HEPARIN FOR THE PREVENTION OF PREECLAMPSIA: THE ROLE OF NON-ANTICOAGULANT MECHANISMS

by

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Abstract

Preeclampsia is a serious disorder of pregnancy characterized by new-onset hypertension with evidence of organ damage. The severe, early-onset form of preeclampsia with fetal growth restriction originates from impaired spiral artery remodeling during early pregnancy, resulting in poor placental function later in pregnancy. Small randomized clinical trials have determined that low molecular weight heparin (LMWH) could effectively prevent preeclampsia, while recent multi-center trials showed no effect. The mechanisms by which LMWH could confer protection against the development of preeclampsia, anticoagulant or non-anticoagulant, have not been thoroughly investigated. Elucidating the mechanisms of LMWH for the prevention of preeclampsia could provide insight into which women may benefit from LWMH therapy.

In this thesis, the hypothesis that anticoagulation-independent mechanisms of LMWH mediate the protective effects against features of preeclampsia in the rat reduced uterine perfusion pressure (RUPP) model was tested using a non-anticoagulant derivative of LMWH,

termed glycol-split heparin. The non-anticoagulant properties of glycol-split heparin were first validated against dalteparin, a clinically utilized LMWH, through a series of *in vitro* experiments. Glycol-split heparin demonstrated similar properties to dalteparin, including stimulating the release of placental growth factor from first trimester human placental villous explants; promoting angiogenic tube formation by cultured endothelial cells; and inhibiting complement activation and leukocyte adhesion. Results from rat studies demonstrated that, although dalteparin was unable to suppress the development of hypertension in the RUPP model of preeclampsia, dalteparin significantly improved fetal growth and normalized placental labyrinth structure disrupted by model induction. However, glycol-split heparin did not elicit the same fetoplacental effects as dalteparin, and was comparable to vehicle-treated RUPP rats.

The data presented in this thesis provide novel insights into the mechanistic effects of LMWH for the prevention of preeclampsia, suggesting that LMWH may mediate improvements in placental function and fetal growth. LMWH therapy may therefore be most beneficial for the subset of patients at risk of the severe early-onset phenotype of preeclampsia, characterized by placental dysfunction and fetal growth restriction. These findings provide the basis for future experimental and clinical studies to elucidate the role of LMWH as a potential preventative therapy against the development of severe early-onset preeclampsia.

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Dedication

To all of my family and friends, who have never stopped believing in me.

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List of Abbreviations

ANOVA - analysis of variance

aPTT - activated partial thromboplastin time

ATBR - antithrombin binding region

ATIII - antithrombin III

ATP - adenosine triphosphate

BSA - bovine serum albumin

DAMPs - damage associated molecular patterns

DNA - deoxyribose nucleic acid

dNK - decidual natural killer

EDTA - ethylenediaminetetraacetic acid

eEVT - endovascular extravillous cytotrophoblast

ELISA - enzyme-linked immunosorbent assay

EMT - epithelial-to-mesenchymal transition

EVT - extravillous cytotrophoblast

FBS - fetal bovine serum

FGF4 - fibroblast growth factor-4

GCM1 - glial cell missing-1

GD - gestation day

GlcA - glucuronic acid

GlcN - glucosamine

gsHep - glycol split LMWH

HELLP - hemolysis, elevated liver enzymes, and low platelet

HMGB1 - high mobility group protein B1

HUVEC - human umbilical vein endothelial cell

IdoA - iduronic acid

iEVT - interstitial extravillous cytotrophoblast

IL - interleukin

INR - International Normalized Ratio

IU - international units

LMWH - low molecular weight heparin

L-NAME - nitro-L-arg-methyl ester

mRNA - messenger RNA

MVM - maternal vascular malperfusion

MW - molecular weight

NO - nitric oxide

PAR - protease activated receptor

PBS - phosphate buffered saline

PCNA - proliferating cell nuclear antigen

PCR - polymerase chain reaction

PDE5 - phosphodiesterase 5

PlGF - placental growth factor

qRT-PCR - quantitative reverse transcription polymerase chain reaction

RNA - ribonucleic acid

ROS - reactive oxygen species

RUPP - reduced uterine perfusion pressure

sFlt1 - soluble fms-like tyrosine kinase-1

SOGC - Society of Obstetricians and Gynaecologists of Canada

TNF-α - tumor necrosis factor alpha

VEGF - vascular endothelial growth factor

VEGFR - vascular endothelial growth factor receptor

1. Introduction

This thesis focuses on elucidating the potential mechanisms by which low molecular weight heparin could prevent the development and progression of preeclampsia, a clinically significant pregnancy disorder that is predominantly mediated by placental dysfunction.

1.1. Pregnancy and the Placenta

Pregnancy is the biological state when a fertilized egg implants into the endometrium of the uterus, initiating the process of embryonic development and progression from fetal life to birth. In normal human pregnancy, this process lasts approximately 40 weeks, during which time the fertilized egg progresses through developmental stages resulting in the formation of a viable fetus prior to delivery. In order to successfully reach this optimal endpoint of pregnancy, fetal growth is supported through coordination of the adaptive maternal physiology and the evolving anatomical and functional aspects of the placenta. These maternal-fetal adaptations are necessary to meet the progressive metabolic demands of the growing fetus, while ensuring the maternal physiology can withstand the significant strain that accompanies pregnancy.

1.1.1. General function of the placenta

The placenta is the main pregnancy organ derived from the cells of the fertilized egg that connects the developing fetus to its host; it is critical for fetal development in humans and most mammals. The primary function of the placenta is to facilitate nutrient, oxygen, and metabolic waste transfer between the maternal circulation and the growing fetus. In humans, this is achieved through direct contact of the placenta with maternal blood. The placenta also

plays additional roles in the maintenance of pregnancy, including acting as an immunological barrier to the maternal immune system, protecting the fetus from potentially noxious substances in maternal blood, and functioning as an endocrine organ that communicates signals of pregnancy to maternal organ systems[1]. Given its crucial role in coordinating pregnancy-related processes, dysregulated placental development and function is associated with adverse maternal adaptations to pregnancy[2], which in turn are associated with adverse clinical outcomes including stillbirth, fetal growth restriction, and preeclampsia.

1.1.2. Human placental development

In humans, the definitive placenta forms during the first trimester; it undergoes rapid growth and specialization to meet the exponentially-growing oxygen and nutrient demands of the fetus[3]. The placenta is derived from cells of the fertilized egg after its migration into the uterine cavity and begins formation at the uterine epithelium.

1.1.2.1. Implantation of the blastocyst

Shortly after fertilization, the egg develops into a blastocyst, a hollow structure that contains an inner cell mass surrounded by an outer trophectoderm layer. While the inner cell mass ultimately develops into the fetus, the trophectoderm gives rise to the placenta. As the outer layer of the blastocyst, the trophectoderm guides the implantation process through a series of three stages: 1) the blastocyst orients itself towards the endometrial implantation site ("apposition"); 2) cells called trophoblasts from the trophectoderm attach to the endometrial epithelium ("adhesion"); and 3) trophoblasts invade into the endometrial stroma to firmly

implant into the uterine wall ("invasion"). Success of the first two stages (days 6-7 post coitus [p.c.]) is dependent on the receptivity of the uterine environment and is critical to establish a viable pregnancy. The last stage is crucial for sustaining a healthy pregnancy; it includes stimulating differentiation of the surrounding endometrium into the decidua, a specialized layer of endometrial tissue that supports the development and function of the mature placenta.

1.1.2.2. Invasion of extravillous cytotrophoblasts into the decidua

After adhesion of the blastocyst to the uterine wall, trophectoderm stem cells generate a primitive syncytiotrophoblast at the site of contact. The syncytiotrophoblast is a single-cell, multinucleated mass formed through proliferation and subsequent fusion of the underlying progenitor cytotrophoblasts. The syncytiotrophoblast initiates the invasive process into the endometrium. As the syncytiotrophoblast invades, it also contributes to the development of lacunae, which will coalesce later to form the intervillous space into which maternal blood is perfused. Meanwhile, as the blastocyst continues to implant deeper into the uterus beyond the uterine epithelium, the syncytiotrophoblast expands to surround the blastocyst, and the lacunae system extends to surround the entire blastocyst.

Cytotrophoblasts emanating from the trophectoderm proliferate into columns that penetrate through the syncytiotrophoblast and anchor the blastocyst into the decidua. At the tip of the columns, cytotrophoblasts also spread laterally to form a cytotrophoblastic shell that situates between the syncytiotrophoblast and the endometrium, and surrounds the blastocyst by approximately day 14 p.c.. Around the cytotrophoblastic shell and at the distal tip of the

anchoring columns, the cytotrophoblasts continue to proliferate and give rise to invasive extravillous cytotrophoblasts (EVTs). Interstitial extravillous cytotrophoblasts (iEVTs) continue to invade into the decidual stroma as far as the inner third of the myometrium, a process that is regulated by cytokines in the tissue microenvironment[4].

1.1.2.3. Remodeling of the uterine spiral arteries

In contrast to the iEVTs, cytotrophoblasts in the columns or the shell that contact maternal uterine spiral arteries migrate down the arterial lumen. These are designated as the endovascular cytotrophoblasts (eEVTs), based on their location within the arteries. Early in pregnancy, the large volume of eEVTs present forms a trophoblastic plug over the distal opening of the spiral artery, blocking premature maternal blood flow into the intervillous space. This eEVT blockage is hypothesized to protect the early embryo from excessive oxidative stress derived from maternal blood flow prior to approximately 8-10 weeks of gestation[4,5]. After this time, the plug dissociates as the eEVTs migrate along the arterial lumen to induce endothelial cell apoptosis and replace the endothelial lining in the spiral arteries[4]. Meanwhile, iEVTs invading the decidua also approach the vessels from the outside, where they can induce apoptosis of the underlying vascular smooth muscle cells of the spiral arteries[6]. These changes transform the spiral arteries from narrow, high-resistance vessels into dilated, low-resistance conduits that are unresponsive to vasoconstrictor molecules[4]. This vascular remodeling allows increased volume of blood to perfuse the placenta at low velocity to adequately supply the developing fetus as pregnancy progresses[7] (Figure 1.1). The remodeling process is complete by

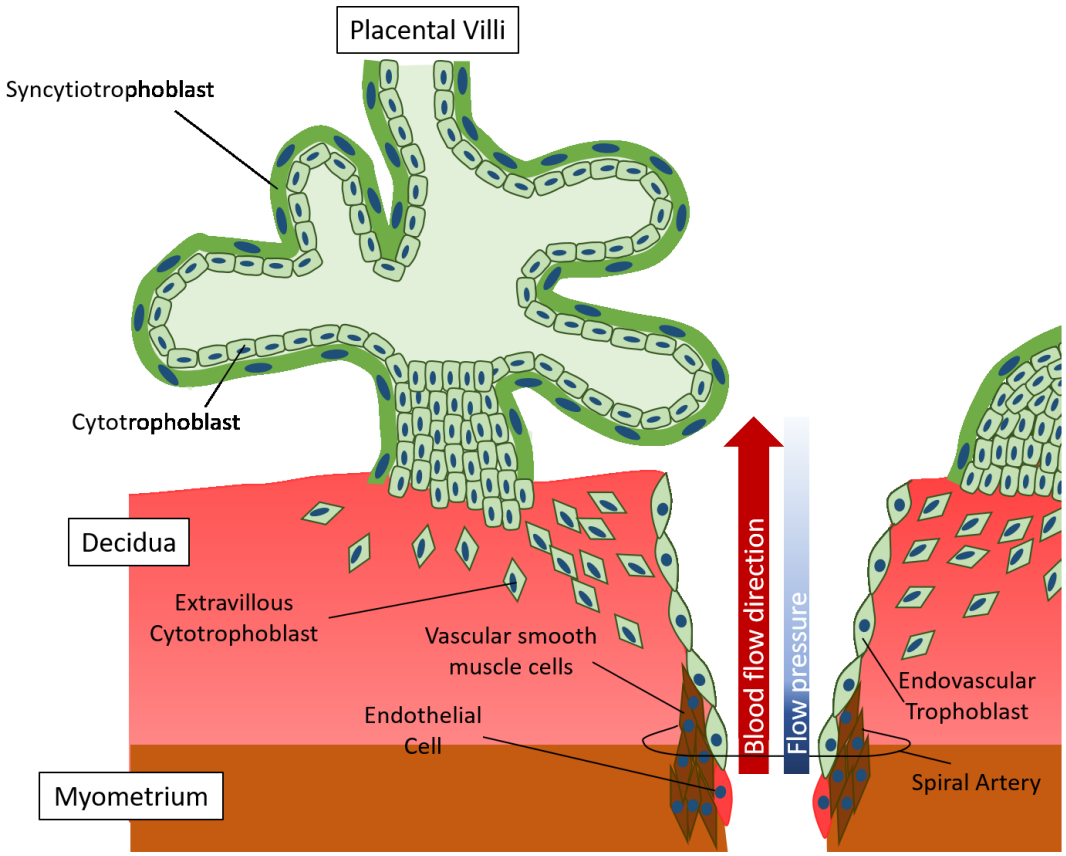

Figure 1.1. Schematic of placental development.

During early placental development, extravillous cytotrophoblasts invade into the decidua towards the myometrium to remodel the maternal spiral arteries, characterized by the replacement of endothelial cells with endovascular cytotrophoblasts and the removal of vascular smooth muscle cells. These changes transfom the distal arteries into low resistance, high capacitance conduits that allow large volumes of blood to perfuse into the intervillous space at low pressure. The placental villi are bathed in maternal blood and transfer nutrients and oxygen from maternal blood to the fetus. (Adapted with permission from Wat et al 2018[9]).

20-22 weeks of gestation, after invading and remodeling deep into the inner third of the myometrium[4].

1.1.2.4. Villous development

As EVT invasion occurs at approximately day 13 p.c., cytotrophoblasts from the columns also proliferate and form side branches which protrude into the lacunae to develop structures called primary villi. The position of these villi therefore places them in direct contact with the lacunae and eventually the intervillous space, where oxygen and carbon dioxide diffuse and nutrients are actively transported across the interface. Over time, mesenchymal cells from the inner cell mass migrate into the primary villi to develop into secondary villi, and fetal vascular formation converts the secondary villi into tertiary villi. Continuous proliferation of the villous cytotrophoblasts contribute to branching of the placental villi. As villous development progresses, the villous cytotrophoblasts also generate and maintain another layer of specialized epithelial syncytiotrophoblast that covers the villi. Similar to the primitive syncytiotrophoblast, the villous syncytiotrophoblast is also formed through asymmetric cell division of villous cytotrophoblast, with subsequent differentiation and syncytial fusion into the outer syncytial layer[8]. The syncytiotrophoblast is an important multi-purpose barrier between the mother and the fetus that maintains overall function of the placenta[10]: the syncytiotrophoblast separates the maternal and fetal circulations, acts as an immunological barrier, releases communication signals to the maternal circulation, and provides a rarefied surface for diffusional exchange of gases and active transport-mediated nutrient exchange between maternal and fetal blood. The

progressive branching of the villi serves to increase the placental surface area available for the efficient gaseous and nutrient exchange.

1.2. Preeclampsia

Pregnancy is a highly regulated process, which is uncomplicated in the majority of women. However, errors that occur during the early period of rapid fetal and placental development can cause major maternal and fetal pregnancy complications. Abnormal development of the placenta can impair its ability to function and support fetal growth which, via aberrant maternal signaling, can manifest as a range of pregnancy-mediated disorders such as preeclampsia.

1.2.1. Clinical and laboratory diagnosis of preeclampsia

Preeclampsia is a hypertensive disorder of pregnancy characterized by new-onset hypertension after 20 weeks of gestation, accompanied by maternal organ injury. The traditional definition of preeclampsia characterized maternal organ injury as renal dysfunction, due to the common presence of proteinuria. However, guidelines published in 2014 by the Society of Obstetricians and Gynaecologists of Canada (SOGC) outlining the diagnosis criteria for preeclampsia have updated the definition of maternal organ injury to include: renal, hematological, hepatic (e.g. nausea or vomiting), cardiorespiratory (e.g. shortness of breath or chest pain), neurologic (e.g. headache or visual symptoms), and placental dysfunction, the latter evidenced *in utero* by fetal growth restriction[11].

Laboratory test results aid in the diagnosis and management of preeclampsia. According to SOGC guidelines[11], blood testing for elevated serum creatinine/uric acid, elevated leukocyte count, low platelet count, low coagulability (determined as increased International Normalized Ratio [INR] or activated partial thromboplastin time [aPTT]), elevated serum liver enzymes, and low plasma albumin can all indicate the risk of severe maternal or perinatal complications associated with preeclampsia. In addition, ultrasound blood flow measurements of the uterine and umbilical arteries can reflect placental function, especially the risk of fetal hypoxia and growth restriction. In women with severe preeclampsia, end-organ injury compromises maternal safety, and can be inferred from low oxygen saturation (<97%), low platelet count (<50 x 10^9/L), elevated serum creatinine >150 µM, abnormal coagulation (INR>2), severe hypertension refractory to common antihypertensive treatments, and seizures (eclampsia). When placental dysfunction is either not recognized or is extremely severe, stillbirth may occur; fetal monitoring and timely iatrogenic preterm delivery may prevent this adverse outcome[11].

1.2.2. Epidemiology and risk factors for preeclampsia

Hypertensive disorders of pregnancy affect approximately 8% of pregnancies[12]. A 2014 World Health Organization review has identified hypertensive disorders of pregnancy as one of the leading causes of maternal mortality, accounting for approximately 14% of all maternal deaths[13]. Preeclampsia affects approximately 3-4% of all pregnancies globally[14,15], with significant variability depending on the region[16]. Women of African descent are at higher risk of developing preeclampsia during pregnancy, while women of Asian descent are at lower risk[17].

The incidence of preeclampsia is increasing[15], in part due to the rising prevalence of risk factors associated with preeclampsia[18].

Maternal risk factors for preeclampsia are diverse, and can be roughly categorized into maternal constitutive, obstetrical, or immune-related factors (Table 1.1). Maternal constitutive risk factors include advanced maternal age, ethnicity, pre-existing medical conditions (e.g. obesity, hypertension, diabetes, renal disease, antiphospholipid syndrome, autoimmune diseases), and family history of preeclampsia; obstetrical risk factors include nulliparity, multiple gestation, previous preeclampsia, and assisted reproduction[18,19]. An immune component may also be present, as paternal contributions (e.g. change in partners, increased interval between pregnancies, limited exposure to seminal fluid prior to pregnancy) also increase the risk of preeclampsia[20,21]. Preeclampsia is therefore a spectrum disorder, where varying risk factors alter the threshold for disease development and result in heterogenous disease severity and presentation[22,23].

1.2.3. Heterogeneity of preeclampsia

Preeclampsia is a heterogeneous disorder that presents differently among individual patients. The clinical presentation of preeclampsia is most common near term (i.e. late-onset preeclampsia), with women exhibiting mild to moderate symptoms. Due to the proximity of disease presentation to the end of pregnancy, late-onset preeclampsia is typically managed with antihypertensive therapy until near-term delivery, and therefore does not threaten fetal health[24]. A minority of women, 10-20% of cases, present with preeclampsia before 34 weeks gestation (i.e. early-onset preeclampsia) with symptoms that are typically more severe. Severe

Table 1.1. Summary of maternal risk factors for preeclampsia.

Category	Risk factor
Constitutive	• Advanced age • Ethnicity (e.g. African descent) • Family history • Antiphospholipid syndrome • Chronic hypertension • Diabetes mellitus • Obesity • Renal disease • Autoimmune disease
Obstetrical	• Nulliparity • Multiple gestation (e.g. twins) • Previous preeclampsia • Conception through assisted reproductive techniques
Immune	• New paternity • Interval between pregnancies • Limited seminal fluid exposure

early-onset preeclampsia is most often associated with fetal growth restriction that necessitates iatrogenic preterm delivery. In more serious cases, preeclampsia can also be associated with hemolysis, elevated liver enzymes, and low platelets – known as "HELLP syndrome" – which is viewed as an exaggerated form of preeclampsia[25]. The heterogeneity of preeclampsia highlights the complex etiological nature of the syndrome, and represents a significant clinical challenge for developing effective therapeutic strategies to prevent, diagnose, and manage the disease.

1.2.4. Clinical management of preeclampsia

Preeclampsia can be predicted with reasonable accuracy in pregnant women through a combination of clinical risk factor assessments (e.g. advanced maternal age, ethnicity, family history, etc.), measurement of early pregnancy placental biomarkers (e.g. pregnancy-associated plasma protein A, placental growth factor), placental shape/texture, and uterine artery Doppler[26,27]. In pregnant women identified as being at high risk of developing preeclampsia, low dose acetylsalicylic acid (150 mg/day) is recommended to be commenced in the early stages of gestation (e.g. before 16 weeks) as prophylaxis against early-onset preeclampsia[28]. However, delivery of the baby and placenta is currently the only definitive cure for women presenting with clinical preeclampsia. Hence, clinical monitoring and treatments associated with preeclampsia are primarily aimed at symptom management. Antihypertensive therapy and vigilant monitoring can potentially extend the duration of pregnancy in affected women, improving fetal outcomes through the avoidance of preterm delivery. In women with late-onset preeclampsia, delivery is associated with good maternal and fetal outcomes; however, in early-

onset preeclampsia where severe symptoms can jeopardize both maternal and fetal health, the goal is to prolong pregnancy to as close to term as possible, so as to maximize fetal survival and minimize the risk of perinatal death or severe handicap, all while ensuring maternal safety[11].

The following are recommended treatment guidelines established by the SOGC[11]. The most immediate objective for a patient presenting with preeclampsia is to control hypertension. Initial antihypertensive therapy can comprise of nifedipine, hydralazine, or labetalol, with oral methyldopa or nitroglycerin as alternative antihypertensive medications. If maternal hypertension becomes uncontrollable despite measures taken to lower blood pressure, especially in the presence of other symptoms (e.g. chest pain, breathlessness), the risk of eclamptic convulsions is increased. Magnesium sulfate in this context is the first-line neuroprotective agent to prevent and/or treat eclampsia, while pregnancies threatened by iatrogenic preterm delivery before 35 weeks are also treated with maternal corticosteroids to promote fetal pulmonary maturation.

When symptoms become unmanageable and either the severity of hypertension and/or organ impairment threatens maternal health, iatrogenic delivery is required. Induction of labour is usually possible in most instances of late-onset disease, while many women with early-onset severe disease merit Caesarean delivery to reduce strain on the underdeveloped fetus.

1.2.5. Theory of preeclampsia pathogenesis

Although the modern description of preeclampsia had been established as early as the early 1900s[29], the precise etiology of preeclampsia remains unclear. Termed the “disease of

theories", various pathological origins of preeclampsia have been proposed, the most widely accepted theory being that preeclampsia is of placental origin.

In 1991, Redman proposed the original two-stage model of preeclampsia, which has remained the mainstay theory for the past 3 decades[30]. Stage 1 of this model involves poor spiral artery remodeling during early placentation, leading to ischemia-reperfusion injury and oxidative stress of the placental villi later in pregnancy. Stage 2 of this model is the clinical stage manifested as maternal hypertension and proteinuria, as a direct consequence from placental dysfunction.

As research progressed and new findings were reported, the two-stage model of preeclampsia remained a stable base, with progressive refinements to the theory proposed. In 1993, Roberts and Redman conjectured that endothelial dysfunction is the link between placental malperfusion and maternal disease: the dysfunctional placenta arising from the Stage 1 insult releases factors that disrupt normal maternal endothelial and vascular function, resulting in Stage 2 clinical presentation[31]. In 1999, Redman et al. integrated the two-stage model of preeclampsia with the concept of "placental" and "maternal" types of preeclampsia, proposed by Ness and Roberts[32,33]. In this refinement, it was proposed that preeclampsia is not necessarily solely caused by placental dysfunction, but that vascular inflammation and endothelial dysfunction caused by stress factors released by the placenta are responsible for the clinical manifestations of preeclampsia. Women with chronic systemic inflammation (e.g. obesity) are therefore predisposed to "maternal" preeclampsia, whereby the stimulus required to reach a critical level of systemic vascular inflammatory dysfunction and elicit signs of preeclampsia is lower and could be in the absence of placentation defects. In 2014, Redman et

al. further proposed two distinct pathways to "placental" preeclampsia: an extrinsic pathway, where early spiral artery remodeling defects preclude normal placental function and result in severe early-onset preeclampsia marked by fetal growth restriction; and an intrinsic pathway, where placental overgrowth relative to uterine capacity compresses the placental villi, impedes normal intervillous perfusion, and induces uteroplacental malperfusion at term[24], thereby contributing to late-onset preeclampsia. Around the same time, Redman also proposed a "six-stage" theory of placental preeclampsia[34], which described pre-conception risk factors (Stage 1); blastocyst implantation (Stage 2); defective placentation (Stage 3); abnormal release of placenta-derived factors (Stage 4); clinical presentation (Stage 5); and atherosis of placental vessels resulting in further reduced uteroplacental perfusion (Stage 6). Finally, Staff proposed a revision in 2019 to the working model of preeclampsia to include risk modifiers to the Stage 1 insult[22], such as inadequate immune tolerance to allogeneic fetal antigens[35], dysfunctional trophoblast-immune cell interactions in the spiral artery remodeling process[36], excessive trophoblast senescence[37], and pre-existing maternal factors such as diabetes, obesity, and advanced age[38].

1.2.6. Physiologic and molecular characteristics of preeclampsia

Despite the widespread acceptance that the placenta is the origin of preeclampsia, the molecular etiology of preeclampsia is unknown. Given the clinical heterogeneity of the disease, it is likely that there is no single pathway leading to the development of preeclampsia, rather various combinations of molecular insults resulting in placental and/or endothelial dysfunction could contribute to disease pathogenesis[33]. To that end, research has identified that

dysregulation in placental, cardiovascular, and immune-related pathways could be involved in mediating placental dysfunction and the associated clinical manifestations of preeclampsia (Figure 1.2).

1.2.6.1. Placental pathologies in preeclampsia

One of the hallmarks of preeclampsia, especially severe early-onset preeclampsia with fetal growth restriction, is poor placental development that is associated with abnormal release of proteins and syncytiotrophoblast debris. Early molecular insults cascade into significant disruption of normal placental function that ultimately results in the preeclampsia syndrome.

1.2.6.1.1. Deficient spiral artery remodeling

Impaired spiral artery remodeling precludes many cases of severe, early-onset preeclampsia. This typically manifests as shallow invasion of invasive EVTs, improper replacement of spiral artery endothelial cells by eEVTs, inadequate removal of vascular smooth muscle cells by iEVTs, or a combination of these[39]. These adaptations result in impaired uteroplacental perfusion necessary for normal placental function and fetal development; pathologists describe this disease as the maternal vascular malperfusion (MVM) spectrum. Although the molecular mechanisms contributing to MVM pathology of the placenta are poorly understood, it is well accepted that defective invasion of EVTs into the decidua and myometrium represent one of the first steps initiating this pathology.

EVT invasion involves epithelial-to-mesenchymal transition (EMT) of EVTs, which includes the acquisition of a motile and invasive phenotype[40]. Although the mechanisms

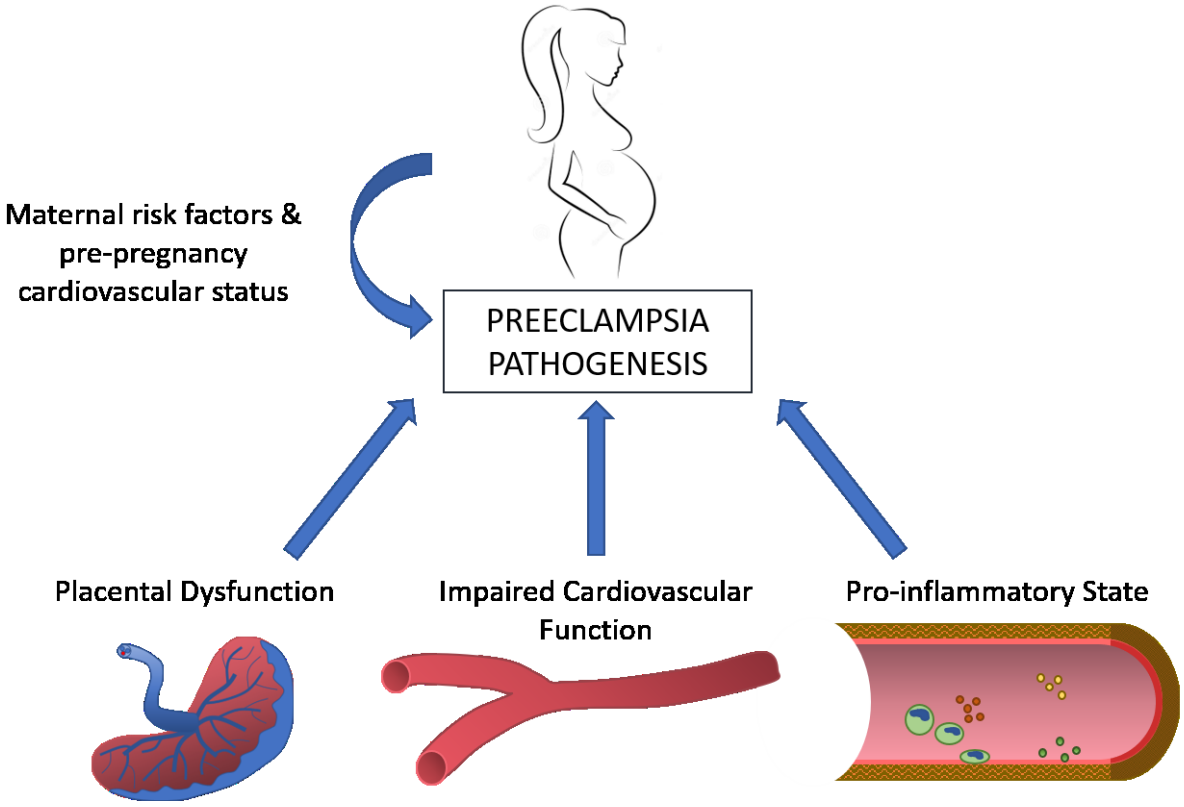

Figure 1.2. Pathways that contribute to the pathogenesis of preeclampsia.

Preeclampsia is a heterogeneous disorder where any or a combination of placental dysfunction, impaired cardiovascular function, and excessive inflammation can contribute to disease pathogenesis. (Adapted with permission from Wat et al., 2018[9]).

controlling EMT in trophoblasts are poorly defined, the downstream effects leading to increased invasiveness involve the differential expression of cell adhesion molecules. For example, the expression of integrins such as α1β1 increases motility through the extracellular matrix; in preeclampsia, the trophoblast-specific expression of this integrin in placental bed biopsies is reduced[41]. Furthermore, EVTs retain elevated expression of E-cadherin in preeclampsia, which is associated with an epithelial-like phenotype with reduced invasiveness[42,43]; the invasive ability of EVTs isolated from preeclamptic placentas is significantly reduced compared to those from healthy controls[44]. Impaired EMT may therefore contribute to reduced invasion of EVTs in preeclampsia, although the mechanism for failed EMT in EVTs has yet to be elucidated.

During the early first trimester, trophoblast plugs prevent maternal blood flow into the placenta to maintain a hypoxic environment until approximately 10 weeks into gestation to keep cytotrophoblasts in a proliferative state. Increased oxygenation stimulates differentiation of the trophoblast pool into the invasive EVT phenotype, thereby promoting their invasion and remodeling process[45–47]. It has been speculated that early trophoblastic plug dissolution could lead to reduced trophoblast proliferation and premature differentiation, leading to impaired spiral artery remodeling[48]. While attractive, this hypothesis has not been fully tested.

Another possible mechanism that could mediate impaired spiral artery remodeling are defects in the decidual environment through which the EVTs invade. In preparation for pregnancy, the endometrium undergoes morphological and functional changes to accommodate for implantation, a process known as decidualization. Part of the decidualization process involves an influx of natural killer (dNK) cells, T regulatory cells, and macrophages that

play a role in facilitating EVT invasion and spiral artery remodeling through the release of cytokines and chemokines[49–51]. dNK cells isolated from first trimester decidual tissues characterized by high uterine artery resistance – which is associated with reduced spiral artery remodeling[52] – exhibited altered gene expression, phenotype, and effects on *in vitro* trophoblast function, compared to those isolated from pregnancies with normal uterine artery indices[53]. Decidual macrophages also exhibit a pro-inflammatory phenotype that could inhibit EVT invasion or even trigger EVT apoptosis[54–57], reducing the extent of spiral artery remodeling. Altogether, aberrations in these processes related to spiral artery remodeling during early pregnancy could contribute to eventual manifestation of preeclampsia.

1.2.6.1.2. Oxidative stress

Oxidative stress occurs as a result of imbalance between the formation and removal of reactive oxygen species (ROS), including superoxide, hydroxyl radicals, and hydrogen peroxide. ROS are generated as part of normal cell metabolism, especially through mitochondrial respiration. ROS are physiologically important and are known to participate in cell signaling, such as oxygen sensing and angiogenic pathways[58,59]. For example, prior to the dissolution of trophoblastic plugs and increased blood flow into the placenta during early gestation, the low oxygen environment results in lower mitochondrial respiration and ROS levels, which stimulate vascular endothelial growth factor expression to promote local angiogenesis[60,61]. Antioxidants, such as superoxide dismutase, catalase, vitamin C, carotenoids, and other scavenger molecules, prevent accumulation of ROS which could disrupt cell and protein function.

Preeclampsia is associated with excess oxidative stress[62]. Failure of proper spiral artery remodeling results in the preservation of the vascular smooth muscle layer, resulting in high-resistance blood flow and ischemia-reperfusion injury[7]. Ischemia-reperfusion injury stimulates the production of superoxide, resulting in damage to the villous cytotrophoblasts and syncytiotrophoblast[63,64]. Chronic excess production of ROS eventually depletes the antioxidant capacity, resulting in damage to the placental villi; this is exacerbated in some cases of preeclampsia where the natural rise in antioxidant activity during pregnancy is absent[65–67]. Ischemia-reperfusion injury increases proliferation of villous cytotrophoblasts, while reducing fusion and formation of the syncytiotrophoblast[68]. This potentially alters the balance of villous cytotrophoblast proliferation and differentiation and disrupts villous integrity and function, which could lead to downstream consequences such as irregular release of angiogenic proteins and reduced support of fetal growth.

1.2.6.1.3. Trophoblast senescence

Chronic oxidative stress is associated with trophoblast aging and senescence through continuous DNA damage; these cells are characterized by significant cellular damage and telomere shortening due to increased turnover[69]. Placental aging is a normal part of pregnancy, with senescence markers such as p21, p16, p53, and Rb proteins expressed by the healthy placenta at term[69]. Overgrowth of the term placenta could lead to malperfusion, which could result in the clinical manifestation of preeclampsia in a manner consistent with the hypothesized intrinsic pathway of preeclampsia[24]. As a result, levels of p21 and p53 are significantly elevated in the placentas from women with preeclampsia, compared to healthy

controls[70]. Preeclampsia is also associated with significantly shorter telomeres in placental trophoblasts relative to uncomplicated pregnancies, although pregnancies complicated by fetal growth restriction in the absence of preeclampsia also exhibit this feature[71].

Emerging evidence suggests that oxidative stress-driven aging of the placenta is partially mediated by mitochondrial dysfunction. The placenta is a highly metabolic organ, supported by enhanced adenosine triphosphate (ATP) production by the trophoblast mitochondria for energy. However, ATP production through oxidative phosphorylation also results in the generation of ROS[72]. As the antioxidant capacity of the trophoblast declines, ROS-mediated damage to the mitochondria increases. Mitochondrial DNA is also more susceptible to ROS-mediated damage due to the absence of histones, leading to mitochondrial dysfunction and deteriorating ATP production[73]. Continued loss of ATP and disrupted metabolism can eventually lead to cellular senescence and apoptosis, contributing to placental dysfunction[74].

1.2.6.1.4. Villous trophoblast pathology

Damage to the placental villi by ischemia-reperfusion injury and oxidative stress accelerates apoptosis and necrosis of the syncytiotrophoblast, which is especially susceptible to oxidative stress due to low antioxidant capacity[65,67]. This results in the excessive formation of "syncytial knots" and the release of syncytial debris and extracellular vesicles into the maternal circulation[75].

