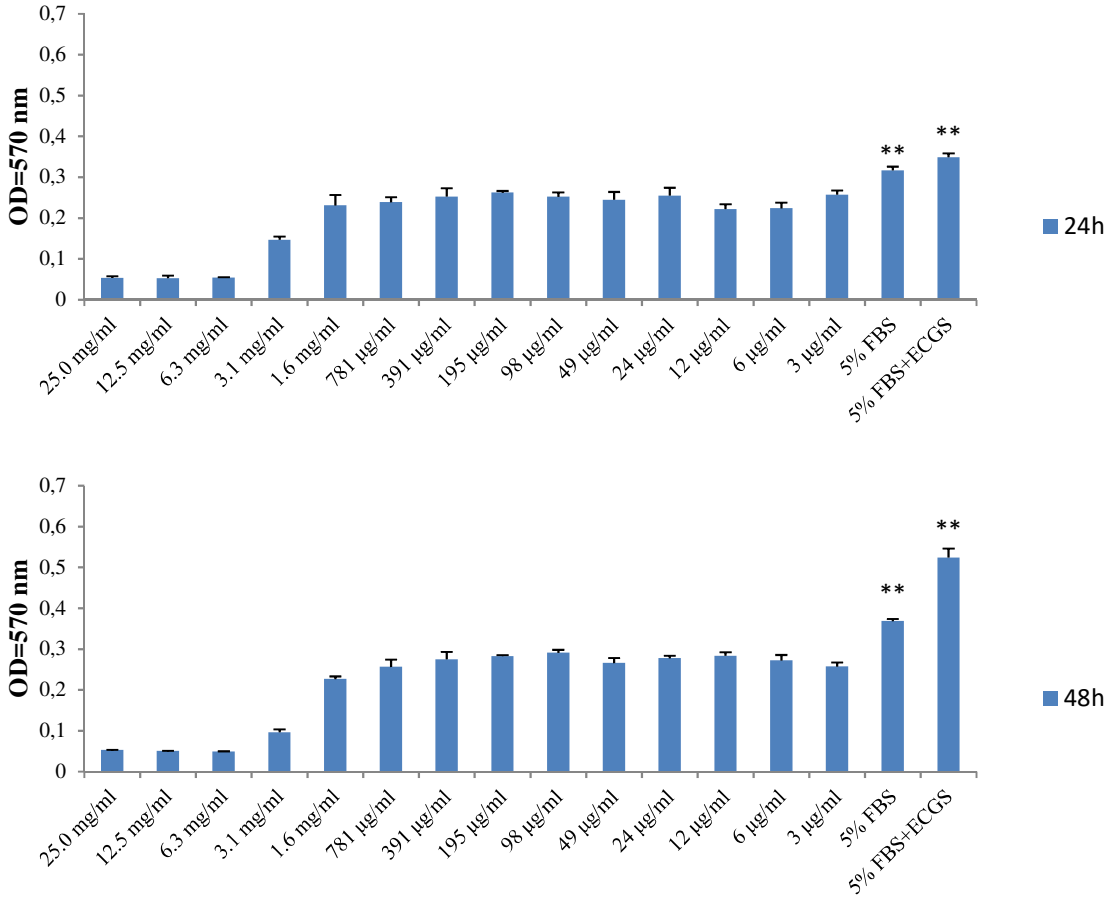


Figure 2.1 HUVEC viability after 24, 48 and 72 hours of culture in different concentrations of *astragalus* injection. 24 h: ** significant difference with respect to all drug groups except 500 mg/ml ($p<0.05$); * Significance difference with respect to 1.0 g/ml, 31.3 mg/ml, 15.6 mg/ml, 7.8 mg/ml, 3.9 mg/ml and 122 µg/ml drug groups ($p<0.05$). 48 h: ** significant difference with other groups ($p<0.01$); * Significant difference with all drug

groups except 500.0 g/ml and 250.0 mg/ml groups. 72 h: ** Significant difference with other groups ($p<0.01$); * Significant difference with respect to other groups except 500 mg/ml group ($p<0.01$).

2.3.2 Effect of puerarin injection on cell growth

Puerarin injection exhibited moderate to high cytotoxicity in all the three periods of time, showing significantly lower optical absorptions than both blank and positive controls ($p<0.05$). High toxicity appeared at 3.1 mg/ml and above. However, it was noticed that at a dose as low as 3 μg/ml HUVECs were significantly suppressed with respect to blank control.



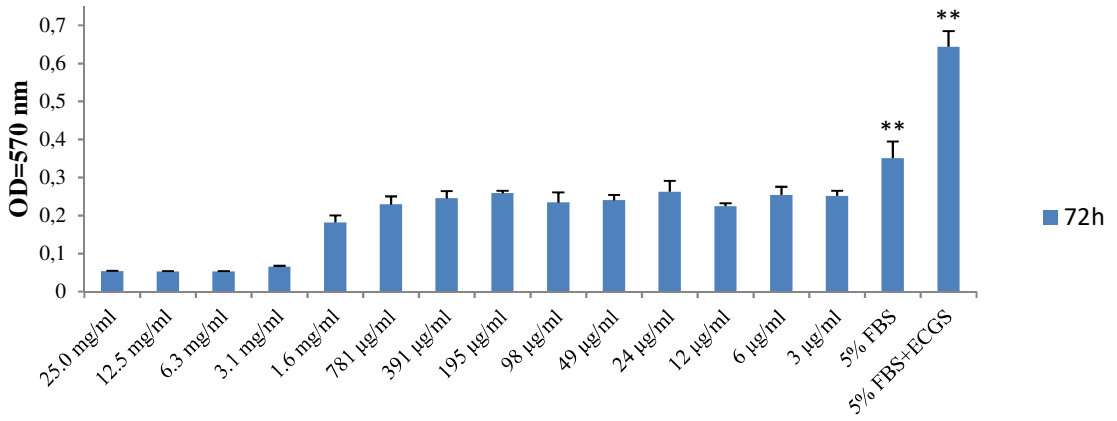
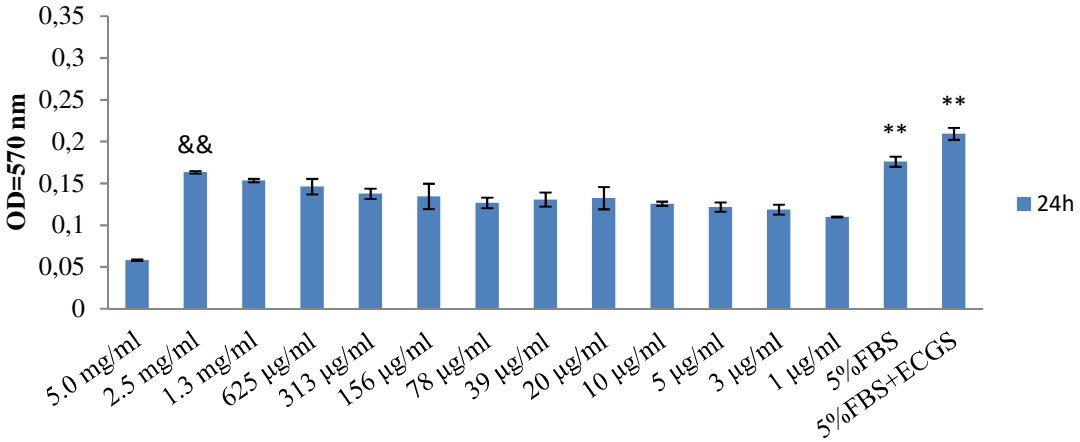


Figure 2.2 HUVEC viability after 24, 48 and 72 hours of culture in different concentrations of puerarin injection, in comparison with blank (5% FBS) and positive (5% FBS + ECGS) controls. **: Statistic significance with respect to all drug groups, $p<0.01$.

2.3.3 Effect of Astragalus extract powder on cell growth

The cytotoxicity of *astragalus* extract powder was slightly higher than that of the *astragalus* injection, showing a moderate cytotoxicity in a wide range of dosage from 6 μg/ml to 1.6 mg/ml, and a high toxicity at 5 mg/ml. Interestingly, the cytotoxicity of *astragalus* extract powder was not proportional to drug concentration, showing a HUVEC activity similar to that of the blank control at 2.5 mg/ml and 1.3 mg/ml.



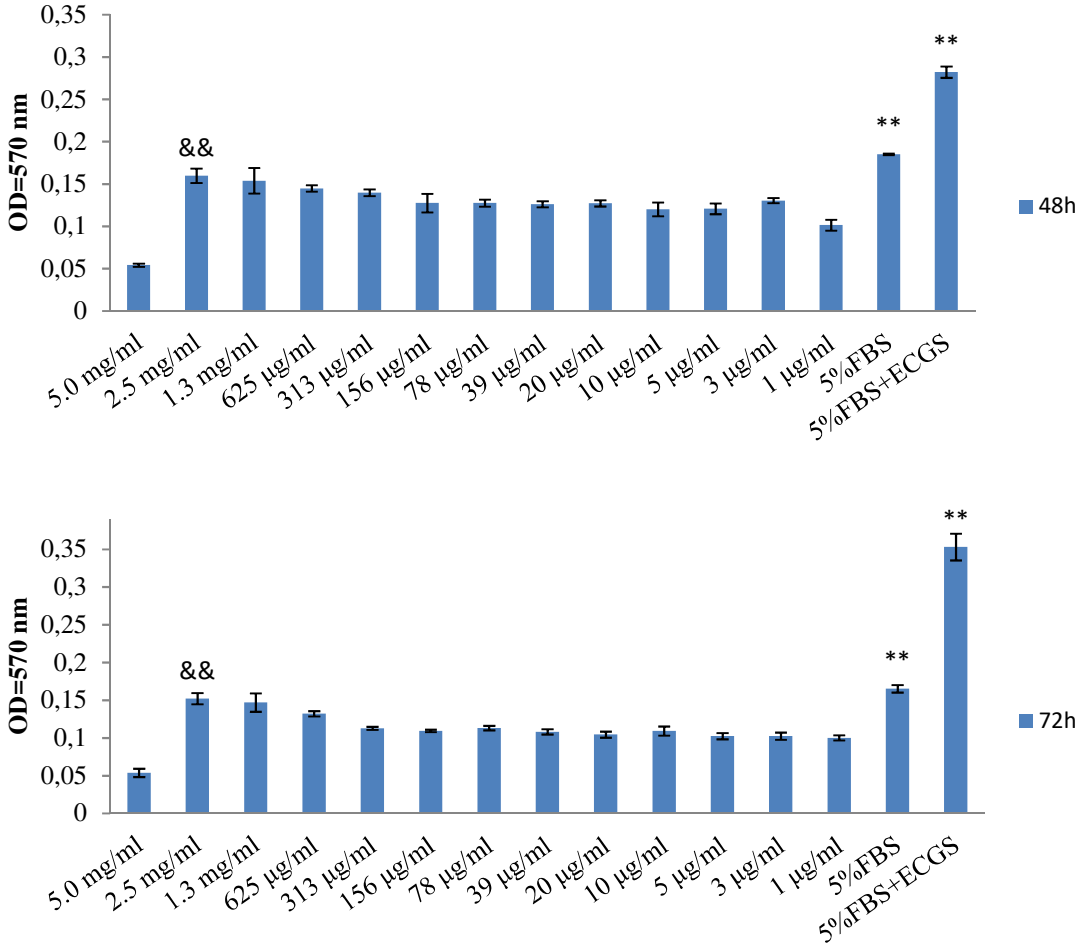
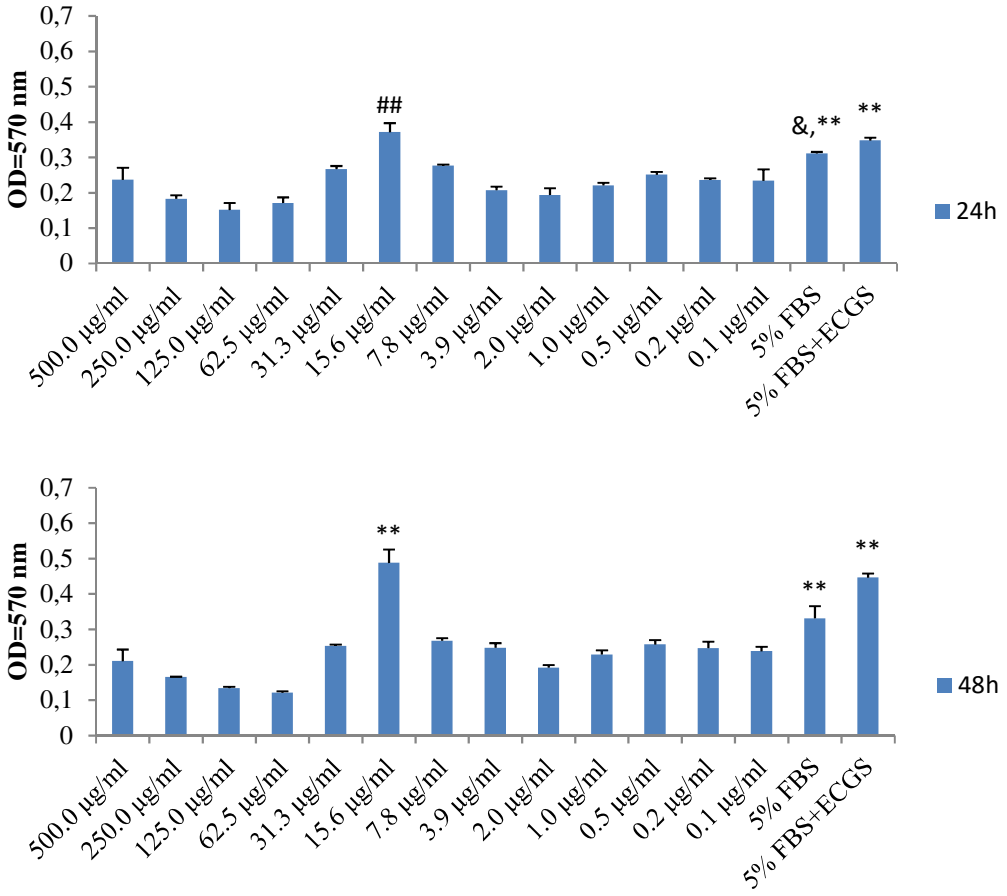


Figure 2.3 HUVEC viability after 24, 48 and 72 hours of culture in different concentration of *Astragalus* extract powder, in comparison with blank (5% FBS) and positive controls (5% FBS + ECGS). **: Significant difference with respect to all drug groups, $p<0.01$. &&: Significant difference with respect to all drug groups except 1.3 mg/ml, $p<0.01$.

2.3.4 Effect of proanthocyanidin on cell growth at low and high FBS levels

At 5% FBS proanthocyanidin presented cytotoxicity in a wide range of dose from 1.0 to 500.0 μg/ml, except at about 16 μg/ml (Fig. 2.4). At this particular dose, the optical absorption was similar to or even higher than that of the positive control. Both positive and blank controls were normal in optical reading, which were significantly higher than the rest of the drug groups at all three time points ($p<0.05$). To verify this extraordinary finding additional experiment was performed at 15% FBS, which again demonstrated the similar phenomenon except for a shift of the effective dose from 15.6 μg/ml to 31.3 μg/ml and 62.5

μg/ml, as showed in Figure 2.5. In addition, all doses below 31.3 μg/ml exhibited cell metabolic activities similar to that of the blank control, without apparent cytotoxicity. High cytotoxicity appeared at 125 μg/ml and above. Figures 2.6 and 2.7 are the line plots of cell viability as a function of time, showing cell proliferation in 3 days. It is clear that at the specific doses mentioned above cells proliferated as well as the positive controls at low FBS, and performed better at high FBS. It also appeared that cells became more tolerable towards proanthocyanidin at high FBS.