Syncytial knots are transcriptionally active, multinucleated structures that form during normal pregnancy[76]. They can also detach from the placental villi and enter the maternal circulation to deposit into capillary beds, such as that of the lungs[77], where they form

biologically active clusters of placenta-derived cells. These placenta-derived debris are present in normal pregnancies and are thought to reflect placental maturity[78]. Placenta-derived debris activate the maternal endothelium and contribute to the systemic inflammatory state of normal pregnancy[79], and there is some evidence that these deported trophoblasts contribute to maternal tolerance to the fetus[80,81]. However, these structures are formed in greater amounts in preeclampsia[77], likely due to greater levels of oxidative stress and enhanced apoptosis of the syncytiotrophoblast, as well as altered hemodynamics at the placental surface consequent to poor spiral artery remodeling causing shear stress on the syncytium and increased liberation of the structures[75]. In addition, preeclamptic syncytial knots produce greater amounts of the anti-angiogenic protein soluble fms-like tyrosine kinase-1 (sFlt1) that can contribute to systemic endothelial dysfunction[82]. Meanwhile, extracellular vesicles can carry damage-associated molecular patterns (DAMPs) such as high mobility group protein B1 (HMGB1) which can trigger inflammatory responses in the maternal circulation. Ischemia-reperfusion injury can also inhibit fusion of the syncytiotrophoblast[68], resulting in syncytial shedding of pro-inflammatory necrotic debris into the maternal circulation. Altogether, increased shedding of placenta-derived debris can potentially overload the maternal capacity to combat basal levels of inflammation, contributing to the clinical manifestation of preeclampsia[83].

1.2.6.2. Cardiovascular impairment

Although the placenta plays a crucial role in the pathogenesis of preeclampsia[22], involvement of the cardiovascular system in the disease is clearly evident[84]. In particular, the

hypertensive feature of preeclampsia is hypothesized to be driven largely by systemic endothelial dysfunction.

1.2.6.2.1. Endothelial dysfunction

In normal physiology, appropriate responses by vascular endothelial cells to vasoactive molecules are necessary to maintain normal hemodynamics, including systemic vascular resistance and blood pressure. This aspect of endothelial function is even more important in pregnancy: dilation of the vasculature is needed to reduce peripheral vascular resistance and ensure adequate circulation of blood throughout the body and to the placenta as cardiac output and blood volume rise during pregnancy[85]. Nitric oxide (NO), a potent vasodilator produced by endothelial cells, is a central mediator of responses to vasoactive stimuli, such as vasodilatory proteins or flow-mediated stress. Pregnant women in normal pregnancy elicit a greater flow-mediated dilation response compared to non-pregnant subjects[86], suggesting enhanced endothelium-dependent NO synthesis and activity during pregnancy. Increases in circulating estrogen may contribute to this adaptation, as estrogen is elevated in pregnancy and can stimulate the production of NO by upregulating expression of NO synthase[87]. By contrast, vascular responses to vasoconstrictor stimuli such as epinephrine are reduced in healthy pregnancy[88].

Endothelial dysfunction occurs when vascular endothelial cells respond to vasoactive molecules in a dysregulated manner. Endothelial dysfunction is associated with exaggerated responses to vasoconstrictor stimuli and diminished responses to vasodilatory stimuli, mediating a pro-inflammatory and pro-thrombotic phenotype. Asymptomatic pregnant women

in early second trimester who subsequently developed preeclampsia demonstrate impaired flow-mediated dilation compared to normotensive women[89]. Small resistance arteries isolated from the omentum of preeclamptic women also responded less to acetylcholine- and bradykinin-mediated vasorelaxation, relative to arteries isolated from normotensive pregnant women[90,91]. Furthermore, abnormal retention of spiral artery endothelial cells during pregnancy is associated with their activation[92]; these vessels would likely contribute to the release of vasoconstrictor molecules. This pathway is hypothesized to accelerate preeclampsia disease pathogenesis in the second trimester, where vasoconstricted spiral arteries can introduce turbulent blood flow into the placenta and damage the placental villi[7], resulting in placental dysfunction.

1.2.6.2.2. Angiogenic imbalance

Normal endothelial function is maintained by the presence of pro-angiogenic and anti-angiogenic proteins in the circulation which are in contact with vascular endothelial cells. These proteins impact the production of NO and mediate vasomotor responses. In pregnancy, the placenta contributes to the maintenance of normal endothelial function by secreting balanced levels of pro- and anti-angiogenic proteins that enter the maternal circulation and mediate maternal cardiovascular adaptations to pregnancy. In general, a healthy pregnancy is characterized by high levels of pro-angiogenic proteins (e.g. placental growth factor [PlGF]) and low levels of anti-angiogenic proteins (e.g. sFlt1)[93].

Vascular endothelial growth factor (VEGF) is one of the most potent and well-characterized family of angiogenic proteins. The VEGF family of ligands include four VEGF

isoforms (VEGF-A, -B, -C, and -D) and PlGF. These ligands interact with one or a combination of its three receptor tyrosine kinases, vascular endothelial growth factor receptor (VEGFR)-1, -2, or -3. These receptors interact with VEGF family ligands with varying affinity and differ in their kinase activity: VEGFR-2 has potent kinase signaling activity, while VEGFR-1 has very weak kinase activity. Indeed, VEGFR-1 is often referred to as a "decoy" receptor that acts as a negative regulator of VEGF-A signaling activity. Vascular endothelial cells express VEGFR-1 and VEGFR-2 receptors, and these receptors can interact with VEGF-A and PlGF. Specifically, VEGF-A interacts with VEGFR-1 and VEGFR-2 with high- and low-affinity, respectively, while PlGF interacts exclusively with VEGFR-1.

Normal healthy pregnancy is associated with high circulating levels of PlGF and VEGF, resulting in a high pro-angiogenic signal on the vascular endothelial cell surface. It is hypothesized that PlGF interacts with the decoy receptor VEGFR-1 and displaces VEGFR-1/VEGF-A interactions, elevating local unbound levels of VEGF-A; VEGF-A is then available for interaction with VEGFR-2 on the endothelial cell surface to mediate strong pro-angiogenic signaling kinase activity and promote normal endothelial function[94]. The importance of PlGF in the maintenance of normal maternal cardiovascular function during pregnancy is highlighted by recent studies demonstrating the predictive value of PlGF for ruling out a diagnosis of preeclampsia[95–97]. PlGF is now endorsed as a diagnostic tool for preeclampsia by the National Institute for Health and Care Excellence in the United Kingdom[98,99] and formally implemented as a hospital-wide program at Mount Sinai Hospital in Toronto, Canada[100].

Endothelial dysfunction is a hallmark of preeclampsia, thought to be contributed by the presence of dysregulated angiogenic proteins in maternal blood. The preeclamptic placenta

releases excess levels of sFlt1[101], a splice variant of the VEGFR-1 receptor lacking the transmembrane and intracellular domains and therefore released into the maternal circulation as a soluble protein. As a soluble form of the VEGFR-1 receptor, sFlt1 lacks kinase signaling activity but can bind PlGF and VEGF-A[101]. In addition, circulating sFlt1 can also potentially displace PlGF and VEGF-A from the cell surface, thereby lowering the local concentration of the pro-angiogenic proteins. sFlt1 is therefore a strong anti-angiogenic signal in preeclampsia that antagonizes the actions of PlGF and VEGF-A, which contributes to systemic endothelial dysfunction and ultimately the hypertensive preeclampsia phenotype[101]. Removing excess sFlt1 via apheresis in early-onset preeclamptic patients was determined to reduce proteinuria and prolong pregnancy[102], highlighting the importance of sFlt1 in the manifestation of preeclampsia and its consequences.

It is unknown why the placentas of women who develop preeclampsia secrete excess amounts of sFlt1. sFlt1 is a splice variant, suggesting that the release of sFlt1 by the preeclamptic placenta is a regulated process. Oxidative stress and hypoxia induces sFlt1 production and release by the placenta[103,104], suggesting the possibility that this mechanism is a maladaptive response to increase maternal vascular resistance in an attempt to sustain perfusion of oxygenated blood into the placenta, so to satisfy the oxygen and metabolic demands of the developing fetus.

1.2.6.2.3. Hemodynamic impairment

In addition to the role of systemic vascular endothelial dysfunction in the manifestation of preeclampsia, there is strong evidence that abnormal cardiac and hemodynamic adaptations

to pregnancy are associated with the development of severe early-onset preeclampsia[84]. Normally, the maternal cardiovascular system adapts to pregnancy through hemodynamic changes to meet the demands of the developing fetus, including increased cardiac output, increased blood volume, and increased heart rate, counter-balanced by reduced total peripheral vascular resistance to maintain normal blood pressure and organ perfusion[85]. Some of these changes are detectable as early as the first trimester[85]. Pregnant women in mid-second trimester destined to develop early-onset preeclampsia exhibit significantly lower cardiac output, lower heart rate, and increased peripheral vascular resistance[105,106]. Interestingly, whereas early-onset preeclampsia was associated with the high vascular resistance and low cardiac output, late-onset preeclampsia was associated with abnormally low vascular resistance and high cardiac output[105,106], suggesting differences in etiologies between the two phenotypes.

Abnormal cardiovascular adaptations to pregnancy led Kalafat and Thilaganathan to postulate the "cardiovascular origin of preeclampsia" theory, which argues that placental dysfunction is secondary to cardiovascular dysfunction[84]. These authors propose that preeclampsia is a result of the maternal cardiovascular system failing to properly adapt to pregnancy, thereby not meeting fetoplacental metabolic demands and triggering placental dysfunction that further accelerates deterioration of the normal vascular function. Although this theory does not currently unify all the phenotypes of preeclampsia observed clinically, the theory highlights the etiological complexity and the multi-system involvement of preeclampsia.

1.2.6.3. Inflammation

Normal pregnancy is associated with a basal level of systemic inflammation[33]. In most healthy pregnant women, pregnancy-induced inflammation is appropriately managed by anti-inflammatory pathways that protect against endothelial activation and/or dysfunction and feedforward pro-inflammatory pathways. However, it is thought that a hyper-inflammatory state can also contribute to the development of preeclampsia, as a result of excess release of placenta-derived factors or pre-existing cardiovascular disease that lowers the threshold to dysfunction[32,33].

1.2.6.3.1. Complement activation and HELLP syndrome

The complement system is part of the innate immune system that targets invading pathogens and foreign matter. Against pathological organisms such as bacteria, complement results in the formation of membrane attack complexes that create pores on the cell surface and allow the influx of water to induce cell lysis. Upon activation of complement, potent pro-inflammatory mediators are also generated, notably the C3a and C5a anaphylatoxins. These mediators bind to their respective cell surface receptors on leukocytes and endothelial cells to elicit various inflammatory responses[107]. However, complement activation does not only occur on bacterial cell surfaces – host antigens can also initiate complement activation. Host antigens can include DAMPs such as HMGB1[108], apoptotic or necrotic cells[109], and cells that do not display adequate complement inhibitory molecules on their cell surface[110], all of which are present in preeclampsia.

As such, preeclampsia is associated with excessive complement activation, potentially contributing to disease pathogenesis. The placental villi of preeclamptic women exhibit complement deposition that is otherwise absent in healthy placenta[110–112]. Increased complement activity can also be observed in the plasma and urine of preeclamptic women, including elevated levels of C3a, C5a, and membrane attack complexes, which can potentially be used as predictors of preeclampsia[113–115]. In animal models of preeclampsia, inhibition of complement activation ameliorated features of preeclampsia, including elevated blood pressure and proteinuria[116,117], thereby highlighting the potential importance of complement activation to endothelial dysfunction.

Furthermore, HELLP syndrome, viewed as an exaggerated form of preeclampsia[25], is also associated with dysregulated complement activation. In humans, genetic polymorphisms to complement inhibitory factors are associated with HELLP syndrome and preeclampsia, including Factor H, Factor I, and membrane cofactor protein[118,119]. In an interesting case study, eculizumab (Soliris®), a neutralizing monoclonal antibody against complement protein C5, normalized blood pressure and halted disease progression in a pregnant women presenting with HELLP syndrome[120]. An additional study determined that incubation of serum from HELLP patients with eculizumab reduced *in vitro* lysis of model cells[121], suggesting that hemolysis observed in HELLP patients is caused by complement activation and membrane attack complex formation on erythryocytes.

1.2.6.3.2. Cytokine dysregulation

The plasma of pregnant women with preeclampsia is characterized by increased pro-inflammatory and reduced anti-inflammatory cytokines and chemokines[122]. Elevated levels of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) have been directly linked to endothelial activation and dysfunction in preeclampsia[123–125], potentially by reducing expression of endothelial NO synthase[126]. TNF-α and IL-6 can also increase trophoblast shedding, which can further stimulate endothelial activation and dysfunction through the production of sFlt1[82,127]. Endothelial activation by TNF-α and IL-6 can result in the expression of cell adhesion molecules and increased vascular permeability[128], contributing to the peripheral edema commonly observed in women with preeclampsia. Meanwhile, IL-10, an anti-inflammatory cytokine that stimulates endothelial NO synthase expression and promotes endothelium-dependent relaxation[129], is reduced in preeclampsia[130]. Supplementing IL-10 in a rat model of preeclampsia reduced blood pressure and normalized pro-inflammatory cytokines[131], demonstrating its potential importance in maintaining the balance of inflammation during pregnancy.

1.2.6.4. Abnormal hemostasis

Compared to non-pregnant women, pregnant women are in a hypercoagulable state[132], which is thought to be required to prevent excessive blood loss during childbirth. In normal pregnancy, markers of hypercoagulation are elevated to levels associated with acute thrombotic events[132]; this risk is reduced by a concomitant elevation of anticoagulant mechanisms[133]. Preeclampsia has been associated with increased risk of thrombosis, including placental thrombosis and venous thromboembolism[134–136]. Compared to normal pregnancy,

preeclampsia is associated with increased levels of coagulant proteins and reduced levels of coagulation inhibitors in the blood[137]. Placental expression of tissue factor, a key cell surface initiator of coagulation, is also elevated[138,139]. Syncytiotrophoblast-derived microvesicles released by the preeclamptic placenta also display elevated tissue factor activity[140]. These observed changes have resulted in the speculation that thrombosis contributes to the pathogenesis of preeclampsia. However, despite significant findings from individual studies[141], meta-analyses have concluded there is no association between thrombophilia and preeclampsia[142–144]. The association of thrombosis and preeclampsia is likely confounded by other factors, such as excess inflammation and antiphospholipid syndrome, in which pregnant women are at increased risk of thrombosis, pregnancy loss, and preeclampsia[145].

1.3. Therapies for the Prevention of Preeclampsia

Delivery of the placenta and fetus is the only definitive cure for preeclampsia. Clinical management of preeclamptic patients involves symptomatic relief and supportive measures, such as antihypertensive and anticonvulsant medications, and corticosteroids for fetal lung maturation. When the disease becomes refractory to treatment or maternal and/or fetal mortality risk increases, iatrogenic delivery is indicated, even at pre-term stages of pregnancy. Hence, therapies to prevent the development of preeclampsia have been explored.

1.3.1. Low-dose acetylsalicylic acid for the prevention of preeclampsia

Acetylsalicylic acid (ASA; e.g. aspirin) is an approved preventative therapy against preeclampsia as established by SOGC guidelines[11]. The first reported case study demonstrating

the therapeutic potential of ASA was in 1978, where restoration of platelet count using ASA significantly improved symptoms at 22 weeks and prolonged pregnancy until 34 weeks[146]. Clinical trials have since revealed a protective effect of low-dose ASA (>75 mg daily dose) for the prevention of preeclampsia by approximately 10-50%[28,147], meaning that ASA does not prevent preeclampsia in at least half of the women on this therapy. Recent meta-analyses demonstrated that treatment may be most effective for the prevention of early-onset preeclampsia when initiated before 16 weeks of gestation[28,148], but the effect of timing is still under debate[149]. Due to well-established safety[150], ease of access, cost effectiveness[151], and patient familiarity of the drug, ASA is currently the only widely used pharmacologic therapy for the prevention of preeclampsia.

1.3.1.1. Mechanism of action of ASA

As a non-steroidal anti-inflammatory drug, the original rationale for using ASA for the prevention of preeclampsia is for its anti-platelet and anti-inflammatory activity. By reducing platelet activation, it was speculated that ASA would reduce platelet adhesion and endothelial activation that would otherwise result in endothelial dysfunction[152]. ASA also reduces cyclooxygenase-mediated production of the pro-inflammatory mediator thromboxane A2, which is increased in high risk preeclamptic patients and involved in mediating vasoconstriction[153]. Recent studies demonstrated that ASA can reduce hypoxia- or preeclamptic serum-induced apoptosis of BeWo choriocarcinoma cells[154]; inhibit sFlt1 secretion[155,156]; improve trophoblast migration and invasion[156,157]; promote endothelial cell tube formation[156]; improve trophoblast-endothelial interactions[158]; increase PlGF from trophoblasts[154]; and

modulate cytokine production from preeclamptic serum-treated BeWo cells[154]. In a lipopolysaccharide model of preeclampsia, ASA reduced trophoblast nuclear factor-kappa B inflammatory signaling[157]. Hence, these newer studies suggest that the effects of ASA in preeclampsia extend beyond that of antiplatelet activity, and provide a possible explanation for why ASA therapy may be most beneficial for patients at risk of early-onset preeclampsia typically presenting with placental dysfunction[159].

Despite its established preventative effects against the development of preeclampsia, not all pregnant women at high-risk of early-onset preeclampsia respond to ASA therapy. Hence, other therapies to complement ASA prophylaxis are being explored.

1.3.2. Experimental therapies for the prevention of preeclampsia

For its vasodilatory activity, the potential use of sildenafil for the prevention of preeclampsia has been explored. Sildenafil is a phosphodiesterase 5 (PDE5) inhibitor that enhances cyclic guanosine monophosphate signaling and NO production. PDE5 is expressed in the uterine vasculature[160]; it is hypothesized that sildenafil could dilate uterine vessels, improve blood flow into the placenta, and promote normal placental function. Inhibition of PDE5 significantly improved vasorelaxation of myometrial arteries isolated from preeclamptic women[161]. In animal models of preeclampsia, sildenafil was determined to improve fetal growth and reduce hypertension[162]. However, sildenafil treatment resulted in several fetal deaths due to lung complications in a recent multinational trial (STRIDER)[163], highlighting safety concerns with this potential therapy that require further investigation.

Use of metformin, an approved drug for treating type 2 diabetes mellitus, during pregnancy has been associated with reduced incidence of preeclampsia[164]. Metformin reduces the production of sFlt1 from endothelial cells, villous cytotrophoblasts, and preeclamptic placental villous explants; improves angiogenic tube formation disrupted by sFlt1; and improves vascular relaxation of vessels impaired by preeclamptic placenta conditioned media[165]. This mechanism may be related to metformin's ability to inhibit mitochondrial electron transport chain activity that is overactive in preeclampsia and may contribute to increased ROS production and related damage[165]. However, most clinical data to date are from trials investigating its effects on gestational diabetes mellitus[164]; randomized clinical trials specifically looking at its effects on preventing preeclampsia have not been conducted but are underway[166,167].

Statins can improve vascular function via heme oxygenase-1-mediated induction of NO synthase expression[168] and reduction of sFlt1 production[169]. Animal studies show favorable results with pravastatin, including increased PlGF production[170]; reduced placental apoptosis[171]; and improved angiogenic and placental hypoxic balance[172]. A recent pilot study demonstrated that pravastatin can improve circulating angiogenic profile in high risk pregnant women[173]. At present, clinical data on the effectiveness of pravastatin for the prevention of preeclampsia are restricted to epidemiological and several pilot studies, and larger randomized clinical trials are needed.

1.3.3. Heparin therapy for the prevention of preeclampsia

Perhaps the longest standing experimental therapy for the prevention of preeclampsia is low molecular weight heparin (LMWH), an anticoagulant drug. The first reported use of heparin for the treatment of preeclampsia dated back to 1948 by Maeck and Zilliacus: treatment of several preeclamptic patients with heparin significantly ameliorated symptoms, including a case of postpartum eclampsia[174]. The hypothesized mechanism of action was related to the reduction of fibrin deposition and thrombotic lesions in the placenta. However, as penned by an author in a subsequent publication, "it is difficult to believe that heparin can remove or dispel the fibrin already deposited in the placenta in severe preeclampsia. The only rational place for heparin therapy would be for incipient pre-eclampsia before there is any significant deposition of fibrin."[175] In 1986, Capetta et al. used heparin in combination with dipyridamole (an anti-platelet drug), demonstrating that prophylactic therapy initiated at 13 weeks of gestation significantly improved pregnancy outcomes in patients with histories of recurrent placental insufficiency, preeclampsia, and/or perinatal death[176]. North et al. in 1995 conducted a retrospective analysis which concluded that heparin in conjunction with ASA was superior in preventing preeclampsia among patients with renal disease compared to ASA therapy alone[177]. Since then, several trials have also concluded that LMWH prophylaxis can augment the effect of ASA and reduce the risk of preeclampsia by as much as 50% relative to ASA alone[178–182]. However, there are also studies, including a few multicenter randomized clinical trials, which demonstrated no effect of heparin therapy on reducing the incidence of preeclampsia[183–186]. With conflicting evidence over the therapeutic potential of heparin therapy for the prevention of preeclampsia, it is imperative to delineate molecular mechanisms of heparin that could play a role in altering the trajectory of disease pathogenesis.

1.3.3.1. Advantages and disadvantages of heparin as a therapy for preeclampsia

Unfractionated heparin and LMWH are well-established therapeutics in the context of hemostasis. Their toxicity profiles are well known, and their anticoagulant effects can generally be reversed using protamine sulfate. Heparin-based therapies are also safe to use during pregnancy as these drugs do not cross the placenta and harm the fetus[11].

However, as an anticoagulant, there is risk of exacerbating bleeding that may occur during pregnancy, such as cases of placenta abruption where the placenta detaches from the uterine wall before delivery (although heparin therapy is associated with reduced risk of placenta abruption[187]). In the absence of major bleeding events, local bruising in the injection area may cause discomfort for the patient and reduce compliance[188]. Heparin-based therapies are also associated with a risk of developing heparin-induced thrombocytopenia, although this risk is significantly reduced with LMWH therapy compared to unfractionated heparin[189]. Prolonged use of heparin-based therapies can reduce bone density and increase the risk of osteoporosis and bone fractures[190], although recent studies have demonstrated negligible risk with LMWH use[191]. Finally, heparin anticoagulation is contraindicated with most anesthesia agents that may be required during an emergency surgical procedure or delivery.

1.3.3.2. Molecular structure of heparin

Heparin is a naturally occurring polysaccharide found predominantly in the mast cells and basophils in humans, while therapeutically used unfractionated heparin is isolated from animal tissues (e.g. porcine intestinal mucosa). Heparin is a heterogeneously long, unbranched,

and highly sulfated glycosaminoglycan composed of repeating disaccharide units of uronic acids and amino sugars, namely glucuronic acid (GlcA), iduronic acid (IdoA), and glucosamine (GlcN). It is structurally similar to heparan sulfate, which is found on most cell surfaces covalently associated with proteoglycans but differs in the proportions of specific uronic acids and level of sulfation. Heparin contains more IdoA residues and is more sulfated than heparan sulfate, while the predominant sequence of heparan sulfate is composed of mostly N-acetylated GlcN and GlcA[192]. Heparin and heparan sulfate often interact with similar molecules; due to the increased level of sulfation, heparin binds to many heparin-binding proteins with greater affinity[193], and can modulate heparan sulfate activity by displacing heparin-binding proteins associated with heparan sulfate on cell surfaces. However, certain molecular interactions of heparan sulfate require a specific balance of sulfation and acetylation that cannot be readily mimicked by heparin without structural modifications[193].

Isolated heparin varies widely in length, with molecular weights ranging from 6-60 kDa and a mean of 14-18 kDa. Certain functions of heparin are chain-length dependent[194], so batch-to-batch variation may yield inconsistent effects on coagulation. Processing of unfractionated heparin into LMWH through chemical means significantly reduces the structural and functional variability, and LMWHs have gradually replaced unfractionated heparin for most indications, due to increased predictability of effects and safety[189]. Different methods of processing unfractionated heparin into LMWH have been used; commonly used LMWH derivatives include dalteparin, enoxaparin, nadroparin, and tinzaparin (Table 1.2).

Table 1.2. Commonly used low molecular weight heparins in North America.

Low molecular weight heparin	Trade name	Manufacturer	Average molecular weight (Da)	Anti-FXa/ anti-FIIa ratio	Specific Activity (IU anti-FXa/mg)	Preparation
Dalteparin	Fragmin	Pfizer	6000	2.2	130	Nitrous acid depolymerization
Enoxaparin	Lovenox	Sanofi-Aventis	4500	4.0	100	Benzylation with alkaline depolymerization
Nadroparin	Fraxiparine	Aspen Pharmacare	4300	3.5	90	Nitrous acid depolymerization
Tinzaparin	Innohep	Leo Pharmaceutical	4500	2.0	110	Enzymatic digestion

1.3.3.3. Role of heparin in anticoagulation

Within the structure of heparin are antithrombin binding regions (ATBRs), composed of specific pentasaccharide sequences that interact with the natural soluble protein antithrombin (Figure 1.3). Using heparin as a cofactor, antithrombin interacts with the major coagulation enzymes, thrombin and Factor Xa, and irreversibly inhibit their activity. Binding of antithrombin is mediated through several critical sulfate groups precisely arranged within the ATBR[195]. Although these pentasaccharide sequences account for less than 1/3 of the molecule[196], its effects on coagulation are profound, leading to its use in the clinic primarily as an antithrombotic drug.

1.3.3.4. Non-anticoagulant effects of heparin

The longstanding hypothesis is that heparin protects against the development of preeclampsia by preventing placental thrombosis[197]. However, Mello et al. in 2005 and Rey et al. in 2009 demonstrated that LMWH prophylaxis effectively protected against the development of preeclampsia in high-risk pregnant women without thrombophilia[182,198]. In addition, a clinical trial from our group failed to demonstrate a reduction of infarcts in the placenta of women treated with prophylactic unfractionated heparin[199]. Together, these studies suggest that heparin protects against preeclampsia through mechanisms unrelated to anticoagulation.

*A modified version of Section 1.3.3.4 was published in a review article: Wat JM, Audette MC, Kingdom JC. Molecular actions of heparin and their implications in preventing pre-eclampsia. *J Thromb Haemost* 2018; 16(8): 1510-22.

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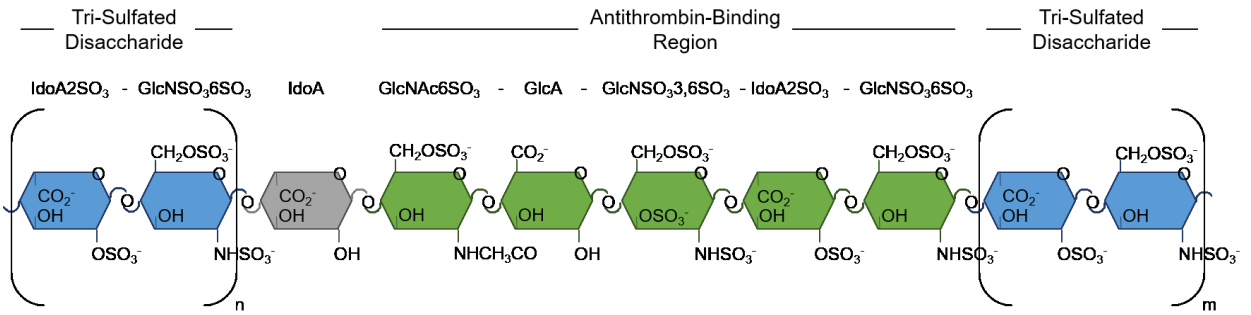

Figure 1.3. Molecular structure of heparin.

Heparin is a glycosaminoglycan of repeating disaccharide units (blue). Its anticoagulant properties mainly reside in the antithrombin-binding region, a specific pentasaccharide sequence (green) responsible for interacting with the serine protease inhibitor antithrombin to catalyze the inhibition of coagulation proteases such as Factor Xa and thrombin. (Adapted with permission from Wat et al 2018[9]).

The concept that heparin has anticoagulation-independent properties is not novel and has been well-explored in the contexts of inflammation and cancer[195]. However, the evidence supporting the non-anticoagulant roles of heparin for the prevention of preeclampsia is limited, and only in recent years has this novel perspective been explored in this specific context, mostly through *in vitro* studies. Heparin's potential non-anticoagulant mechanisms in preeclampsia can be broadly categorized into three pathways – placental, vascular, and anti-inflammatory – each of which may contribute to prevention of the preeclampsia syndrome.

1.3.3.4.1. Placental effects of heparin

Considering the importance of abnormal spiral artery remodeling in the pathogenesis of preeclampsia, several *in vitro* studies have demonstrated that LMWH may be capable of promoting spiral artery remodeling by regulating EVT protein expression and function. JAR choriocarcinoma cells and isolated EVTs from first trimester placental tissues treated with enoxaparin or tinzaparin demonstrated increased expression of matrix metalloproteinases and decreased expression of tissue inhibitors of metalloproteinases; these findings correlated with increased invasion of EVT cells into Matrigel[200]. Enoxaparin-treated EVT-like cells also underwent integrin switching from α6β4 to α1β1, which is associated with increased invasiveness; this effect was dependent on heparin-binding epidermal growth factor signaling[201]. In healthy pregnant rats, LMWH reduced decidual expression of E-cadherin, a cell-adhesion molecule that is elevated in preeclamptic placentas and associated with reduced invasive phenotypes[43,202].

LMWH treatment can also affect placental trophoblast growth and survival. LMWH treatment increased proliferation of primary placental villous cytotrophoblasts and cytotrophoblast-like cells[203–206], which was accompanied by elevated expression of glial cell missing-1 (GCM1), a transcription factor that is required for cytotrophoblast differentiation[205,207]. This effect is thought to be related to the ability of heparin to interact with fibroblast growth factor-4 (FGF4) to stimulate proliferation of villous cytotrophoblasts[208], which can then undergo asymmetric cell division and differentiation to replenish the syncytial layer. These findings suggest that LMWH can promote the healthy turnover of the syncytium and maintain integrity of the placental villi. Interestingly, this *in vitro* effect peaked at sub-therapeutic plasma levels of heparin relative to anticoagulant activity[203,206], suggesting that LMWH may elicit specific effects at varying concentrations. In addition to stimulating proliferation, heparin also protected against apoptosis of trophoblasts[204,209,210] by increasing the expression of the anti-apoptotic factor Bcl-2[210]. Heparin-binding epidermal growth factor was demonstrated to be important for this cell survival mechanism[201,203], the production of which was stimulated by LMWH treatment[211]. These positive effects on placental development could potentially impact overall function, as suggested by elevated secretion of human chorionic gonadotropin and PlGF from LMWH-treated placental villous explants[205].

1.3.3.4.2. Vascular effects of heparin

Given the role of placenta-derived angiogenic proteins mediating systemic vascular dysfunction in the pathogenesis of early-onset preeclampsia, methods to manipulate circulating levels of these proteins have been considered. Administration of recombinant PlGF in a baboon

model of preeclampsia alleviated features of preeclampsia[212], while sFlt1 apheresis in pregnant women with early-onset preeclampsia and high circulating levels of sFlt1 significantly reduced proteinuria and prolonged pregnancy[102]. However, recombinant PlGF therapy would likely be prohibitively expensive as a preventative therapy for a relatively common disorder, while apheresis to remove excess circulating sFlt1 may be more appropriate as an acute treatment since sFlt1 levels rise much later in pregnancy[213]. Hence, a more cost-effective and convenient therapy that can replace these intensive treatments is needed.

In this context, LMWH therapy may promote both PlGF production and sFlt1 elimination. LMWH increased PlGF release from cultured endothelial cells and healthy first trimester placental villous explants[27,205]. Interestingly, LMWH therapy also elevated sFlt1 levels in the blood and in placenta conditioned media[205,214–216]. Consistent with these findings, women undergoing heparin therapy also exhibit elevated levels of both PlGF and sFlt1 during pregnancy[217,218]. The implication of this effect is unknown; while this could be due to *de novo* stimulation of sFlt1 expression from the placental villi[205], LMWH could also be displacing sFlt1 from the surface of vascular endothelial cells[215] and contribute to its urinary elimination[214].

Despite the apparent simultaneous increases in both PlGF and sFlt1 release, conditioned media generated by dalteparin-treated 1st trimester placental villi increased endothelial cell tube formation[219], a surrogate assay of angiogenesis and endothelial function. Microvesicles derived from LMWH-treated healthy pregnant women also significantly increased endothelial cell tube formation[210]. Enoxaparin improved endothelial function when acutely administered to asymptomatic pregnant women at high-risk of developing preeclampsia in the 2nd trimester[27]. Uterine artery resistance index was also reduced in dalteparin-treated pregnant women with a

history of preeclampsia[182]. Collectively, these results suggest that the overall effect of LMWH therapy could restore angiogenic balance to normalize endothelial function, favor angiogenesis, and promote vascular dilation.

Furthermore, virtually all angiogenic growth factors interact with heparins[220], suggesting that the effects of heparin therapy on angiogenic balance in the blood is not restricted to PlGF and sFlt1; heparins can also interact with VEGF-A, the natural ligand for VEGFR1/2[194]. However, chain length may have substantial effects on heparin's activity. When the length of heparin is insufficient to bring two molecules into close proximity for function, heparin may function as an antagonist. In the context of VEGF-A, long chain unfractionated heparin can form a ternary complex with VEGF-A and its receptors, thereby augmenting cell signaling. However, heparin chains below 20 sugar residues long, or approximately 6 kDa in molecular weight, sterically hinder the ligand-receptor interactions, resulting in impaired signaling[194]. For example, while dalteparin (average MW = 6 kDa) induced a pro-angiogenic response using the rat mesenteric artery assay[221], tinzaparin (average MW = 4.5 kDa) was anti-angiogenic in the same experimental system[222]. Furthermore, sulfation – which may be altered from LMWH processing – can also affect heparin activity[223]. Altogether, these findings suggest that the activity of specific LMWHs should not necessarily be extrapolated to others.

1.3.3.4.3. Anti-inflammatory effects of heparin

As a naturally occurring molecule, endogenous heparin is specifically localized to immune mast cells in humans, leading to the postulation that heparin's physiologic function is modulation of inflammatory pathways[224]. One of the most established non-anticoagulant

effects of heparin is anti-inflammation via suppression of complement activation. The specific heparin-complement protein interactions are well-documented and include inhibition of C1q (initiator of the classical complement pathway), augmentation of Factor H (soluble inhibitor of the alternative pathway), and inhibition of various components of the membrane attack complex[224]. In a mouse model of antiphospholipid syndrome, a condition which is highly associated with preeclampsia[145], both unfractionated heparin and LMWH prevented fetal loss by inhibiting complement activation[225]. Accordingly, this effect was not reproduced through administration of fondaparinux (a molecular mimetic of heparin's ATBR) or hirudin (a thrombin inhibitor), indicating that the anticoagulant effects of heparin were not likely responsible for protection against fetal loss in this model. Administration of 40 mg/day LMWH to pregnant women with a history of thromboembolic events during previous pregnancies elicited a reduction in complement activity[226], indicating that prophylactic doses of LMWH are sufficient to elicit anti-complement activity *in vivo*. Thus, heparin may suppress symptoms of preeclampsia caused by dysregulated complement activity.

Preeclampsia is also characterized by widespread endothelial activation caused by dysregulated release of angiogenic factors from the preeclamptic placenta, resulting in increased expression of cell adhesion molecules which permit the adhesion and transmigration of leukocytes[227,228]. Heparin can directly inhibit leukocyte adhesion to the activated endothelial cell surface by interacting with cell adhesion molecules, including endothelial P-selectin and leukocyte L-selectin and CD11b/18[229–231]. By doing so, heparin sterically inhibits their interactions with ligands on the target cell. While heparin does not appear to directly affect expression of these adhesion molecules[232], it is possible that heparin can indirectly influence

their expression by modulating the activity of proteins that do affect their expression, such as cytokines and chemokines[233]. Certain cytokines such as IL-8, which is elevated in the circulation of women with preeclampsia, play a role in endothelial activation[234], and rely on interactions with cell surface heparan sulfate proteoglycans to exert their effects[235]. Soluble heparan sulfate proteoglycans and heparin can competitively inhibit IL-8 interactions with the cell surface receptor, thus suppressing its inflammatory effects[236]. Extracellular histones, a DAMP, may be released as a result of tissue injury and stimulate a pro-inflammatory response that results in endothelial activation and expression of adhesion molecules; heparin is able to interact with these histones and inhibit their activity[237,238]. The presence of extracellular histones has been observed in preeclampsia, forming a complex with neutrophil extracellular traps[239]; this suggests its potential to be a heparin target in preeclampsia. In addition, both heparin and LMWH can inhibit extracellular histone-mediated endothelial cell death and antiangiogenic activity[240].

Another DAMP, the HMGB1 protein, has been implicated in the pathogenesis of preeclampsia. Preeclamptic sera increased trophoblast expression of HMGB1 from first trimester placental explants, which were then carried via extracellular vesicles and contributed to endothelial activation[241]. Heparin can block extracellular vesicle entry into target cells, which may negate the endothelial activation response[242,243]. Furthermore, as shown with murine macrophages, an O-desulfated heparin derivative, which has significantly reduced anticoagulant properties, inhibited the release of HMGB1 during inflammation thereby dampening the inflammatory response[244]. However, it is unknown if heparin can elicit the same effect in trophoblast cells.

Overall, there is strong evidence that heparin exhibits anti-inflammatory functions that may be relevant to preeclampsia, but currently there are limited studies that have directly addressed the potential for these anti-inflammatory properties of heparin to suppress the severity of preeclampsia. Further research is needed to evaluate the role of heparin's anti-inflammatory properties for the prevention of preeclampsia.

1.3.3.5. Non-anticoagulant derivatives of heparin

Considering the possibility that the non-anticoagulant properties of heparin can be specifically exploited for various pathologies including inflammatory diseases, cancer, and preeclampsia, several methods of chemically disrupting the ATBR and producing non-anticoagulant heparins have been devised. Such derivatives of heparin could overcome the dosing limitation that its anticoagulant actions would impose. These methods can be categorized as desulfation-dependent mechanisms or glycol splitting.

1.3.3.5.1. N- and O-desulfation of heparin

The binding of antithrombin to heparin is mediated by several critical sulfate groups within the ATBR[195]. Eliminating these sulfate groups would therefore disrupt the ability of heparin to bind antithrombin and abolish its anticoagulant properties. One method to achieve this is through the chemical removal of N- and O-linked sulfate groups followed by stabilization of these sites with acetyl groups. However, with the goal of producing a heparin-like molecule that lacks anticoagulant activities while preserving all of its other activities, this approach may not be ideal due to the fact that the majority of heparin, including regions outside of the ATBR,

is highly sulfated and thus would be susceptible to the non-specific desulfation process. Extensive modification of heparin's sulfation status could have dramatic effects on its ability to modulate protein activity[193].

1.3.3.5.2. Glycol-splitting of heparin

To circumvent the issue of specificity that plagues the desulfation method, glycol-splitting was devised as a way of specifically disrupting the ATBR without altering the sulfation status of heparin[245]. Rather than targeting sulfated groups, the glycol-split reaction oxidizes all non-sulfated uronic acid residues (mostly GlcA, but also IdoA) that are only found in select locations along the molecule such as the ATBR, thereby "splitting" the C-C bond between the two adjacent hydroxyl groups. While this method does not remove the sulfate groups in the ATBR, the splitting of the uronic acid residues alters the conformation of the ATBR, rendering it non-conducive to antithrombin binding.

As a result of glycol splitting of native heparin, the anticoagulant properties of glycol-split heparins are dramatically reduced, while most of its other functional activities are preserved as observed in experiments in the context of cancer[195]. Interestingly, glycol-split heparins have greater structural flexibility which can enhance protein interactions[245]. The therapeutic potential of glycol-split heparins is highlighted by the discovery and development of tafoxiparin, a glycol-split LMWH currently in Phase IIb clinical trial for enhancing myometrial contractility and cervical ripening during protracted labor[246].

1.4. Animal Models of Preeclampsia

While there are various mechanisms of heparin that could be relevant for the prevention of preeclampsia, the current findings are largely restricted to *in vitro* studies, and the available data surrounding the effects of heparin in *in vivo* models of preeclampsia are sparse. An objective of this thesis was therefore to evaluate the effects of heparin in an animal model of preeclampsia. However, preeclampsia is a disease that only occurs naturally in humans and higher order primates[247,248], making the study of this disease difficult. Nevertheless, various animal models of preeclampsia have been devised. While none fully reproduce the molecular pathways leading to the pathogenesis of preeclampsia or the clinical symptoms and presentation, these animal models have helped in the understanding of how modulation of molecular processes during pregnancy can result in a preeclampsia-like syndrome.

1.4.1. Genetic models of preeclampsia

Several genetic models of preeclampsia have been described, including NO synthase knockout mouse; rodent model between transgenic females expressing angiotensinogen and transgenic males expressing renin[249]; p57kip2 heterozygous mouse[250]; catechol-O-methyltransferase knockout mouse[251]; and a borderline hypertensive mouse strain, BPH/5[252]. The advantage of these models is that they are performed in the mouse, which is genetically well-defined and is amenable to a host of molecular techniques and analyses. However, the disadvantage is that these models assume a single underlying molecular pathway that leads to

disease, and many of these pathways' relevance to the pathogenesis of clinical preeclampsia are unclear.

1.4.2. Pharmacologic models of preeclampsia

Preeclampsia-like symptoms can also be induced in animals through administration of pharmacological agents. One of the oldest and most commonly used rodent models utilizes nitro-L-arg-methyl ester (L-NAME) to inhibit NO synthase from synthesizing NO. L-NAME administration to pregnant animals elicits hypertension, proteinuria, reduced glomerular filtration rate, renal damage, and fetal growth restriction[253,254]. Other models involve artificially elevating proteins found to be increased in preeclampsia, including sFlt1[101], soluble endoglin[255], TNF-α[123], IL-6[125], and angiotensin II type 1a receptor autoantibodies[256], achieved via infusion of the relevant proteins or via adenovirus-mediated gene transfer. Inversely, a preeclampsia-like phenotype can also be induced in animals using agents that antagonize protective pathways such as suramin (inhibitor of several angiogenic growth factors such as VEGF, platelet-derived growth factor, and basic fibroblast growth factor)[257] and antibody against IL-10 (anti-inflammatory cytokine)[258]. However, similar to the limitations of genetic models of preeclampsia, these models rely on the assumption that the root cause of preeclampsia is due to a defect in one of these pathways, while upstream deficiencies are not evaluated.

1.4.3. Reduced uterine perfusion pressure models

One of the most prevalent theories of preeclampsia involves defective placental perfusion, which then triggers a cascade of events that ultimately leads to placental

dysfunction, release of placenta-derived molecules into the maternal circulation, and systemic endothelial dysfunction. This is supported by animal models where reduced perfusion of blood to the uteroplacental circulation simulated a preeclampsia-like syndrome during pregnancy. These reduced uterine perfusion pressure (RUPP) models of preeclampsia have been described in various species including the dog[259], rabbit[260], rhesus monkey[261], baboon[262], sheep[263], and rodent[264–266]. Reduction of uterine perfusion is typically achieved by partial occlusion of the infra-renal abdominal aorta and the utero-ovarian arteries with silver clips during pregnancy. In the rat where much of the RUPP model has been utilized, the result is preeclampsia-like features by gestation day 19, including new-onset hypertension[267], proteinuria[267], decreased glomerular filtration rate[267], reduced renal plasma flow[268], increased inflammation[123,125,269], oxidative stress[270], mitochondrial dysfunction[271], angiogenic imbalance in the blood[272,273], placental hypoxia[273], angiotensin II type 1a receptor autoantibodies[274], and fetal growth restriction[275].

1.4.3.1. Advantages and disadvantages of the RUPP model

The advantage of the RUPP model is that it does not rely on a single or predetermined molecular pathway that leads to disease pathogenesis. While the RUPP model assumes that the pathogenesis of preeclampsia stems from reduced perfusion and resistive blood flow into the placenta, which mimics severe early-onset preeclampsia[24], it also recapitulates many molecular features of severe early-onset preeclampsia such as oxidative stress[270], circulating angiogenic imbalance[272,273], and inflammation[123,125,269]. If the molecular mechanism of a potential pharmacologic therapy involves normalization of a pathway that has been knocked out in a

genetic model or over-induced in a pharmacologic model, then the beneficial effects of therapy could be masked. A model that does not overwhelm a specific pathway is more suitable to test the effect of an experimental therapy for which a precise mechanism of action is unknown.

However, the RUPP model is not without limitations. A key disadvantage is that this model relies on mechanical manipulation of uteroplacental perfusion by using silver clips to reduce blood flow. This contrasts with the clinical syndrome whereby physiologic defects in the spiral artery remodeling process result in altered placental perfusion.

1.4.3.2. Evaluating experimental therapies with the RUPP model

The RUPP model of preeclampsia in the rat has been used to evaluate the effects of numerous experimental therapies, ranging from pharmaceutical drugs (e.g. pravastatin[276], sildenafil[277], recombinant proteins/antibodies (e.g. IL-10[131], PlGF[278], VEGF[279]), and others (e.g. vitamin D[280], sodium tanshinone IIa sulfonate[281]). Many of these studies have provided support for further investigation in human clinical trials. However, despite the importance of the placenta in the pathogenesis of severe early-onset preeclampsia, the effects of treatment on placental structure or function have received limited attention. In addition, the effects of low dose ASA and LMWH – two of the most commonly administered agents for prophylaxis against preeclampsia – have not been evaluated in the RUPP model of preeclampsia.

1.4.4. Morphological similarities and differences between the human and rat placenta

Preeclampsia is a uniquely human disorder of pregnancy; naturally observed cases of preeclampsia in non-humans are very rare and confined to higher order primates[282]. Why

preeclampsia does not naturally occur in other mammals is unknown, but presumably the anatomy, development, or function of the placenta plays a role[283]. Given the diverse structural differences in the placentas between species, it is therefore a technical challenge to study preeclampsia in animal models and extrapolate findings to the human condition.

Of all the standard model organisms used in biomedical research, the rat is considered a good animal model to study placenta-mediated complications of pregnancy because of similarities between human and rat placentation[284]. Both species rely on hemochorial placentation whereby placental trophoblasts are directly exposed to maternal blood to transfer oxygen and nutrients to the fetus; this developmental process therefore relies on successful vascular remodeling of maternal uterine spiral arteries to increase uteroplacental perfusion. Uterine trophoblast invasion in the rat is deep and extends beyond the decidua into the mesometrial triangle; this contrasts with the arrangement in the mouse whereby invasion is superficially restricted to the decidua. Thus, rat placentation is comparatively more similar than the mouse to human placentation, where spiral artery remodeling by EVTs occur at the decidual-myometrial interface[285].

However, there are also important differences between human and rat placentas. While both humans and rats have hemochorial placentas, humans have hemo*mono*chorial while rats have hemo*tri*chorial placentas. This reflects the number of trophoblast cell layers that maternal nutrients must past through before entering the fetal bloodstream. In humans, maternal nutrients are transported through a continuous syncytiotrophoblast layer that covers the placental villi, through the stroma, past a fetal capillary endothelial cell layer, and into the fetal bloodstream. In rats, nutrients are transported through two syncytiotrophoblast layers as well

as a layer of underlying sinusoidal giant cells before entering the adjacent fetal capillary. Furthermore, human placental villi are bathed in maternal blood, whereas in rats the maternal blood traverses along a labyrinth of sinusoidal channels formed by columns of trophoblasts to increase the surface area for maternal-fetal transfer of nutrients and oxygen. Finally, rat pregnancies are typically multigestational and multichorial, while human pregnancies typically involve one fetus and one placenta. This has implications when considering potential compensatory effects in multichorial pregnancies.

1.4.5. Placental regulation of fetal growth

One of the most serious and strongly associated co-morbidity of severe early-onset preeclampsia is fetal growth restriction[286], likely caused by defective spiral artery remodeling-induced placental dysfunction leading to impaired ability of the placenta to support fetal growth. In addition, abnormal development or function of any of the placental structures such as the junctional zone, labyrinth, and interhemal barrier between trophoblast and fetal vessels can all contribute to impaired fetal growth.

1.4.5.1. Junctional zone defects

The junctional zone of rodent placentas is a specialized area in between the decidua and the placental labyrinth. The three main cell types in this area – spongiotrophoblasts, glycogen cells, and trophoblast giant cells - primarily play supporting roles in pregnancy such as hormone and growth factor production and glycogen storage for energy[287]. A small junctional zone is linked to fetal growth restriction, even when there are no apparent defects in the labyrinth[288].

This could be a result of, or result in, changes to glycogen content[289], glycogen cell localization[289], or spongiotrophoblast development[290]. Glycogen usage may especially be important for fetoplacental development later in pregnancy[287].

Despite these interesting findings in rodents, especially those of glycogen storage and release, the clinical implications are unclear because the human placenta does not have specialized glycogen cell types. However, EVTs do accumulate glycogen, and glycogen content in the human placenta increases in the first trimester and declines toward term[291], suggesting the use of glycogen energy stores later in pregnancy when nutrient transfer may be reduced by placental overgrowth[24].

1.4.5.2. Labyrinth defects

The placental labyrinth in the rat, which is the equivalent of the human placental villi, mediates transfer of nutrients and oxygen from maternal blood to the fetus. A small labyrinth has reduced nutrient exchange area, just as a human placenta with diminished villous branching[292], which could result in fetal growth restriction. A small labyrinth could be a consequence of poor placental growth as a whole or failure of the specific compartment to proliferate[293]. In addition to intrinsic factors such as genetics[294,295], external factors such as maternal nutritional status can also impair placental labyrinth morphogenesis[296]. Furthermore, factors that disrupt labyrinth function, such as abnormal expression or function of glucose, amino acid, and fatty acid transporters, can impair fetal growth[297].

1.4.5.3. Interhemal barrier defects

In addition to labyrinth size and blood flow through the labyrinth, the interhemal barrier thickness can also regulate fetal growth. The interhemal barrier thickness reflects the distance that nutrients and oxygen must traverse from maternal blood to the fetal vasculature, and therefore is involved in placental transfer efficiency: the greater the thickness, the less efficient transport is. Altered expression of the transcription factor GCM1 increases interhemal thickness and is associated with excess development of the syncytium[298]. In humans, placentas from pregnancies complicated by fetal growth restriction show increased acetylation of GCM1, which can enhance its stability[299], compared to those from healthy controls[300]. Interestingly, wild-type pregnant mice carrying heterozygous *Gcm1+/-* conceptuses developed gestational hypertension with elevated plasma sFlt1[298], suggesting a potential link between dysregulated GCM1 function and hypertensive disorders of pregnancy.

1.5. Overall Objective and Hypothesis

The therapeutic potential of LMWH for the prevention of preeclampsia remains unclear. Furthermore, the mechanisms by which LMWH could alter the trajectory of preeclampsia pathogenesis are poorly understood. Given the broad spectrum of mechanistic properties that LMWH possesses, it is possible that the non-anticoagulant actions of LMWH can influence the trajectory of disease development. However, to date, studies investigating the anticoagulation-independent actions of LMWH in models relevant to preeclampsia have been confined to the *in vitro* context; limited *in vivo* studies utilizing animal models of preeclampsia that can provide important insights into the mechanism of LMWH have been conducted.

The overall objective of the collective studies outlined in this thesis was to evaluate the effect of LMWH in an animal model of preeclampsia, and the molecular mechanisms that could be involved, with specific focus on placental function. Specific aims include to:

1) Validate a non-anticoagulant, glycol-split LMWH (gsHep) as a molecular tool that can be used to investigate the role of LMWH's anticoagulation-independent mechanisms in an animal model of preeclampsia. It is hypothesized that gsHep will be non-anticoagulant but will exhibit similar placental, vascular, and anti-inflammatory properties as dalteparin (a clinical LMWH) in *in vitro* assays relevant to preeclampsia;
2) Evaluate the effects of dalteparin and gsHep on blood pressure and fetal growth in the rat RUPP model of preeclampsia. It is hypothesized that both dalteparin and gsHep will reduce blood pressure and restore fetal growth in the RUPP model; and
3) Characterize the effects of dalteparin and gsHep on placental growth and development in the RUPP model of preeclampsia. It is hypothesized that both dalteparin and gsHep will promote placental function that would be associated with improved fetal growth.

2. General Methods

2.1. The Selection of Heparins

A specific aim of this thesis was to investigate non-anticoagulant actions of heparin that could contribute to the prevention of severe early-onset preeclampsia in high-risk pregnant women, through a combination of animal and molecular models. Several heparin derivatives are used clinically, including unfractionated heparin and LMWH such as dalteparin, enoxaparin, tinzaparin, and nadroparin (Table 1.2).

2.1.1. Low molecular weight heparin vs unfractionated heparin

In the projects outlined in this thesis, low molecular weight derivatives of heparin were utilized. LMWHs are used clinically for both the treatment and prophylaxis of venous thromboembolism during pregnancy. In addition to these accepted anticoagulant actions, LMWHs have been evaluated in clinical trials for the prevention of pregnancy-mediated complications in high-risk populations, specifically for the prevention of the early onset form of preeclampsia[187]. Clinician preferences for the use of LMWHs over unfractionated heparin are based on ease of use (subcutaneous vs intravenous), longer duration of action (24 vs 12 hourly injections) and their superior safety profile[301], including predictable pharmacokinetics and pharmacodynamics, and reduced risk of heparin-induced thrombocytopenia. Evaluating the effects of LMWH in the current studies will therefore yield results that can be more readily interpreted clinically compared to using unfractionated heparin.

2.1.2. Glycol-split heparin vs other non-anticoagulant derivatives of heparin

To assess the non-anticoagulant mechanisms of heparin, a glycol-split derivative of heparin was selected for this series of experiments. The chemical process of glycol-splitting heparin is more selective at disrupting the ATBRs without widespread modifications to other areas of the molecule, in comparison with desulfation techniques[245]. Since the mechanism(s) by which heparin could confer its protective effects against the development of severe preeclampsia in high-risk pregnant women is unknown, a non-anticoagulant derivative that had undergone the least modification would in theory reduce unintended off-site effects as much as possible, thereby focusing investigation on the role of the ATBRs of LMWH. Specifically, a glycol-split heparin derivative was acquired from Dilafor, a Swedish pharmaceutical company, for the experiments outlined in this thesis. Dilafor is currently conducting clinical trials evaluating the use of glycol-split LMWH for protracted labor by stimulating uterine contractions[302]. Because of a material transfer and confidentiality agreement, the identity of the heparin derivative can not be revealed in this thesis and is generically referred to as "glycol-split heparin" (gsHep).

2.1.3. Dalteparin vs other clinically used LMWH

Based on proprietary data supplied by Dilafor, the average molecular weight of gsHep provided is approximately 6 kDa. Of the various LMWHs commonly used clinically in North America, dalteparin (Fragmin®, Pfizer) shares a similar molecular weight distribution. This was independently confirmed with gel electrophoresis and subsequent staining of different glycosaminoglycans with Alcian blue (Figure 2.1). Dalteparin and gsHep exhibited comparable staining patterns, while the LMWH enoxaparin (Lovenox®, Sanofi-Aventis) migrated lower into

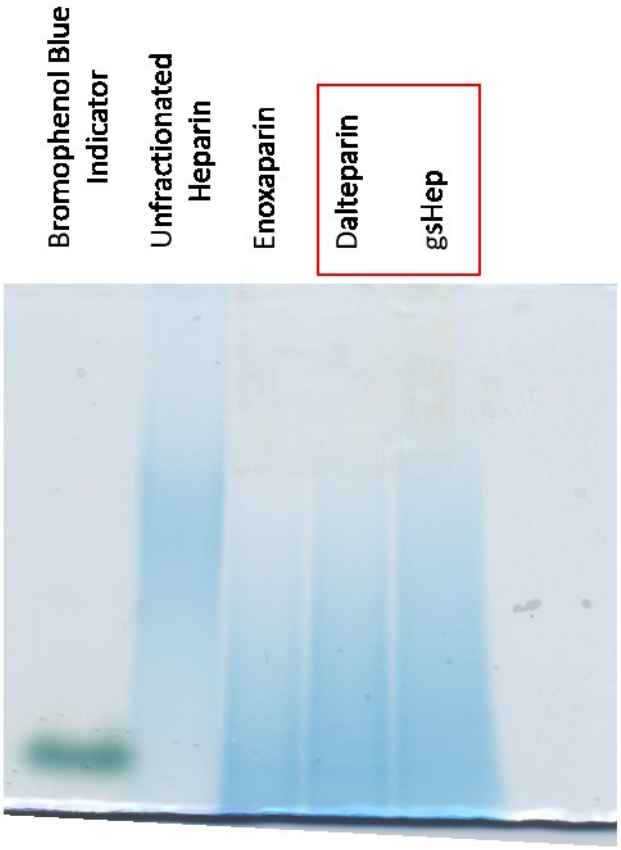

Figure 2.1. Gel electrophoresis of heparin preparations.

Fifty micrograms of unfractionated heparin, enoxaparin, dalteparin and gsHep were separated by gel electrophoresis in a 10% tris-borate-EDTA gel and stained with a 3% solution of Alcian blue to visualize sulfated glycosaminoglycans. Lower molecular weight entities migrate farther down the gel.

the gel, indicating lower molecular weight; this is consistent with the manufacturer-reported average molecular weight of 4.5 kDa for enoxaparin[303]. By comparison, unfractionated heparin migrated slower and was localized higher in the gel, indicating higher molecular weight. Hence, dalteparin was determined to be an appropriate LMWH to compare with gsHep for the experiments described in this thesis.

2.1.4. Standardization of measurement units between dalteparin and gsHep

Concentrations of LMWH are typically expressed clinically as anti-Xa units per mL, or simply IU/mL, in reference to the anticoagulant activity of LMWH and its ability to inhibit the proteolytic activity of coagulation enzyme Factor Xa. Such a designation is required because LMWH is a heterogeneous preparation, and a given mass of LMWH could have different anti-Xa units. However, since gsHep has minimal anticoagulant activity, using a similar approach to quantify its concentration is impractical. Hence, it was determined that the standard weight:volume unit of concentration is more appropriate. Since the molecular weight distribution of dalteparin and gsHep are similar, weight:volume units can be used to compare the two molecules on a molar basis. According to the product monograph, the specific activity of dalteparin is approximately 150 IU/mg; this value was used to calculate and interchange units of measure for dalteparin (e.g. 0.3 IU/mL = 2 μg/mL).

2.2. Selecting the Animal Model of Preeclampsia

Since LMWH's mechanism of action in preeclampsia is unknown, it would be disadvantageous to use a genetic knockout model of preeclampsia (e.g. catechol-O-

methyltransferase knockout mouse[251]), where LMWH could potentially exert effects on a protein or pathway absent in the model, thereby masking a biological effect. Pharmacologic models oversimplify the pathogenesis of the preeclampsia phenotype by overloading the experimental animal with a specific molecule or protein such as L-NAME[253,254] or sFlt1[101], which is not truly reflective of the complex disease pathogenesis of early-onset preeclampsia. Given LMWH's broad range of actions and its hypothesized actions on the placenta, an animal model that mimics an early insult in the pathogenesis of early-onset preeclampsia while conserving the various dysfunctional pathways leading to preeclampsia development would be most appropriate for evaluating the effects of dalteparin and gsHep. The RUPP model of preeclampsia in the rat was therefore selected to evaluate and compare the effects of dalteparin and gsHep. Although a key disadvantage of this model is reliance on an irreversible and non-physiologic surgical method of reducing uteroplacental perfusion, the RUPP model recapitulates the impaired uteroplacental perfusion often observed in women with severe early-onset preeclampsia. The placentas of such women commonly express MVM pathology, which is characterized by defective spiral artery remodeling, leading to chronic ischemia-reperfusion injury to the developing preeclampsia[7]. The RUPP model preserves many of the pathologic downstream pathways that ultimately lead to the manifestations of severe early-onset preeclampsia, especially severe hypertension, organ injury, and fetal growth restriction[304].

2.3. Surgical Induction of the RUPP Model of Preeclampsia

The RUPP model of preeclampsia is induced via mechanical manipulation of blood flow into the uteroplacental circulation, based on a protocol established by Crews et al[265]. As part of this project, I visited the laboratory of Dr. Joey Granger, a leader in the RUPP procedure, at the University of Mississippi Medical Center to learn firsthand the surgical technique.

2.3.1. Preparation of occlusion clips for RUPP

Reduced placental perfusion is achieved by using silver clips of defined gap widths to partially constrict arterial blood vessels and therefore restrict blood flow leading into the uterus. In total, 3 clips were introduced – a clip with a gap width of 0.200 mm for the abdominal aorta, and a clip with a gap width of 0.100 mm for the left and right uterine artery proximal to the first segmental artery that feeds into the first placenta. These clips were crafted in-house from sheets of silver foil 0.25 mm thick (Sigma-Aldrich, Oakville, Canada; cat no. GF51993322). The abdominal clips were made to the correct gap width by bending and flattening over commercially available feeler gauges, and then slid across the aorta during surgery. Due to the delicate nature of the uterine vessels, however, sliding these clips across the uterine vessels risks damaging the arteries. The uterine artery clips were therefore not bent in advance but were positioned as an open clip across the artery before clamping down to the correct gap using a modified pair of hemostats. The tip of the hemostat was ground down such that, when it clamped down on the clip, a final gap width of 0.100 mm was achieved (i.e. since the foil thickness was 0.25 mm, and the clip occupied twice this width plus the desired 0.100 mm, the tip of the hemostat was ground down to result in a 0.600 mm gap). All the clips were filed down

at the edges to prevent nicking and damaging the blood vessels. A photograph of all the clip-related materials is shown in Figure 2.2.

2.3.2. Surgical procedure for RUPP

All procedures were approved by the University Health Network Animal Care Committee (Toronto, Canada) and followed the guidelines established by the Canadian Council on Animal Care. Rats were maintained on a 12:12-hour light-dark cycle at 23 ^{o}C and were provided food and water ad libitum. Virgin, female Sprague Dawley rats (190-250 grams; Charles River Laboratories, Saint Constant, Canada) were time-mated with male rats overnight; copulation was confirmed by the presence of sperm plugs the next day, which was designated gestation day (GD) 0. On GD14, rats were induced with 5% isoflurane and maintained with 2% isoflurane (supplied by the University Health Network Animal Research Centre) at a flow rate of 1.5 L/min with O_2 carrier. Rats were placed onto a warmed padded surface and administered a subcutaneous injection of 0.05 mg/kg buprenorphine (Vetergesic®; supplied by the Comparative Medicine Animal Resources Centre at McGill University, Montreal, Canada) for pre-operative analgesia. The abdominal region was shaved and prepped using a three-stage cleaning regimen of betadine/iodine scrub, alcohol, and betadine/iodine solution, and the eyes were kept lubricated with tear gel. After testing for effective anaesthesia via loss of pedal and corneal reflexes, a 1-inch abdominal midline incision was performed using surgical scissors, and the gravid uterine horns were exteriorized onto sterile gauze pads to allow access to the posterior peritoneal wall. Using sterile cotton swabs, connective tissues surrounding the abdominal aorta above the iliac arterial bifurcation were gently dissociated. Curved forceps

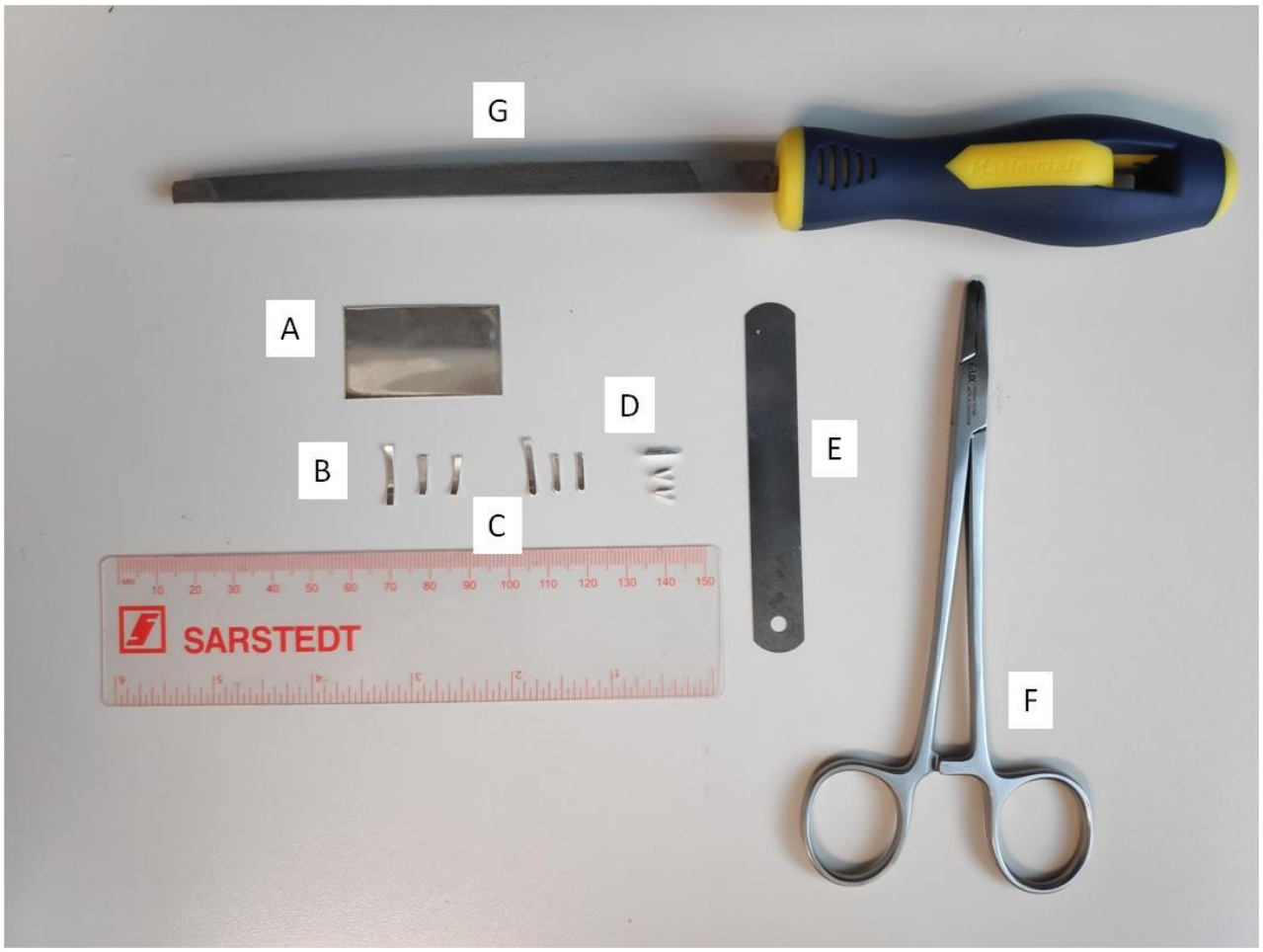

Figure 2.2. Materials and equipment used for RUPP clip making.

A: Silver foil, 0.25mm thick; B: 1.5 cm aortic clip and 1.0 cm uterine clips approximately 2 mm in width; C: clips were ground down to remove sharp edges and burrs; D: folded aortic clip and open-ended uterine clips; E: feeler gauge of 0.20 mm thickness used to fold aortic clip; F: modified hemostat with the tips ground to 0.60 mm gap width; G: filing rod used to smooth the edges and burrs of clips.

were then slipped under the aorta and resurfaced between the artery and the inferior vena cava to separate the vessels. While gently lifting the abdominal aorta with this forcep, another set of forceps were used to slide the abdominal clip across the vessel (Figure 2.3).

The uterine clips were introduced distally to the ovarian-uterine artery junction and proximal to the first segmental artery. A curved forcep was used to isolate this area of the uterine arcade, and an open-ended uterine clip was slipped across the vessel. The ground down, modified hemostat was then used to clamp down on the clip to yield a gap width of 0.100 mm across the vessel. This was performed on both left and right uterine arteries (Figure 2.4). For sham surgeries, the relevant vessels were isolated in a similar fashion as RUPP animals but without introduction of the clips.

Following the procedures, the abdominal cavity was filled and hydrated with 2.5 mL of sterile saline before closing the abdominal wall and skin with 3-0 nylon sutures and an interlocking horizontal mattress stitch. The rats were also given a single dose of 1 mg/kg subcutaneous meloxicam for post-operative analgesia. After rats regained consciousness from anesthesia, they were housed in separate cages and monitored for the next 48 hours.

2.4. Measuring Blood Pressure in RUPP Animals

2.4.1. Non-invasive vs invasive blood pressure measurements

Ideally, blood pressure would be measured in the conscious animal, since anesthesia has the potential to depress hemodynamic parameters, such as blood pressure and cardiac output[305]. A common technique to measure blood pressure is to use a non-invasive tail cuff device on a restrained and conscious rat. This device relies on adequate blood flow to the tail

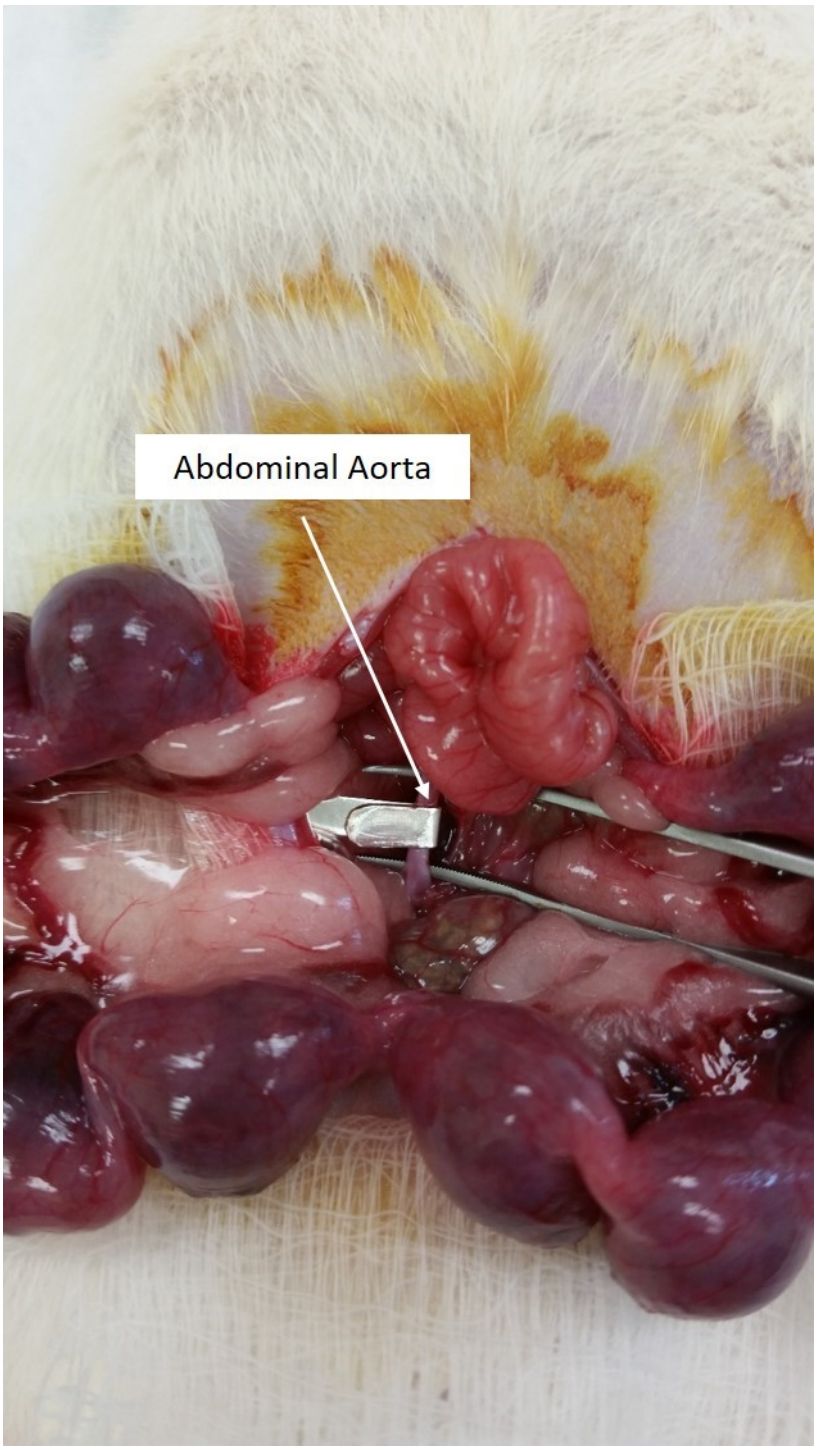

Figure 2.3. Positioning of the abdominal aortic clip.