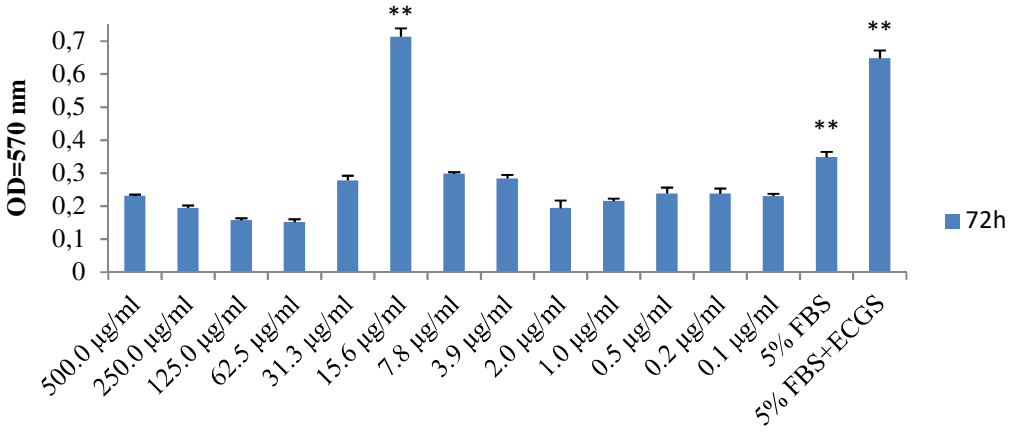
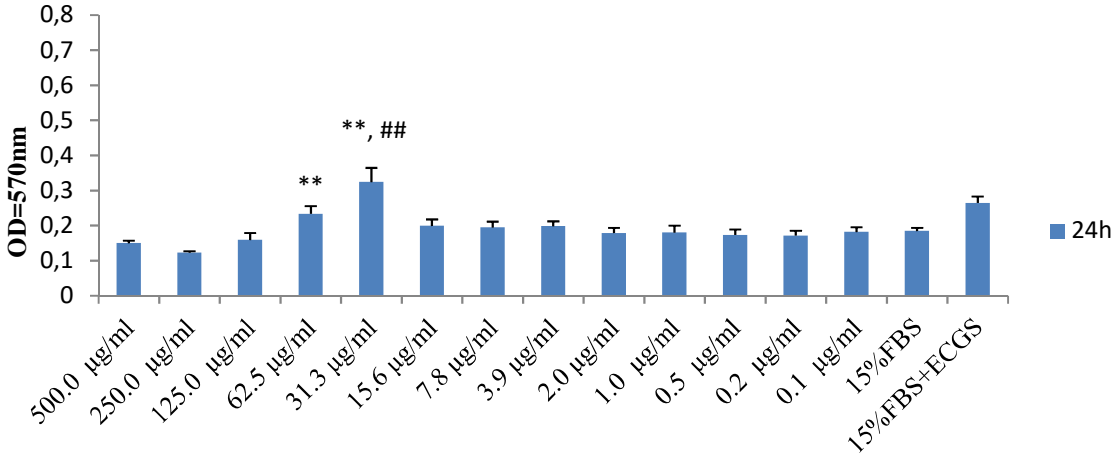


Figure 2.4 HUVEC viability after 24, 48 and 72 hours of culture in different concentrations of proanthocyanidin, in comparison with positive controls (5% FBS + ECGS). 24 h: ** Significant difference with respect to all drug groups except 15.6 µg/ml, $p<0.01$. & Significant difference with respect to 7.8 µg/ml group, $p<0.05$. ## Significant difference with respect to other groups except the positive control, $p<0.01$. 48 h & 72 h: ** Significant difference with respect to other drug groups, $p<0.01$.



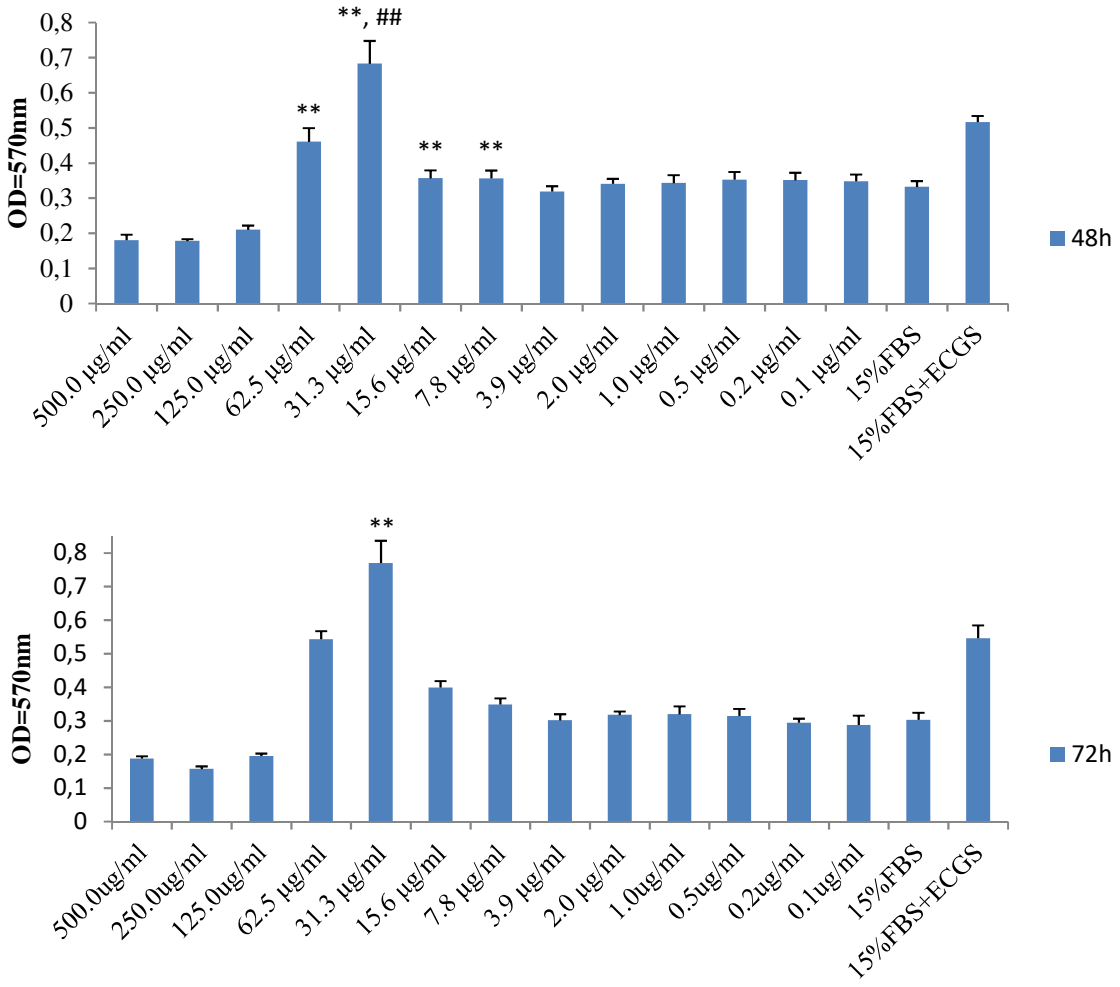


Figure 2.5 HUVEC viability after 24, 48 and 72 hours of culture in different concentrations of proanthocyanidin, in comparison with blank (15% FBS) and positive controls (15% FBS + ECGS). ** Significant difference with respect to other groups, $p<0.01$. && Significant difference with respect to other groups except 31.3 µg/ml, $p<0.01$. ##: Significant difference with respect to other drug groups except 31.3 µg/ml, $p<0.01$.

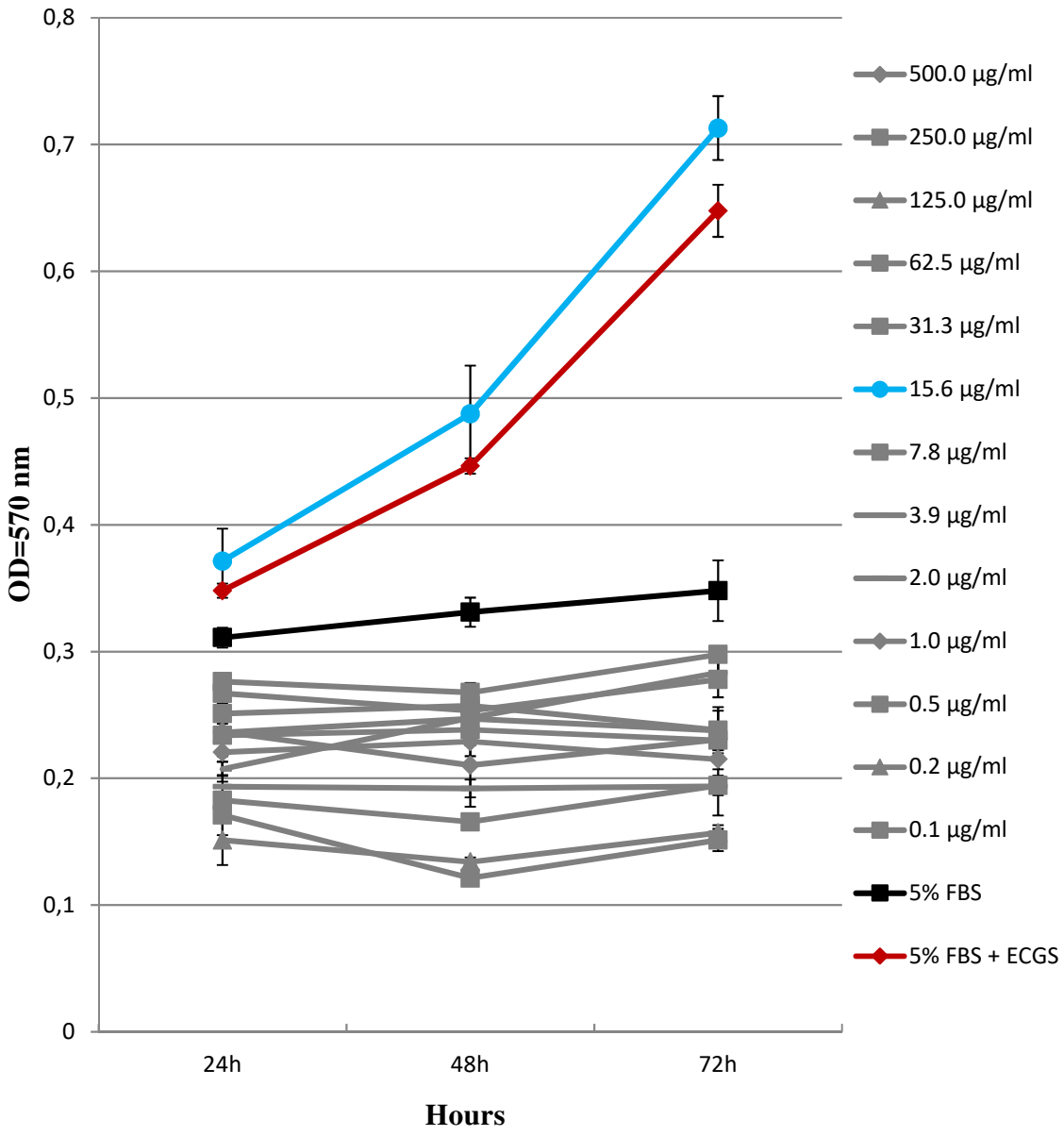


Figure 2.6 HUVEC proliferation in different concentrations of proanthocyanidin at 5% FBS, in comparison with blank and positive controls.

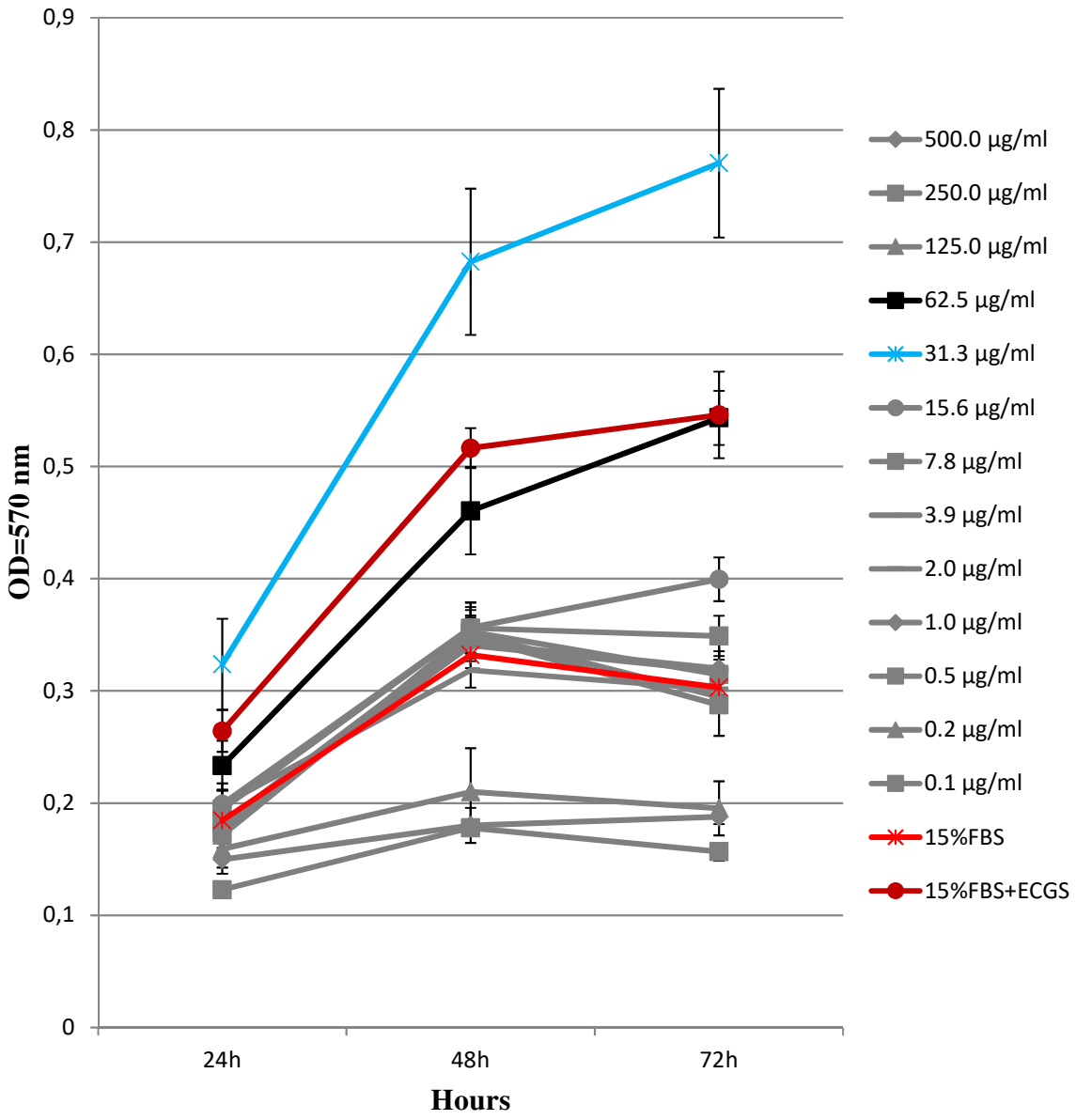


Figure 2.7 HUVEC proliferation in different concentrations of proanthocyanidin at 15% FBS, in comparison with blank and positive controls.

Figure 2.8 and 2.9 present the number and Hoechst stain of the cells cultured for 3 days in the presence of 31.3 µg/ml proanthocyanidin and 15% of FBS. The cell density in drug groups appeared higher than that of the blank control but lower than that of the positive control.

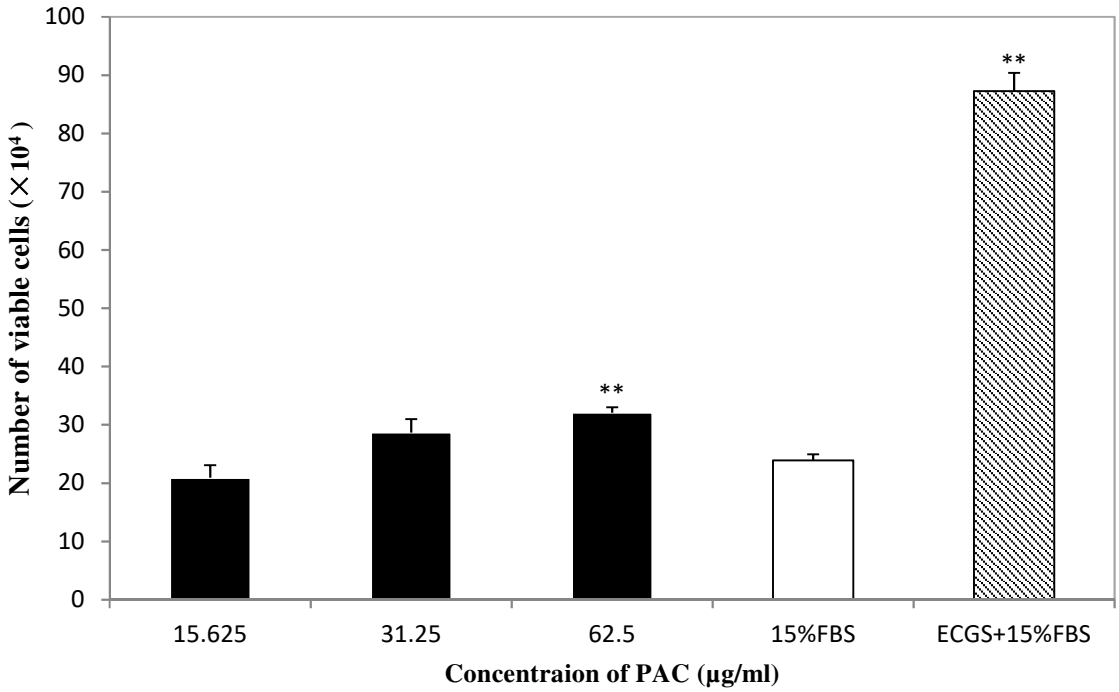


Figure 2.8 Number of viable cells after 3 days of culture in the presence of proanthocyanidin and 15% of FBS in comparison with blank and positive controls. ** Significant difference, $p < 0.01$.