The premade folded clip was slid across the abdominal aorta above the iliac bifurcation after dissociating the connecting tissue between the aorta and the vena cava with sterile cotton swabs and isolating the aorta with curved forceps.

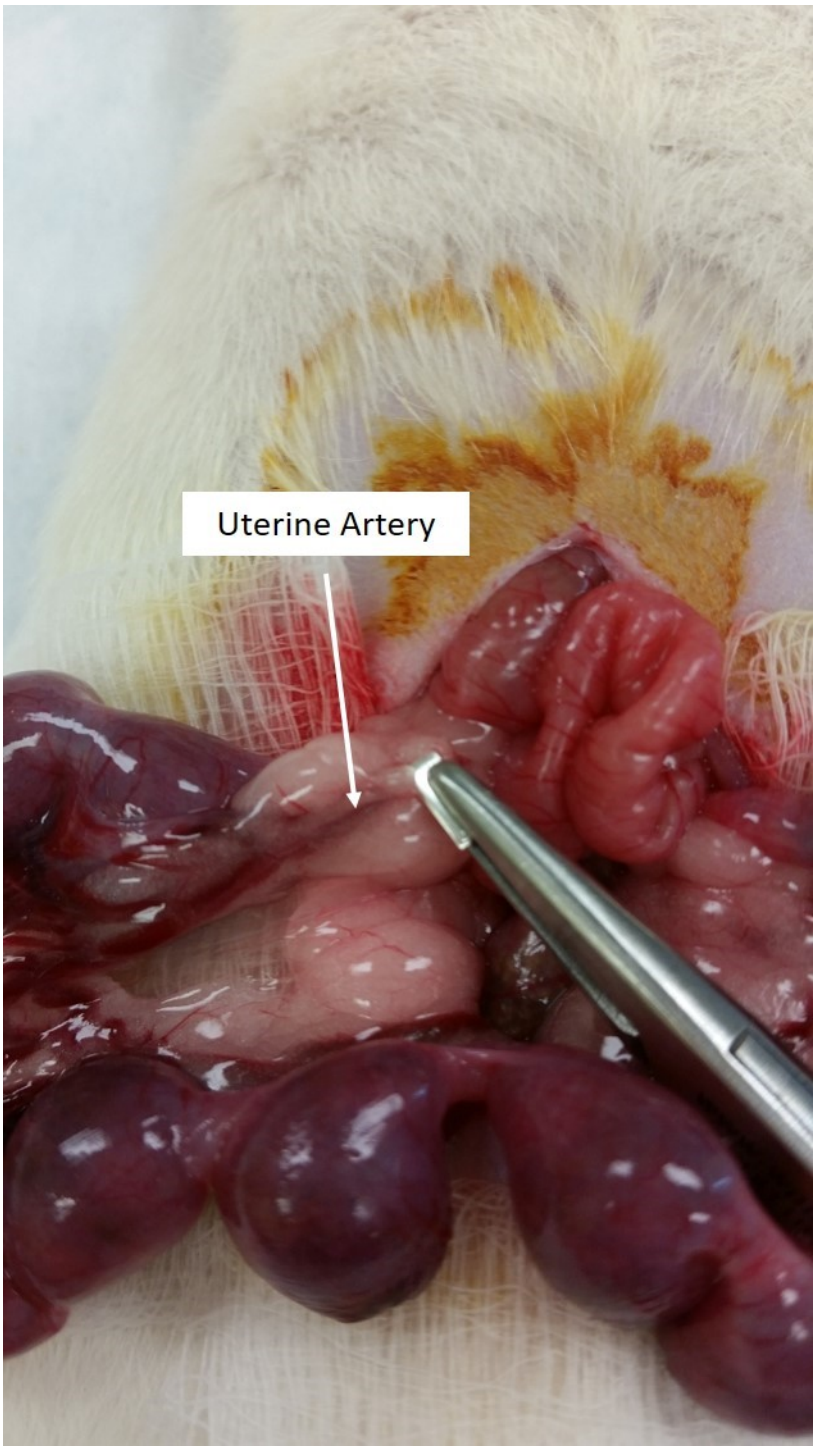

Figure 2.4. Clamping of the uterine clips.

The uterine clips were clamped onto the uterine arcades proximinal to the first segmental artery with modified hemostats to reduce the risk of tearing the vessels.

region of the animal. However, since the RUPP model involves constricting the abdominal aorta, tissue perfusion to the hind region of the rat is reduced. Early troubleshooting experiments identified poor signal-to-noise ratio in the tail, leading to inconsistent and inaccurate blood pressure measurements; this issue was also acknowledged by authors in a subsequently published study[266].

Due to these limitations, blood pressure recording in the conscious RUPP rat is typically accomplished by introducing a carotid cannula that is exteriorized to the back of the neck and connected to a pressure transducer that can record blood pressure while the animal is freely mobile. This procedure is usually performed the day prior to blood pressure measurement such that any effects of anesthesia, surgical pain, or stress would have passed[265]. However, this procedure requires the cannula to remain patent overnight, which is achieved by filling the cannula with an anticoagulant locking solution such as heparin saline. Given that the current set of experiments involves assessing the potential effects of heparin in the RUPP model, and there is a risk of leaking the locking solution into the animal's circulation, this technique for measuring blood pressure in conscious RUPP animals was not used in order to reduce the risk of introducing heparin into sham or vehicle control RUPP animals. Blood pressure was instead measured in the anesthetized rat via carotid cannulation immediately prior to sacrifice on GD19.

2.4.2. Surgical procedure for carotid cannulation and blood pressure measurement

After the animal had been induced with 5% isoflurane and maintained with 2% isoflurane, a 1-inch midline incision was performed on the anterior neck region. Using forceps,

the sternomastoid and sternohyoid muscles were teased apart to expose the right carotid artery. After a 0.5 cm segment of the artery had been isolated from the surrounding connective tissue, the cranial end of the artery was tied with suture, while the caudal end was temporarily occluded with a bulldog clamp. A 24-gauge 3/4" Becton Dickinson Insyte Autoguard Shielded IV Catheter (cat no.: 381412; Mississauga, Canada) was used to puncture the artery in between the clamp and the suture and insert the catheter (Figure 2.5). The open end of the catheter was then connected to a pressure transducer via silicone tubing filled with saline. The bulldog clamp was then removed, the catheter was inserted deeper towards the aortic arch, and the catheter was secured with additional suture. Air bubbles in the line were eliminated via a three-way stopcock and a syringe, and involved the removal of approximately 0.4 mL of blood.

Blood pressure measurements were recorded with a data acquisition and amplification device (National Instruments, Austin, TX, USA; model USB-6003) programmed in-house that was calibrated to an aneroid sphygmomanometer before each use (Figure 2.6). Data were sampled at 100 Hz over approximately 20-30 seconds, and mean arterial blood pressure was derived by averaging all data points across 5 seconds of stable reading (Figure 2.7). In total, the surgical and data acquisition procedures were completed within 20 minutes, to minimize the effects of anesthesia on hemodynamic parameters. In circumstances where there were aberrant blood pressure readings (e.g. absence of distinct peaks and troughs) or surgical complications (e.g. failed catheter access to the carotid artery), the data were omitted.

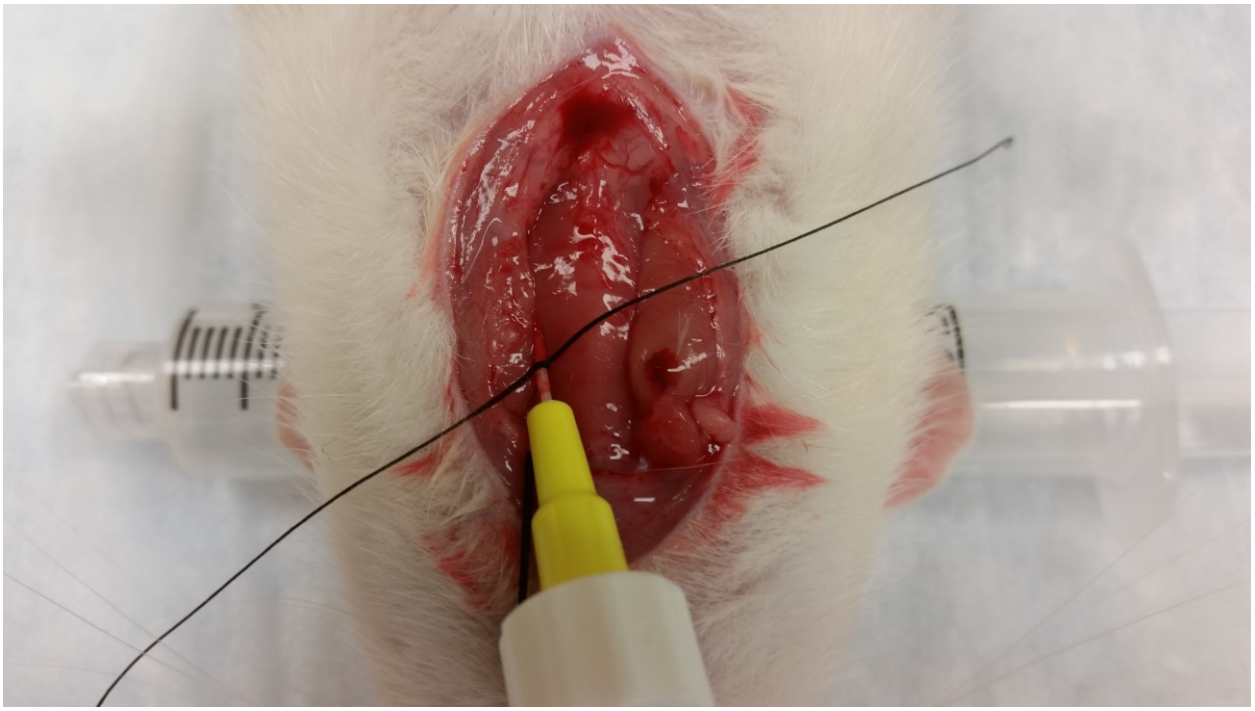

Figure 2.5. Insertion of the catheter into the carotid artery.

For blood pressure measurements, the left carotid artery was isolated and punctured with a 24G catheter with a needle insert to gain access to the vessel. Suture was used to secure the catheter, which was connected to a pressure transducer and data acquisition device. The syringe was used to arch the neck to elevate the carotid for easier access.

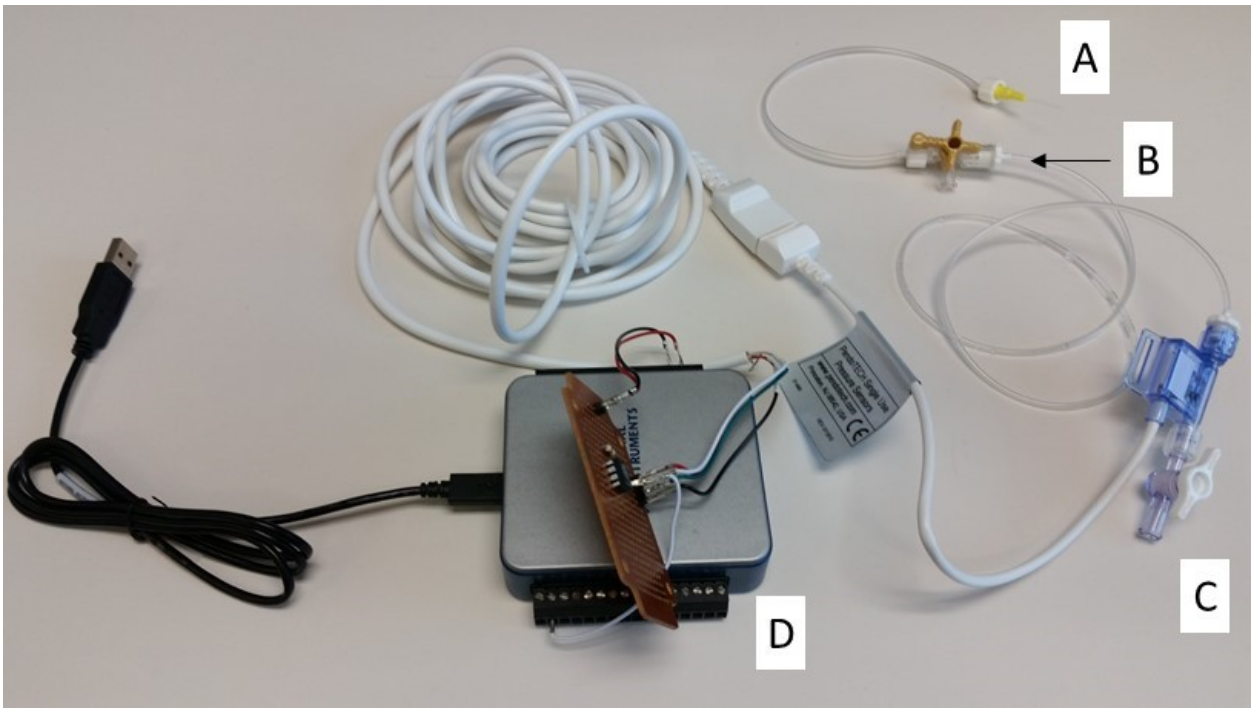

Figure 2.6. Materials and equipment for measuring blood pressure.

A: catheter with needle insert (needle removed in picture); B: three-way stopcock for removing air bubbles in the line; C: pressure transducer; D: data acquisition device with amplifier to be connected to a computer.

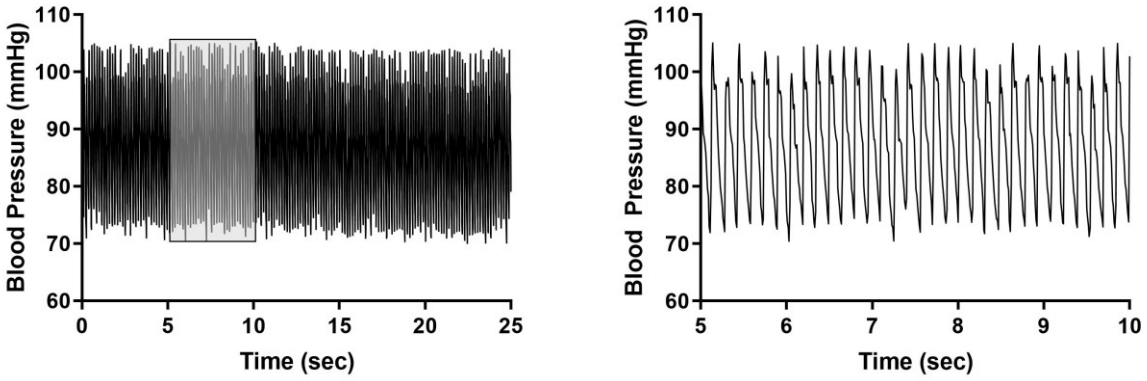

Figure 2.7. Example of blood pressure outputs.

Mean arterial blood pressure was derived from the averaged carotid pressure readings over a 5-second interval.

3. Comparing the Effects of Dalteparin and gsHep on Placental, Endothelial, and Anti-Inflammatory Pathways Relevant to Preeclampsia

3.1. Introduction

The objective of this chapter was to establish the functional similarities and differences between dalteparin and gsHep to support the use of gsHep as a molecular tool to specifically examine the role of non-anticoagulant properties of LMWH in the RUPP model of preeclampsia. To address this objective, the effects of dalteparin and gsHep were compared in several placental, endothelial, and anti-inflammatory assays relevant to preeclampsia. It was hypothesized that both dalteparin and gsHep can support placental function, interact with the placenta to stimulate angiogenesis, inhibit complement activation, and reduce leukcoyte adhesion, as previously described for LMWH[205,206,219,224,230], while differing in their anticoagulant capacity.

3.2. Methods

Low Molecular Weight Heparins

Non-anticoagulant heparin (glycol-split heparin, gsHep) was provided by Dilafor (Stockholm, Sweden), chemically generated by periodate oxidation of pig intestinal mucosa heparin, followed by alkaline β-oxidation of the product as previously described[306]. The average molecular weight of gsHep was approximately 6 kDa as determined by high performance gel permeation chromatography according to manufacturer's data. This is similar to the average molecular weight distribution of dalteparin[307] (Figure 2.1), which is being used as the

*A modified version of Chapter 3 was published: Wat JM, Hawrylyshyn K, Baczyk D, Greig IR, Kingdom JC. Effects of glycol-split low molecular weight heparin on placental, endothelial, and anti-inflammatory pathways relevant to preeclampsia. *Biol Reprod* 2018; 99(5): 1082-90.

comparator for this study to allow direct comparisons at molar equivalency. Concentrations of dalteparin and gsHep used for functional experiments were within the clinical range used for dalteparin prophylaxis and acute therapy (2-10 μg/mL, equivalent to 0.3-1.5 anti-Factor Xa IU/mL)[308].

Tissue Culture

Human umbilical vein endothelial cells (HUVECs; Lonza, Walkersville, MD, USA) were maintained in EGM-2 growth media (CC-3162). Where appropriate for experiments, fetal bovine serum (FBS; Wisent Inc, St-Bruno, QC, Canada) was replaced with 5% patient serum and the heparin included in the growth media kit was replaced with dalteparin or gsHep. Cells were passaged at 90% confluency. Experiments performed on HUVECs utilized cells that were less than passage 8. THP-1 monocytes (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 media (Thermo Fisher Scientific, Burlington, ON, Canada) containing 10% FBS and 0.05 mM β-mercaptoethanol as described by the manufacturer, and maintained at a density no greater than 1.0×10^6 cells/mL.

Anticoagulant Assays

The ability of the heparins to inhibit Factor Xa, the major coagulation protease target of LMWHs, was assessed using the Biophen Heparin Anti-Xa 2-stages kit (Aniara Diagnostica, West Chester, OH) according to manufacturer instructions for assessing purified heparin activity, using dalteparin of known concentrations as the standard. Activated partial thromboplastin

time (aPTT) assay was performed using the Pacific Hemostasis APTT-XL kit (Thermo Fisher Scientific) according to manufacturer's instructions.

Differential Scanning Fluorimetry

Differential scanning fluorimetry assesses the thermal stability of proteins in the presence or absence of non-peptide small molecules, where a melting curve shift indicates a positive interaction[309]. 20 µL reaction mixtures consisting of 1.2 µg of protein, heparin (in molar excess), and 5x SYPRO Orange (Thermo Fisher Scientific) were loaded into Biorad hard-shell 96-well polymerase chain reaction (PCR) plates (Mississauga, ON, Canada). Samples were heat denatured using a Biorad CFX96 Real-Time PCR system using a ramp configuration starting at 25 °C and increasing at 1 °C/min to 95 °C. Fluorescence was measured every 30 sec using the HEX filter configuration. Data for each curve were fitted against a 4-parameter logistic curve to determine the melting temperature of the protein, defined as the temperature at half-maximal fluorescence of the curve. Recombinant sFlt1, FGF1, and FGF2 were from BioLegend (San Diego, CA, USA), and recombinant antithrombin III was from Haematologic Technologies (Essex Junction, VT, USA).

Placental Villous Tissue Explant Model

Healthy 1st trimester placental tissue was obtained from elective terminations via the Mount Sinai Hospital Biobank with research ethics board approval and written consent from the patient (REB #11-0248-E). The placental villous explant model was employed as previously described[205]. Villous trees from placenta samples of 8-12 weeks of gestation were dissected in

cold sterile phosphate buffered saline (PBS) and cultured in a 24-well plate containing DMEM Ham F12 media supplemented with 1x insulin-transferrin-selenium, 1x penicillin-streptomycin-glutamine, 0.25 μg/mL fungizone, and 500 μg/mL gentamicin (all from Thermo Fisher Scientific). Explants were acclimated overnight in a humidified 37°C incubator kept at 8% O_2/5% CO_2 before treating with heparin compounds prepared in fresh media for 24 hours. The treated tissues and conditioned media were stored at -80 °C until further use. Conditioned media were quantified for sFlt1 and PlGF levels through enzyme-linked immunosorbert assays (ELISAs; R&D Systems, Minneapolis, MN, USA) to determine protein release into the conditioned media from control and treated explants, as per manufacturer's instructions.

Quantitative Polymerase Chain Reaction

RNA extraction and quantitative reverse transcription PCR (qRT-PCR) was performed as previously described[27]. RNA extraction was performed using the RNeasy Plus Universal Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. 1 μg of isolated RNA was reversed transcribed using Biorad iScript Reverse Transcription Supermix with an Eppendorf Mastercycler nexus (Mississauga, Canada) according to manufacturer's instructions. qRT-PCR reaction mixes contained 6 ng of cDNA, 1:2 LuminoCt SYBR Green (Sigma Aldrich), and 0.3 μM forward and reverse primers (Integrated DNA Technologies, Coralville, IA) performed in a Biorad hard-shell 384-well PCR plate. Primers used in this study are provided in Table 3.1. Gene expression was normalized to the geometric mean of the housekeeping genes *YWHAZ*, *TOP1*, and *HPRT*.

Table 3.1. List of primers used for qRT-PCR studies in placental explants.

SFLT1	F: 5'-GCA CGT TTG GAT TTG GAG GA-3' R: 5'-GGA AAG GAT CAT CCC AAG TTG TT-3'
PLGF	F: 5'-CCA GCA GTG GGC CTT GTC T-3' R: 5'-ACA CTT CCT GGA AGG GTA CCA-3'
PCNA	F: 5'-TTG AAG CAC CAA ACC AGG AGA-3' R: 5'-TGC AAA TTC ACC AGA AGG CA-3'
YWHAZ	F: 5'-ACT TTT GGT ACA TTG TGG CTT CAA-3' R: 5'-CCG CCA GGA CAA ACC AGT AT-3'
TOP1	F: 5'-GAT GAA CCT GAA GAT GAT GGC-3' R: 5'-TCA GCA TCA TCC TCA TCT CG-3'
HPRT	F: 5'-TGA CAC TGG CAA AAC AAT GCA-3' R: 5'-GGT CCT TTT CAC CAG CAA GCT-3'

Endothelial Tube Formation Assay

Fifty microliters of growth factor-reduced Matrigel were added to a 96-well tissue culture plate and allowed to polymerize at 37 °C for 20 min. 17,000 HUVEC cells/well were seeded on top of the Matrigel. The seeded HUVECs were treated with either endothelial growth media containing dalteparin or gsHep, or with 50% conditioned media from placental explants treated with dalteparin or gsHep (as described above) and diluted in endothelial basal media. Cells were incubated in a humidified 37 °C incubator at 20% O_2/5% CO_2 for 16 hours before imaging with light microscopy and manual analysis by ImageJ Software assessing total tube length in the central field of view.

Serum Samples

Serum samples from preeclamptic patients were collected upon admission for delivery at Mount Sinai Hospital (Toronto, ON) upon informed consent (REB #11-0248-E). All samples were collected from patients clinically diagnosed with early-onset preeclampsia at 26-28 weeks with fetal growth restriction. Whole blood was collected into BD Vacutainer SST tubes (#367988) and centrifuged at 2,000g for 15 min; the separated serum was stored at -80 °C until use.

Complement-mediated Hemolysis Assay and ELISA

Five percent sheep erythrocytes (Cedarlane, Burlington, ON, Canada), in HEPES-buffered saline, which are sensitive to lysis by activation of the classical pathway of complement[310], were mixed, in order, with 1:100 dilution of anti-sheep erythrocyte IgM (Cedarlane), 1 mM $CaCl_2$, 1

mM $MgCl_2$, heparin, and 3.5% patient serum to induce complement-mediated hemolysis. The reaction was incubated for 30 min at 37 °C and stopped with 330 mM EDTA. Unlysed cells were removed by centrifuging at 600 g for 5 min, and 100 µL of the supernatant was mixed with 100 µL water in duplicates. The absorbance of this mixture at A_{405nm} was used to determine hemoglobin release into the supernatant via lysis of the erythrocytes. In the absence of treatment, the reaction conditions resulted in 80-90% lysis of erythrocytes compared to total lysis with water. For treatment effects, lysis was relative to the control conditions. An aliquot of the hemolysis reaction supernatant for C5a measurements was stored at -80 °C until use. A C5a ELISA kit (BioLegend) was used to measure C5a generation.

Leukocyte Adhesion Assay

HUVECs were seeded onto 96-well plates at a density of 50,000 cells/well and allowed to adhere overnight in the absence of heparin, and then stimulated with 5% preeclamptic serum in EGM-2 media without FBS for 2 hours. Following treatment, cells were washed two times with PBS and then incubated with heparin compounds in EGM-2 media for 1 hour. Meanwhile, THP-1 cells were labelled with calcein-AM according to manufacturer instructions and resuspended in EGM-2 media. Following heparin incubation, labelled THP-1 cells were added directly on top of the activated HUVECs and allowed to adhere for 45 min. Non-adherent cells were removed through a series of 3 washes with PBS, and adhered cells were quantified by measuring the fluorescence intensity at 480 nm ex/520 nm em using the Tecan microplate reader. Values were normalized to FBS control in growth media.

Statistical Analyses

All experiments were performed as technical triplicates. Data are presented as mean ± standard deviation (SD). One-way ANOVA with Bonferroni's post-hoc was used to compare melting temperature changes in the absence or presence of treatment. Two-way ANOVA with Bonferroni's post-hoc was used to compare dose-dependent differences between dalteparin and gsHep. Repeated measures ANOVA with Bonferroni's post-hoc was used to compare the dose-dependent effect of treatment to the untreated condition. GraphPad Prism 5.0 software (La Jolla, CA, USA) was used for analyses and $p \leq 0.05$ was considered statistically significant.

3.3. Results

3.3.1. gsHep has negligible anti-Factor Xa and anticoagulant activity compared to dalteparin

The anticoagulant properties of dalteparin and gsHep were assessed using clinically-relevant assays for anticoagulant activity. The anti-Factor Xa activity assay is a chromogenic assay that measures the ability of heparin to inhibit Factor Xa proteolytic activity. As expected, dalteparin dose-dependently inhibited Factor Xa-mediated activation of the chromogenic substrate; by contrast, gsHep exerted negligible anti-Factor Xa activity compared to dalteparin ($p < 0.001$; Figure 3.1A). In addition, an aPTT assay was used to evaluate time to plasma fibrin clot formation *in vitro*. Plasma fibrin clot formation was significantly prolonged by exogenously added dalteparin, indicative of anticoagulant activity, while gsHep demonstrated significantly reduced ability to prolong aPTT fibrin clot formation ($p < 0.001$; Figure 3.1B). These results confirm that gsHep has substantially reduced anticoagulant activity compared to dalteparin.

3.3.2. gsHep interacts with sFlt1, FGF1, and FGF2, but not ATIII

Differential scanning fluorimetry was used to assess the ability of dalteparin and gsHep to interact with various proteins (Figure 3.2). Dalteparin and gsHep both induced a significant shift in the melting curve of sFlt1, indicative of a positive interaction ($p < 0.001$). The magnitude of the shift was not significantly different between dalteparin and gsHep ($p > 0.05$). Other classic heparin-binding growth factors, such as FGF1 and FGF2, also interacted with both dalteparin and gsHep. While dalteparin induced a curve shift for ATIII, no significant shift was observed in the presence of gsHep. This confirms that gsHep does not bind ATIII, while

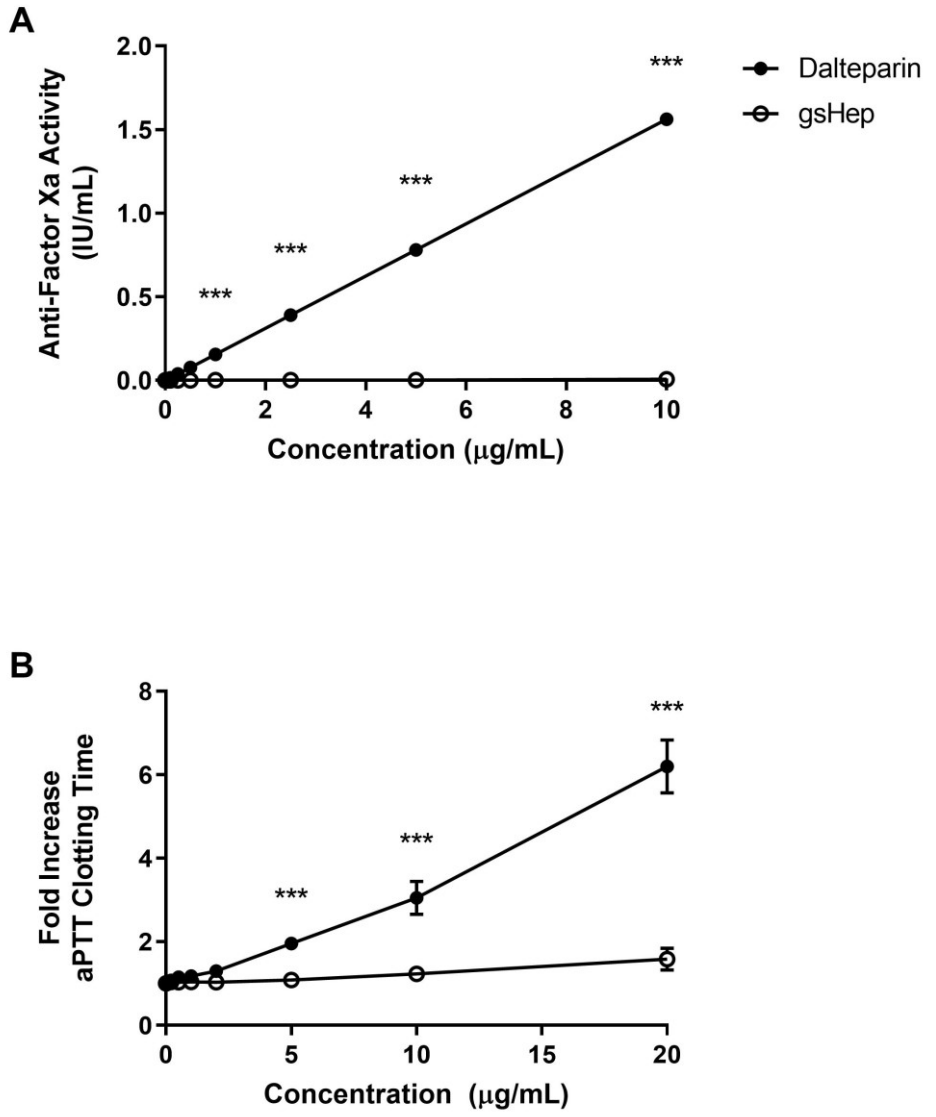

Figure 3.1. Anticoagulant activities of dalteparin and gsHep.

(A) Effects of dalteparin and gsHep in the anti-Factor Xa activity assay evaluating the proteolytic activity of recombinant Factor Xa to cleave and activate a chromogenic substrate. Dalteparin was used as a standard curve using known concentrations provided by the manufacturer. (B) Effects of dalteparin and gsHep in the activated partial thromboplastin time assay assessing *in vitro* clot formation using plasma from healthy pregnant women. Two-way ANOVA with Bonferroni's multiple comparison test, *** $p < 0.001$ compared between treatments at each concentration, n = 3 independent experiments. Data presented as mean +/- SD. aPTT: activated partial thromboplastin time.

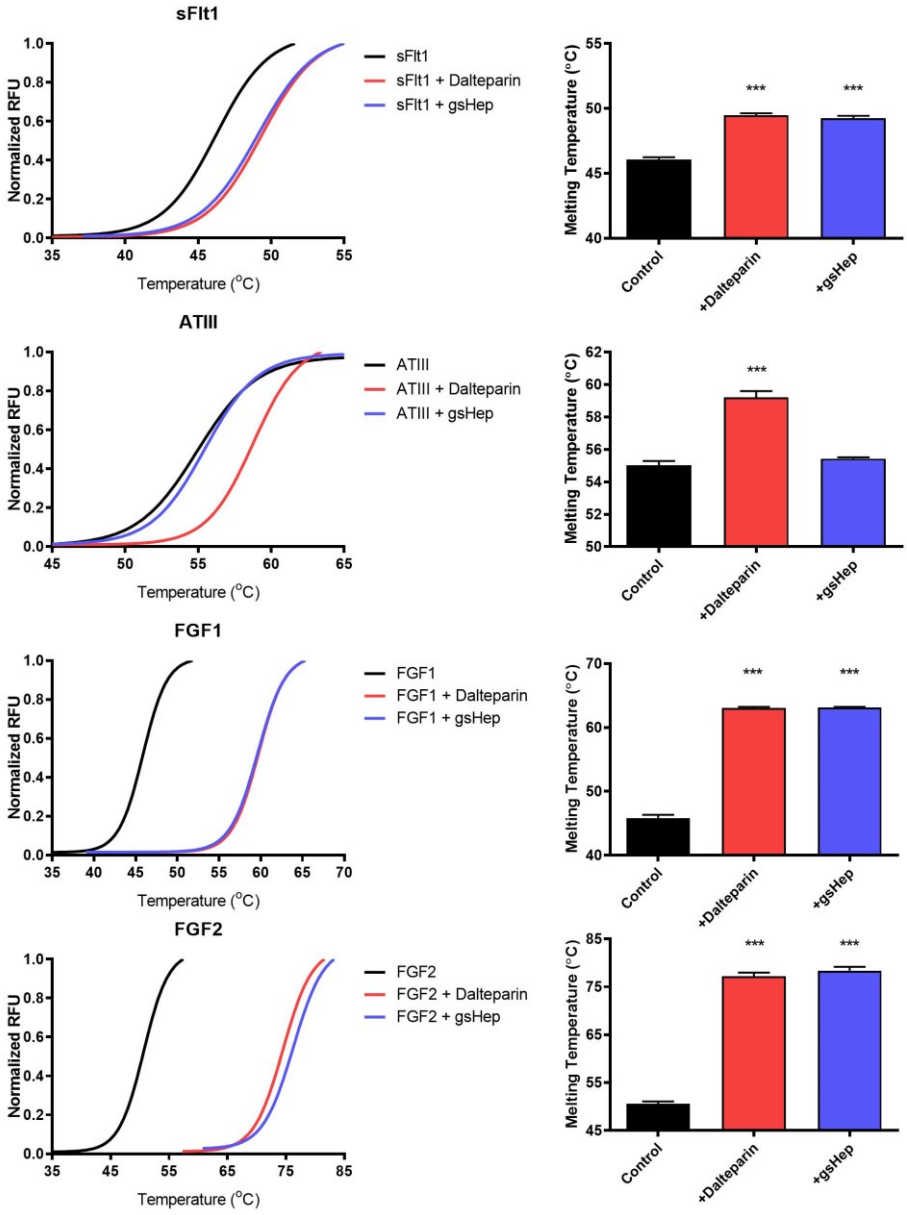

Figure 3.2. Interactions between dalteparin or gsHep with known heparin-binding proteins.

Differential scanning fluorimetry was used to evaluate dalteparin and gsHep interactions with recombinant proteins. Shifts in melting curve (left panels) and changes in melting temperature (right panels) indicate positive interactions. *** $p < 0.0001$ compared to no heparin control. n = 3 independent experiments. Data presented as mean +/- SD. RFU, relative fluorescence units; sFlt1: soluble fms-like tyrosine kinase-1; FGF: fibroblast growth factor; ATIII: antithrombin III.

maintaining interaction with additional growth factors.