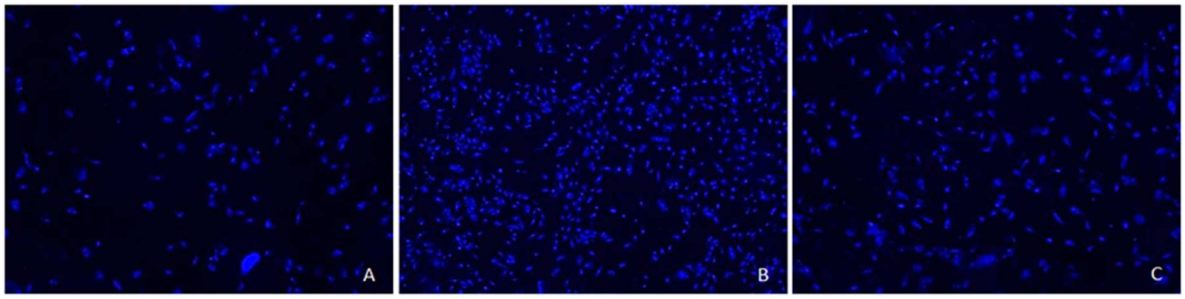


Figure 2.9 Hoechst stained HUVECs after 72 hours culture. A: Blank control (15% FBS); B: Positive control (15% FBS, ECGS); C: Proanthocyanidin (31.3 μg/ml), x100.

2.3.5 vWF expression

The following Figure 2.10 shows the results of immunocytochemical staining of HUVEC after being incubated with 31.3 μg/ml of proanthocyanidin and 15% FBS for 72 h. vWF was detected. This confirmed that the treatment with proanthocyanidin didn't affect

the production of vWF that is characteristic of endothelial cells. A higher number of cells in drug group was also noticed, indicating a better proliferation of HUVECs.

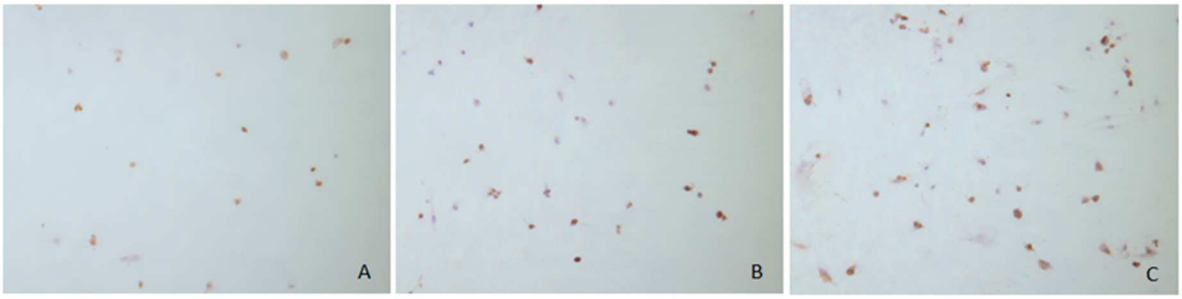


Figure 2.10 Positive vWF stain showed that the treatment of proanthocyanidin didn't affect the expression of this characteristic factor by endothelial cells. A: Blank control, B: Positive control, C: Proanthocyanidin (31.3 μg/ml). Cells were cultured for 72 hours in medium supplemented with 15% FBS, x 100.

2.4 Discussion

Vascular endothelial cells play critical roles in tissue regeneration and treatment of ischemic diseases. To maintain the normal function and the growth of endothelial cells in vitro, growth factors such as VEGF, EGF, bFGF or tissue preparations are required. These growth factors are also widely used in tissue engineered products to induce angiogenesis in vivo. However, protein products are expensive and their activities are sensitive to environment and processing. This often means a short shelf life and a quick loss of activity once used in vivo. Therefore there would be many advantages if botanic drugs of same or similar effect could be identified as substitutes. Compared with growth factors, botanic drugs can be easily resourced, inexpensively processed, and transported and stored with much less effort. They are also much less vulnerable to the hostile hydrolytic enzymes that often present in high concentration in an inflammatory environment such as a healing wound or implanted scaffold.

The major discovery of this study is the effectiveness of proanthocyanidin in maintaining endothelial cell culture or stimulating cell metabolic activity/proliferation at both low and normal serum levels without adding growth factors. The significance is the

possibility of using proanthocyanidin to substitute VEGF, at least to support a short period of culture, e.g., up to 3 days. Cell biologists may find this useful if they want to maintain endothelial cells in a growth factor depleted environment or under serum starving conditions. What could be important to biomaterial researchers is the potential of using proanthocyanidin as an angiogenic reagent to create new vasculature in tissue engineered products. What we can expect immediately is that proanthocyanidin is much cheaper and stable than growth factors and should be easily loaded into carriers.

There is a long history of research on proanthocyanidins, with most of the literature focusing on their antioxidative properties (130). As a naturally occurred antioxidant, they can be easily found in daily consumptions, such as in red win and some dark fruits, especially in the pericarps of the fruits in dark color. There are also many reports about proanthocyanidins and endothelial cells or angiogenesis. However, most of these studies reported the suppression of endothelial cell activity and growth (131,132), which are in line with our data at doses higher than 63 μg/ml. For example, Kim et al. reported reduced viability of HUVECs treated with proanthocyanidins isolated from cranberry in the range of 12.5 to 100 μg/ml (133). They also found that proanthocyanidin increased the intracellular level of reactive oxygen species and caused SKOV-3 cells (human ovarian adenocarcinoma) apoptosis. Another research used the proanthocyanidin from the same supplier as in this work reported an increasingly reduced viability of human microvascular endothelial cells after 72 hours treatment in 50 μg/ml or higher concentrations of the drug (134). They only tested one dose below 50 μg/ml, which was 20 μg/ml, and didn't find any cytotoxicity. Procyanidin B2 was also reported to reduce HUVEC viability (135). In fact there are only a few research reporting that proanthocyanidins protect endothelium or endothelial cells. Lu et al. used indoxyl sulfate to induce oxidative injury to HUVECs and found that proanthocyanidin in the range of 2 to 10 μM protected cells by reducing the level of reactive oxygen species (136). Proanthocyanidin was also found to inhibit PCK activation and so prevent high glucose induced ICAM-1 and VCAM-1 and endothelium dysfunction (137). The stimulatory effect of proanthocyanidin on cell growth was found on human gastric mucosal cells and murine macrophage cells (138) and on human skin keratinocytes (139). However, there has been no report about the upregulation of endothelial cell

viability/proliferation by proanthocyanidin, not even mentioning the effect as potent as VEGF or endothelial cell growth supplements. The discrepancy between our data and what reported in the literature is likely because of the dosage of the drug. In fact, our data revealed that proanthocyanidin has three zones of action to HUVECs: down-regulation of viability or cytotoxicity at 125 μg/ml or above, up-regulation of viability/proliferation between 15 to 63 μg/ml, and ineffectiveness below 8 μg/ml.

The effective component(s) in the proanthocyanidin used in this work is not clear. Proanthocyanidin obtained commercially often contains different chemical compositions, e.g., different ratio of oligomers. Different sources and even batch of extractions also contribute to variations. One may notice from the above data that the promotive effect of proanthocyanidin on HUVECs was sometimes less effective compared with ECGS, likely reflecting the variations between batches of the drugs. Therefore significant effort is required to identify the effective component(s).

Different from what was originally expected, the other three preparations failed to show any beneficiary effect on cell growth even though they were found either only slightly cytotoxic or no cytotoxicity to endothelial cells when used at an appropriate dose.

2.5 Conclusion

The proanthocyanidin tested in this work was found capable of maintaining and promoting endothelial cell growth under low and normal serum conditions in the absence of growth factor supplement. This effect on HUVECs proliferation is as potent as the commercially available endothelial cells growth supplements. The endothelial cells treated by proanthocyanidin keep the expression of the characteristic vWF. Therefore this work suggests the potential use of proanthocyanidin in endothelial cell culture and angiogenesis. Batch variations were noticed among proanthocyanidins from the same supplier. On the other hand, the three other botanic drugs selected, *Astragalus* injection, puerarin injection and *Astragalus* extract powder failed to show any promotive effect on HUVEC viability and proliferation.

Chapter 3

Mechanistic study of the promotive effect of proanthocyanidin on HUVEC proliferation

3.1 Introduction

Vascular ECs play a critical role in promoting angiogenesis. Regulation of the survival of ECs therefore becomes a central issue in tissue engineering (pro-angiogenesis) and cancer treatment (anti-angiogenesis). The survival of ECs depends on growth factors such as VEGFs, bFGF and angiopoietin-1 (140), among them VEGFs are the most frequently used and most potent promotive growth factors. There are three VEGF tyrosine kinase receptors that have been identified, of which the vascular endothelial growth factor receptor 2 (VEGFR-2) appears to be the most important active receptor during angiogenesis (141). In addition to VEGFs, EGF also induces EC proliferation through membrane receptor EGFR (142). In the previous chapter, proanthocyanidin showed the capacity to maintain EC survival/proliferation in vitro. However the mechanism of such actions is unknown. In order to investigate the possible mechanism of how proanthocyanidin promotes EC survival and proliferation, it was hypothesized that proanthocyanidin regulates EC through VEGFR and EGFR. This chapter is to test this hypothesis by blocking these two membrane receptors.

Proanthocyanidins are polymers of various flavan-3-ol derivatives containing different forms of procyanidins. Since the exact composition of the proanthocyanidin used in this work was unknown at the time of the work, it was decided to compare it with two important procyanidins available from Sigma-Aldrich, namely procyanidin B1 and procyanidin B2. Procyanidin B types are more abundant than A types in proanthocyanidins.

3.2 Materials and Methods

3.2.1 Materials

Human primary umbilical vein endothelial cells line (PCS-100-013) (HUVECs) was purchased from ATCC (Burlington, Ontario, Canada); VEGFR2 inhibitor vandetanib, VEGFR inhibitor axitinib and EGFR inhibitor PD168393 were purchased from APExBIO (Houston, MA, USA). Procyanidin B1, procyanidin B2, culture medium 199, endothelial cell growth supplement (ECGS), heparin, 3-(4,5-dimethyl thiazole-2)-2,5-diphenyl thiazolyl blue (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Company (Oakville, Ontario, Canada). Fetal bovine serum was order from Fisher

Scientifique (Saint-Laurent, Quebec, Canada). Penicillin-streptomycin, L-glutamine and trypsin were ordered from Gibco (Chelmsford, MA, USA).

3.2.2 Methods

3.2.2.1 Inhibition of VEGF and EGFR

HUVECs were suspended in culture medium and seeded on 1% gelatin-coated 24-well plate at a concentration of 1×10^4 cells/well in 1ml of complete medium. After 6 h the cells were refreshed of medium to remove the non-adhesive cells and kept incubated in 5% CO_2 at 37°C overnight. To test the role of membrane receptors, cultures were divided into the following groups: vandetanib + VEGF, vandetanib + proanthocyanidin, axitinib + VEGF, axitinib + proanthocyanidin, proanthocyanidin, blank control and positive controls (ECGS, VEGF). FBS was 15% in all groups. Vandetanib and axitinib were added into cell culture medium at 10 μmol/L for 45 min and 30 min, respectively (140-142). EGFR inhibitor was added into cell culture medium at 10 μmol/ml and incubated for 30 min (143). After inhibition to the receptors, the media were replaced with test and treatment media containing 15% FBS. VEGF, proanthocyanidin and EGF were added at the final concentrations of 20 ng/ml, 31.3 μg/ml and 5 ng/ml, respectively. Positive controls contain either 5 ng/ml VEGF or 25 μg/ml ECGS. All tests were in triplicate.

3.2.2.2 Test of procyanidins B1 and B2

Procyanidins B1 and B2 were tested using the same procedure as that used for proanthocyanidin test. Dose effect was performed by choosing a range of concentrations that were found optimal for proanthocyanidin, which was 100, 50, 25, 12.5 and 6.3 μg/ml.

3.2.2.3 MTT test

After 72 h of culture, the test medium in the wells was removed and replaced with 1.0 ml fresh complete medium containing 15% FBS, to avoid the potential effect of the drugs in MTT test. Then, 100 μl of 5 mg/ml MTT was added to each well of the plate. After being incubated for 4 h at 37 ℃, the medium was carefully removed with a pipette and 1.0 ml DMSO was added to each well to dissolve the formazan crystals. Then, 100 μl of the solution was collected in triplicate from each well and transferred to a μQuant microplate

reader (μQuant Bio-Tek Company, Winooski, VT, USA) for absorbance measurement at 570 nm.

3.2.2.4 Statistical analysis

Data were expressed as mean ± S.D. A Student t-test was used to determine the statistical difference between control and test groups. A $p<0.05$ was considered statistically significant and $p<0.01$ was considered statistically highly significant.

3.3 Results

3.3.1 Effect of VEGFR inhibitors

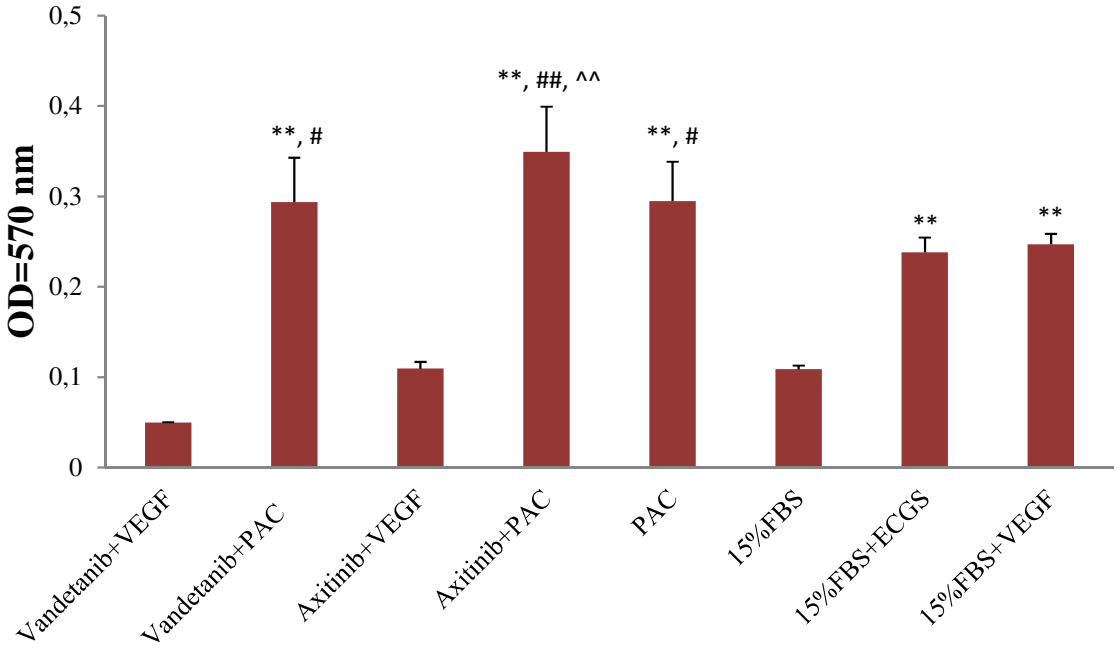


Figure 3.1 The effect of VEGFR inhibitors on HUVECs proliferation after 72 h culture in the presence of proanthocyanidin (PAC, 31.3 μg/ml) and 15% FBS, in comparison with blank (15% FBS) and positive controls (15% FBS + VEGF or ECGS). ** Significant difference compared with the blocker treated and blank control, $p < 0.01$; # Significant difference compared with 15% FBS + ECGS, $p < 0.05$; ## Significant difference compared with 15% FBS + ECGS, $p < 0.01$; ^^ Significant difference compared with positive controls, $p < 0.01$.