3.3.3. Dalteparin and gsHep elicit similar mRNA expression and protein release from healthy 1st trimester placental villous explants

Healthy 1st trimester placental explants were treated with dalteparin and gsHep for 24 hours to evaluate gene expression and protein release. Dalteparin and gsHep dose-dependently downregulated placental explant *PlGF* and *sFLT1* mRNA ($\rho < 0.001$ and $\rho < 0.0001$, respectively) compared to untreated controls (Figure 3.3A). Steady-state mRNA expression of the proliferation gene *PCNA* was increased with both treatments ($\rho < 0.01$), indicating the absence of toxicity. The effects of dalteparin and gsHep on *PlGF* ($p = 0.78$), *sFLT1* ($p = 0.65$), and *PCNA* ($p = 0.38$) mRNA expression were not significantly different between treatments. PlGF protein levels were significantly increased in conditioned media from 5- and 10 µg/mL dalteparin and gsHep-treated placental explants ($\rho < 0.01$); the heparin treatments did not significantly impact sFlt1 protein levels compared to media from untreated explants (Figure 3.3B). The effects of dalteparin and gsHep on PlGF and sFlt1 levels in the conditioned media were not significantly different between treatments. These results indicate that gsHep and dalteparin elicit similar angiogenic regulator mRNA level changes and protein release from placental villous explants.

3.3.4. Endothelial tube formation is stimulated by media conditioned by dalteparin- and gsHep-treated placenta explants

Endothelial tube formation assays were performed to evaluate the angiogenic effect of dalteparin and gsHep. When added directly to endothelial cell growth media, dalteparin had a

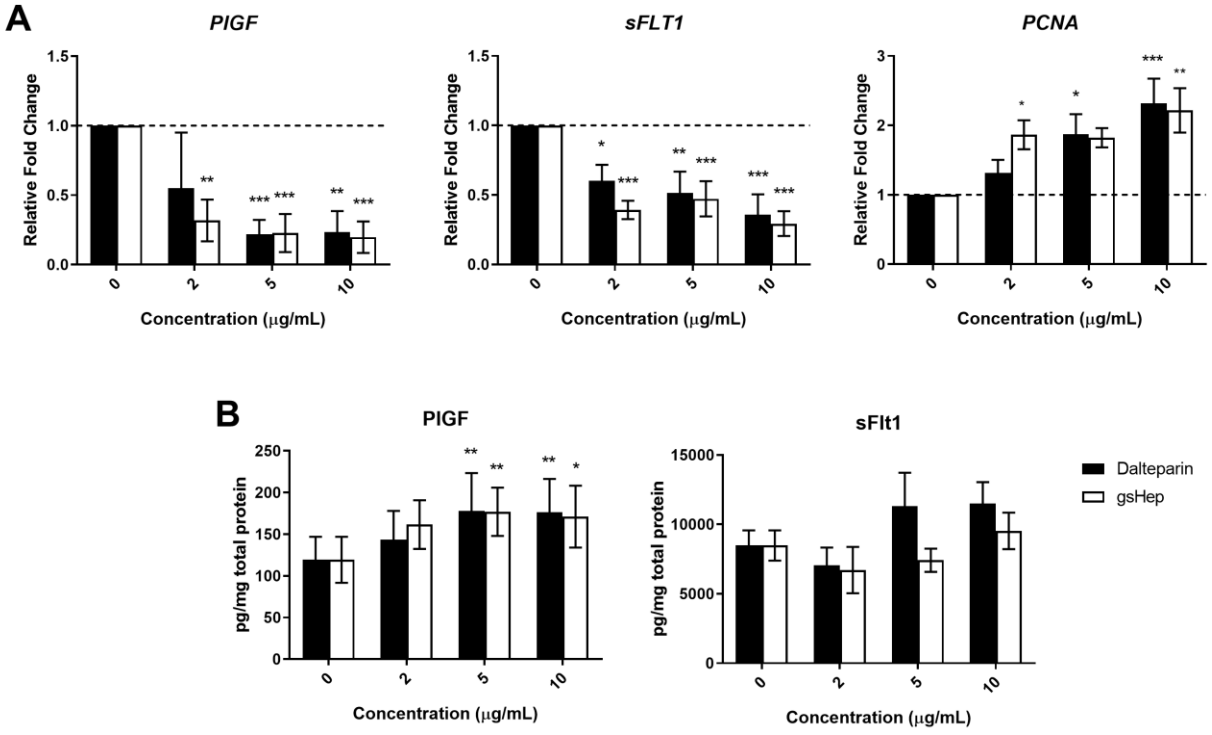

Figure 3.3. Effects of dalteparin and gsHep on steady-state mRNA expression and protein release from 1st trimester placental villous explants.

Placental explants were treated with dalteparin or gsHep for 24 hours before conditioned media were collected for analyses. (A) Total RNA was isolated from explant tissues and quantitative reverse transcription PCR was performed to evaluate steady-state mRNA expression of angiogenic genes *PlGF* and *sFLT1*, as well as the proliferation gene *PCNA*. (B) ELISA analysis of conditioned media derived from treated explants for PlGF and sFlt1. *P < 0.05, **P < 0.01, ***P < 0.001 compared to no heparin controls. n = 6 placenta and media samples. Data presented as mean +/- SD. PlGF: placental growth factor; sFlt1: soluble fms-like tyrosine kinase-1; PCNA: proliferating cell nuclear antigen.

small stimulatory effect on angiogenic tube formation at 2 μg/mL, increasing tube formation by 1.2 ± 0.16 fold compared to untreated controls ($p < 0.05$; Figure 3.4A). Lower and greater concentrations of dalteparin did not significantly impact tube formation compared to untreated controls. By contrast, gsHep did not elicit a significant effect at any concentrations tested ($p = 0.31$)

LMWH can condition placental villous explants to modulate angiogenic balance in the conditioned media[219]. Compared to conditioned media from untreated placental explants, conditioned media from 2 μg/mL dalteparin- and gsHep-treated placental explants increased total tube lengths by 1.42 ± 0.14 and 1.41 ± 0.14 fold, respectively ($p < 0.05$; Figure 3.4B); the effects on tube length were not significantly different between dalteparin and gsHep treatments ($p = 0.59$). Representative images of the tube formation experiments are shown in Figure 3.4C. These data suggest that dalteparin and gsHep can promote the stimulation of endothelial cell tube length formation in a similar manner.

3.3.5. Dalteparin and gsHep inhibit complement activation

Dalteparin and gsHep were evaluated for the ability to inhibit complement activation. Both dalteparin and gsHep dose-dependently inhibited complement-mediated hemolysis of sheep erythrocytes; this was irrespective of whether serum from normal pregnant or preeclamptic patients were used as the source of complement ($p < 0.0001$; Figure 3.5A). No significant difference was observed between dalteparin and gsHep treatments ($p = 0.62$ and $p = 0.26$ for normal pregnant and preeclamptic serum, respectively). Inhibition of 50% of lytic

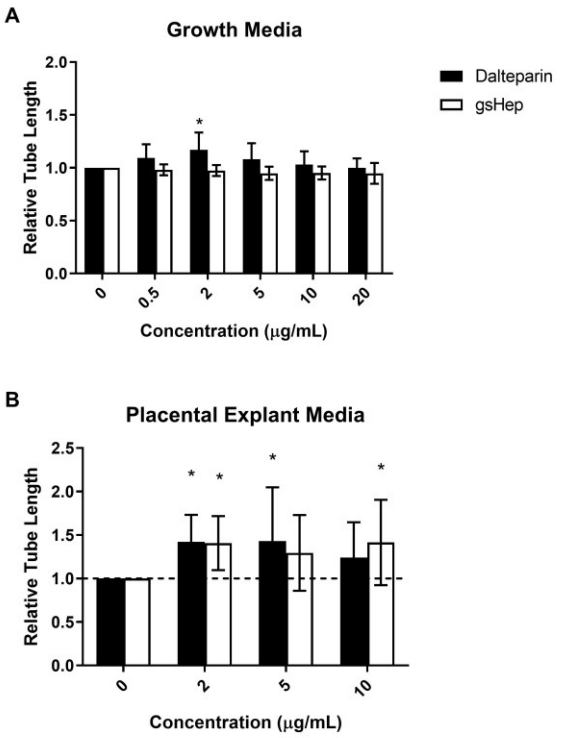

Figure 3.4. Angiogenic effects of dalteparin and gsHep on cultured endothelial cells.

(A) Effects of dalteparin and gsHep on human umbilical vein endothelial cell (HUVEC) tube formation on growth factor-reduced Matrigel and cultured in growth media. (B) Effects of conditioned media derived from dalteparin- and gsHep-treated placental villous explants on HUVEC tube formation on growth factor-reduced Matrigel. (C) Representative images of tube formation were taken from HUVECs treated with media conditioned with 2 μg/mL treatment. *$p < 0.05$ compared to no heparin controls. n = 5 media samples. Data presented as mean +/- SD. Scale bars = 200 μm.

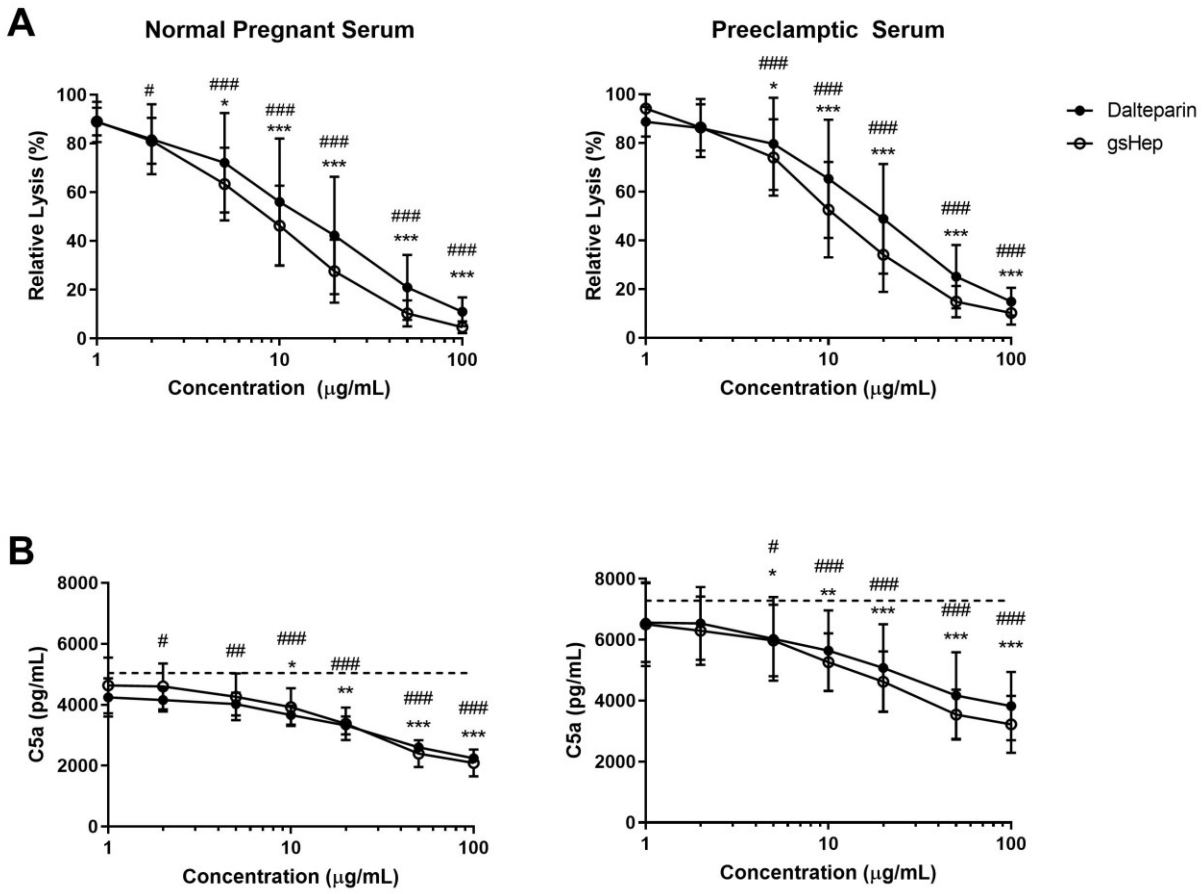

Figure 3.5. Anti-complement activities of dalteparin and gsHep.

(A) Effects of dalteparin and gsHep in a complement-mediated hemolysis assay using sheep red blood cells. 3.5% serum from normal pregnant or preeclamptic patients were used for the source of complement proteins. (B) ELISA analyses of the reaction supernatant for the C5a pro-inflammatory mediator. Dotted line represents no heparin control. * = dalteparin; # = gsHep. *,#p < 0.05, **,##p < 0.01, ***,###p < 0.001 compared to no heparin control. n = 3 serum samples. Data presented as mean +/- SD.

activity was achieved with 10 µg/mL dalteparin or gsHep treatment. To determine whether dalteparin and gsHep also reduced the generation of the C5a pro-inflammatory anaphylatoxin, a C5a ELISA was performed on the hemolytic reaction supernatant. There was a dose-dependent decrease in the generation of C5a for both treatments, also achieving approximately 50% inhibition at 10 µg/mL treatment concentration ($p < 0.0001$; Figure 3.5B). No significant difference between dalteparin and gsHep was observed ($p = 0.45$ and $p = 0.86$ for normal pregnant and preeclamptic serum, respectively). These results suggest that both dalteparin and gsHep impact complement activation relating to complement-mediated hemolysis and C5a generation in a comparable manner.

3.3.6. Dalteparin and gsHep prevent leukocyte adhesion to preeclamptic serum-activated endothelial cells

The leukocyte adhesion assay was used to investigate if the heparins can reduce the inflammatory effects of endothelial activation. As previously reported, preeclamptic serum activated cultured endothelial cells and increased THP-1 monocyte adhesion to the endothelial monolayer[227]. Both dalteparin and gsHep interfered with the adherence of THP-1 monocytes onto activated endothelial cells ($p < 0.01$; Figure 3.6). No significant difference between dalteparin and gsHep was observed ($p = 0.85$). These data suggest that dalteparin and gsHep can inhibit inflammation mediated by leukocyte adhesion to activated endothelial cells in preeclampsia.

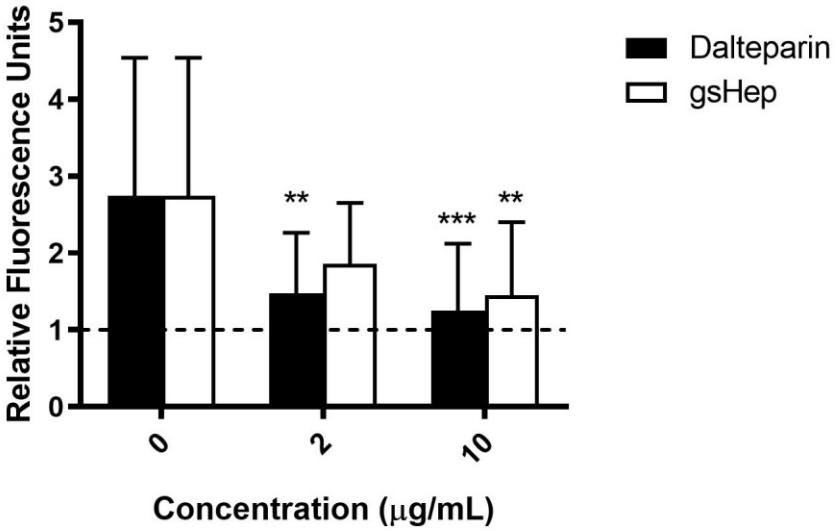

Figure 3.6. Inhibition of leukocyte adhesion onto endothelial cells by dalteparin and gsHep.

Effects of dalteparin and gsHep on adhesion of fluorescently-labelled THP-1 monocytes to HUVECs stimulated with 10% preeclamptic serum. Dashed line represents FBS control. **p < 0.01, *** p < 0.001 compared to no heparin control. n = 8 serum samples. Data presented as mean +/- SD.

3.4. Discussion

The experimental findings from this chapter confirm that dalteparin and gsHep exert similar *in vitro* placental, endothelial and anti-inflammatory effects. Both dalteparin and gsHep stimulated PlGF release from 1st trimester placental explants, promoted endothelial tube formation in the presence of placenta conditioned media, inhibited complement activation, and inhibited leukocyte adhesion to endothelial cells activated by preeclamptic serum. Importantly, it was confirmed that gsHep has negligible anticoagulant properties compared to dalteparin, due to the lack of functional ATBR. Overall, these collective actions suggest that both dalteparin and gsHep may be capable of exerting non-anticoagulant effects on several pathways that could impact the pathogenesis of preeclampsia.

3.4.1. Placental and angiogenic effects of dalteparin and gsHep

Placental ischemic injury is a hallmark of severe preeclampsia[22], and therefore a therapeutic strategy designed to support or promote normalized physiologic trajectory of placental development is an attractive approach for the prevention of severe early-onset preeclampsia. In this study, both dalteparin and gsHep increased gene expression of *PCNA* (Figure 3.3A), a proliferation antigen, in whole 1st trimester placental explants; this is consistent with a previous study published by our group where dalteparin was demonstrated to increase cytotrophoblast proliferation in 1st trimester placental villous explants[205]. Restoration of the physiologic turnover of the villous trophoblast compartment may be an effective pathway to prevent preeclampsia, as healthy syncytiotrophoblast secretes PlGF into the maternal circulation[311], which is hypothesized to be protective against this disease[312,313].

Angiogenic protein imbalance is commonly observed in preeclampsia, and is thought to contribute to the pathogenesis of preeclampsia by inducing endothelial dysfunction[101]. In this study, both dalteparin and gsHep increased the angiogenic potential of the conditioned media derived from dalteparin- and gsHep-treated placental explants, observed as an increase in PlGF levels in the conditioned media (Figure 3.3B). Treating HUVECs seeded onto Matrigel with the dalteparin- and gsHep-treated placenta conditioned media also improved tube formation, a surrogate assay of angiogenesis (Figure 3.4). These data support the hypothesis that LMWH favorably normalizes angiogenic protein release by the placenta, which may reduce the impact of placental dysfunction on maternal endothelial and cardiovascular function in women at high risk of preeclampsia[101].

Interestingly, despite an increase in PlGF release and in the angiogenic potential of the conditioned media, the steady-state mRNA expression of *PlGF* was dose-dependently inhibited by dalteparin and gsHep (Figure 3.3A). Although heparin-mediated regulation of PlGF expression has not been elucidated, it is possible that the predominant effect of treatment in this experimental model was stimulation of proliferation. Both syncytiotrophoblast differentiation and PlGF expression are controlled by GCM1 and its activity is suppressed by proliferation[207,314]. Asymmetric cellular division followed by differentiation of the villous cytotrophoblast is required to replenish the syncytiotrophoblast layer, and a persistent mitogenic signal due to constant dalteparin and gsHep exposure *in vitro* could limit the differentiation pathway in favor of increased proliferation. This could therefore result in a relative reduction in *PlGF* mRNA expression and a relative increase in *PCNA* mRNA expression after 24 hours in culture. *In vivo*, the cyclical availability of LMWH from daily subcutaneous

injections followed by its clearance from the circulation could therefore stimulate both proliferation and differentiation, and mitigate the undesired constant mitogenic signal.

LMWH can bind to and displace sFlt1 associated with the extracellular matrix on endothelial cell surfaces[214,216]. Although the *in vivo* implications of this effect remains unclear, the interaction of heparins and sFlt1 could disrupt the decoy action of sFlt1 at the endothelial cell surface, and contribute to the clearance of excess sFlt1[214]. Here, it was shown that gsHep can still interact with sFlt1 in the absence of functional ATBRs (Figure 3.2). Therefore, a non-anticoagulant heparin could, in theory, be used to facilitate the efficient removal of excess sFlt1 from the endothelial cell surface to be eliminated in the urine of pregnant women at high risk of preeclampsia[214]. This mechanism may be equivalent to apheresis of sFlt1 using a heparin-binding column[102], yet could be safer, more feasible and cost-effective.

3.4.2. Anti-inflammatory effects of dalteparin and gsHep

Widespread host inflammation and complement activation is present in women with preeclampsia. Activated complement fragments are observable in biological fluids[113,115,315] and on the placenta, which can play a role in placental damage[110,112]. Excess complement activation also occurs in HELLP syndrome, inhibition of which could lead to disease resolution[120]. Elevated serum levels of the complement anaphylatoxins such as C5a can also contribute to endothelial dysfunction[116]. This study demonstrated that both dalteparin and gsHep can suppress complement activation, reducing lytic damage to sheep erythrocytes and reduced generation of C5a (Figure 3.5). Clinically relevant doses of LMWH have been shown to suppress complement

activation *in vivo*[226]. Suppression of complement-mediated inflammation is therefore a potential mechanism by which LMWH can confer protection against preeclampsia.

The premise of LMWH therapy for the prevention of preeclampsia has traditionally been to prevent thrombosis-mediated placental damage. However, the strength of the association between placental thrombotic disease and preeclampsia is controversial[316], with recent data suggesting only a 25% contribution of placental thrombosis to disease burden[2]. A wide variety of other factors, including placental dysfunction, cardiovascular status, and inflammation, also contribute to increased preeclampsia risk, which in turn may be blocked by non-anticoagulant actions[198]. Interestingly, a recent meta-analysis suggests that LMWH may be most beneficial for pregnant women at risk of preeclampsia who do not have a pre-existing thrombophilia disorder[187], suggesting that LMWH confers protection against preeclampsia via non-anticoagulant pathways. This concept has not been directly investigated in the clinical setting. The validation of gsHep in this study as a LMWH mimetic without anticoagulant properties serves as a justification for further *in vivo* experiments to determine specifically whether the non-anticoagulant effects are therapeutically relevant in the context of preeclampsia prevention. The hypothetical advantage of a non-anticoagulant therapeutic is that it may permit the use of such heparin derivatives in pregnant women for the prevention of preeclampsia without incurring significant risk of bleeding complications.

3.4.3. Limitations to the study

There are limitations to the interpretation of results related to this investigation. First, a major limitation is the inherent disadvantage of *in vitro* studies, the results of which do not

necessarily translate to the *in vivo* or clinical settings. The current study provides a strong basis for further investigation into the *in vivo* relevance of the observed effects and the anticoagulation-independent actions of heparin for the prevention of preeclampsia. Second, while gsHep is a very close approximation to native heparin in biochemical structure, there may be minor differences that may impact its non-anticoagulant functions. The process of glycol-splitting that yields gsHep specifically modifies non-sulfated uronic acid residues, which mainly localizes to the ATBR but may also be found scattered throughout the molecule[195]. It is not known whether these modifications outside of the ATBR could also impact non-anticoagulant properties not assessed in this study. Furthermore, disruption of the ATBR prevents the inactivation of the major coagulation enzymes Factor Xa and thrombin by heparin-antithrombin complexes. However, these enzymes may also contribute to additional anticoagulation-independent effects such as by activating protease-activated receptors on cell surfaces or regulating the activity of other proteins through proteolytic cleavage, among other effects[317]. Thus, these non-anticoagulant effects may not be fully evaluated by gsHep.

3.5. Conclusion

In summary of this chapter, dalteparin and gsHep are confirmed to exert similar *in vitro* placental, endothelial and anti-inflammatory effects, while gsHep exhibits negligible anticoagulant activity compared to dalteparin. These results validate the use of gsHep as a molecular tool to specifically examine the potential role of non-anticoagulant properties of LMWH in the RUPP model of preeclampsia.

4. Evaluating the Effects of Dalteparin and gsHep in the RUPP Model of Preeclampsia

4.1. Introduction

In the previous chapter, it was determined that gsHep retained similar *in vitro* placental, angiogenic, and anti-inflammatory effects as dalteparin, while the ability of gsHep to mediate anticoagulant activity was significantly diminished due to its inability to interact with antithrombin. However, limited *in vivo* or clinical studies have investigated the effects of LMWH or gsHep on placental function. The objective of this chapter was to compare the effects of dalteparin and gsHep in the RUPP model of preeclampsia, with the hypothesis that both dalteparin and gsHep will ameliorate features of preeclampsia induced by the model including hypertension and fetal growth restriction.

4.2. Methods

Treatment and Sample Collection

The RUPP model was induced as described in Section 2.3. For treatment, daily subcutaneous injections of dalteparin or gsHep were administered beginning 24 hours after surgery to allow for ample recovery time from surgery and reduce the risk of bleeding (Figure 4.1). Two doses for each treatment were selected: a low-dose of heparin (67 IU/kg dalteparin or 0.42 mg/kg gsHep) and high-dose of heparin (300 IU/kg dalteparin or 1.92 mg/kg gsHep). These dosages were selected to reflect clinically-relevant prophylactic and therapeutic doses,

*A modified version of Chapter 4 has been accepted for publication at the time of submission: Wat JM, Baczyk D, Kingdom JC. The antithrombin binding regions of heparin mediate fetal growth and reduced placental damage in the RUPP model of preeclampsia. *Biol Reprod*.

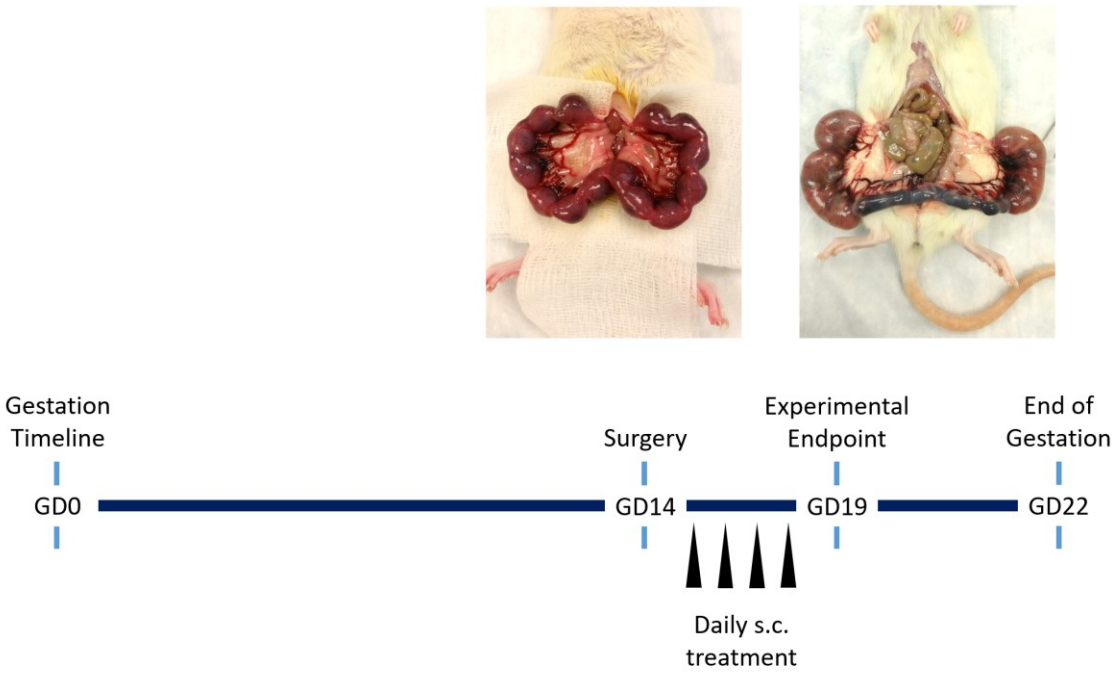

Figure 4.1. Schematic of the RUPP experimental setup.

On gestation day (GD) 14, clips were surgically implanted to induce RUPP. The number of viable fetuses (upper left photograph) were counted at this time to compare with at the experimental endpoint. Treatments were subcutaneously administered once daily beginning on GD15 and ending on GD18 for a total of 4 injections. Experimental endpoint was on GD19, when features of preeclampsia including hypertension and fetal growth restriction were apparent. Fetal resorption (upper right photograph) was a feature in this RUPP model.

between approximately 0.2 and 1.0 IU/mL peak anti-Factor Xa activity, respectively. The appropriateness of the selected doses was verified in a time course experiment (Figure 4.2). Saline of equivalent volumes to treatment doses were used as the vehicle control.

Following blood pressure measurements on GD19 as described in Section 2.4.2, Sarstedt S-monovettes (Numbrecht, Germany) were connected to the catheter to collect serum and plasma-EDTA samples. Serum blood samples were allowed to clot for 20 min at room temperature before centrifuging at 2000 x g for 10 min to collect serum. Samples were stored at -80 ^{o}C until further analyses. PlGF levels in the plasma was measured using a commercially available ELISA kit from R&D Systems (Minneapolis, MN, USA; cat no.: MP200). To measure the coagulability of rat blood, the aPTT assay was performed using the Pacific Hemostasis APTT-XL kit (Thermo Fisher Scientific) according to manufacturer's instructions, similar to previously described in Section 3.3.1, but for plasma samples without addition of exogenous heparin.

To perform fetal measurements and collect placenta samples, viable fetuses and placentas were dissected from the uterus. Amniotic fluid was drained, and the fetuses and placentas were blotted on to sterile gauze before weighing. Weights were averaged within the litter per data point. Half of each placenta were then formalin-fixed overnight and embedded into paraffin blocks for histological analyses, while samples of the remaining placenta were flash frozen in dry ice for RNA analyses before transferring into -80 ^{o}C for long term storage.

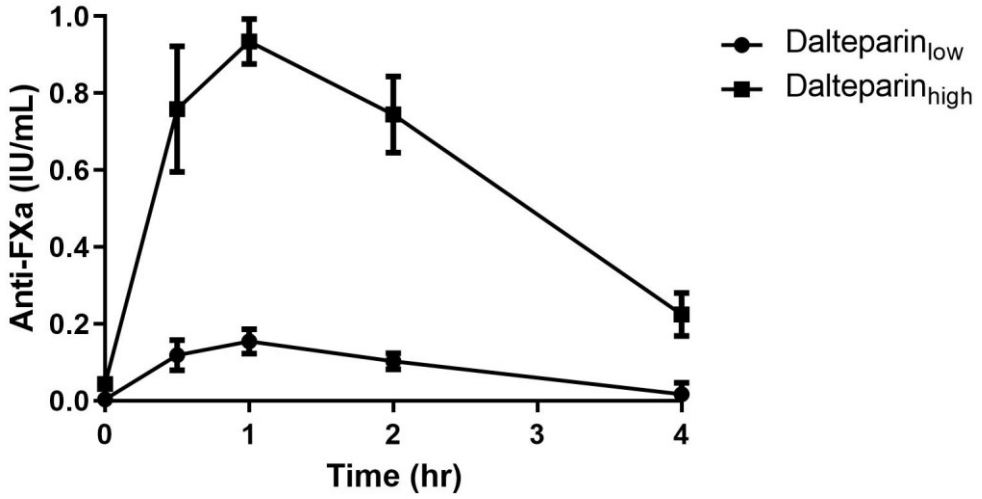

Figure 4.2. Evaluation of the selected doses of dalteparin in rats.

Non-pregnant rats were subcutaneously injected with either a low (66.7 IU/kg) or high (300 IU/kg) dose of dalteparin, and serial plasma samples were collected at t = 0, 0.5, 1, 2, and 4 hours. Anti-Factor Xa (Anti-FXa) activity assay was performed on the plasma to evaluate the anticoagulant activity of dalteparin. N = 3 rats for each experiment.

Morphometry Analysis

Morphometry of the placental labyrinth bisected at the midpoint was analyzed using Visiopharm stereology software. 12.5% of the labyrinth area was quantified at 40x magnification, approximately 50-70 randomly sampled frames per placenta. In each frame, 6 points marked by the software were categorized as either maternal blood space, fetal blood space, or placental tissue, which included syncytiotrophoblast, sinusoidal trophoblast giant cells, and fetal vascular endothelial cells. Frames and points that sampled non-regions of interest, such as the junctional zone, the central canal, and trophoblast giant cells were excluded. One histological section from an average of 4 placentas from each litter were quantified and averaged as one biological replicate per dam. 6 animals in each of sham, RUPP, RUPP + dalteparin$_{low}$, and RUPP + gsHep$_{low}$ were included in this analysis.

To quantify the area of the junctional zone and labyrinth, the area was manually demarcated and measured with stereology software. Each data point is expressed as an average of the area quantified from each section on the slide (average of 4 sections per slide), representing one biological replicate per dam. 6 animals in each of sham, RUPP, RUPP + dalteparin$_{low}$, and RUPP + gsHep$_{low}$ were included in this analysis.

Immunohistochemistry

Five micrometer thick sections of placentas were hydrated in xylene (10 min x 2 repeats) and successively lower concentrations of ethanol (100% x 3, 95% x 1, 90% x 1, 80% x 1, 70% x 1, 50% x 1 repeat) until cleared. Antigen retrieval was performed in a solution of 10 mM sodium citrate with 0.05% Tween-20, pH 6.0, for 20 min heated to 95 ^{o}C in a microwave.

Permeabilization was achieved by incubating sections with 0.2% Triton X-100 in PBS for 10 min. After blocking sections with 1% bovine serum albumin in PBS containing 0.02% Triton X-100 (BSA/PBS-T) for 30 min, sections were incubated with primary antibody diluted in BSA/PBS-T overnight at 4 ^{o}C in a humidified chamber. Sections were washed with PBS for 10 min and then incubated in a solution of 3% hydrogen peroxide diluted with methanol to block endogenous peroxidase activity for 30 min. Samples were then incubated with 1:300 biotinylated secondary antibody diluted in BSA/PBS-T for 1 hour at room temperature, washed with PBS, incubated with 1:2000 horseradish peroxidase-streptavidin in BSA/PBS-T, and then washed thoroughly in PBS for 30 min. 3,3'-Diaminobenzidine was used to develop the stain, and a hematoxylin counterstain was applied. Samples were then dehydrated and coverslipped for analysis.

Tissue Processing for Micro-Computed Tomography

For a subset of animals, placentas were collected and processed for micro-computed tomography to examine fetoplacental arterial tree branching. The uterine horn with fetuses and placentas were placed in ice-cold PBS to preserve viability while individual samples were being processed. An individual pup and placenta with intact umbilical cord was transferred to a clean petri dish containing PBS. Drops of pre-warmed PBS were slowly applied to the placenta, umbilical cord, and fetus through a syringe to stimulate circulation and vasodilation of the umbilical artery. Once the umbilical artery had dilated, drops of 3% paraformaldehyde were applied onto the length of the umbilical artery to prevent vasospasm. Once the vessel was fixed, Microfil MV-122 contrast agent (Flow-Tech Inc.) was prepared according to manufacturer instructions and loaded into a 1 mL syringe. Using a 24-gauge 3/4" Becton Dickinson Insyte

Autoguard Shielded IV Catheter similar to that used for blood pressure measurements described in Section 2.4.2, the umbilical vein was nicked to create a vent for blood to drain, and then the catheter was introduced into the umbilical artery using the needle to gain access. The needle was then removed from the catheter, and prewarmed perfusate (1% xylocaine, 0.45% sodium chloride, 0.75 IU/mL heparin) in a 1 mL syringe was then connected to the catheter and gently infused to flush blood out of the placenta. The syringe was then replaced with the syringe containing the Microfil contrast agent, and the contrast was gently infused into the placenta just until the agent reached the venous capillaries. The umbilical artery and vein bundle were then clamped with bulldog clamps to maintain pressure and allow the contrast agent to polymerize and set, which took approximately 45 min. The placenta was then excised and fixed in formalin for 24 hours, stored in PBS, and then mounted in 1% agarose gel.

To acquire 3D images of the fetoplacental arterial vasculature, the agarose-mounted, contrast-infused placentas were scanned using a Skyscan 1272 micro-CT scanner (Skyscan, Belgium). With the X-ray source at 50 kV and 201 µA, the samples were rotated 360° in 0.4° increments, generating 900 views over approximately 2 hours, which were reconstructed into data blocks with a 13.4 µm voxel size. MNI Display tool was used to generate a 3D render of the arterial vascular surface, and an automated image analysis algorithm was then applied to identify vessel segments and branches, and measure vessel lengths and diameters. Samples where perfusion of the contrast agent was incomplete or had disrupted the integrity of the arterial vasculature were omitted.

Quantitative Polymerase Chain Reaction

For each dam, viable placentas were harvested, pooled, flash frozen with dry ice, and stored at -80 ^{o}C until processing. Frozen tissue was finely ground with a mortar and pestle, and then RNA extraction and qRT-PCR was performed as described in Section 3.2. Primers used in this study are provided in Table 4.1. Gene expression was normalized to the geometric mean of the housekeeping genes *YWHAZ* and *HPRT*.