Figure 3.1 shows that both VEGFR blockers, vandetanib and axitinib, effectively suppressed the viability of the HUVECs. To be more specific, the viability of the group treated with axitinib was reduced to approximately the same level of the 15% FBS group (negative control); and the vandetanib group was reduced to an even lower viability. At the same time the viability of the positive controls, those supplemented with either VEGF or ECGS, was normal and more than double of the negative control. Since the only difference between the negative and positive controls is the supplementary growth factors, these data validated the necessity of the growth factors to HUVEC proliferation and the effectiveness of the two blockers. Surprisingly, the positive effect of proanthocyanidin on HUVEC proliferation was still significant: the three groups supplemented with proanthocyanidin recorded the highest viability among all groups regardless the presence of either type of VEGFR blockers. No significant difference was found among the three drug groups. Qualitative observation of cell density under optical microscope supports MTT data.

3.3.2 Effect of EGFR inhibitor

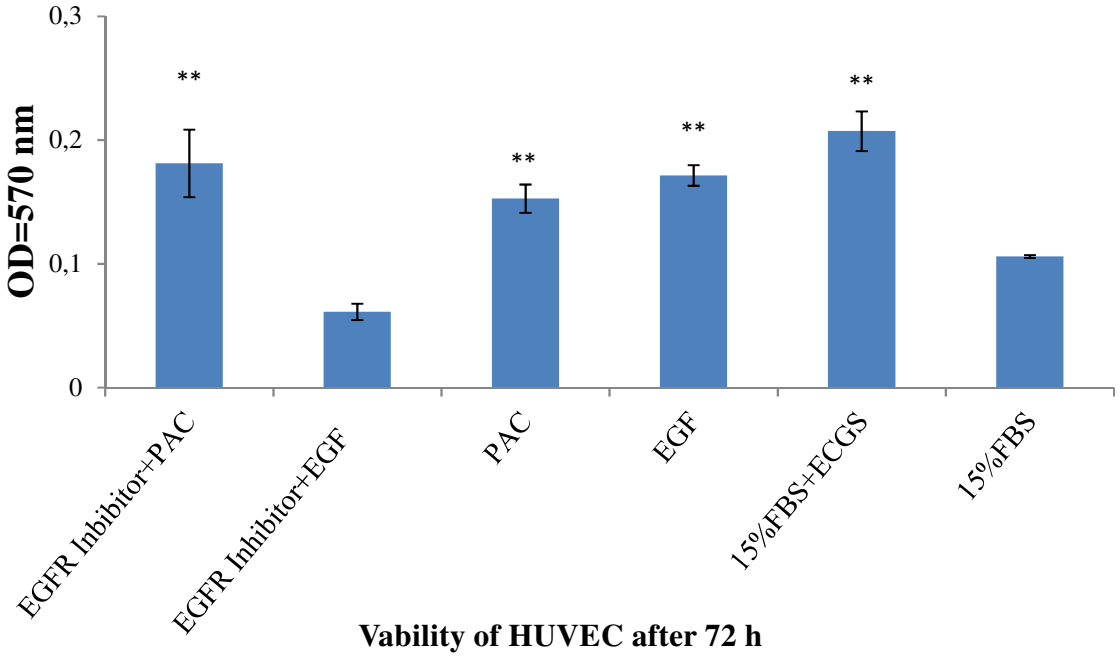


Figure 3.2 The effect of EGFR inhibitor on HUVECs proliferation after 72 h culture in the presence of proanthocyanidin (PAC, 31.3 μg/ml) and 15% FBS, in comparison with blank

(15% FBS) and positive controls (15% FBS + ECGS or EGF).** Significant difference compared with EGF blocker + EGF and blank control (15% FBS).

Figure 3.2 shows the effect of EGF inhibitor on HUVEC viability in the presence or absence of 31.3 μg/ml proanthocyanidin. The results show that the blockers successfully suppressed EGF receptors and inhibited the effect of EGF on cell proliferation. The number of viable cells in the presence of EGF blockers was lower than that of the black control (15% FBS) and was only about one third of the positive control (15% FBS + ECGS). However, the growth of the cells in the presence of proanthocyanidin was similar to that of the positive controls (15% FBS + ECGS or EGF), with or without the presence of EGF blocker. Among the groups that had normal cell growth, the positive control and the group with proanthocyanidin plus EGF locker appeared superior to others.

3.3.3 Procyanidins B1 and B2

The effect of procyanidin B1 and B2 is presented in Figures 3.3 and 3.4.

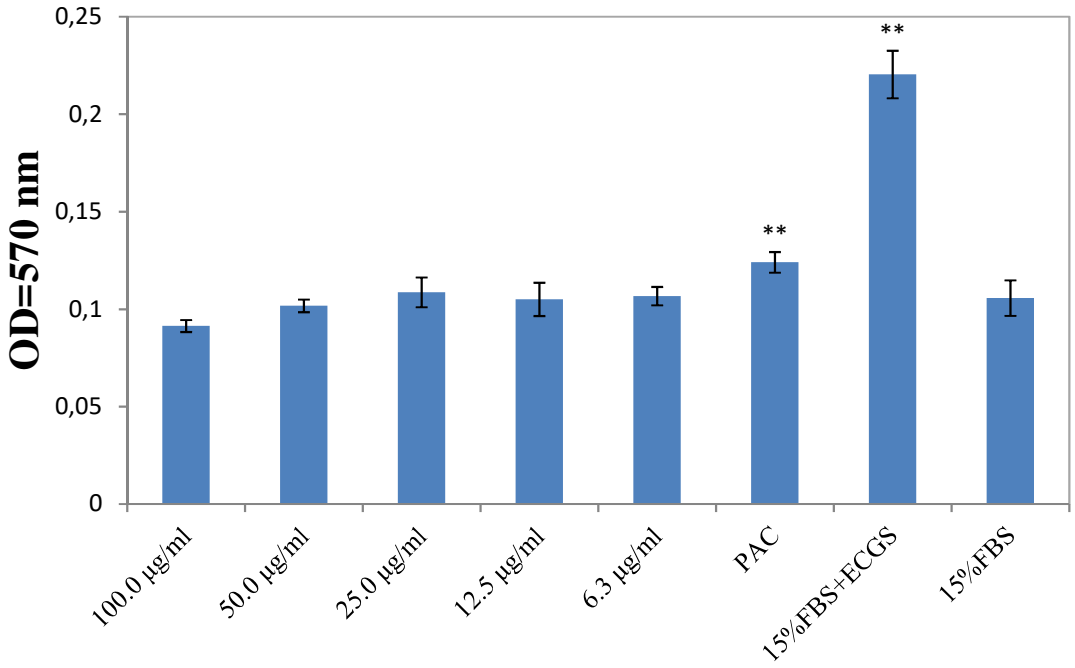


Figure 3.3 Effect of procyanidin B1 on HUVEC proliferation after a 72 h culture in 15% FBS, in comparison with positive drug control (PAC: 31.3 μg/ml proanthocyanidin),

positive control (15% FBS + ECGS), and blank control (15% FBS). ** Significant difference with respect to other groups, $p < 0.01$.

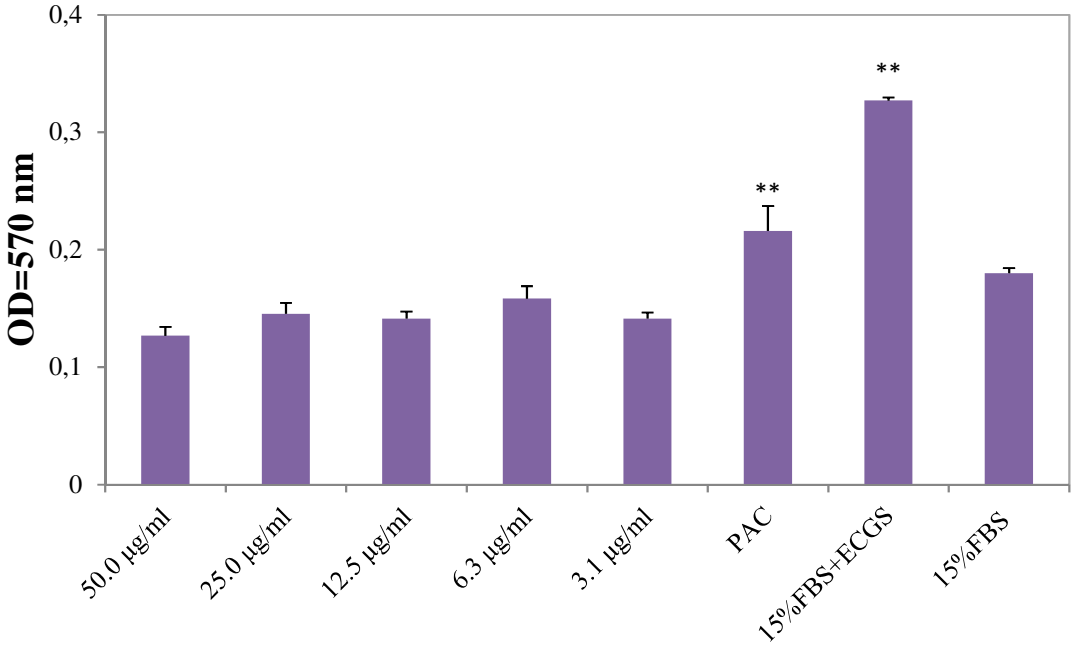


Figure 3.4 Effect of procyanidin B2 on HUVEC proliferation after a 72 h culture in 15% FBS, in comparison with positive drug control (PAC: 31.3 µg/ml proanthocyanidin), positive control (15% FBS + ECGS), and blank control (15% FBS). ** Significant difference with respect to other groups, $p < 0.01$.

The results show that both B1and B2 didn't have any positive effect on HUVEC proliferation. In fact, in all the doses tested, from 6 to 100 µg/ml, the viability of the HUVECs in the presence of procyanidins was similar or appeared lower than the negative control group. The difference within the drug groups was small and mostly insignificant. It was noticed that the effect of proanthocyanidin, used positive drug control, was not as strong as in other tests, showing the variation in a different batch of proanthocyanidin.

3.4 Discussion

The hypothesis of this chapter is that the stimulatory effect of proanthocyanidin to the growth of HUVECs is mediated by VEGFR and EGFR. This hypothesis was made because that the addition of proanthocyanidin compensated the negative effect of growth

factor depletion, and that ECGS is rich in VEGF and EGF. However, both VEGFR and EGFR blockers failed to inhibit the stimulatory effect of proanthocyanidin on HUVECs. Vandetanib is a potent tyrosine kinase inhibitor known to block VEGFR2, VEGFR3 and EGFR at the concentration used in this work (144,145). Axitinib on the other hand blocks multiple VEGF receptors including 1 to 3 and platelet-derived growth factor receptor beta (PDGFR-β). PD168393 selectively blocks EGFR. In fact, the data of this experiment also support the efficacy of these inhibitors by showing that the three inhibitors significantly suppressed cell growth. Consequently, the data demonstrate that the promotive effect of proanthocyanidin on HUVEC proliferation is mediated through mechanisms not related with VEGFR, EGFR and PDGFR-β. Obviously, additional work is warranted to identify the pathways through which proanthocyanidins act on HUVECs.

While there have been extensive studies about the biological functions of proanthocyanidins in literature, the mechanistic study about how proanthocyanidins interact with human endothelial cells remains very limited. A Medline search with the following combination (proanthocyanidin AND endothelial cell AND (mechani* OR signalling OR pathway OR cascade)) on December 22, 2017 returned only 19 publications. Among them only 9 are relevant research articles. Among those 9 publications, 3 focused on the antioxidant and anti-inflammatory property of the proanthocyanidins (146-148), 4 studied the production of nitric oxide and effect on hypertension (149-152), and 2 investigated the inhibitory effect of proanthocyanidins on endothelial cell migration and viability (153,154). The focuses of these mechanistic studies are in fact in agreement with most of the reports in literature about proanthocyanidins, which have been about anti-oxidation, anti-inflammation, anti-angiogenesis, regulation of vascular permeability and functions, prevention of damage caused by stress, etc. These properties are largely related with the free radical scavenging property of proanthocyanidins, particularly those extracted from grape seeds. However, the anti-oxidative and free radical scavenging properties of proanthocyanidins are apparently not relevant to the discovery in this work. The HUVECs used in this work were normal cells not challenged by any stress factor. The positive effect of the drugs on HUVEC proliferation was found in both low (5%) and normal (15%) serum levels, meaning an up-regulation of normal endothelial cell proliferation instead of any

protection from stress factors. The molecular mechanisms reported in literature were mostly about cancer cells or cells under the challenge of stress factors such as hydrogen peroxide, which was unlikely to explain the findings in this work.

The biological functions and therapeutic potential of proanthocyanidins have been extensively studied and reviewed (155-157). However, in all these works there are only very limited reports on the promotive capacity of proanthocyanidins to the growth of normal cells. Proanthocyanidins prepared from grape seeds were reportedly promoted the growth of mouse hair follicle cells; and they were found most effective as oligomers than monomers in a concentration of 3 μM in a 5 day culture (158,159). Ye et al. reported a specific type of grape seed proanthocyanidins extract IH636 that promoted the growth of human gastric mucosal cells and murine macrophages in 72 h while suppressing the growth of several types of cancer cells (138). The concentration of the drug in the experiment was 25 and 50 mg/lit (20 and 50 μg/ml), which is similar to the optimal dosages in this work. IH636 was found to increase the expression of an anti-apoptotic gene bcl-XL (160). Another report by Deters et al. looked at the effect of the proanthocyanidins extracted from the bark of *Hamamelis virginiana* (161). They found that two fractions of the proanthocyanidins, the fraction B of 1500 Da and fraction C of 3400 Da, significantly stimulated the proliferation of human skin keratinocytes. The effective dose of the fraction B is 1 and 10 μg/ml and that of the fraction C is 10μg/ml. These dosages are lower than the effective dose used in this work. Nevertheless these dosages are in the same order as what we identified. On the other hand, the vast majority of the research are about the suppression of cancer cell growth and cell apoptosis. This work therefore is the first report on the stimulatory effect of proanthocyanidins on human endothelial cells.

Proanthocyanidins are oligomers of catechins and epicatechins with variations in structure and polydispersity (meaning monomer vs. dimer vs. trimer, etc). Among the components, procyanidin B1 and B2 were proven not the active ingredient. Other constituent components available in the market need to be tested as well. There is also the possibility that there is no any single active component but the synergy of more than one components in this particular type of proanthocyanidins. Further investigation is needed.

3.5 Conclusion

This experiment failed to support the hypothesis that proanthocyanidin act on HUVECs through VEGFR and EGFR mediated pathways. PDGFR-β is also likely unrelated. Procyanidins B1 and B2 are excluded from the active component. Further investigation is necessary to study the pathway and active constituent(s).

Chapter 4

Proanthocyanidin promotes angiogenesis in chick chorioallantoic membrane model

4.1 Introduction

Angiogenesis is a process of generating new capillaries from existing blood vessels. This process is highly indicated in wound healing to supply blood cells to the battlefield of inflammation and to nourish the regenerated tissues. On the other hand, angiogenesis also plays a key role in tumor growth, stimulated by the mediators secreted by tumor and inflammatory cells. Proanthocyanidins are reported anti-angiogenic. In a review article of 2006, proanthocyanidins particularly that from grape extract were listed as an inhibitor to endothelial cell proliferation and as anti-angiogenesis (162). A mechanistic study showed that procyanidins of 3.9 in mean degree of polymerization inhibited endothelial cell proliferation and migration by modulating eight genes important to angiogenesis (163). Interestingly, (-)-epicatechin and procyanidin B2 were found not effective. Another work studied cinnamon extract and identified procyanidins of trimer and tetramer as the active components that inhibit endothelial cell proliferation through reducing the activity of VEFGR2 (164). This reduced VEGFR2 activity was confirmed later with oligomeric proanthocyanidins (165), showing dose effect on reducing endothelial cell proliferation. The authors also suggested that proanthocyanidins exhibited cytotoxicity by inducing elevated intracellular reactive oxygen species and modulating AKT pathway. Similarly, a specific proanthocyanidin (2S)-4',5,7-trihydroxyflavan-(4 β →8)-afzelechin was identified with potent inhibitory effect on endothelial cell growth in vitro and on angiogenesis in a chick embryo chorioallantoic membrane (CAM) assay (166). Another recent work also identified a new type of proanthocyanidin GC-(4→8)-GCG that inhibited the migration and tubule formation of endothelial cells in a dose dependent fashion, and reduced angiogenesis in vivo. They found that such anti-angiogenic property was related with the ability of the drug to reduce the phosphorylation of ERK, p38 and Akt (129). Most recently, two research groups reported the inhibitory ability of two different types of procyanidins on endothelial cell growth, both linked to the modulation of VEGFR2 activity (135,167).

In contrary to literature, the in vitro data of this thesis demonstrated that the proanthocyanidin investigated in this work supported endothelial cell growth at both low and normal serum levels, suggesting that proanthocyanidins are potentially pro-angiogenic. This chapter is therefore to test this hypothesis.

Because of its rapid development of extensive vasculature, easy accessibility and visibility, chick embryo chorioallantoic membrane (CAM) is considered an in vivo model of angiogenesis and has been broadly used to investigate the efficacy and mechanisms of a variety of drugs (168). This experiment was designed to study the effect of proanthocyanidin on angiogenesis using CAM as an in vivo angiogenesis model.

4.2 Materials and Methods

4.2.1 Materials

Proanthocyanidine was purchased from Jianfeng Natural Product R&D, (Tianjin, China). Day-0 fertilized chicken eggs were provided by Couvoir de la Coop Fédérée, (Victoriaville, Quebec, Canada). The filter paper of median pore size (Catalog 28320-041) was purchased from VWR (Ville Mont-Royal, Quebec, Canada). VEGF was purchased from ATCC (PCS-100-041™).

4.2.2 Methods

The filter paper was cut into circular form of 6 mm in diameter and sterilized by autoclaving. They were used as drug carriers and implanted on top of CAM.

Proanthocyanidin solutions were freshly prepared before implantation by dissolving the drug in PBS at concentration of 31.3, 62.5 and 125.0 μg/ml. Ten microliters (10 μl) of the dissolved drug solutions were added to the filter paper per implant. These dosages were found to promote endothelial cell growth in vitro. Angiogenic molecule VEGF was also prepared in PBS at 20 ng per 10 μl per implant, which was used as the positive control.

4.2.2.1 Establish of CAM model

All the eggs were cleaned with 70% ethanol and incubated for 7 days in a Pro-FI egg incubator fitted with an automatic egg turner before being transferred to a Roll-X static incubator (Lyon Electric, San Diego, CA, USA) for the rest of the incubation time. The eggs were kept at 37°C in a 60% humidity atmosphere for the entire incubation period. On the day 7 of incubation, the eggs were transferred to a laminar-flow hood under sterile

conditions, where a small hole was drilled (Dremel hobby drill, model: RTD35ACL) at the shell concealing the air sac and a second hole was drilled on the broad side of the egg, the place of which should be kept away from the main visible blood vessels. A negative pressure was applied to the second hole to create a false air sac directly over the CAM, allowing its dissociation from the egg shell membrane. Then, a square window (approximately 10 × 10 mm) was created on the shell over the dropped CAM with the aid of a drill and dissecting scissors to reveal the underlying embryo and CAM vessels. The windows were covered with transparent adhesive tapes to prevent contamination. The eggs were randomly divided into 5 groups comprising 3 drug groups and two control groups, with 10 egges in each group. A sterilized filter paper of 6 mm in diameter was implanted onto the freshly exposed CAM tissue through the window. Ten microliters (10 µl) of freshly prepared PBS solution containing 31.3, 62.5 or 125 µg of proanthocyanidin was pipped onto the implanted paper. Twenty nanograms (20 ng) VEGF in 10 µl of PBS was used as positive control while the same volume of PBS as the negative control. After the windows being sealed, the eggs were returned to incubator and incubated for additional 3 days. On day 10, all the embryos were harvested by placing at 4°C to decapitate. The CAMs exposed at the air sac were collected and pictures were taken at the same magnification.

4.2.2.2 Evaluation of the angiogenic response

The angiogenic response was evaluated by analyzing the numbers of branching blood vessels to generate a semi-quantitative vascular index. To do this, a 1 cm diameter ring was drawn and superposed on the image of CAM with the implant in the middle. All the vessels that converged toward the implant were enumerated. This ring was drawn around the implant in such a way that it forms an angle of less than 45° with respect to the edge of the implant, as illustrated in Figure 4.1. Vessels that branch dichotomously outside the ring were not counted. Only those that branched inside the ring or not branched but formed an angle of less than 45° to the edge of the implant were scored.

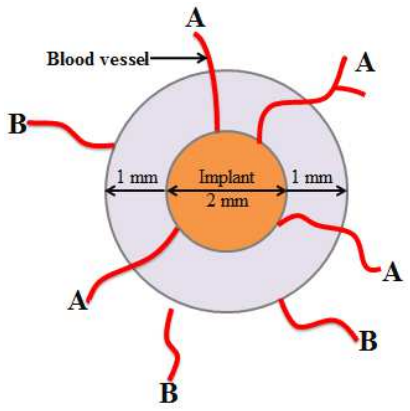


Figure 4.1 Evaluation of a proangiogenic response by macroscopic vessel branching. The drawing illustrates representative examples of different branching responses.

These evaluations were carried out on all explants harvested at day 10 of incubation. The vascular index for individual specimen was defined as the sum of the scored blood vessels.

4.3 Results

Figure 4.2 shows the effect of proanthocyanidin on angiogenesis in comparison with positive and blank controls. The upper panel shows the representative images of CAM explants. One can easily identify the newly formed blood vessels either near or converged to the circular implant. Both drug and positive groups showed more vessels than blank control did. The number of blood vessels that converging towards implant was counted and presented in Figure 4.3. VEGF group has an average slightly above 30 vessels, in contrast to about 12 vessels of the blank control. All drug groups recorded more vessels than that of the blank control, with the 62.5 μg group reached an average of 26 vessels that are significantly more than the blank control and not significantly different from the VEGF group.

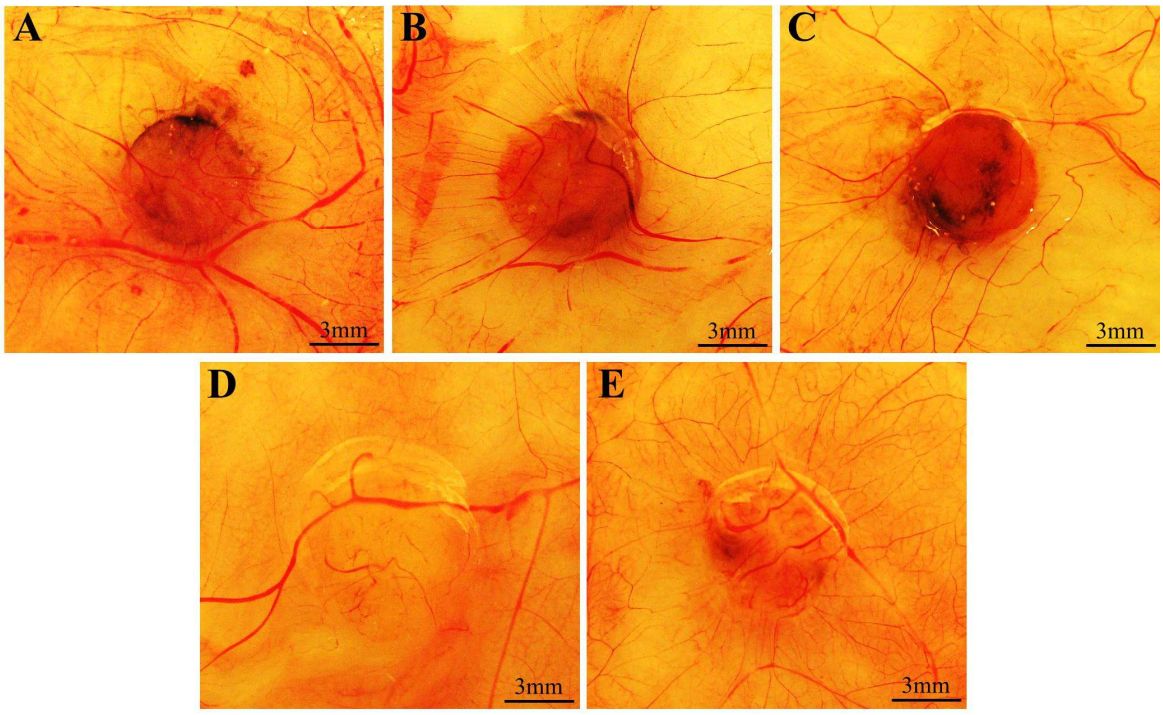


Figure 4.2 Effect of proanthocyanidine on CAM vascularization after implantation for 3 days. A: 31.3 µg/egg, B: 62.5 µg/egg, C: 125.0 µg/egg, D: PBS, 10 µl/egg, E: VEGF, 20 ng/egg.

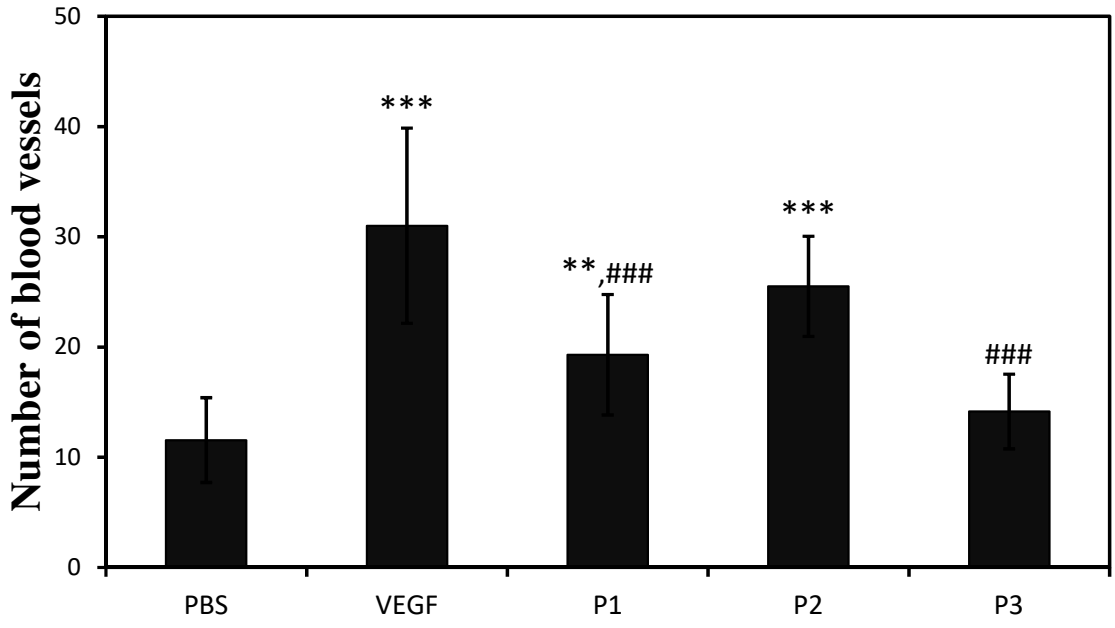


Figure 4.3 The numbers of vessel branch points at day 3 on CAM, showing the angiogenic effect of proanthocyanidin. P1: 31.3 µg/egg, P2: 62.5 µg/egg, P3: 125. µg/egg, **

Significant difference with respect to negative control (PBS), $P < 0.01$; *** Significant difference with respect to negative control (PBS), $P < 0.001$; ### Significant difference with respect to positive control (VEGF), $P < 0.001$.

4.4 Discussion

CAM is a proven model of in vivo angiogenesis, which has been used extensively in basic research (169) and in screening drugs (170,171). Compared with mammals such as mouse model CAM is less expensive, easy to operate, relatively fast and allowing direct observation in a well maintained system. Despite the fact that CAM has been widely used to study a variety of tumor cells and anti-tumor drugs, proanthocyanidins have rarely been tested in this model. In literature there is only one publication that used CAM to study the anti-angiogenic activity of proanthocyanidin (165). CAM model has not been reported to investigate the pro-angiogenicity of proanthocyanidins. In this work, it was found that proanthocyanidin can be easily loaded onto the filter paper that was used to confine and localize the diffusion of the drugs. The proanthocyanidins released from the filter paper into CAM remained bioactive as evidenced by the higher number of the newly formed blood vessels, showing that the filter paper as a substrate appeared not interfering with proanthocyanidins in terms of both activity and dosage. Indeed, for the first time, this study shows that proanthocyanidin has a significant pro-angiogenic activity in vivo, as suggested by the in vitro data showing the proliferative effect of the proanthocyanidin on endothelial cells. In scientific literature, proanthocyanidins are mostly reported as anti-angiogenic, anti-inflammatory, and suppressing tumor cell growth. Oligomeric proanthocyanidins (OPC) from various sources are marked as a dietary supplement with anti-oxidation function. Orally administrated procyanidin has been reported to appear as intact molecules in plasma and urine in pig model (172,173) as well as in human (174,175). The perceptions on the antiangiogenic roles of proanthocyanidins and their bioavailability through oral administration either as a dietary supplement or as a beverage should cause worrisome in populations where pro-angiogenesis is considered unfavorable, such as in cancer patients.

This experiment clearly showed the dose effect. Among the three dosages, i.e., ca. 31, 63 and 125 μg per implant, only the median recorded a similar number of blood vessels

as the positive control ($p \geqslant 0.05$). Nevertheless, other two doses were also found pro-angiogenic with respect to PBS control ($p<0.05$). One may notice that these dosages are slightly higher than the optimal concentration used in endothelial cell culture, which was between ca. 15 to 30 μg/ml. While drug concentration can be accurately controlled in cell culture medium, the exact concentration of the drugs in CAM tissue was unknown. Such concentration depends on how fast the drugs were diffused from the filter paper to the tissues, and the extent of diffusion, among other things. Even so, one may agree that an optimal dose likely exists to maximize the effect of proanthocyanidins on angiogenesis.

The analysis on angiogenesis was performed 3 days after implantation of the drug loaded filter paper. It is unknown whether the drugs were mostly released in the first few hours or stayed available during the entire period of 3 days. In either case the efficacy on angiogenesis remained evident 3 days following the first administration of the drugs. This lasting effect appears advantageous should the drugs be used in clinic, which would reduce the frequency of administration. Because of the non-protein nature, proanthocyanidins are expected less vulnerable to the hydrolytic and oxidative environment one may encounter in many pathologies, consequently being more stable.

Angiogenic therapy has great potential in treating ischemic diseases such as infarcted heart and lower extremity ischemia. Clinic trials include injection of bone marrow derived CD133+ progenitor cells (176), injection of endothelial growth factor encoding genes (177), and infusion of angiogenic growth factors such as basic fibroblasts growth factor (178). These remedies are complicated by their instability, short shelf life and high cost. It is very important to continue seeking pro-angiogenic drugs and methods that are safe, effective and easy to use. Proanthocyanidins are stable, inexpensive and generally considered safe. From this sense the clinic potential of the angiogenic functions of proanthocyanidins warrants further investigation.

CAM as an angiogenesis model has its limitations. The study period is short and the tissue reactions differ from that in immunocompetent mammals. The observations

identified in this study therefore must be validated in other models before the angiogenic function of proanthocyanidins could be ascertained.

4.5 Conclusion

The proanthocyanidins used in this study were found to promote angiogenesis through stimulating high number of blood vessels in CAM. This effect was dose related and had an optimal dosage. The efficacy of angiogenesis was similar to that of VEGF, which warranty further investigation. This work also shows that CAM can be used to study the angiogenesis of polyphenols such as proanthocyanidins.

Chapter 5

Slow release of proanthocyanidin promotes HUVEC growth in vitro and possibly angiogenesis in rat subcutaneous implantation model

5.1 Introduction

In previous chapters, proanthocyanidin has been showed to stimulate HUVEC growth in culture and to promote angiogenesis in CAM model. While CAM is a well-established in vivo model to study angiogenesis, particularly in screening drugs, it has limitations such as short duration and more importantly the missing of mature immune reactions. Any medical implant including tissue engineering scaffold invariably induces inflammation and reaction of the host immune system. Immune cells like lymphocytes, monocytes and macrophages are involved in the different stages of inflammation, helping cleaning foreign materials and guiding the direction of wound healing (179). Therefore any study of angiogenesis must not omit inflammation in immune intact animal models. Subcutaneous implantation in the rodent is a model widely used for a various purpose including tissue compatibility, material toxicity and angiogenesis (180,181). It also becomes the animal model used in this thesis to study the angiogenesis of proanthocyanidins in normal wound healing.