Proliferation Assay

BeWo choriocarcinoma cells (ATCC) were seeded onto 96-well culture plates at a density of 10,000 cells/well and incubated with 5% serum from preeclamptic patients overnight. Cells were washed with PBS and then incubated with growth media for 72 hours in the presence of agonists for protease activated receptor (PAR)-1 (10 µM), PAR-2 (100 µM), or recombinant Factor Xa (10 U/mL). Cells were approximately 80% confluent by the end of the incubation period. The sulforhodamine B assay (Sigma Aldrich), which estimates total biomass based on total cellular protein content, was then performed according to manufacturer's instructions.

Statistical Analyses

All experiments were performed as technical triplicates. Data are presented as median [interquartile range]. Multiple comparisons used vehicle-treated RUPP rats as the comparison group. Parametric (one-way ANOVA with Dunnett's post-hoc) or non-parametric (Kruskal-Wallis test with Dunn's post-hoc) analyses were used where appropriate, based on normality. GraphPad Prism 8.0 software (La Jolla, CA, USA) was used for analyses and $p \leq 0.05$ was considered statistically significant.

Table 4.1. List of primers used for qRT-PCR studies in rat placentas.

SNAT1	F: 5'-CTCAAAGACGGTGTACGCTT-3' R: 5'-TTGAGCTCGCTGTAGATTGG-3'
SNAT2	F: 5'-CTCACAGACTGTGTATGCTG-3' R: 5'-GCTCTTCAGCTCTTCATAGATGG-3'
SNAT4	F: 5'-GGTACCTCAATGGCAACTAC-3' R: 5'-GGTGTAGCCAAGGTAGCCTA-3'
LAT1	F: 5'-GCTGTGACCTTTGCAGATCA-3' R: 5'-CTAGAAGCAGCCACAATGGA-3'
LAT2	F: 5'-GGATCCTTACAAGAACCTTCC-3' R: 5'-GGACATTGCAGTGACATAGG-3'
GLUT1	F: 5'-TTCACTGTGGTGTCGCTGTT-3' R: 5'-ATGGTCATGAGCACAGCAC-3'
GLUT3	F: 5'-CAGACGCAACTCCATGCTT-3' R: 5'-CCAGGATCAGCATTTCAACC-3'
GLUT4	F: 5'-CCAGCATCTTTGAGTTAGCTG-3' R: 5'-TACTAAGAGCACCGAGACC-3'
GCM1	F: 5'-GAAGCTGCCTCAGAATGTGA-3' R: 5'-TGTGCGCTCCTGTCGTCTGA-3'
SFLT1	F: 5'-GGAAGACTCGGGCACCTATG-3' R: 5'-CTGCAGTGCTCACCTCTAAC-3'
SYN	F: 5'-CATTCGTGTTAGCCACAACC-3' R: 5'-GCCTAGAACGATGACTATGG-3'
PAR1	F: 5'-GTGCGGCCCGCTGTTGTCT-3' R: 5'-GTTGCTTTTGATTCTGGCCTGC-3'
PAR2	F: 5'-CAGTGGCACCATCCAAGGAA-3' R: 5'-TCCAGTGAGGACAGATGCAG-3'
PLGF	F: 5'-CAGCCAACATCACTATGCAG-3' R: 5'-CAGAATAGGCCTGCATTCGC-3'
HPRT	F: 5'-CAGTCCCAGCGTCGTGATTA-3' R: 5'-CAAGTCTTTCAGTCCTGTCC-3'
YWHAZ	F: 5'-CTTGACATTGTGGACATCGG-3' R: 5'-GGCAGACAAAGGTTGGAAGG-3'

Sample Size Calculation

Sample size was estimated based on the potential of LMWH treatment to reduce blood pressure in RUPP-induced animals. Preliminary experiments from n = 3 sham-operated rats determined an average mean arterial blood pressure reading of 80 ± 7.8 mmHg; previous studies have demonstrated a 25% increase in blood pressure in RUPP-induced rats[267]. Using G*Power Statistical Power software[318], it was estimated that 72 animals in total (n = 12 in each group) would be required to detect a 10% reduction in blood pressure in heparin-treated RUPP rats, compared to vehicle-treated animals. Sample size estimates assumed 1 - β = 0.2 and two-sided α of 0.05.

4.3. Results

4.3.1. Maternal and pregnancy outcomes

The first specific aim of this study was to evaluate the maternal features and pregnancy outcomes of the RUPP model, such as blood pressure and fetal growth.

4.3.1.1. RUPP-induced hypertension was not reversed by dalteparin or gsHep

Reducing uterine perfusion pressure in the RUPP animals significantly elevated diastolic and mean arterial blood pressure compared to the sham-operated group (Figure 4.3). Neither dalteparin nor gsHep, at low or high doses, had a significant impact on systolic, diastolic, or mean arterial blood pressure compared to the vehicle-treated group ($p > 0.05$).

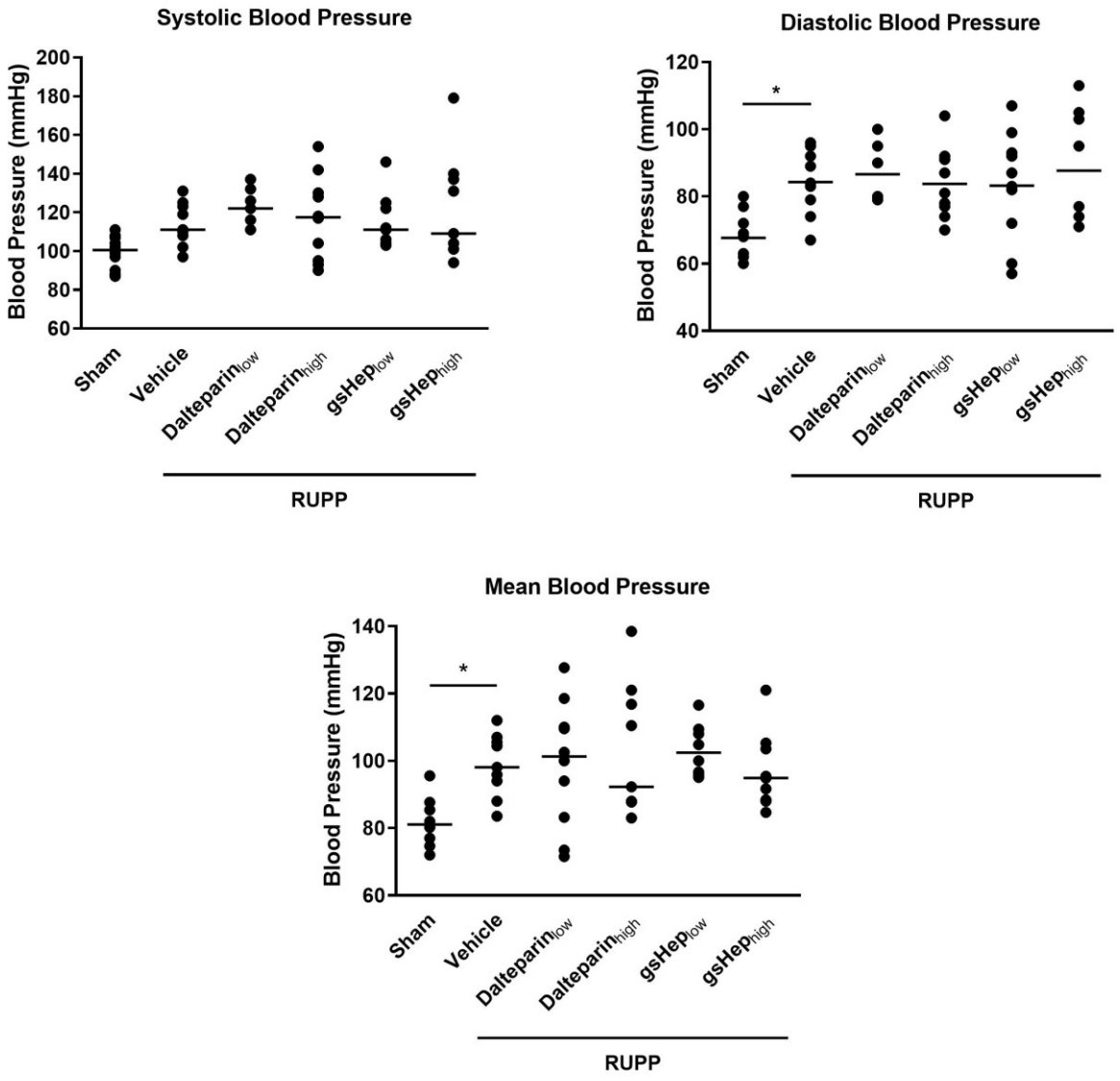

Figure 4.3. Effects of RUPP and treatments on maternal blood pressure.

Blood pressure measurements were performed on GD19 prior to sacrifice via carotid catheterization. Kruskal-Wallis test with Dunn's multiple comparison test, * $p < 0.05$ compared to RUPP-vehicle group, n = 8-11 dams per group. Line represents the median.

4.3.1.2. Low-dose dalteparin improved fetal growth stunted by RUPP

Reduction in uterine perfusion pressure induced on average 60% fetal resorption rate compared to sham (sham: 0.0 [0.0-0.0] %; RUPP: 60.4 [50.0-79.7] %; $p < 0.0001$; Figure 4.4), there was no significant difference in fetal loss between the RUPP control and treatment groups ($p > 0.05$). RUPP also significantly impaired fetal growth; fetuses from sham animals were 17% larger than the viable fetuses from RUPP animals (sham: 2.57 [2.42-2.71] grams; RUPP: 2.19 [2.12-2.33] grams; $p < 0.001$; Figure 4.5A). Low-dose dalteparin treatment significantly improved the growth of viable fetuses compared to the RUPP group (2.48 [2.33-2.49] grams, $p < 0.05$); none of high-dose dalteparin, low-dose gsHep, or high-dose gsHep had a statistically significant impact on fetal growth ($p > 0.05$). Placental weights were not significantly different between the groups, including between sham and vehicle-treated RUPP ($p = 0.09$; Figure 4.5B), although changes in placental weights trended in the same directions as fetal weight changes. Altogether, this suggests that improved fetal growth in the low-dose dalteparin group cannot be attributed to compensatory blood flow to fewer viable pups.

4.3.2. Characterization of the placenta

As fetal growth was restored through low dose dalteparin treatment, the mechanism responsible for this effect was hypothesized to be related to the placenta. This specific aim therefore focused on characterizing the phenotype of the placenta. Since only low dose dalteparin exerted an observable effect, these analyses were only performed on the low dose dalteparin and gsHep conditions.

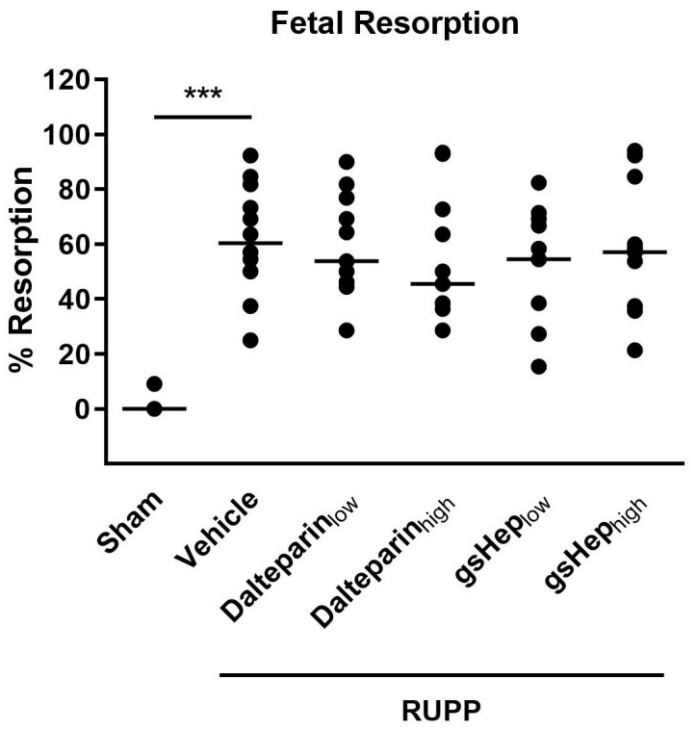

Figure 4.4. Effects of RUPP and treatments on fetal resorption.

The number of viable fetuses were counted at surgery and at sacrifice; viable fetuses are expressed as percent fetal resorption. Kruskal-Wallis test with Dunn's multiple comparison, *** $p < 0.0001$ compared to RUPP-vehicle group, n = 11-12 dams in each group. Lines represent the median.

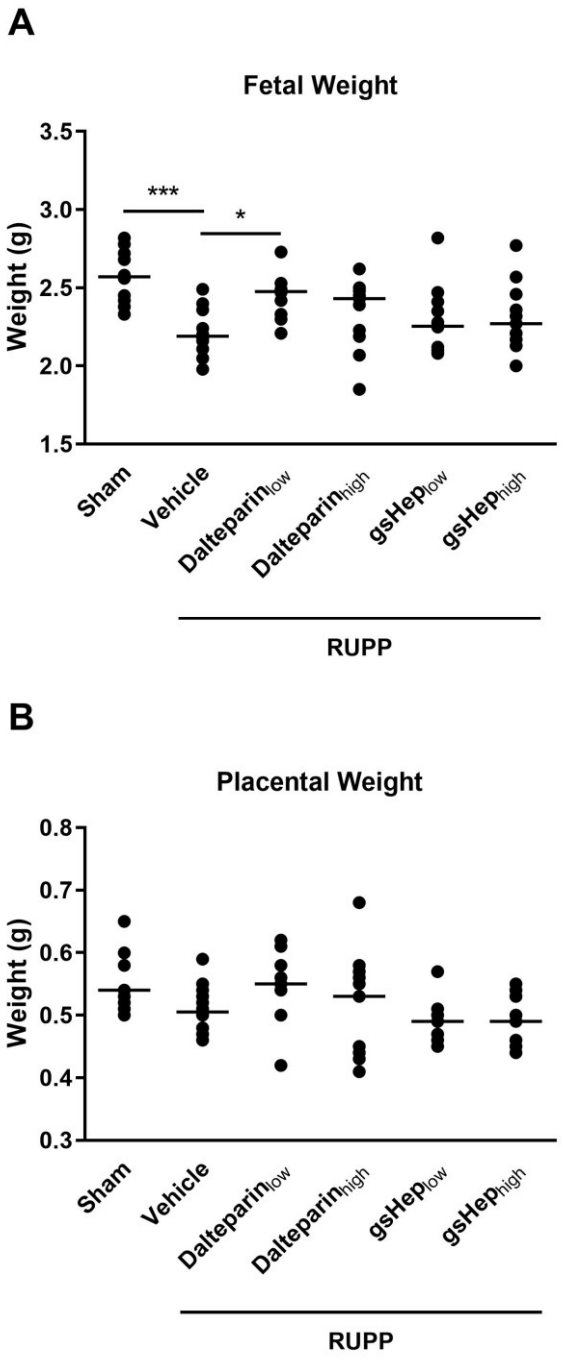

Figure 4.5. Effects of RUPP and treatments on fetoplacental weight.

Average viable (A) fetal and (B) placental weights were measured after sacrifice at GD19. One-way ANOVA with Dunnett's multiple comparison test, *$p < 0.05$, *** $p < 0.001$ compared to RUPP-vehicle group. n = 11-12 dams in each group. Lines represent the median.

4.3.2.1. RUPP placentas exhibited a histologically abnormal placental structure that was restored by dalteparin$_{low}$ treatment

Histological sections of the placenta were examined under the light microscope for structural changes. Compared to placentas from sham animals, those from RUPP animals showed perturbed features, especially in the labyrinth where maternal sinusoidal blood spaces appeared abnormally expanded (Figure 4.6). Morphometric analyses revealed that the relative area occupied by sinusoidal blood spaces in RUPP placentas was greater than in sham placentas (sham: 0.34 [0.28-0.37]; RUPP: 0.43 [0.39-0.47]; $p < 0.01$; Figure 4.7A); correspondingly, placental cell area was reduced in RUPP placentas compared to sham placentas (sham: 0.60 [0.58-0.65]; RUPP: 0.50 [0.46-0.54]; $p < 0.01$; Figure 4.7B). Placentas from RUPP animals treated with dalteparin$_{low}$ exhibited a restorative effect, with reduced sinusoidal area (0.35 [0.30-0.38]; $p < 0.05$) and increased placental cell area (0.58 [0.56-0.63]; $p < 0.01$), resembling those of sham placentas. RUPP placentas treated with gsHep$_{low}$ exhibited a mixed phenotype that was not statistically significant (sinusoidal area: 0.38 [0.34-0.42]; placental cell: 0.56 [0.53-0.60]; $p > 0.05$).

In addition, the junctional zone area of the RUPP placenta, normalized to the area of the labyrinth, was significantly smaller than that of sham placentas (Figure 4.8). Dalteparin$_{low}$-treated RUPP animals exhibited a trending increase in junctional zone size, although this was not statistically significant. However, the variation observed in the dalteparin$_{low}$ group was mainly contributed by 1 of 6 data points, which may contribute to a Type II statistical error. By

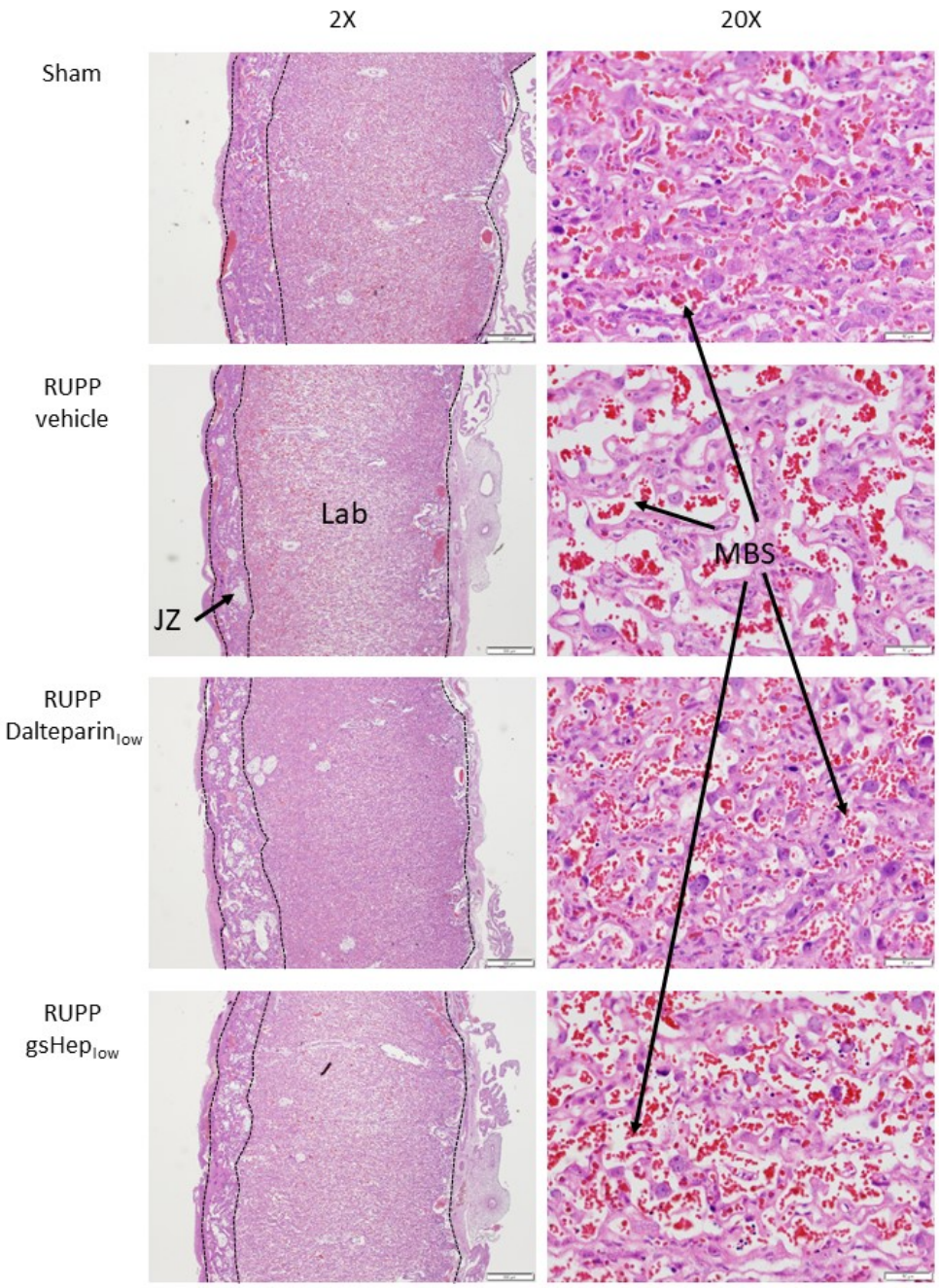

Figure 4.6. Representative images of H&E-stained sections of rat placentas.

Formalin-fixed placentas were bisected, embedded into paraffin blocks, sectioned, and stained with H&E for light microscopy analysis. Dotted lines in 2X magnification delineates borders of the junctional zone and labyrinth areas. 20X magnification shows placental labyrinth structure. JZ: junctional zone; Lab: labyrinth; MBS: maternal blood space. Scale bars: 2X = 500 µm; 20X = 50 µm.

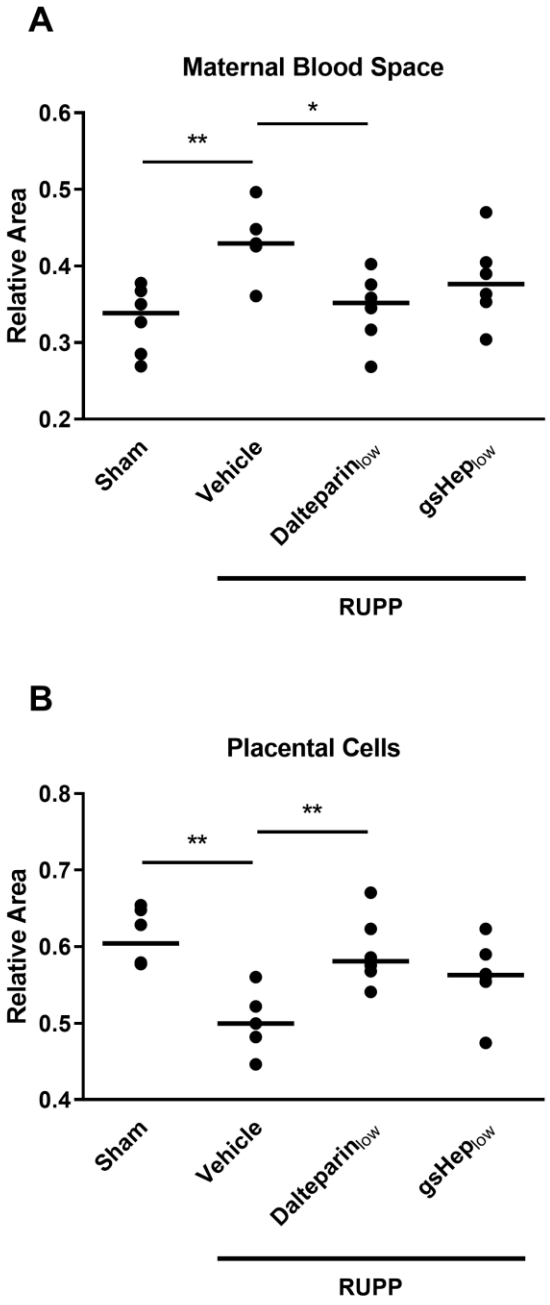

Figure 4.7. Morphometric analyses of the rat placental labyrinth.

Results from morphometric relative quantification of H&E sections for (A) maternal sinusoidal blood spaces and (B) placental cells in the rat placental labyrinth. One-way ANOVA with Dunnett's multiple comparison test, *$p < 0.05$, **$p < 0.01$ compared to RUPP-vehicle group. Each data point is derived from the average of 2-6 viable placentas from n = 5-6 dams in each group. Lines represent the median.

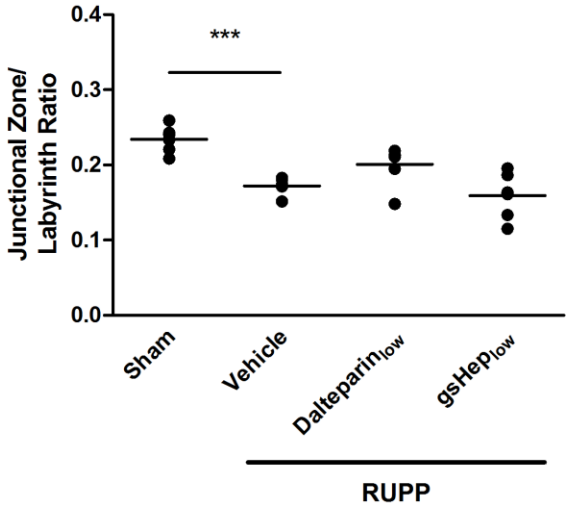

Figure 4.8. Quantification of the junctional zone and labyrinth areas of the rat placentas.

The junctional zone and labyrinth areas of H&E-stained placental sections were quantified using a calibrated computer software and expressed as a ratio. One-way ANOVA with Dunnett's multiple comparison test, *** $p < 0.001$ compared to RUPP-vehicle group. n = 6 dams in each group. Lines represent the median.

contrast, the size of the junctional zone in placentas from gsHep$_{low}$-treated animals do not appear to be different from vehicle-treated RUPP animals.

To examine whether proliferation of placental trophoblasts differed between vehicle- and heparin-treated RUPP, immunohistochemical stainings of rat placental sections for Ki67, a proliferation marker, were performed. Ki67 staining was absent in the labyrinth of GD19 placentas, suggesting that placental development was complete by this timepoint (Figure 4.9). Therefore, a separate set of animals sacrificed at GD17 was used for this analysis. By gross observation, there was no evidence that Ki67 staining in the labyrinth differed between sham and RUPP animals (Figure 4.10). Since the size of the junctional zone was smaller in vehicle-treated RUPP placentas compared to sham, Ki67 staining was further quantified in the junctional zone by morphometry. Morphometry analysis revealed no significant difference between sham, vehicle-treated RUPP, dalteparin$_{low}$-treated RUPP, and gsHep$_{low}$-treated RUPP in the number of Ki67 positive cells per square area of the junctional zone ($p = 0.22$; Figure 4.11).

Collectively, these results suggest that RUPP-mediated impairment of fetal growth was mediated by disruption of normal placental development; dalteparin but not gsHep treatment may mitigate the placenta-damaging effects of the RUPP procedure and promote fetal growth.

4.3.2.2. Fetoplacental arterial vasculature development was not affected by RUPP induction

Detecting changes to fetal blood space area through point counting morphometry proved challenging, as this technique was limited in sensitivity for small structures. Micro-computed tomography was therefore utilized to visualize the fetal arterial vasculature in the

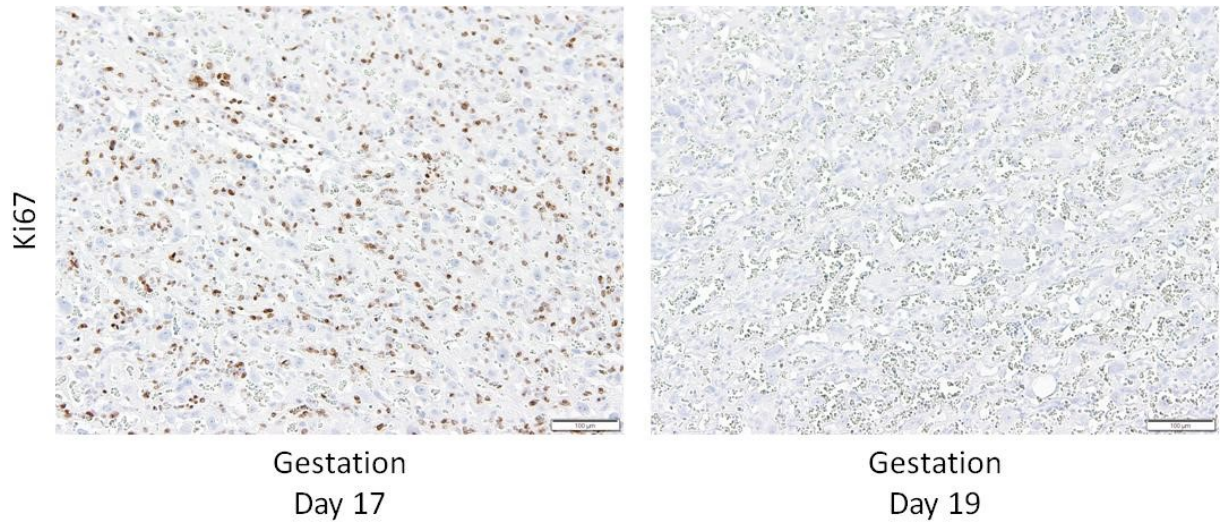

Figure 4.9. Representative images of Ki67 stained sections in GD17 and GD19 placental labyrinths from sham rats.

Sections were stained simultaneously to reduce the likelihood of procedural error. Scale bar = 100 μm.

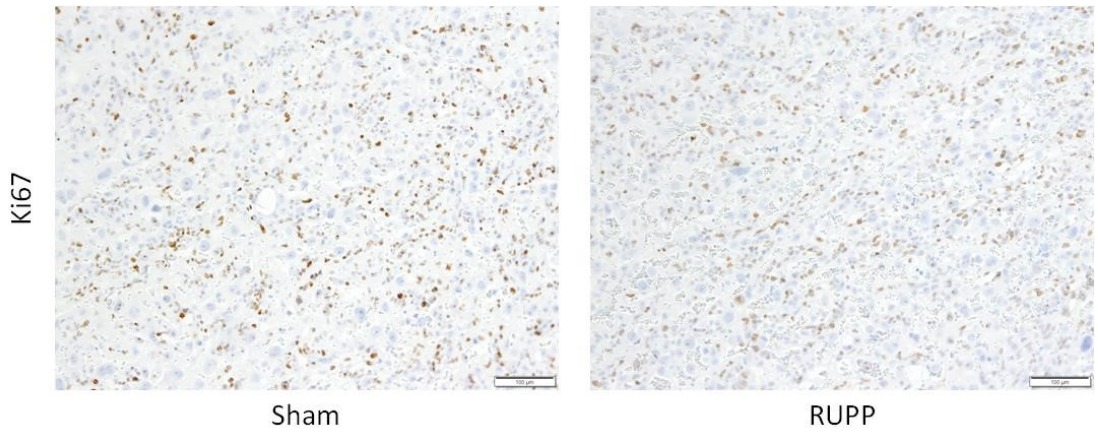

Figure 4.10. Representative images of Ki67 stained sections in the labyrinth of GD17 sham and RUPP placentas.

Sections were stained simultaneously to reduce the likelihood of procedural error. Scale bar = 100 µm.

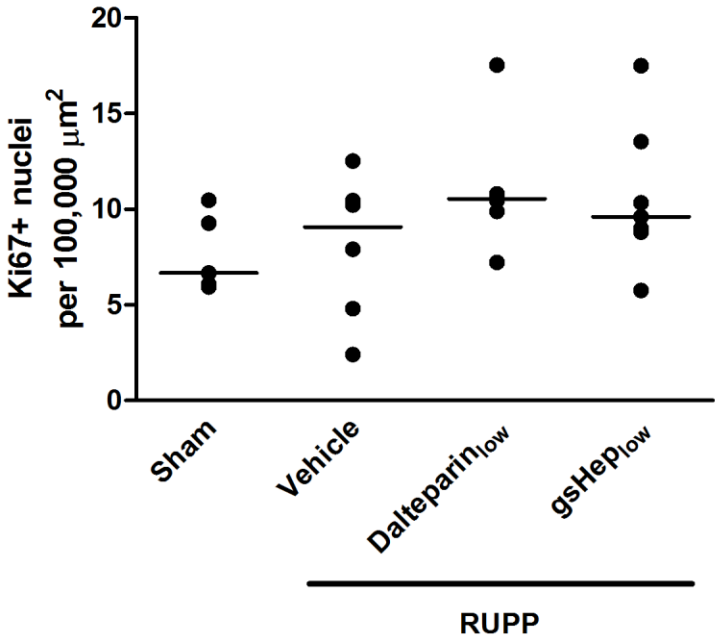

Figure 4.11. Morphometric quantification of Ki67+ cells in the junctional zone of GD17 rat placentas.

Stereology software was used to select five regions of interest to count Ki67+ cells and normalize to the area of interest. One-way ANOVA, n = 2-4 placentas from 4-6 rats in each group. Lines represent the median.

placenta in three dimension and assess whether changes to fetoplacental arterial vasculature could explain changes in fetal development. There was no evidence that fetoplacental arterial vasculature development was affected by the RUPP procedure (Figure 4.12 & Figure 4.13), suggesting that fetal growth restriction was mediated by impairment of the maternal placental compartment. There was a small but statistically significant difference in arterial lengths between vehicle and treated RUPPs, but the implication of this effect is unclear. Considering that fetal growth was improved with dalteparin treatment, it is unlikely that the small decrease in arterial density in the treated groups translates to impaired nutrient transport.

4.3.2.3. Placental mRNA expression of genes related to nutrient transport and placental function were not significantly different between experimental groups

Based on the finding that low dose dalteparin treatment resulted in improved fetal growth and reduced placental damage, mRNA expression of genes related to nutrient transport and placental function was examined. A panel screen of 11 different genes relating to nutrient transport, placental development, and placental function revealed no significant difference between sham, vehicle-treated RUPP, dalteparin$_{low}$-treated RUPP, and gsHep$_{low}$-treated RUPP (Figure 4.14). The lack of a statistically significant difference in mRNA expression between experimental groups could potentially be due to a dilution effect from isolating mRNA from whole tissue compared to specific cells or tissue region.

4.3.3. Potential mechanisms influencing features of the RUPP model

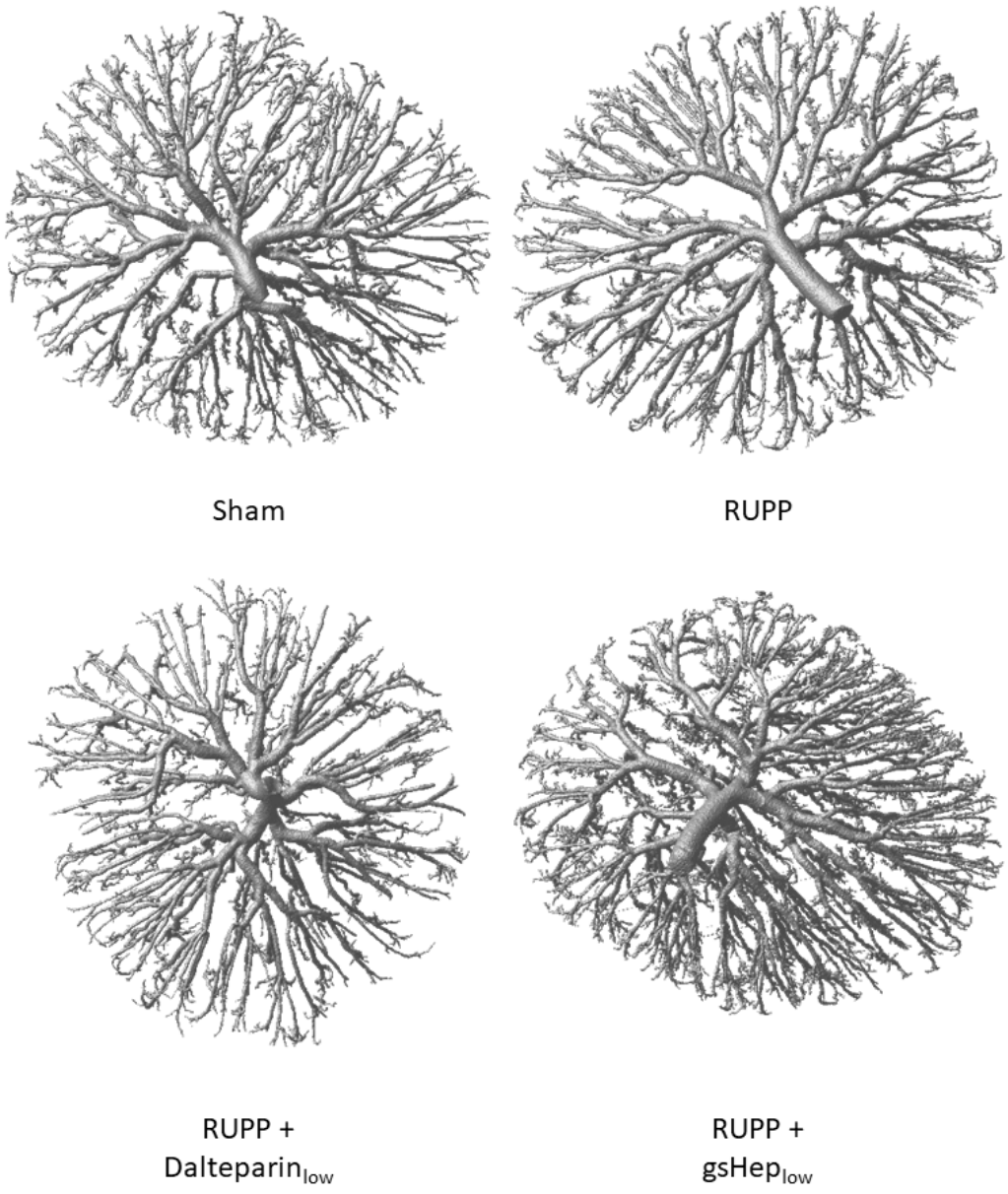

Figure 4.12. Representative micro-computed tomography renderings of the fetoplacental arterial vasculature from rat placentas.