Soluble drugs injected into soft tissue are quickly diffused into tissues, making it difficult to sustain long term efficacy of the test drugs in a specific location. Drug carriers are therefore often used to slowly release test drugs into tissue (182). A drug carrier is a drug-containing substance made of tissue or blood compatible materials, which is either implanted or injected into circulation. The drug molecules are then slowly released from the carrier into tissue or circulation. Drug carrier materials used as implant must not be cytotoxic and not induce strong inflammation. To test angiogenesis of released drugs the material used to fabric drug carrier is preferred non-biodegradable otherwise degradation of carrier itself will interfere inflammation and angiogenesis as well.

Polyvinyl alcohol (PVA) is a synthetic polymer of high hydrophilicity because of the abundant hydroxyl side groups, as showed in Figure 5.1A. It is generally considered safe, biocompatible and has been used in clinic as embolization particles such as Contour® of Boston Scientific Inc. PVA can dissolve in water and form insoluble gel or cryogel upon cycling between frozen and room temperatures (183). The water crystals formed at low temperature push PVA molecules together to the extent that those PVA hydroxyl groups

eventually form stable hydrogen bonds, i.e., physical crosslinks. The voids occupied by water crystals at low temperature become water filled space at room temperature. Because of the high hydrophilicity of PVA molecules, the physically crosslinked PVA appears like hydrogels and is called cryogels for the reason of how it is formed. PVA can also be chemically crosslinked by formaldehyde. PVA cryogels have been studied to construct medical implants such as heart valve (184) and blood vessels (185). PVA has also been studied as drug delivery carrier (186). PVA is generally considered non-biodegradable.

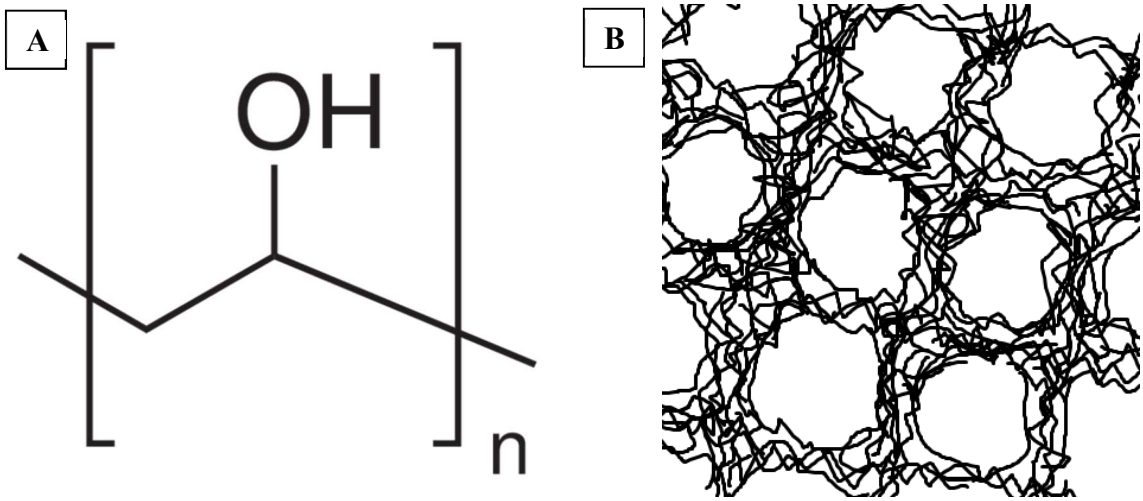


Figure 5.1 Chemical structure (A) of PVA and physical crosslinks (B) upon freeze-thaw cycling.

In this chapter proanthocyanidins were loaded into PVA cryogels for an in vitro release test. They were then implanted subcutaneously in rats. At different time points the implants and the tissues around were harvested with the angiogenic effects analyzed through histology.

5.2 Materials and Methods

5.2.1 Materials

Polyvinyl alcohol (Mw 89000-98000, 99+% hydrolyzed, catalogue number: 341584) was purchased from Sigma.

5.2.2 Methods

5.2.2.1 Cryogel preparation

Membranes of PVA cryogel were prepared through a cyclic freeze-thaw process. PVA powder was dissolved in deionized water at elevated temperature (ex. 50 ℃) to obtain 5% and 10% (w%) aqueous solutions. The solution was poured into a container to reach a desired thickness. The container was then frozen for at least 16 h at -20 ℃ followed by thawing at room temperature. This freeze-thaw process was repeated for 5 time to achieve desired physical cross-link. After the 5 cycles, the membrane cryogels were formed and stored at 4 ℃ until used.

To prepare drug loaded cryogels, proanthocyanidins were dissolved in water and mixed into PVA solution before freeze-thaw cycling. To achieve the desired dose determined in previous in vitro cell culture experiment, preliminary experiment was performed using various drug loads to test the quantify of drug released into water. Based on the preliminary experiment, 78 and 122 μg of proanthocyanidins were loaded into 5% and 10% of PVA solutions, respectively, to prepare cryogels of 12mm in diameter × 2 mm in thickness. Each membrane cyrogel weighs about 200 mg.

5.2.2.2 Releasing test

The drug loaded cryogels were individually weighed and randomly divided into five groups. Each group contains five gels loaded with the same amount of proanthocyanidin. The specimens were individually incubated in 2 ml of PBS at 37 ℃ for 72 h. At the time points of 6, 12, 24, 48 and 72 h, 1.0 ml of the soaking liquid was sampled and measured for optical density (OD) at 280 nm to detect proanthocyanidin using a UV/VIS spectrometer (SHIMADZU, UV-1601, SHIM ADZU EUROPA GmbH). *Proanthocyanidin* concentration was calculated against a standard curve that was established by measuring a series of solutions with known drug concentrations. Specimens without drug were used as the control to deduct background.

5.2.2.3 HUVEC viability in presence of drug loaded PVA cryogel

HUVECs were diluted to 1×10^4 cells/ml by a completed medium with 15% FBS and 25 μg/ml ECGS. Two milliliters of cell suspension were added into each well of a 12-well plate and incubated overnight. After, the media were aspirated and 1.5 ml fresh complete medium without ECGS was added to each well. A piece of cryogel was placed into a culture plate insert that was then put into a well. Half an ml of fresh complete medium without ECGS was added into the insert to make sure that the gel was completely bathed. Two ml of complete medium containing 15% FBS was used a blank control; and two ml of complete medium containing 31.3 μg/ml proanthocyanidins was used as drug control; and same volume of complete medium containing 15% FBS and 25 μg/ml ECGS was set as positive control. The assay was in triplicate.

After a 72 h incubation without changing culture medium, the inserts were lifted and experimental media were removed. Two ml of fresh medium with 15% FBS were added to avoid potential interference of the drugs to MTT test. Then, 200 μl of 5 mg/ml MTT was added to each well. After an incubation of 4 h at 37 ℃, the media were carefully removed and 1 ml DMSO was added to each well. The optical density (OD) at 570 nm was measured to quantify the number of viable cells.

5.2.2.4 Subcutaneous implantation

Twenty-five female Sprague-Dawley rats, each weighing 200-250 g, were randomly divided into five groups according to the duration of implantation that is 2, 3, 7, 14 and 28 days. These time points were selected to investigate the tissue reaction and angiogenesis at acute (2 and 3 days), transition (7 and 14 days), and chronic phases (28 days). PVA cryogels with and without proanthocyanidin were implanted in subcutaneously.

Following an intraperitoneal anesthesia using 10% chloral hydrate, back of the rat was shaved and skin disinfected first with iodine tincture then with 70% ethanol, for two times. Three 1 cm long incisions were performed longitudinally along each side of the spine, with each incision separated by about 3 cm. After separating the subcutaneous tissue to create a small pocket using blunt-nosed forceps, a piece of cryogel (10 mm diameter, 1.5

mm in thickness) was implanted under the skin with a 5 mm distance to the incision. The incision was then closed with a disposable skin stapler and disinfected again. After awakening, the animals were returned to the cage and fed an unrestricted diet. The use animals complies with the guidelines of the institutional ethical committee.

At the end of the prescheduled times, the animals were sacrificed and implants harvested together with the surrounding tissues. The explants were photographed and then fixed in 10% formalin. The fixed specimens were dehydrated in a series of alcohol solution of increasing concentration, embedded in paraffin, and cut into slices of about 5 μm in thickness. The slices were collected on glass slides, deparaffined and hydrated before being stained with hematoxylin & eosin (H&E) and Masson's trichrome.

5.3 Results

5.3.1 PVA cryogels

After 5 freeze-thaw cycles, PVA solutions became physically cross-linked and appeared as hydrogels (cryogels). The formed specimens are uniform in size and thickness (ca. 2.5 mm), as showed in Figure 5.2. They are easy to cut and manipulate.

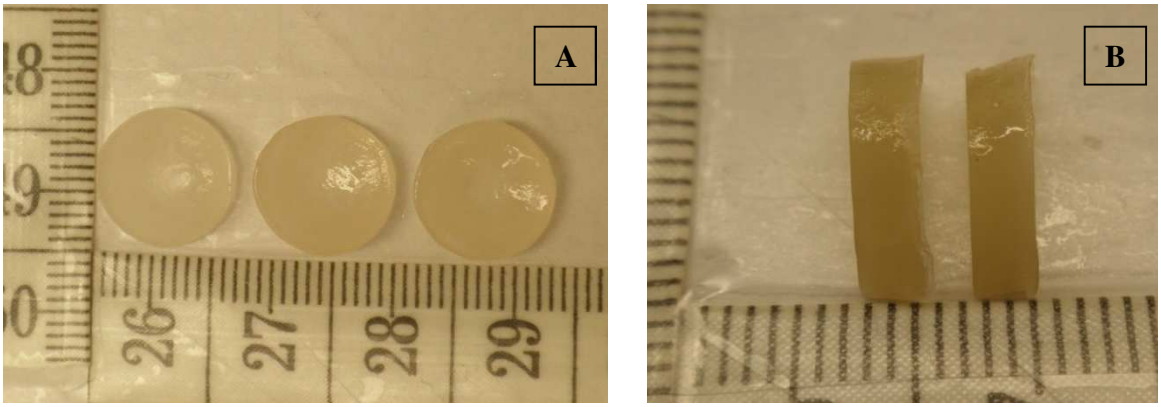


Figure 5.2 PVA cryogels showing uniform diameter (A) and thickness (B) of cryogels.

5.3.2 Releasing ratio

The amount of proanthocyanidin released into water over a period of 72 hours was shown in Figure 5.3. A burst release was observed in the first 6 h, reaching about 27 and 24 μg/ml of the drug in solution for the specimens prepared from 5% and 10% of PVA,

respectively. Between 6 and 72 h, the concentration of proanthocyanidin released from the 5% PVA cryogels increased steadily but slightly as well, being 31.2 μg/ml at 72 h. For the 10% PVA group the drugs in the solution increased slightly and became 35.4 μg/ml at the end of incubation.

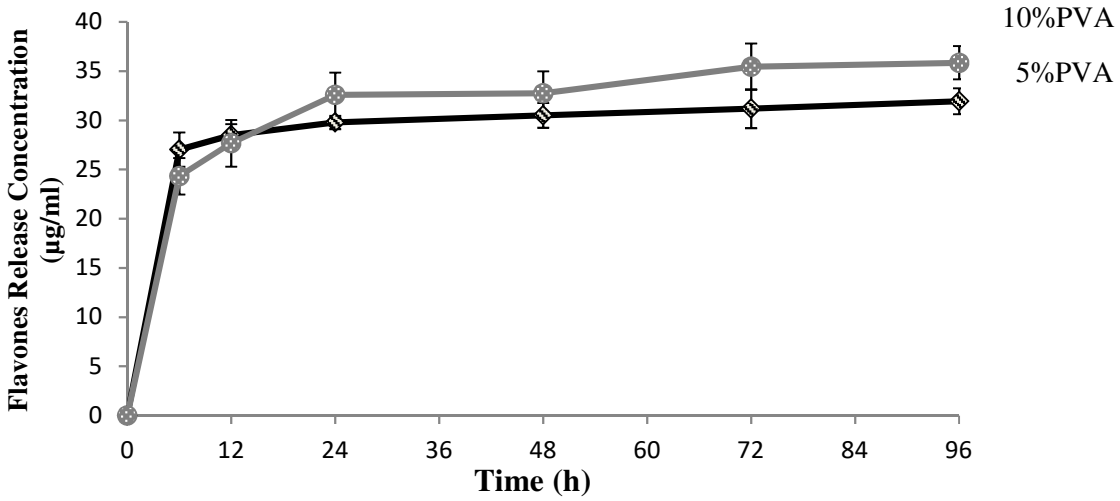


Figure 5.3 Concentration of proanthocyanidin released into solution in 72 h. The results represent the mean±S.D. from five gels per time point.

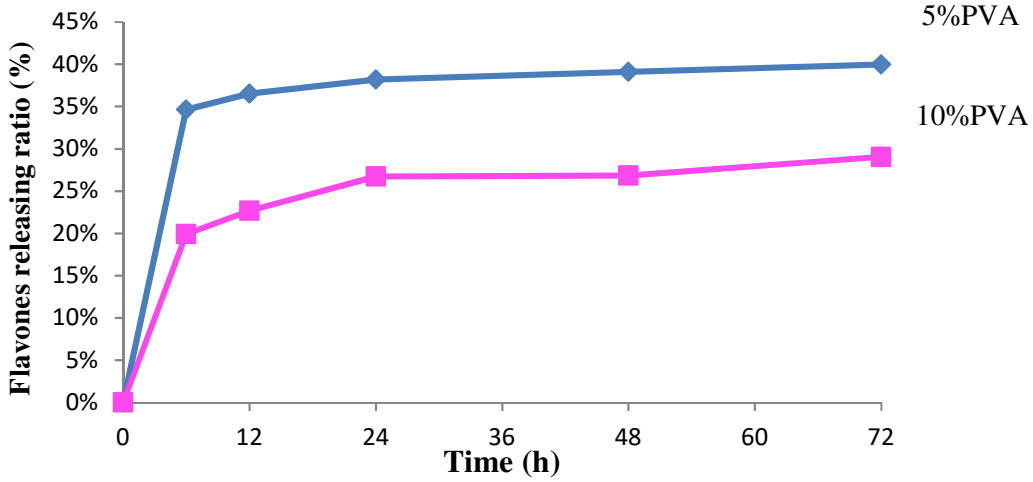
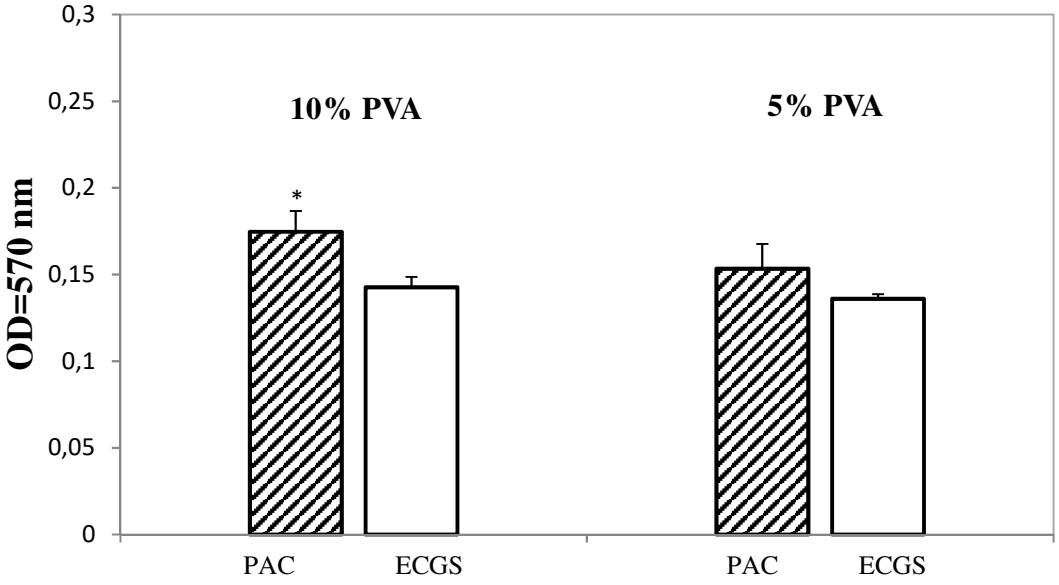


Figure 5.4 Releasing ratio of proanthocyanidin from PVA cryogels in 72 h.

The releasing ratios were calculated by dividing the amount released into solution with the total loading, as shown in Figure 5.4. The cryogels of 5% PVA released 35% of its load in the first 6 h; and the total release was about 40% in 72 h. In comparison, the burst release from the 10% PVA cryogels was about 20% in first 6 h, which was 15% lower than that of the 5% gels. The total amount released from the 10% cryogels at 72 h was about 30% of the total loaded drug, which was lower than that from the 5% gels.

5.3.3 Cell viability with inserts

Figure 5.5 shows the cell viability/proliferation measured with MTT absorption.



compare with ECGS group $^{*}p<0.05$, $^{**}p<0.01$

Figure 5.5 HUVEC viability after 3 days of culture with hydrogels containing proanthocyanidin in comparison with positive control (ECGS).

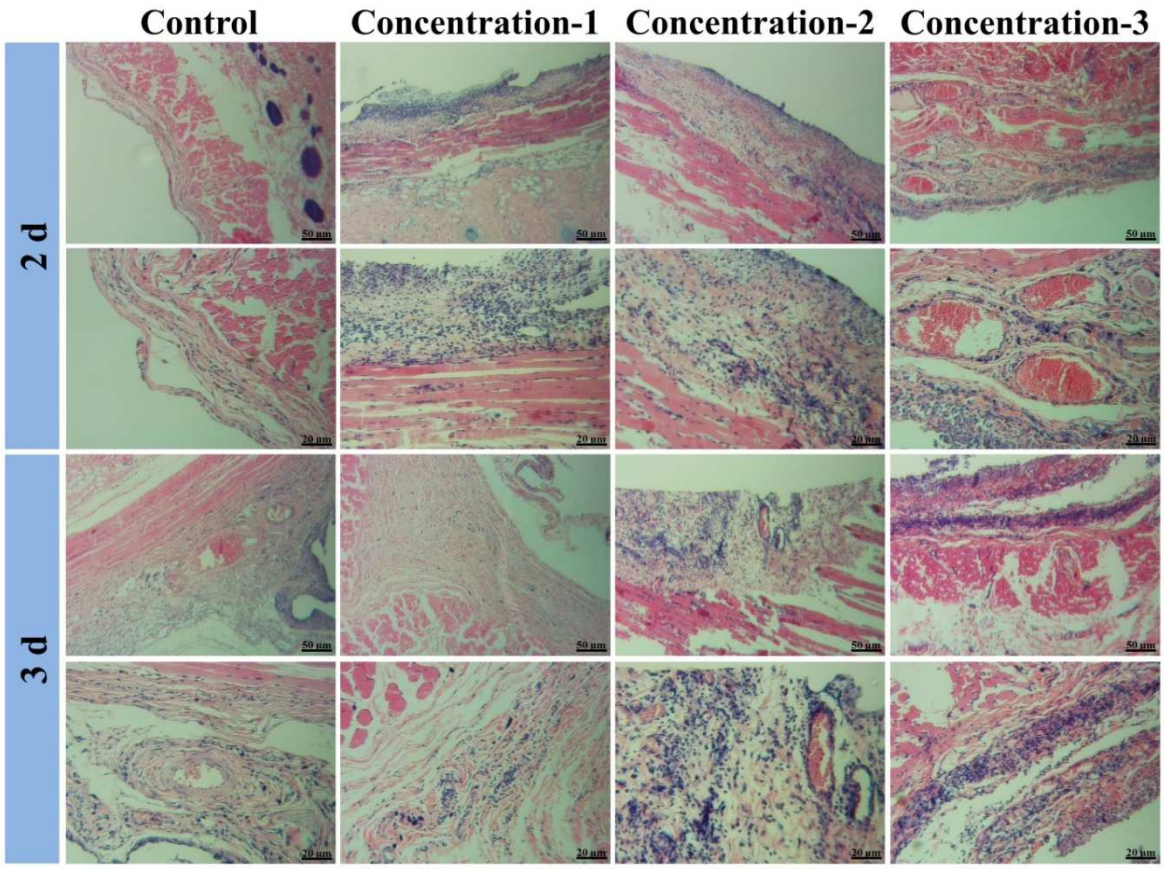
The results show that the cells cultured in the presence of the drug loaded PVA cryogels recorded higher MTT absorptions. In particular, there is a significant difference between the group of the 10% PVA loaded with drug and the ECGS group that is used as positive control. This positive effect on cell growth is most likely because of the proanthocyanidins released from the gels, meaning that proanthocyanidins released from the 5% and 10% PVA gels can promote endothelial cell proliferation.

5.3.4 Clinic and gross observations

All animals survived operation and the pre-determined periods of experiment. They were fund eat well and behave normally, showing normal wound healing and no body weight reduction. The explants at harvest appeared blunt at edge, indicating some degradation. By 28 days, the implants were encapsulated by dense tissue.

5.3.5 Histology analysis

As showed by the H&E stained images in Figure 5.6 and Masson's trichrome stained images in Figure 5.7, high cellularity stained dark violet was found at the implant/tissue interface during the acute phase of inflammation (2 and 3 days), which then gradually diminished over time but remained stronger than that in normal tissues distant to the implant. Fibrous capsules formed as early as 2 days, stained blue with trichrome, and became better organized over time. Compared with control group (gel without drug), the drug loaded gels showed a generally stronger inflammation at acute phase and more collagen tissue in capsules. The capillaries were labelled by arrows. However, there was no visible difference between the drug loaded groups and controls in terms of capillary density and capsule thickness.


Control
Concentration-1
Concentration-2
Concentration-3
2 d
3 d
50 µm
20 µm

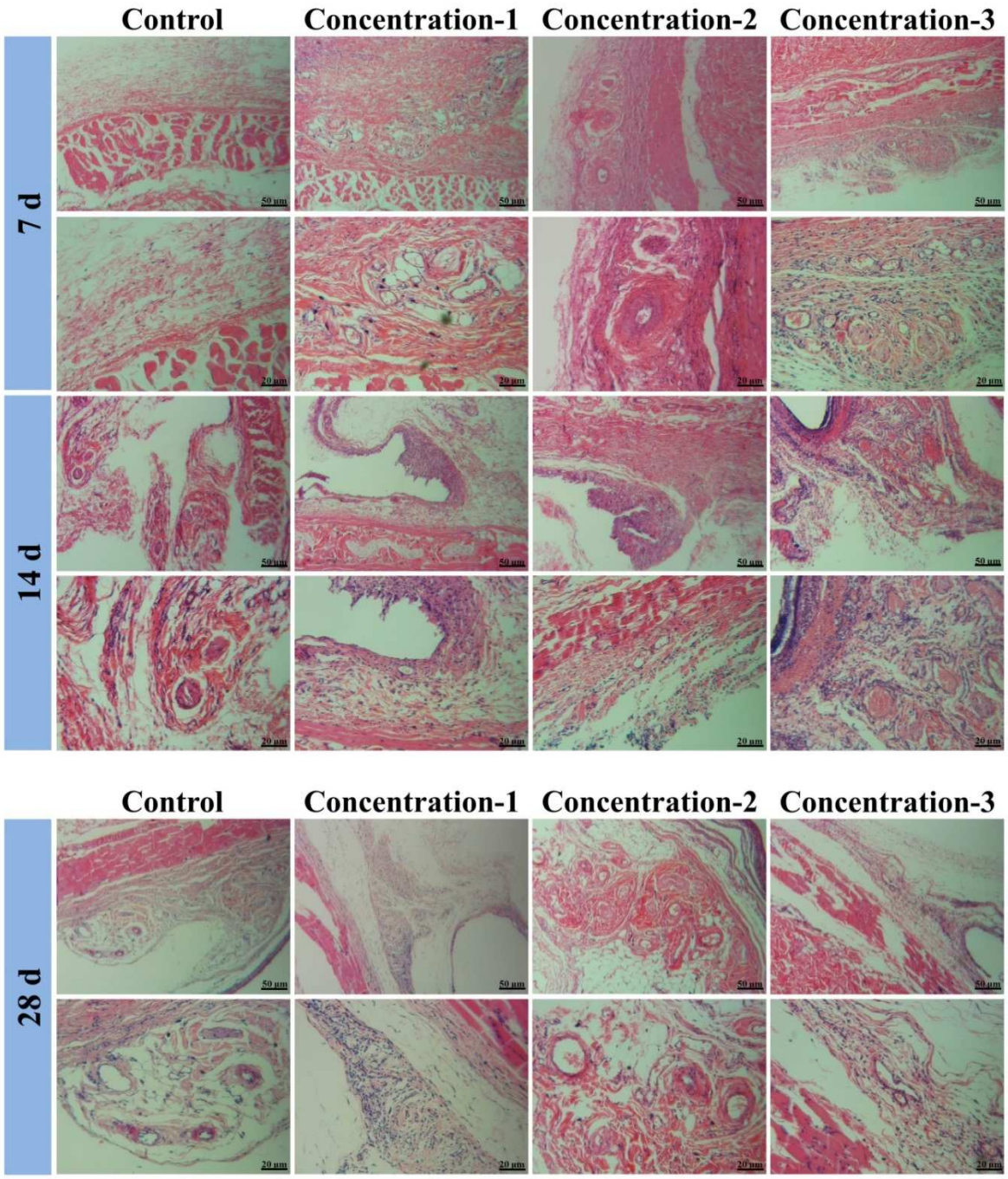
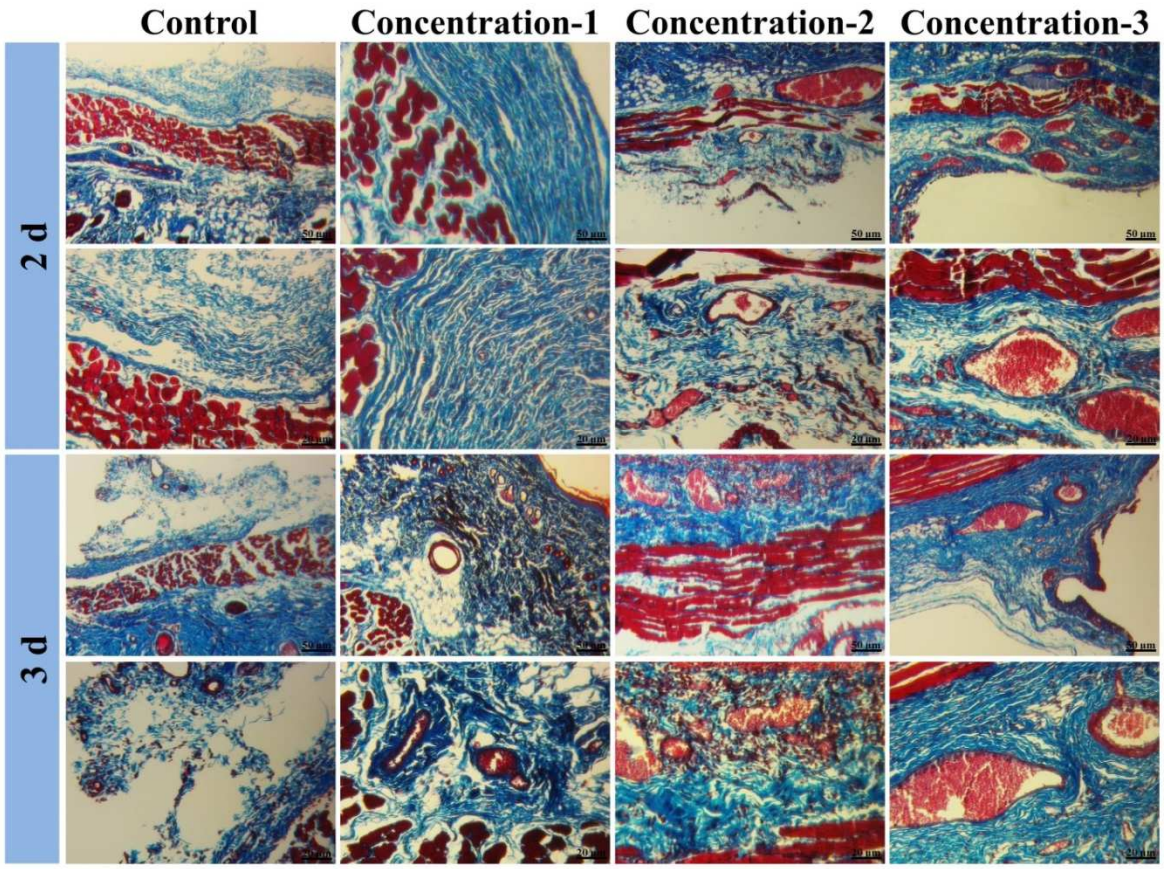


Figure 5.6 Tissue reactions to the PVA cryogels implanted subcutaneously in a rat subcutaneous model at various time points (H&E stain)


Control
Concentration-1
Concentration-2
Concentration-3
2 d
3 d

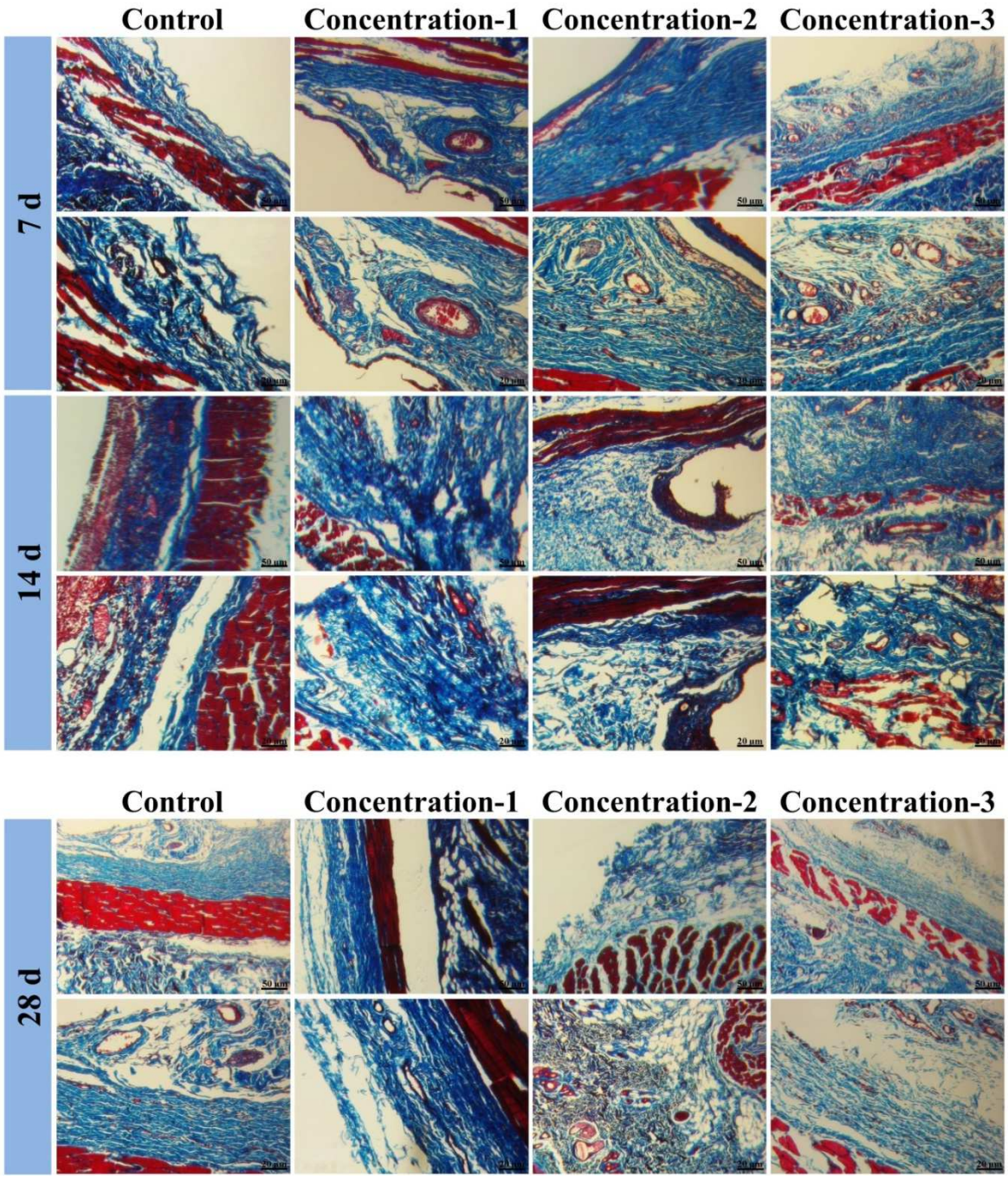


Figure 5.7 Tissue reactions to the PVA cryogels implanted subcutaneously in a rat subcutaneous model at various time points (Masson's Trichrome stain).