Contrast agent was perfused into the placentas and scanned 360^{o} to produce layered images that were then compiled and rendered using MNI Display.

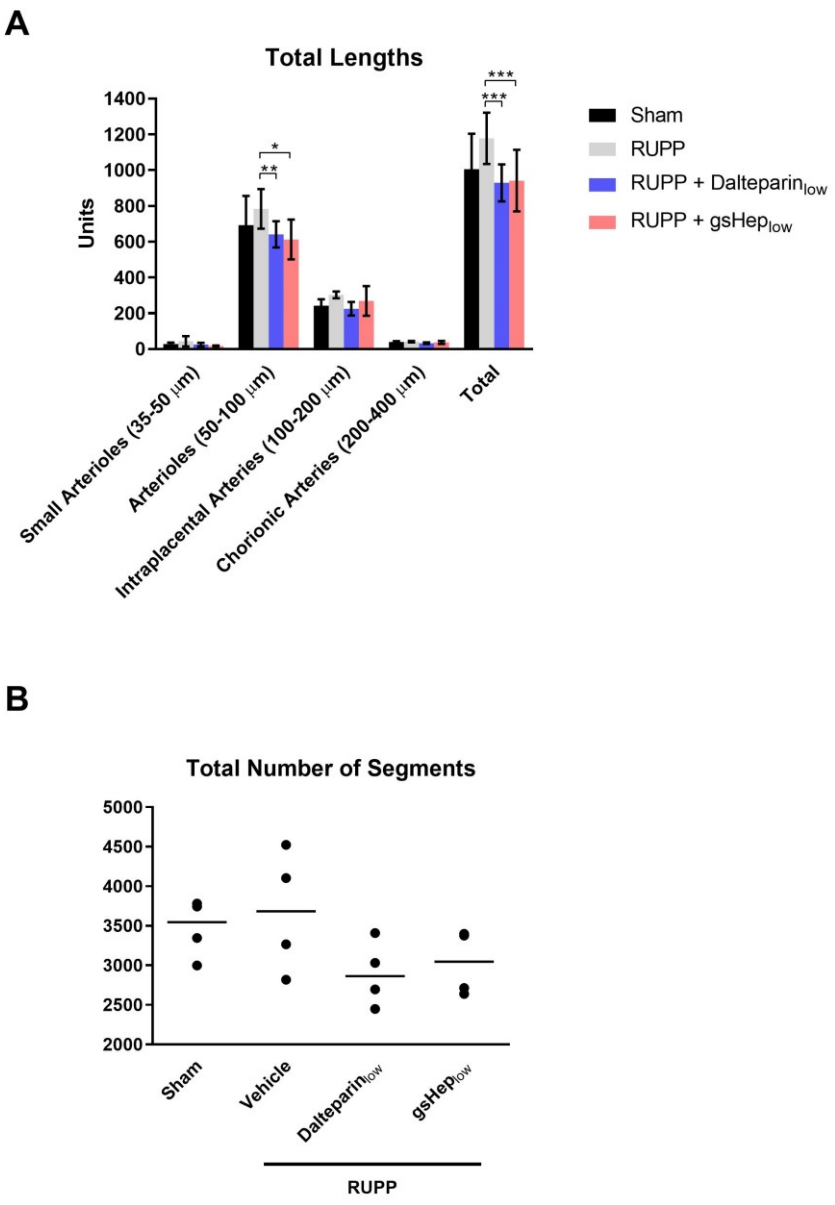

Figure 4.13. Metrics from micro-computed tomography analysis of the fetoplacental arterial vasculature.

An automated image analysis algorithm was used to measure (A) the lengths of arteries stratified by arterial diameter and (B) the total number of branching segments. Two-way ANOVA with Dunnett's multiple comparison, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to RUPP-vehicle group, n = 4 dams in each group from the average of analyses from two placentas from each dam. Bars represent mean +/- SD, lines represent median.

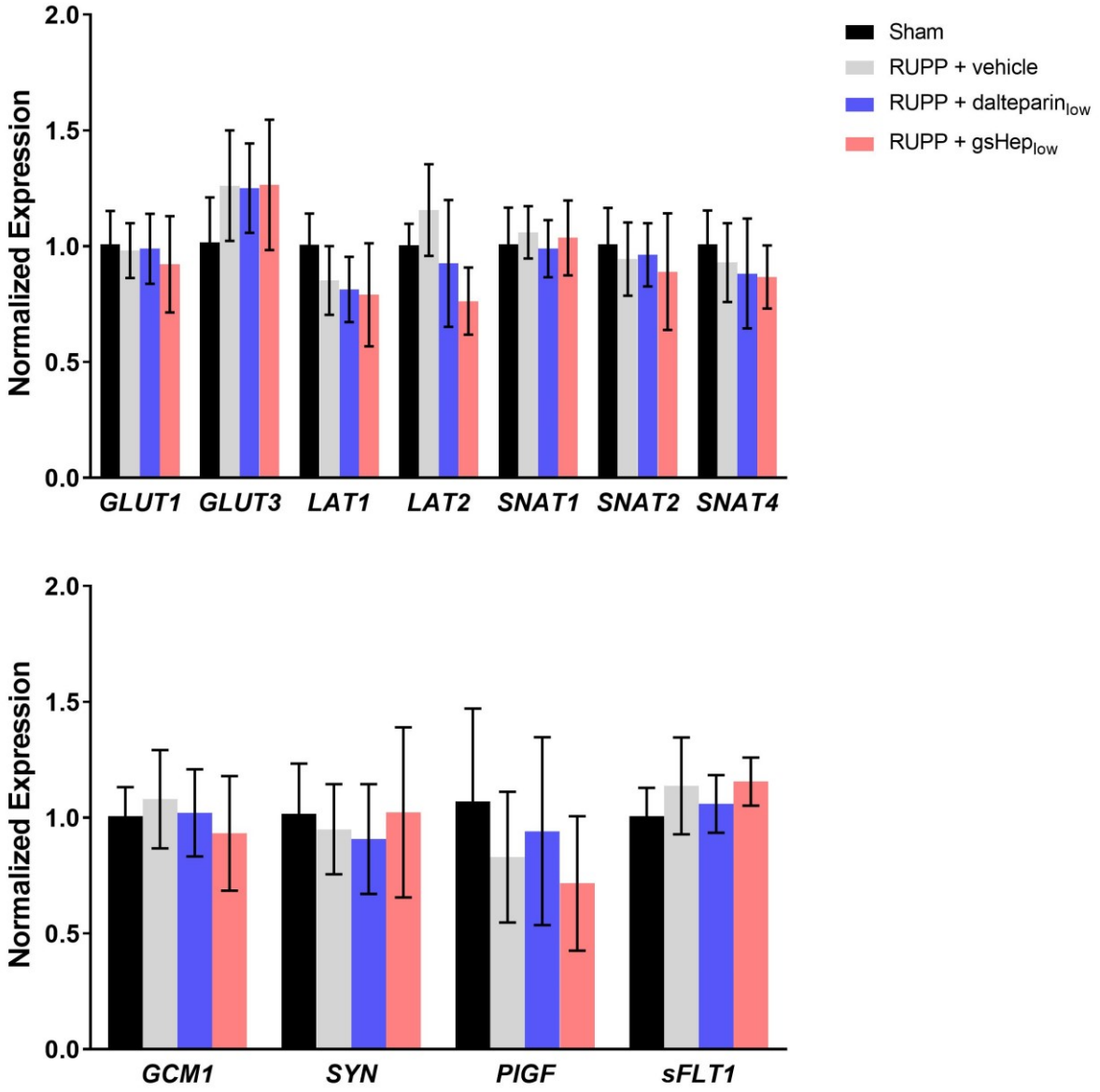

Figure 4.14. Effects of RUPP and treatment on steady-state placental mRNA expression.

Quantitative RT-PCR was performed on total RNA isolated from whole rat placental tissue to examine the steady-state mRNA expression of various genes relating to nutrient transport and placental development and function. Two-way ANOVA, n = pooled samples from 6 dams in each group. Bars represent mean +/- SD.

In this final aim, potential mechanisms that could be involved in influencing hypertension, fetal growth, and placental development in the RUPP model were investigated.

4.3.3.1. RUPP-mediated reduction in plasma PlGF was not impacted by dalteparin or gsHep

PlGF is emerging as an important angiogenic protein that is effective for the prediction of preeclampsia development[97,312]. In this study, levels of PlGF in the plasma of vehicle-treated RUPP animals were significantly lower compared to sham by 63% (sham: 40.5 [23.8-51.0] pg/mL; RUPP: 15.04 [7.7-25.7] pg/mL, $p < 0.01$; Figure 4.15A). There were no significant differences in plasma PlGF levels in RUPP rats treated with dalteparin or gsHep compared to control RUPP animals. The changes in plasma PlGF between vehicle and treated RUPPs were inversely correlated with blood pressure (Figure 4.15B), providing evidence that low PlGF is associated with vascular function in this animal model of preeclampsia.

However, the possibility that levels of PlGF in the plasma could be influenced by the number of viable fetoplacental units was also considered. After normalizing for the number of viable fetoplacental units, there were no significant differences in plasma levels of PlGF between the groups ($p = 0.44$; Figure 4.15C). To determine whether there was a relationship between PlGF levels and fetoplacental viability, a linear regression analysis was performed. For this analysis, all RUPP-induced animals were pooled together (i.e. including vehicle- and heparin-treated animals) to determine if PlGF levels were affected by RUPP-induced fetal loss irrespective of treatment. There was a significant positive correlation between PlGF levels and number of viable fetuses in RUPP-induced animals ($r^2 = 0.43$, $p < 0.0001$, $n = 39$; Figure 4.15D), while there was no relationship between these two variables in the sham group

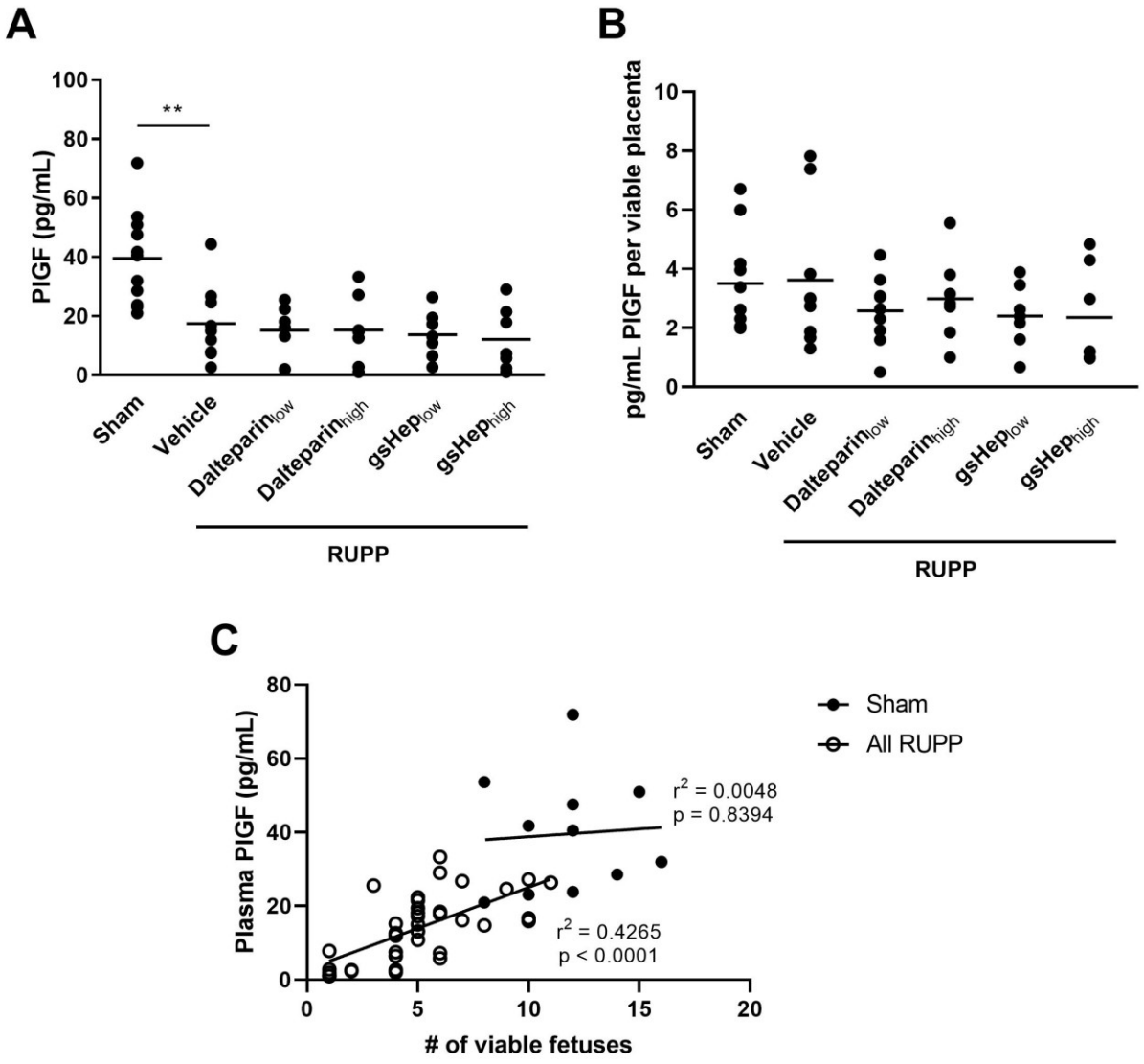

Figure 4.15. Effects of RUPP and treatments on maternal plasma PlGF levels.

(A) ELISA analyses for PlGF were performed on plasma collected from sham and RUPP rats. (B) PlGF levels were adjusted for number of viable fetoplacental units (one-way ANOVA with Dunnett's multiple comparison, ** $p < 0.01$ compared to RUPP-vehicle group, n = 7-11 dams in each group; lines represent the median). (C) Linear regression analysis determining the correlation between PlGF levels and number of viable fetuses in the sham and pooled RUPP groups (n = 11 vs 39 samples).

(p = 0.84, n= 11). These data suggest that plasma PlGF levels were influenced by the number of viable fetoplacental units.

4.3.3.2. RUPP animals exhibited a thrombophilic phenotype that was not reversed by treatment

It has been hypothesized that the potential effects of LMWH in preventing preeclampsia includes prevention of placental thrombotic lesions[319]. This study therefore investigated whether dalteparin treatment chronically altered the hemostatic state of animals that had undergone the RUPP procedure. Using the aPTT assay on plasma collected at sacrifice, it was determined that plasma from RUPP animals exhibited significantly reduced aPTT clotting time compared to plasma from sham animals (RUPP: 59.4 [56.7-66.8] sec; sham: 89.8 [77.9-92.3] sec, $p < 0.001$), indicating a thrombophilic state in RUPP animals. However, neither low nor high doses of dalteparin or gsHep impacted aPTT clotting time by the time of plasma collection ($p > 0.05$; Figure 4.16). Paraffin-embedded sections of placenta were also stained with Martius-Scarlet-Blue stain, which allows visualization of fibrin deposition. Fibrin deposition was not detected in any of the placenta samples (Figure 4.17), suggesting that placental thrombosis is not relevant in this model of preeclampsia. These data suggest that dalteparin promoted fetal growth independently of its anticoagulant properties.

4.3.3.3. Protease-activated receptor signaling does not influence BeWo proliferation

The observation that dalteparin but not gsHep treatment restored placental labyrinth structure suggests that the ATBRs, the molecular feature of heparin responsible for its

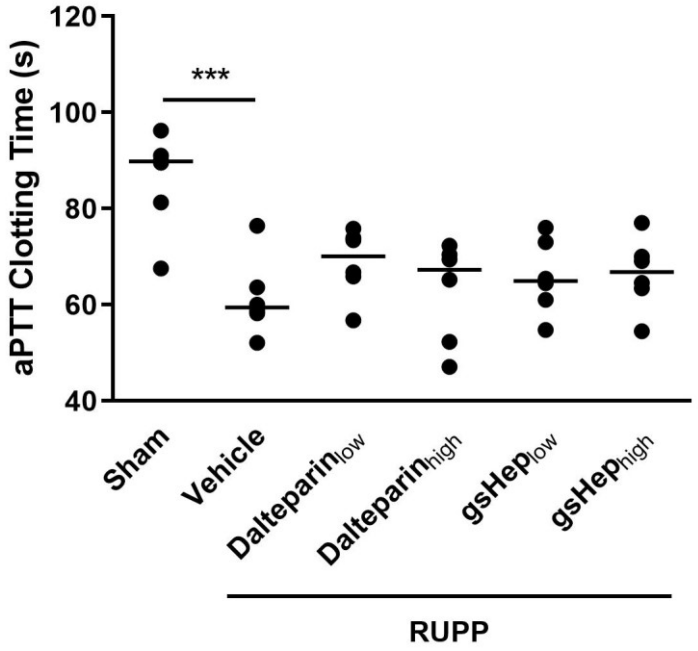

Figure 4.16. Hemostatic state of sham and RUPP rats.

Activated partial thromboplastin time assays were performed on plasma from sham and RUPP animals. One-way ANOVA with Dunnett's multiple comparison test, *** $p < 0.001$ compared to RUPP-vehicle group. n = 6 dams in each group. Lines represent the median. aPTT: activated partial thromboplastin time.

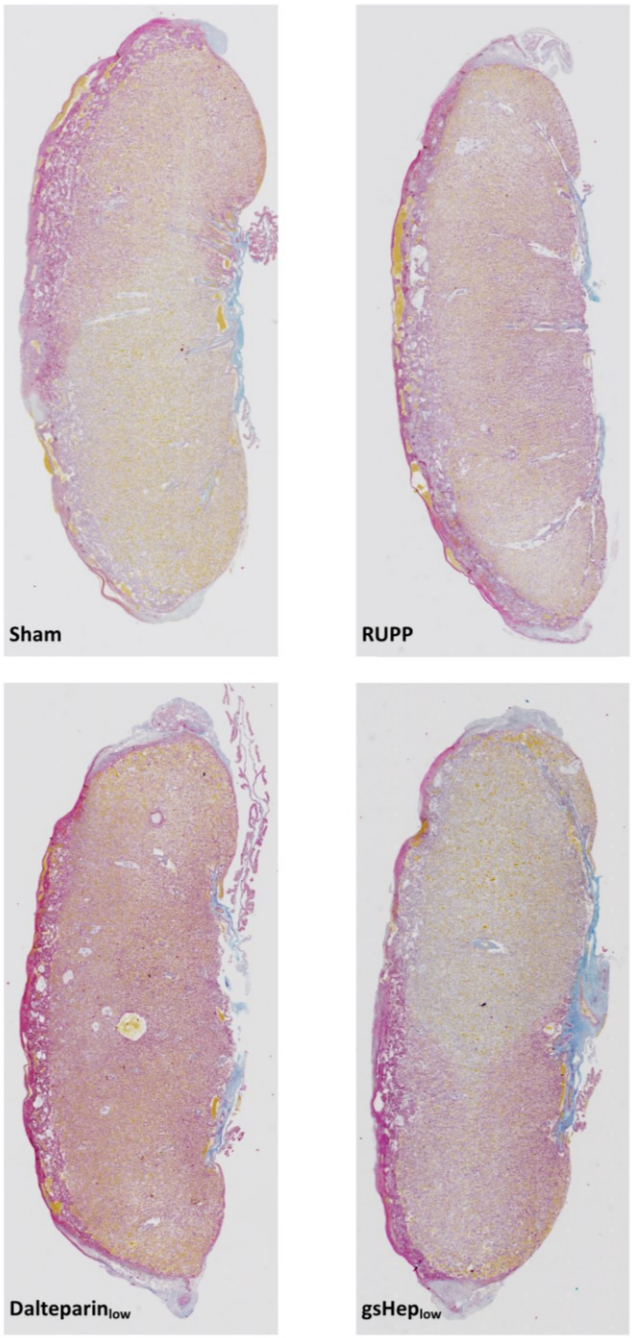

Figure 4.17. Representative images of Martius-Scarlet-Blue staining of rat placentas.

Paraffin sections of rat placentas were stained with Martius-Scarlet-Blue to detect the presence of fibrin. Blue staining is indicative of collagen; yellow staining is indicative of erythrocytes; red staining is indicative of fibrin deposition; nuclei are stained purple.

anticoagulant properties that is disrupted in gsHep, is important for the observed placental effect. In addition to anticoagulation, antithrombin-heparin interactions can also potentially inhibit Factor Xa-mediated activation of protease-activated receptors (PARs) on the placenta, possibly leading to improved proliferation of placental trophoblasts.

It was therefore hypothesized that PAR signaling, which is dysregulated in preeclampsia[320], can impair trophoblast proliferation. To test this hypothesis, the human BeWo choriocarcinoma cell line was utilized, due to these cells' cytotrophoblast-like qualities and ability to fuse to form a syncytium. First, it was confirmed that BeWo cells expressed both PAR1 and PAR2 by RT-PCR (Figure 4.18). Next, BeWo cells were treated using 10% serum from preeclamptic patients for 24 hours; cells were then treated with PAR-specific agonists or recombinant Factor Xa for 72 hours. Using the sulforhodamine B assay which measures total biomass and therefore acts as an indicator of proliferation, it was determined that none of the PAR agonists or Factor Xa significantly impacted BeWo proliferation ($p = 0.51$; Figure 4.19). This suggests that PAR1/2 activation does not influence BeWo proliferation, and that this mechanism may also not be responsible for abnormal development of the RUPP placenta.

4.4. Discussion

The objective of this study was to determine whether dalteparin, an anticoagulant LMWH, could reverse the preeclampsia-like phenotype induced in the RUPP model of this disease in the rat. The effects of gsHep, a heparin derivative rendered non-anticoagulant due to chemical disruptions to the ATBR, were also assessed in the RUPP model of preeclampsia.

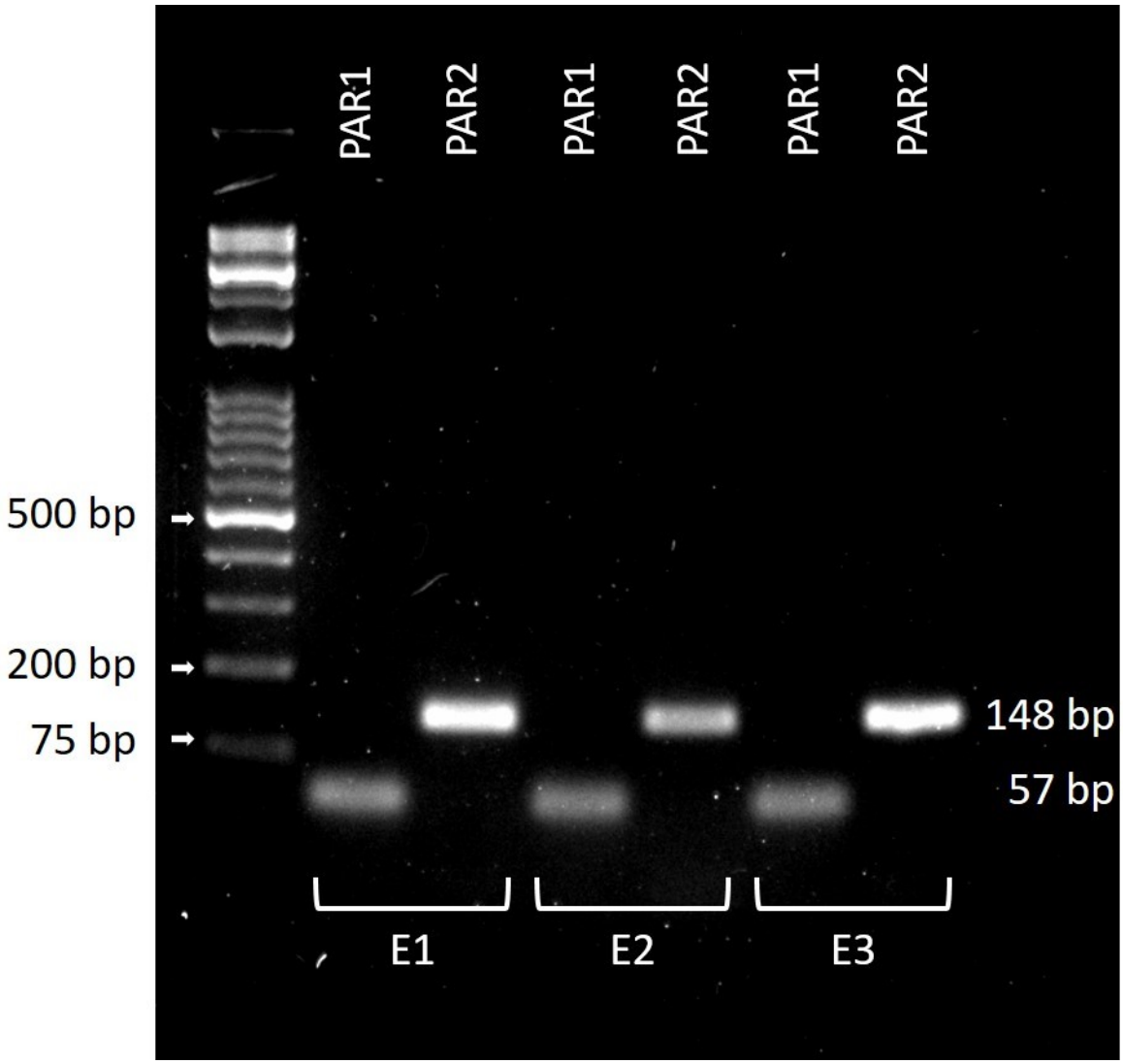

Figure 4.18. Expression of PAR1 and PAR2 receptors in BeWo choriocarcinoma cells.

Reverse transcription PCR coupled with 1.5% agarose gel electrophoresis was used to detect the mRNA expression of PAR1 and PAR2 in BeWo choriocarcinoma cells. Image shows data from 3 independent experiments.

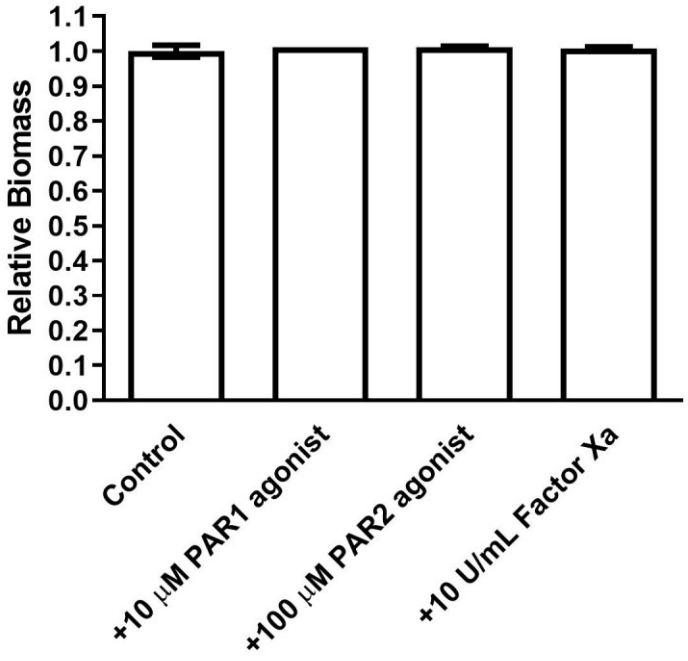

Figure 4.19. BeWo proliferation in response to PAR agonists and recombinant Factor Xa.

Preeclamptic serum-stimulated BeWo cells were treated with SFLLRN-NH2 (PAR1 agonist peptide), SLIGKV-NH2 (PAR2 agonist peptide), or recombinant Factor Xa for 72 hours before performing the sulforhodamine B assay to measure total biomass. One-way ANOVA, $p = 0.51$, $n = 3$ independent experiments. Bars represent mean +/- SD.

Although neither heparin treatments reduced blood pressure, low dose dalteparin but not gsHep significantly improved fetal growth; this selective effect of dalteparin was associated with preserved placental structure.

4.4.1. Impact of dalteparin and gsHep on blood pressure and angiogenic balance in the RUPP model of preeclampsia

The current series of experiments determined that neither dalteparin nor gsHep significantly impacted maternal blood pressure in the RUPP model of preeclampsia (Figure 4.3). These findings are in contrast with previous studies, which determined that LMWH such as enoxaparin and nadroparin can mitigate the increase in blood pressure in the L-NAME model of preeclampsia[321–323]. L-NAME elicits preeclampsia-like symptoms in pregnant rats by inhibiting NO production in endothelial cells, thereby inducing endothelial dysfunction. Although it is unclear why LMWH reduced blood pressure in the L-NAME model of preeclampsia but had no effect in this study utilizing the RUPP model of preeclampsia, it is possible that the mechanism of blood pressure dysregulation differs between the two models (i.e. direct inhibition of NO synthesis vs angiogenic imbalance). Nevertheless, these studies indicate that LMWH may exert an effect on endothelial function and blood pressure that is not applicable in the RUPP model.

Preeclampsia, especially cases defined by the severe early-onset phenotype, is characterized by profound angiogenic imbalance involving excess circulating levels of sFlt1 and reduced levels of PlGF[101]. Overexpression of sFlt1 in animals can elicit preeclampsia-like symptoms[324], while PlGF supplementation in animal models of preeclampsia can ameliorate hypertension[313]; the circulating balance of angiogenic proteins during pregnancy are therefore

hypothesized to mediate systemic maternal vascular function, and possibly maternal blood pressure. It has been speculated that LMWH therapy can enhance PlGF availability[218] and promote sFlt1 urinary clearance[214], thereby providing potential mechanisms by which LMWH could impact angiogenic balance. In the current study, it was only possible to detect a reduction in plasma levels of PlGF in RUPP rats compared to sham (Figure 4.15A); sFlt1 levels were not detectable in any of the serum and plasma samples using commercially available ELISA kits. Therefore, a thorough conclusion regarding the impact of dalteparin and gsHep on placenta-derived circulating angiogenic protein balance in the RUPP model cannot be made.

In addition, although angiogenic balance is well described in both the clinical syndrome and the RUPP model, this study uncovered a potentially important difference in the mechanism by which this imbalance is generated. While women at risk of developing early-onset preeclampsia and the RUPP model of preeclampsia exhibit reduced circulating levels of PlGF, the plasma PlGF levels did not significantly differ between sham and RUPP animals after normalizing for the number of viable placentas in the RUPP groups (Figure 4.15). Indeed, there was a positive correlation between PlGF levels and the number of viable fetoplacental units in the RUPP. This finding suggests that, in contrast to the clinical disease where low PlGF is likely contributed by impaired placental function[325], defective PlGF production in the RUPP model may be due to placental loss. The mechanism of reduced PlGF levels in the RUPP rat may therefore only be relevant to the multi-chorionic nature of the model organism and not in humans, where most cases of severe early-onset preeclampsia occur in singleton pregnancies. Furthermore, in contrast to the implied protective role of chorionicity in the rat, twin pregnancies are at higher risk of preeclampsia than singleton pregnancies[326], and dichorionic

twins are at even greater risk compared to monochorionic twins[327]. Hence, the translatability of experimental data with regards to angiogenic balance in the RUPP model to the clinical disease will need to be further examined.

In support of the potential effects of LMWH on modulating angiogenic balance and blood pressure in humans, the data in Chapter 3 demonstrated that both dalteparin and gsHep can increase PlGF release by 1st trimester placental villous explants, and the resulting conditioned media can enhance angiogenic tube formation of HUVECs on Matrigel (Figure 3.3 & Figure 3.4). This is supported by several studies demonstrating that LMWH therapy can elevate PlGF levels in pregnant women[218] and promote urinary clearance of sFlt1[214]. Acute administration of enoxaparin, a clinically used LMWH, to women at high risk of severe preeclampsia also improved brachial flow-mediated dilation, an indicator of endothelial function[27].

Altogether, the current studies determined that dalteparin or gsHep treatment did not impact maternal blood pressure or circulating angiogenic balance in the RUPP model of preeclampsia, but the effects of heparin on angiogenic balance deserves further investigation.

4.4.2. Impact of dalteparin and gsHep on placental development and fetal growth in the RUPP model of preeclampsia

The RUPP procedure resulted in damage to the placentas, characterized by expanded sinusoidal blood spaces and reduced placental cell density in the labyrinth compared to placentas from sham-operated animals (Figure 4.7). These structural changes were associated with fetal growth restriction, defined as a reduction in the average birthweight of viable fetuses

(Figure 4.5). Treatment with dalteparin, but not gsHep, ameliorated the RUPP-induced structural changes to the placentas and led to improved fetal growth.

The maternal sinusoidal blood spaces in the placental labyrinth serve as channels through which maternal blood flows, and the syncytiotrophoblast in contact with maternal blood draws nutrients and oxygen from the blood into the fetal vasculature to support development of the fetus. It is unclear why reduced placental cell density in the labyrinth in this study correlated with impaired fetal growth. Although not evaluated in the current study, it is possible that expanded sinusoidal blood spaces could result in congested blood flow through the labyrinth, while reduced placental cellularity could decrease accessibility to nutrients and oxygen.

Alternatively, it is possible that the reduction in placental cell density and impaired fetal growth is secondary to impaired development of the junctional zone. The junctional zone – composed of spongiotrophoblasts, glycogen cells, and trophoblast giant cells – plays a supporting role in placental and fetal development through hormone signaling and energy storage and release. Glycogen cells store and release glycogen to supply energy to the growing placenta and fetus. It is thought that the release of stored glycogen plays a role in labyrinth expansion and fetal growth, especially later in gestation[287]. Impaired glycogen storage or release can therefore impact fetal development even in the absence of labyrinth defects[328]. This is most often associated with a general reduction in junctional zone area[329], an observation that was observed in the vehicle-treated RUPP group compared to sham (Figure 4.8). Reduction of junctional zone size after reducing uterine perfusion was also recently reported in a mouse RUPP model[330]. Although there was no statistically significant effect of dalteparin treatment on

junctional size compared to control RUPP, large variability was noted in the dalteparin group, suggesting the possibility of a Type II statistical error resulting in a false negative conclusion. The decision to not further increase sample size by analyzing additional existing samples was made on the basis that the level of fetal resorption and/or average fetal weight among the remaining samples would be significantly deviated from the mean of the group; this could result in the inclusion of placentas that were subjected to extreme low or high model induction intensity and potential compensatory mechanisms, which could contribute to additional variability within the data.

While human placentas do not have a defined equivalent to the junctional zone of rodent placentas, the human cellular equivalent to the rodent glycogen cells is decidual EVTs. EVTs similarly store and release glycogen throughout pregnancy, presumably to contribute to placental and fetal growth; altered glycogen deposition and metabolism has been associated with preeclampsia and fetal growth restriction[331]. However, it is currently unknown whether glycogen stores provide energy to the placenta itself or to the fetus when resources from maternal blood are low.