5.4 Discussion

Because of its non-toxic, hydrophilic and flexible properties, among other things, PVA is not only clinically used as embolization reagent but also has been widely studied as

a biomaterial for a variety of potential applications. Moreover, PVA is a chemically stable material resisting hydrolysis, even though it can be degraded by bacteria (187). This biostability makes PVA not an ideal candidate of carrier for in vivo drug release. Naturally, it is preferred that a drug carrier is degraded and metabolized in vivo. In this work, however, drug carrier degradation was intentionally avoided by choosing PVA. Material degradation in vivo will inevitably stimulate inflammation and the consequent angiogenesis to clean degradation products. The use of non-biodegradable PVA is expected to reduce the interference of carrier degradation to the angiogenic effect of loaded drugs. Another advantage of using PVA is that PVA is water soluble, making it easy to incorporate water soluble drugs. In this work, the drugs were dissolved in PVA water solution, which was then entirely converted into cryogel. This process also ensures all drug being loaded into the gel. In vitro drug release experiment showed that for PVA cryogels of 1.5 mm thick, there was about 30 to 40% of the drug released into culture medium during 72 hours. In other words, there was still 60% to 70% of the drug not released. Figure 5.3 is an accumulated release showing a burst increase in the first 6 h, following by a plateau in case of the 5% gels or a small increase in drug concentration in case of the 10% gels. From drug release point of view, such burst release is undesirable. However, this burst release permits the drug concentration in culture medium quickly reach the range of almost optimal dose, i.e., between 15 to 30 μg/ml, which has been identified in previous work. As the incubation time increases very little drug was further released into medium, particularly after 24 h. Considering that there was still a majority of the drug unreleased, this phenomenon may imply strong interaction between proanthocyanidins and PVA molecules. Evidently, further experiment of long time release will be required to demonstrate how many drugs will be ultimately retained inside of the PVA gel.

The drug constituents released into culture medium could be different from that of the original composition. Proanthocyanidins include molecules of different molecular weight owing to the polydispersity of the constituents. The difference in mass among these molecules is large. For example, the size of a dimer is two third of a trimer. It is well-known that drug release is controlled by the diffusion coefficient of the constituent in polymer matrix; and larger molecules normally have smaller diffusivity. In view of the

incomplete release of the drugs during 72 hours, it is possible that the drug constituents released into culture medium differ from that in original loading. It may be further deduced that the released drugs have lower average molecular weight than those retained in PVA gel. Another factor potentially contributing to this "differential release" is the concentration of PVA solution. Based on literature about PVA cryogels of different concentrations (183), cryogels prepared from 10% vs. 5% PVA solutions are expected to have different crystallinity, pore size, hydrogen bonding and cross-link degree. All these parameters are expected to impact the diffusion coefficient of drugs and such impact could be size-dependent. Proanthocyanidins are molecules with rich hydroxyl groups, as PVA. One may logically anticipate hydrogen bonding between proanthocyanidins and PVA. Should such non-covalent interactions become significant it would restrain drug diffusion and release. All these parameters potentially important in regulating proanthocyanidin release from PVA cryogel warrant further investigation.

Proanthocyanidins released from PVA cryogels stimulated HUVEC growth superior to or as good as growth factors (ECG) did (Fig. 5.4). This demonstrates that despite the anticipated interactions between drug and PVA molecules sufficient amount of bioactive drugs were still released. This means that PVA can be used as drug release carrier of proanthocyanidins and potentially as a carrier for other types of flavonoids as well. Another important message is that the released constituents were bioactive despite the possibility that they are different from the original composition. This information is useful to identify the active constituent(s) of the proanthocyanidins used in this study.

The drug loaded PVA cryogels were found to stimulate stronger acute inflammation and more evident angiogenesis in subacute and chronic phases. The elevated inflammatory cell population in the proximity of drug loaded implants was likely caused by proanthocyanidins because controls showed less inflammatory cells. Proanthocyanidins are generally reported as anti-inflammatory owning to their anti-oxidant property. However, there has been little report concerning the tissue reaction of proanthocyanidins. Literature search on Medline using keywords ((proanthocyanidin OR procyanidin) AND subcutaneous), Nov. 18 2016) only scored 12 returns. The combination of

((roanthocyanidin OR procyanidin) AND tissue reaction), Nov. 18 2016) returned only 22 scores. Among all these scores only one article dealt with subcutaneous injection of procyanidin. In fact this phenomenon is quite normal because proanthocyanidins are considered nutrient compliment with therapeutic effect and administrated orally. One article reported subcutaneous injection of high dose of procyanidin B2 in rats, ranging from 500 to 2000 mg/kg. The injections of 1000 and 2000 mg/kg were found inducing granulomatous inflammation at 14 days (188). When the dose reduced to 500 mg/kg the abnormality was limited to the change of color of duodenum mucosa together with the accumulation of macrophages. These dosages are however much higher than what were used in this work. Nevertheless, this study on subcutaneous injection of procyanidin B2 shows that high dose procyanidin B2 only causes localized tissue inflammation and no systemic toxicity. Cellular activity gradually declined during the subacute phase and became similar among all implants at 28 days. The slow disappearance of high cellular activity around the drug loaded implants suggests a continuous release of proanthocyanidins up to 14 days. However, visual inspection on capillaries didn't reveal a clear difference between drug loaded and control gels. Therefore further animal experiment is required to verify the angiogenic property of proanthocyanidins.

5.5 Conclusion

Proanthocyanidins were successfully loaded into and released from PVA cryogels. By controlling total load and gel density, the profile of drug release can be modified. PVA cryogels prepared from higher PVA concentration can slow down release rate and reduce burst release. The proanthocyanidins released from PVA remained bioactive and stimulated in vitro growth of endothelial cells. When released subcutaneously, proanthocyanidins induced intensive acute inflammation with respect to controls. The inflammation declined over time and became similar to controls at 28 days. However, a further animal experiment is required to verify the angiogenicity of proanthocyanidin. This work demonstrated for the first time that bioactive proanthocyanidins can be slowly released from a polymeric carrier and can stimulate endothelial cell growth.

Chapter 6

General conclusions & perspectives

This thesis was to demonstrate the potential of botanic drugs in wound healing in the context of tissue repair and tissue regeneration. Based on literature four drugs in TCM were selected to test the hypothesis that they can stimulate endothelial cell growth and encourage angiogenesis. Both in vitro and in vivo experiments were performed, leading to the following general conclusions.

Firstly, proanthocyanidin can promote HUVEC growth in vitro and stimulate angiogenesis in vivo. Contrary to the widely reported inhibitory effect, this stimulatory effect takes place in a relatively narrow window of dosage. Even under a low serum condition, proanthocyanidin can maintain cells viability up to 72 h. In particular, this stimulatory effect on HUVEC proliferation can be as potent as that of the commercial endothelial cell growth supplements. Therefore it is likely that one may use proanthocyanidin to replace VEGF and EGF to maintain a short-term endothelial cell culture.

Secondly, proanthocyanidin can be safely loaded into and released from PVA cryogels, without losing its bioactivity on endothelial cells. By controlling total drug load and gel density, the profile of drug release can be modified. These findings demonstrated the feasibility of using botanic drugs in tissue engineering scaffold or release them from drug carriers.

Thirdly, proanthocyanidin promoted angiogenesis in developing CAM. This effect was dose related and showed an optimal dosage. The efficacy of proanthocyanidin on CAM angiogenesis was similar to that of VEGF, showing the potential of using proanthocyanidin in tissue engineering and in treating ischemic diseases. This also raises concerns about the consumption of proanthocyanidins as health supplements.

Fourthly, neither VEGFR nor EGFR is responsible for how proanthocyanidin works, calling for further mechanistic studies. The effective component in the proanthocyanidin is also not clear, which may explain the inconsistency in drug effectiveness in some

experiments. This work compared the proanthocyanidin under test with procyanidins B1 and B2 from Sigma-Aldrich and found that neither of them is the active component.

Finally, the other three drugs selected from TCM, i.e., Astragalus injection, puerarin injection and Astragalus extract powder, didn't show any positive effect on HUVEC proliferation or cell viability under the experimental conditions used in this thesis. Cytotoxicity was observed at high dosages for all the three drugs.

In addition to the general conclusions mentioned above, some future work is suggested as following.

A mechanistic study is critical to identify how proanthocyanidin works. Because currently there is no clear clue either from the experiment or from literature, one possibility is to look at the activation of cell proliferation genes using gene arrays, followed by confirmation of some key proteins coded by the activated genes. This may eventually lead to the discovery of particular signaling pathways related to the proanthocyanidin modulated endothelial cell activities.

The effective component(s) in proanthocyanidin must be identified. The proanthocyanidin used in this thesis was extracted from grape seeds, with a 99% purity of proanthocyanidin in total extract. However, proanthocyanidin is a broad name given to a group of chemicals of similar structure but different molecular weight and side groups. Proanthocyanidins from different sources vary in compositions as well. Therefore it will be important to analyze the proanthocyanidin from the same supplier.

The other three drugs didn't show any effect on HUVEC proliferation under the experimental conditions in this thesis. However, their roles in wound healing may be related with other type of cells, not endothelial cells. Therefore their functions may deserve further study.

Promoting neovascularization at the ischemic site with angiogenic molecules is considered one of the most promising methods in the treatment of ischemic cardiovascular diseases. However, the unsatisfactory results from trials have pointed to the short life of growth factors in vivo. TCM has a long history and many traditional Chinese medicines were reported to improve blood circulation and myocardial functions. Many research about TCM have found that some traditional medicines can promote angiogenesis. Promoting angiogenesis might be one of the mechanisms that TCM has played in treating cardiovascular diseases. However, the mechanisms of how TCM promotes angiogenesis could be very complex. It might not be a single factor that plays the role, but the collective effort of multiple components on multiple targets. The same drug in different diseases may play diametrically opposed roles. Different doses may have different effects through different mechanisms. So, finding out the mechanisms is particularly important, which can only be achieved through well designed and controlled experiment and clinical trials.

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Appendix

1. A Calibration curve of *proanthocyanidin*

Ten (10) ml of 200 μg/ml proanthocyanidin dissolved in deionized water were prepared freshly before the test. A series of double dilution was applied till 6.25 μg/ml. In such a way proanthocyanidin standard solutions of 200, 100, 50, 25, 12.5 and 6.25 μg/ml were prepared. To measure OD, 1.0 ml of each proanthocyanidin solution was sampled in triplicate and observed. The OD value was read at 280 nm.

The proanthocyanidin concentrations were taken as abscissa while the absorbance values as the vertical axis, to draw a standard calibration curve as below in Figure 1. A linear regression was made to obtain the standard equation Y=0.00556X-0.0117, showing a high linearity (R^2 = 0.998).

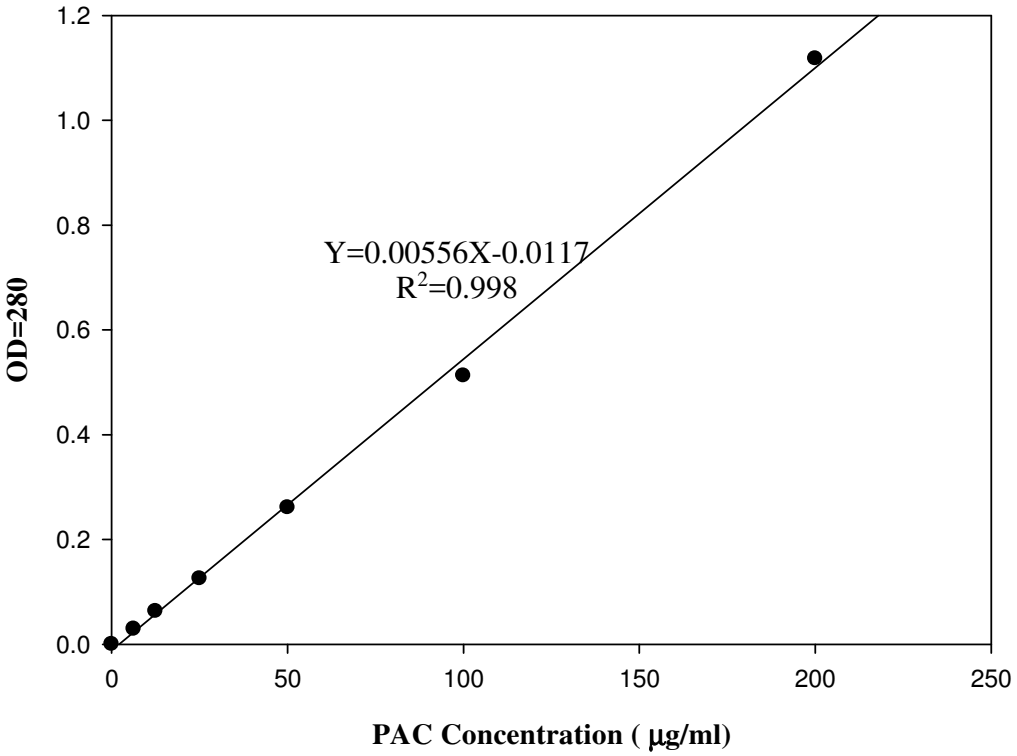

Figure 1. The standard curve of proanthocyanidin dissolved in deionized water

Thus, proanthocyanidin concentrations can be calculated from experimental OD against the curve.

2. Photos of the four botanic drugs selected in this study

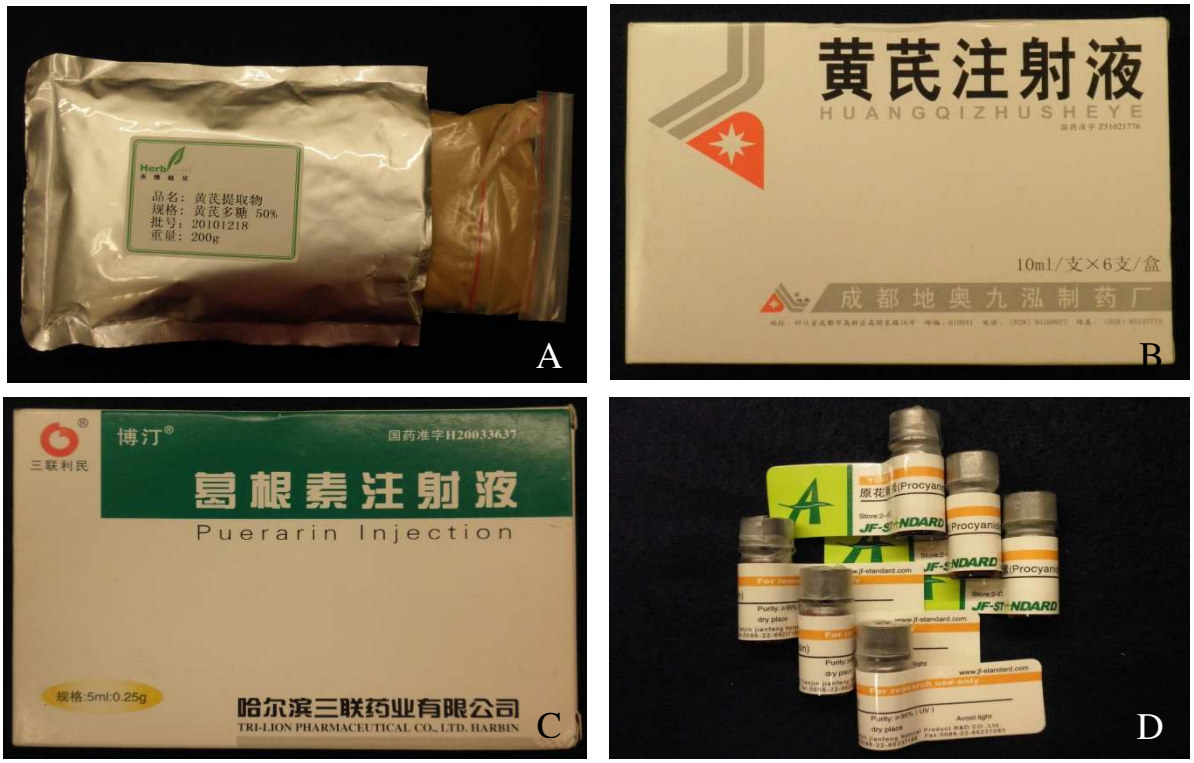

Figure 2. The four botanic drugs selected in this study. A: *Astragalus* powder extract, B: *Astragalus* injection, C: Puerarin injection, D: Proanthocyanidin.