The effects of LMWH on cells in the junctional zone have not been thoroughly explored. However, recognizing the similarity between glycogen cells and EVTs, hypothesis-generating speculations with regards to its potential effects can be made. EVT-like cells subjected to ischemic-reperfusion injury exhibit reduced apoptosis and enhanced survivability when treated with LMWH compared to untreated controls; this effect was found to be mediated by heparin-binding epidermal growth factor-like growth factor signaling[201]. It is therefore possible that, in the RUPP model, dalteparin treatment protected glycogen cells against oxidative stress

generated by RUPP. In turn, increased survival of glycogen cells could provide an additional source of energy that the fetus can use for growth. In support of this hypothesis, enoxaparin administered to healthy pregnant rats does not enhance fetal growth[332], suggesting that LMWH does not directly augment fetal growth but reduces the impact of extraneous insults to fetal growth.

4.4.3. Is anticoagulation required for the effects of LMWH in preeclampsia prevention?

Dalteparin, but not gsHep, normalized placental development and fetal growth in the RUPP model of preeclampsia. Given that gsHep has negligible anticoagulant properties compared to dalteparin through the disruption of the ATBR, results from the current studies would suggest that the anticoagulant actions of dalteparin mediate the effects seen in the RUPP model. However, this interpretation is only supported if 1) the ATBR is the only region modified by glycol splitting, and 2) if the ATBR only mediates anticoagulant functions.

4.4.3.1. Is the ATBR the only region of LMWH modified by glycol splitting?

Glycol-splitting targets unsulfated uronic acid residues of the heparin molecule, which is mostly present as part of the pentasaccharide ATBR[195]. However, there may be other unsulfated uronic acid residues distributed throughout the prevalent sequences as well (Figure 1.3). It is possible that the glycol-splitting process had off-target effects on other areas of the molecule that could have influenced the non-anticoagulant function of the resulting molecule, such as modifying protein interactions. For example, it is thought that the C-C cleavage in the uronic acid residues elicited by glycol-splitting could increase overall flexibility of the heparin

molecule, which could impact its ability to interact with proteins[245]. Although the activities of dalteparin and gsHep were compared through various *in vitro* assays related to preeclampsia in Chapter 3, these presumably constitute only a portion of potential mechanisms that could modulate the pathogenesis of preeclampsia. Hence, additional studies will need to be conducted to further evaluate the similarities and differences between dalteparin and gsHep.

4.4.3.2. Is the ATBR only mediating the anticoagulant actions of LMWH?

Assuming any off-target effects of glycol-splitting did not alter function, there are alternative anticoagulation-independent explanations for why dalteparin, and not gsHep, exerted an effect on placental development and fetal growth in the RUPP model of preeclampsia.

4.4.3.2.1. Effect of dalteparin and gsHep dosing in the RUPP model of preeclampsia

In this study, an interesting effect of dosing was observed; clinically-relevant low but not high dose dalteparin elicited an effect on placental development and fetal growth. This observation challenges the notion that the antithrombotic actions of dalteparin mediates the fetoplacental effects in the RUPP model of preeclampsia, as the higher dose would impart greater anticoagulant activity. Consistent with this notion, our group previously demonstrated that prophylactic doses of heparin, similar to the low dose used in this study, does not significantly reduce placental thrombotic lesions[199], suggesting that higher doses would be necessary to elicit a greater antithrombotic effect in the placenta.

Furthermore, previous reports investigating the causality between thrombophilia and the pathogenesis of preeclampsia are conflicting[333]. The trial conducted by Rey et al. demonstrated a protective effect of LMWH against preeclampsia even in the absence of thrombophilia[198]. The Lancet meta-analysis also revealed that LMWH may be most effective at reducing the primary outcome (i.e. early-onset/severe preeclampsia, small-for-gestational age <5th percentile, placental abruption, pregnancy loss >20 weeks) in thrombophilia-negative women[187]. These data collectively challenge the perspective that anticoagulation plays a significant role in the prevention of preeclampsia.

Understandably, one might expect the mechanism responsible for improved fetal growth, anticoagulant or not, would also be more pronounced at higher concentrations. However, LMWH can elicit actions that are inversely dose-dependent. Unfractionated long-chain heparin typically enhances growth factor signaling by facilitating complex formation between receptor and ligand, such as VEGF and VEGFR2[194]. However, at short lengths, LMWH cannot sufficiently bridge the two proteins together and in turn sterically hinders the ligand-receptor interaction, resulting in impaired signaling. For VEGF-VEGFR2 interactions, the critical length is approximately 20 sugar residues, or approximately 6 kDa in molecular weight[194]. As a heterogeneous mixture, dalteparin has an average molecular weight of approximately 6 kDa[307], meaning that short-chained molecules which can inhibit growth factor-receptor interactions are present. At high concentrations, these inhibitory molecules may function in a dominant manner, as seen by heparin's anti-angiogenic effects on cancer cells at high doses[195]. The predominance of inhibitory shorter chains at higher concentrations of LMWH may also explain the previous observation that low concentrations of LMWH mildly stimulated angiogenesis,

whereas higher concentrations did not (Figure 3.4). Therefore, a plausible explanation for the effects observed in the RUPP model is that, at higher doses, LMWH began interfering with growth factor signaling in the placenta resulting in a different pathway to dysfunction and impaired fetal growth.

Another potential explanation for why lose dose treatment may be more effective for the normalization of placental function in preeclampsia could be related to the mitogenic effect of LMWH as discussed in Section 3.4. The requirement of asymmetric cell division and differentiation for the healthy maintenance of the syncytiotrophoblast layer suggests that mitogenic and differentiating signaling need to be delicately balanced. At higher doses of LMWH, the dominant proliferation signal could impair proper formation and function of the syncytiotrophoblast layer, resulting in suboptimal placental development and fetal growth.

Nevertheless, the unexpected effect of heparin dosing may have potential clinical implications regarding the safe use of LMWH during pregnancy, since LMWH dosing is not currently routinely monitored during pregnancy (e.g. using the anti-Factor Xa activity assay). The concept that the non-anticoagulant actions of LMWH does not necessarily behave in a linear dose-dependent manner should be carefully explored in future studies.

4.4.3.2.2. Potential non-anticoagulant mechanism mediated by the ATBR of LMWH

Based on the current set of experiments, it is unknown why gsHep did not elicit a similar effect on placental development and fetal growth as dalteparin in the RUPP model of preeclampsia. Considering that the difference between dalteparin and gsHep is the presence of functional ATBR, and assuming that anticoagulation is not responsible for the effects, the

current findings would suggest that the ATBR itself is capable of mediating non-anticoagulant actions of LMWH that could lead to improved placental development and fetal growth. One potential non-anticoagulant mechanism that could be modulated by the ATBR is the activation of PARs by the coagulation protease Factor Xa.

PARs belong in a family of G-protein coupled receptors (PAR1-4) that are activated through proteolytic cleavage of a tethered ligand on the extracellular N-terminus of the receptor by proteases. The first PAR to be described was activated by thrombin[334], hence the early nomenclature of these receptors being "thrombin receptors". Since then, PARs have been demonstrated to be activated by other proteases, especially those involved in coagulation pathways and include Factor Xa, although these proteases often require cofactors for PAR activation. As G-protein coupled receptors, PARs are involved in signal transduction and participate in pathways related to cell metabolism and growth[335].

A small number of studies have investigated the potential role of PARs in the placenta of healthy pregnancy and in preeclampsia. PAR1-3 are expressed by cytotrophoblasts, EVTs, and the syncytiotrophoblast in human placentas[336,337]. Expression of PAR1-3 are detectable in the first trimester but decline by the start of the second trimester[336], suggesting their role in early placental development. Treatment of EVT cell lines and cells isolated from first trimester placentas with thrombin or agonistic peptides for PAR1 and PAR2 agonist all promoted their invasion into Matrigel[337,338]. In addition, activation of PAR1 stimulated proliferation of cytotrophoblasts derived from mouse placentas, while activation of PAR2 and PAR4 suppressed proliferation[339]. In the placentas of women with preeclampsia, placental PAR1 is overexpressed[320], and suppression of this upregulated expression or suppressed activation by

thrombin reduced sFlt1 expression[340,341]. PAR2 activation also results in elevated sFlt1 secretion by an immortalized human trophoblast cell line[342]. Finally, a recent study demonstrated that inhibition of PAR1 overexpression in the L-NAME model of preeclampsia improved placental vascular development, reduced circulating sFlt1, and reduced blood pressure[341]. Hence, although existing data on the role of PARs in the placentas of normal and preeclamptic pregnancies are limited, it is evident that PAR signaling play a role in placental development and function.

Factor Xa, a candidate activator of PARs, is amenable to LMWH-mediated inhibition via the ATBR, thereby constituting a potential mechanism by which the ATBR could participate in a non-anticoagulant role. Since the ATBRs have been disrupted in gsHep, gsHep would be unable to catalyze the inactivation of Factor Xa and prevent its signaling through PARs. It is possible that this difference between dalteparin and gsHep could explain the dissimilar effects observed in this study.

Therefore, whether PAR activation could compromise cytotrophoblast proliferation, and whether Factor Xa can mediate this effect was explored. BeWo human choriocarcinoma cells, which is a villous cytotrophoblast-derived cell line capable of fusion into a syncytium, were used for these experiments. Through RT-PCR analysis, it was determined that BeWo cells express PAR1 and PAR2 (Figure 4.18). However, despite previously published observations[339], neither PAR1/2 agonists or recombinant Factor Xa impacted BeWo cell proliferation (Figure 4.19). It is possible that other non-proliferation pathways are involved, such as maturation (e.g. BeWo fusion), or that their effects are more pronounced in other cell types. Considering the observation that junctional zone area is reduced in the RUPP model of preeclampsia, PAR

signaling could be dysregulated in other trophoblast lineages not assessed in this thesis, such as spongiotrophoblasts, glycogen cells, or trophoblast giant cells. Thrombin, a coagulation protease and an activator of PAR signaling, enhanced sFlt1 expression in decidual cells[343], identifying another potential area of PAR activity and regulation by LMWH, although whether this response was due to PAR activation was not directly explored. The potential requirement for circulating cofactors for Factor Xa-mediated activation of PARs may also explain the lack of an observed effect in this study. Further studies on the role of PAR receptors in the placenta and in preeclampsia are needed.

4.5. Conclusion

The specific results from these studies support the conclusion that the ATBR is required to mediate the effects of LMWH on placental development and fetal growth in the RUPP model. However, it would be premature to conclude that the anticoagulant actions of LMWH protect against features of preeclampsia in the RUPP model of preeclampsia. Additional mechanistic experiments, in conjunction with appropriate animal studies, are needed to further understand the precise pathways that LMWH could confer its protective effects in preeclampsia in pregnant women at high risk of developing preeclampsia.

5. General Discussion

The objectives of this thesis were to evaluate the effects of LMWH in an animal model of preeclampsia, and to compare the effects between LMWH and a non-anticoagulant derivative of LMWH in assays and models relevant to preeclampsia to explore the potential relevance of heparin's non-anticoagulant mechanisms for the prevention of preeclampsia.

In Chapter 3, the similarities and differences between dalteparin and gsHep that could be pertinent to the pathogenesis of preeclampsia were explored, through the assessment of pathways relating to anticoagulation, placental function, vascular function, and anti-inflammation. It was determined that both heparin molecules interact with sFlt1, promote PlGF release from healthy 1st trimester placental villous explants, increase the angiogenic potential of placenta-conditioned media, inhibit complement activation, and reduce leukocyte adhesion. In contrast to dalteparin, however, gsHep was unable to interact with antithrombin, exert significant anti-Factor Xa activity, or significantly prolong aPTT clotting time. These experimental findings support the hypothesis that dalteparin and gsHep share similar anticoagulation-independent functions, while gsHep has negligible anticoagulant properties due to chemical disruption of its ATBRs. These results therefore validated the use of gsHep to explore the hypothesis that the non-anticoagulant mechanisms of LMWH confer the protective effect against the development of preeclampsia.

The effects of dalteparin and gsHep in the RUPP model of preeclampsia were compared in Chapter 4. Neither dalteparin nor gsHep prevented the hypertensive phenotype in rats induced by RUPP. However, low dose dalteparin significantly normalized fetal growth impaired by RUPP, which was associated with improved placental development in the maternal

compartment. This effect was only observed with low dose dalteparin treatment; gsHep treatment did not elicit similar fetal and placental effects.

5.1. Validity of the RUPP Model of Preeclampsia

The work in this thesis resulted in the establishment of the RUPP model of preeclampsia for the first time at our institution. As such, it was important to evaluate whether the model described in this thesis accurately recapitulated the characteristics of RUPP that were previously described in published studies.

5.1.1. Experimental validity of the RUPP model of preeclampsia

The original RUPP model of preeclampsia was defined by elevated mean arterial blood pressure (in the conscious animal), proteinuria, impaired renal function, fetal loss, and fetal growth restriction by sacrifice on GD19[265]. Another important characteristic of the RUPP model that is relevant to preeclampsia, but only later identified in the model, is the presence of a circulating imbalance of placenta-derived angiogenic proteins. Specifically, the RUPP model was characterized by a reduction in plasma levels of proangiogenic molecules such as PlGF and VEGF and an increase in antiangiogenic molecules such as sFlt1[272]. Consistent with previously published studies, the current experiments demonstrated that RUPP rats exhibited elevated blood pressure (in the anesthetized animal; refer to Section 2.4.1 for a detailed explanation), fetal loss, and fetal growth restriction compared to sham rats. The RUPP rats in this study also exhibited signs of angiogenic imbalance in the blood, as indicated by decreased plasma PlGF levels compared to sham animals. However, sFlt1 was undetectable in any serum or plasma

samples using commercially available ELISA kits, and angiogenic profiling of the RUPP rats was therefore incomplete.

Proteinuria is a common clinical characteristic of preeclampsia, as well as a feature in the RUPP model of preeclampsia[267]. The evaluation of proteinuria in the current experiments was therefore considered. Metabolic cages are typically employed to allow sufficiently large volumes of urine to be collected after 24 hours to minimize the influence of contaminants on proteinuria assessments. However, the animal facility where the rats were housed in for this study was unequipped with metabolic cages to collect high quality urine samples for proteinuria analysis. Cystocentesis was considered as an alternative method to collect urine samples at the time of sacrifice, but there is significant risk of protein contamination from needle aspiration, especially in small volumes. In addition, previous studies identified that renal pathology, defined by glomerular endotheliosis and podocyte shedding which are commonly observed in preeclampsia[344], is absent in the RUPP model, indicating a natural limitation to the interpretation of renal data. Since the observed effects on fetoplacental growth and development by dalteparin provided strong rationale for a focused investigation into the effects of dalteparin and gsHep on the placenta, and considering the limitations addressed above, the renal characteristics in the RUPP model were not evaluated in the current study.

Based on the presence of hypertension, fetal loss, fetal growth restriction, and reduced maternal plasma PlGF in the RUPP rats, it was concluded that the establishment of the RUPP model of preeclampsia at our institution was successful and was valid for addressing the objectives and hypotheses outlined in this thesis.

5.1.2. Clinical validity of the RUPP model of preeclampsia

Preeclampsia is a heterogeneous disorder that does not occur naturally occur in non-primates[282], which presents a challenge for generating animal models of preeclampsia that resembles the clinical disease.

5.1.2.1. Is the RUPP model of preeclampsia representative of mild late-onset preeclampsia or severe early-onset preeclampsia?

Despite its widespread acceptance as a model, the clinical relevance of the RUPP model of preeclampsia has been extensively debated. It has been suggested that the RUPP model is not representative of severe preeclampsia, due to the absence of liver damage and HELLP syndrome-like features[304], while others have argued that the RUPP model does capture many of the clinical manifestations of severe early-onset preeclampsia[345].

Severe early-onset disease typically occurs prior to 34 weeks gestation and is strongly associated with impaired uteroplacental blood flow, resulting in abnormal placental growth, placental injury, and fetal growth restriction due to reduced placental function[24]. In contrast, late-onset preeclampsia is typically more mild and manifests near term with diverse symptom etiology such as pre-existing maternal constitutional risk factors or placental overgrowth that does not impact placental or fetal development[24]. In the current experiments, fetuses from RUPP rats were growth restricted compared to those from sham animals and placentas were also trending smaller; these are features of the severe early-onset phenotype of preeclampsia. The RUPP model is based on restriction of blood flow into the uteroplacental circulation, which is also characteristic of the severe early-onset and not the late-onset form of preeclampsia[24].

However, it is also important to note that, unlike the placentation defects associated with severe early-onset preeclampsia, there are no spiral artery remodeling defects in the RUPP model as the RUPP surgery is performed after the remodeling process has completed[304,346]. In addition, as will be discussed in Section 5.3.3, it is believed that the mechanism of placental damage in this model is likely to be related to hypoxia rather than pressurized shear stress in the clinical disease[7,273]. From these perspectives, the model may be more mechanistically aligned with the late-onset preeclampsia phenotype.

Altogether, it is likely that the RUPP model is a hybrid model with features of both early- and late-onset preeclampsia, characterized by abnormalities in placental development and fetal growth caused by an external factor unrelated to spiral artery remodeling defects.

5.1.2.2. Does the heparin treatment regimen in the RUPP model reflect clinical applications?

In most clinical trials investigating LMWH for the prevention of preeclampsia, LMWH has been prescribed to patients at prophylactic doses – between 0.2 and 0.5 IU/mL at peak plasma levels[308] – administered once daily via subcutaneous injections. A similar dosing regimen was therefore adapted in the current studies and was expanded to include higher doses of heparin to investigate if efficacy of treatment was dose dependent. The validity of the selected doses of dalteparin was confirmed by injecting non-pregnant rats with a single dose of the drug subcutaneously, collecting serial blood samples, and then performing anti-Factor Xa assays on the plasma. Peak anti-Factor Xa activity levels for the low and high doses were approximately 0.2 and 1.0 IU/mL (Figure 4.2), respectively, which is in agreement with clinically-appropriate

values for prophylactic and therapeutic uses. It is therefore believed that the dosing regimens chosen for the current RUPP experiments are clinically relevant.

However, one of the experimental designs that does not reflect clinical application is the timing and duration of treatment. LMWH prophylaxis during pregnancy for the prevention of venous thromboembolism and for the prevention of preeclampsia in clinical trials is typically initiated early in pregnancy, before 17 weeks of gestation[187]; this is due to the increased risk of thrombosis in high-risk patients as early as the first trimester[347]. Early initiation of LMWH therapy could impact ongoing placentation, potentially modulating early placental development events such as spiral artery remodeling. Indeed, *in vitro* evidence suggests that LMWH could potentially modulate EVT invasion[43,200–202], which in turn could influence spiral artery remodeling. In the current experiments, dalteparin and gsHep treatments were initiated on GD15, when the trophoblast invasion and spiral artery remodeling process in the pregnant rat is complete[346]. This was limited by the timing of model induction on GD14. The short therapeutic window between surgical induction and sacrifice on GD19 is therefore a limitation to the model.

5.2. Comparing Current Results to Published Studies

The experiments outlined in this thesis were the first to explore the effects of dalteparin in the RUPP model of preeclampsia, and also the first to evaluate the potential utility of gsHep as a tool to investigate the role of heparin's non-anticoagulant effects. However, previous work has explored the non-anticoagulant effects of heparin *in vitro* and in animal models of preeclampsia.

5.2.1. Exploring the non-anticoagulant actions of dalteparin and gsHep *in vitro* in assays relevant to preeclampsia

The experiments in Chapter 3 explored the effects of a glycol-split heparin in the context of preeclamspia and placenta-related assays. The concept that LMWH has non-anticoagulant properties thus far is largely unexplored in the field of placentation and preeclampsia, but is much more well known in other areas of studies such as cancer and inflammation[193]. From research in these areas, LMWH has been shown to have anti-angiogenic and anti-inflammatory properties. Glycol-split derivatives of heparin were generated to harness these actions of LMWH without being limited by its anticoagulant actions which would incur bleeding risks in patients.

In contrast, the current experiments determined that dalteparin and gsHep are not necessarily anti-angiogenic, and can both stimulate a pro-angiogenic response under specific conditions. For example, conditioned media from 1st trimester placental villous explants treated with dalteparin or gsHep promoted angiogenic tube formation when incubated with HUVECs; by contrast, treating the endothelial cells with dalteparin or gsHep directly had minimal or no effect at the same concentrations (Figure 3.4A). These findings suggest that the non-anticoagulant actions of LMWH in general are highly context dependent. The effects of both LMWH and gsHep should be extensively explored in the specific context of preeclampsia to further elucidate mechanisms that could contribute to protection against the disease.

5.2.2. Exploring the effects of dalteparin and gsHep in animal models of preeclampsia

The set of experiments outlined in Chapter 4 explored the effects of LMWH in an animal model of preeclampsia. Two previous studies investigated the effects of LMWH in the L-NAME model of preeclampsia.[321,348] In these studies utilizing the L-NAME model of preeclampsia in the rat, LMWH reduced L-NAME-induced gestational hypertension by GD20 and GD21, respectively[321,348], with conflicting effects on fetal survival and growth.

In contrast to these previous studies, the current experiment determined that dalteparin did not significantly reduce RUPP-induced hypertension nor improve fetal viability, but significantly improved fetal growth in the RUPP model of preeclampsia. Firstly, as discussed in Section 5.2.1, the mechanism by which hypertension is induced may differ between the RUPP and the L-NAME model, where dalteparin or gsHep could potentially exert an effect in the L-NAME model but not the RUPP model of preeclampsia. Secondly, it is also likely that the pathway to fetal loss and growth restriction also differ between the two models. In the RUPP model, fetal loss is likely caused by inadequate blood perfusion to the fetoplacental units caused by restriction with the silver clips. L-NAME induces endothelial dysfunction in pregnant rats by inhibiting NO production and signaling, likely resulting in vasoconstriction of the myometrial arteries that reduce supply of blood to the uteroplacental circulation and causing fetal loss and growth restriction[349]. Hence, the pathway to vascular dysfunction in RUPP is mechanical and mostly irreversible, while the L-NAME model is biochemically induced with the potential to be reversed by LMWH. In support of this, LMWH improved flow-mediated dilation in asymptomatic women at high-risk of preeclampsia, a process which is dependent on endothelial production of NO[27]. Therefore, the effect that LMWH can exert may differ between these two models.

5.3. Limitations of the Studies

The experiments presented in this thesis were designed to evaluate and compare the effects of two heparin derivatives in a well-characterized animal model of preeclampsia. However, there are several important limitations to the studies, particularly surrounding the RUPP model of preeclampsia.

5.3.1. Timing of the RUPP surgery

There is an inherent limitation when using the RUPP model of preeclampsia which is induced late in gestation through a surgical and mechanical method. Unlike clinical prophylaxis against severe early-onset preeclampsia, LMWH therapy in the RUPP model is initiated much later in pregnancy for a short duration, and would be physically unable to alleviate the mechanically-induced reduction in uteroplacental perfusion pressure. This is an important limitation, since LMWH therapy initiated early during pregnancy could have an effect on processes related to EVT invasion and spiral artery remodeling[43,200–202] that cannot be evaluated in this model.

5.3.2. Multiple pregnancy in the rat

Although the rat is one of the most relevant non-primate model organisms with respect to human hemochorial placentation, there are significant differences between humans and rats with regards to placental development and function and fetal growth. First, preeclampsia is a pregnancy disorder of humans and great apes[282]; rodents have not been observed to develop

preeclampsia naturally. Secondly, rats have multigestational pregnancies, while humans have primarily singleton pregnancies. Twin pregnancies in humans are associated with higher risk of preeclampsia[350], but this is not true with rats, clearly indicating differences in the biological mechanisms present to avert hypertensive disorders in pregnancy. Furthermore, compensatory mechanisms in the rat could redirect blood supply towards remaining viable fetuses to support their growth, thereby potentially complicating results and interpretations. Lastly, evidence is presented in this thesis that the reduction in PlGF in the RUPP model is caused by loss of viable placentas, which is not likely to be relevant to the clinical disease. Hence, despite the rat being a well-accepted model for human pregnancy, the multi-chorionicity presents a major challenge in the interpretation and translation of experimental data to the human condition.

5.3.3. Rheological difference between RUPP and preeclampsia

It had been assumed that impaired spiral artery remodeling leads to chronic hypoxia of the placenta due to underperfusion[351]. However, computer modeling of impaired spiral artery conversion suggested that the overall volume of intervillous blood flow remains unchanged with defective spiral artery remodeling, and it is the rate and pressure at which maternal blood flows into the placenta that ultimately damages the placenta through shear stress and ischemia-reperfusion injury[7]. In this sense, the RUPP model likely fails to mimic this mechanism of damage to the placenta. Because the clips are positioned well away from individual placentas in the RUPP model, it is not expected to create the turbulent blood flow that would be seen with impaired spiral artery remodeling. This is also evident by the fact that resorption in the model occurs beginning with the distal and not the proximal fetuses to the clips (Figure 4.1),

suggesting that RUPP damages the placentas and fetuses through reduced blood flow and hypoxia and not through shear stress and ischemia-reperfusion injury.

5.3.4. Lack of concomitant low dose ASA treatment

In many clinical trials for the prevention of preeclampsia, LMWH prophylaxis is accompanied by low dose ASA[148]. It is possible that LMWH and ASA treatments work synergistically to lower the risk of preeclampsia, since the effect of LMWH was found to be more prominent with daily ASA compared to no ASA[187]. For example, ASA could exert stronger effects on the endothelium and reduce blood pressure in preeclampsia, while LMWH therapy may reduce placental dysfunction and promote angiogenic balance in the blood. While low dose ASA has become a mainstay in high-risk pregnancy care due to its well-established safety in pregnancy and efficacy[11], ASA prophylaxis in high-risk pregnancies is at best only effective in 2/3 at-risk women[352]. The focus of future therapy discovery and development is to develop therapies to supplement the effects of ASA for the improved prevention of early-onset preeclampsia. In this thesis, the objective was to evaluate the specific effects of LMWH treatment on the preeclamptic phenotype in the RUPP model, and ASA treatment was therefore omitted. Further work assessing the combined effects of LMWH and low dose ASA in an animal model of preeclampsia would be of clinical significance.

5.3.5. Incomplete assessment of the RUPP phenotype

The RUPP model recapitulates various aspects of preeclampsia, including changes to placental, cardiovascular, and inflammatory function[304], which dalteparin and gsHep can all

impact[9]. The current studies focused on the effects of dalteparin and gsHep treatment on the placenta in the RUPP model due to its specific importance in severe early-onset preeclampsia, but the treatments might impact the other pathways as well which could have clinical relevance. Given the relevance of angiogenic balance in the pathogenesis of preeclampsia, experiments were performed to measure sFlt1 in plasma samples, but the protein was not detectable using commercially available ELISA kits, thereby prohibiting conclusions about the effects of dalteparin and gsHep on angiogenic balance in the RUPP model. Additional studies will need to be performed to investigate the effects of dalteparin and gsHep on the cardiovascular and inflammatory pathways that could contribute to disease pathogenesis.

5.4. Clinical implications of findings

The therapeutic potential of LMWH for the prevention of preeclampsia is controversial. Small randomized clinical trials have demonstrated an augmentative effect of LMWH to protect against severe early-onset preeclampsia when used in conjunction with low dose ASA in high-risk pregnant women[178–182,184,198], while larger trials have failed to demonstrate a beneficial effect for the prevention of preeclampsia[183,185,186]. A reasonable explanation for this discrepancy is that LMWH is only biologically important for a subset of patients at risk of developing preeclampsia due to severe placental disease. Preeclampsia is a syndrome with multiple etiologies, involving one or more abnormalities in placental, cardiovascular, or immune function[9]. It is therefore conceivable that a single therapeutic would be unable to protect against all subtypes of preeclampsia. Therefore, the findings from the current study and previous data from *in vitro* and clinical studies generate the hypothesis that LMWH may

prevent a specific phenotype of preeclampsia in a certain patient population, rather than all types of preeclampsia.

5.4.1. LMWH may be most useful for the prevention of preeclampsia in pregnant women at risk of severe early-onset preeclampsia

The observations generated from the current set of experiments, whereby LMWH therapy improved placental function in the RUPP model, could provide a mechanistic explanation as to why several clinical trials have failed to observe a protective effect of LMWH for the prevention of preeclampsia. The TIPPS, HEPEPE, and EPPI trials[183,185,186] did not focus on enrolling women at risk of preeclampsia originating from a specific cause (i.e. severe preeclampsia mediated by placental dysfunction causing maternal systemic endothelial dysfunction). Rather, these trials included "high-risk" pregnant women, broadly defined as a history of preeclampsia in previous pregnancy, small-for-gestational age in previous pregnancy, placental-mediated disorders in previous pregnancy, fetal loss in previous pregnancy, major abruption in previous pregnancy, unexplained pregnancy loss, and thromboembolic events or risk factors. Women were not necessarily differentiated based on history of preeclampsia phenotype, including early versus late, mild versus severe, with or without fetal growth restriction. In the HEPEPE trial, the percentage of recruited patients with a history of severe preeclampsia before 34 weeks of gestation – a phenotype associated with impaired placentation – was less than 5%[185]. In the EPPI trial, although more than 40% of recruited patients had a history of early-onset preeclampsia requiring iatrogenic delivery, a significant proportion of recruited patients also had thrombophilia positive status, preexisting

hypertension, and high BMI, which can all contribute to the development of early-onset preeclampsia in the absence of placental defects[186]. By contrast, smaller trials which reported that LMWH effectively prevented preeclampsia, such as that of Rey et al., specifically recruited patients with a history of placenta-mediated complications during pregnancy, of which severe preeclampsia with delivery before 35 weeks constituted 52.6% of the study population[198]. Broad inclusion criteria could mask the true therapeutic potential of LMWH for the prevention of a specific subset of patients at risk of developing severe, early-onset preeclampsia emanating from placentation defects.

Hence, a mechanism by which LMWH could reduce the incidence of severe early-onset preeclampsia may be through stabilization of placental development and function, leading to reduced impact on the systemic maternal endothelium and cardiovascular system. However, the current set of experiments failed to see a normalizing effect of dalteparin on blood pressure despite improved placental development. A plausible explanation for this observation is that the RUPP model is too robust to observe an indirect effect on blood pressure via improved placental function. Induction of RUPP by surgical means is a significant insult that results in partial or even total fetal loss. This is accompanied by a sharp reduction in plasma PlGF (Figure 4.15) that would be expected to create a strong disturbance in endothelial function. As discussed, this decline in PlGF may be mediated by placental loss, which would be difficult to reverse by LMWH therapy in the RUPP model and may not even be clinically relevant.

In addition, as a limitation to the study in Chapter 4 and discussed in Section 5.3.4, LMWH therapy was not used in conjunction with low dose ASA; combination LMWH and ASA therapy could potentially exert synergistic effects on the maternal vasculature. This is

supported by the Lancet meta-analysis, which found that LMWH prophylaxis alone was ineffective at reducing incidence of the primary outcome (i.e. early-onset/severe preeclampsia, small-for-gestational age <5th percentile, placental abruption, pregnancy loss >20 weeks) compared to LMWH with daily ASA[187]. This suggests that LMWH and low-dose ASA potentially act synergistically to promote placental function, thereby impacting maternal vascular function to confer protection against the development of preeclampsia.

5.4.2. Potential safety considerations for the use of LMWH during pregnancy

A potentially important observation from the current studies was the absence of a simple dose-dependent effect of dalteparin on fetal growth in the RUPP model. Although LMWH therapy during pregnancy is generally regarded as safe and does not incur significant bleeding risks especially when used for prophylaxis, only the low prophylactic dose, and not high therapeutic dose of dalteparin, improved fetal growth in the RUPP model of preeclampsia in the current study (Figure 4.5). The potential of a biphasic response by LMWH in the context of preeclampsia has not been examined, but the prospect of undesired effects of LMWH independent of anticoagulation at higher doses warrants further investigation. Currently, the dose of LMWH for preeclampsia prophylaxis in clinical trials is typically a fixed dose (e.g. 5,000 U/day dalteparin[181]) for all patients, or adjusted based on the range of body weight (e.g. 4,000 IU/day for women < 60 kg, 5,000 IU/day for women 60-90 kg, 6,000 IU/day for women > 90 kg[198]). Subsequent clinical trials should adopt an adjusted dosing regimen with monitoring of anti-Factor Xa activity in blood samples at randomization to establish safe and consistent dosing

to further delineate the treatment requirements for LMWH to confer protective effects against preeclampsia.

Another interesting but speculative mechanism of LMWH could also be related to the frequency of dosing. Clinically, LMWH is subcutaneously administered once daily typically at a subclinical prophylactic dose. However, the terminal half-life of dalteparin in humans is approximately 4 hours; in rats, the half-life is approximately 1.5 hours[353] (Figure 4.2). In both cases, one would expect that the concentration of LMWH would fall below effective concentrations within several half lives and well before the next injection; yet, higher therapeutic concentrations, which would hypothetically prolong the length of time LMWH can function, did not elicit the same effects as prophylactically dosed LMWH in the current study. As observed in Chapter 3 and previously described, LMWH can stimulate cytotrophoblast proliferation[205]. It can therefore be hypothesized that prophylactic LMWH stimulates proliferation of progenitor cytotrophoblasts at peak concentrations, while the fall in LMWH concentration coincides with differentiation of the daughter cells to maintain a healthy syncytial barrier. In such cases, efficacy could therefore depend on very precise and careful monitoring of LMWH dosing through pregnancy.

5.5. Future Directions

Additional information regarding the mechanisms by which LMWH could prevent preeclampsia is required prior to the expansion of LMWH therapy in clinical care for the prevention of severe preeclampsia.

5.5.1. Investigating the role of anticoagulation in the RUPP model of preeclampsia

Based on the findings that dalteparin, but not gsHep, promoted placental development and fetal growth in the RUPP model of preeclampsia, it is reasonable to conclude that the ATBR is required for the observed effects. However, whether these placental and fetal effects are mediated by the anticoagulant or the non-anticoagulant properties of the ATBR remains unclear (as discussed in more detail in Section 4.4.3.2). A logical next step to delineate this difference is to design a study investigating which mechanistic component of the LMWH is required to promote placental development and fetal growth. A potential study would compare the effects of dalteparin, gsHep, fondaparinux (a synthetic pentasaccharide that mimics the ATBR), and hirudin (a heparin-independent anticoagulant) in the RUPP model of preeclampsia. This approach was previously used to demonstrate that anticoagulation and the ATBR was not sufficient to elicit heparin's protection against fetal loss in a mouse model of antiphospholipid syndrome[225]. This experimental design would inform: 1) whether anticoagulation alone is sufficient for the effect (based on hirudin); 2) whether mechanisms mediated by the ATBR alone is sufficient for the effect (based on fondaparinux), or 3) whether the ATBR and the heparin chain together is required for the effect (based on dalteparin). In this experimental design, gsHep could serve as a negative control to elucidate the exact impact of the ATBR based on the results of the current study.

5.5.2. Expanding mechanistic insight of LMWH for the prevention of preeclampsia

Although the RUPP model of preeclampsia is one of the most well-characterized and relevant models of preeclampsia currently available, interpreting the data generated from

these models is still challenging given the model limitations (as discussed in Section 5.3). Hence, other animal models of preeclampsia should also be used to diversify the tools to interpret the mechanistic *in vivo* effects of LMWH for the prevention of preeclampsia. As previously mentioned, the RUPP model does not recapitulate early placental defects, which is a major drawback especially when considering the *in vitro* evidence surrounding the potential positive effect of LMWH on placentation. While there are currently no well-established animal models that naturally mimic early placental development impairments seen in preeclampsia, genetic models that result in placental hypoxia and ischemia such as the catechol-O-methyltransferase knockout model can be used to elucidate the placental effects of LMWH throughout the duration of pregnancy[251].

5.5.3. Validating the therapeutic potential of LMWH in a specific population of patients at high risk of developing severe early-onset preeclampsia

Another reasonable step in future research would be to evaluate the effects of LMWH in a niche clinical population who are confirmed to be at risk of severe early-onset preeclampsia despite initiating ASA prophylaxis in the first trimester, assessed through clinical obstetrical history, placental ultrasound, and PlGF testing[27,97]. Rather than testing the effects of LMWH in patients at risk of developing general preeclampsia based on a wide range of criteria, a clinical trial designed to investigate the preventative effects of combination ASA and LMWH in a specific population of patients at the highest risk of developing severe early-onset preeclampsia characterized by placental dysfunction which mediates systemic endothelial dysfunction could be of greater significance, based on the findings of the current study. These patients can be

identified with high accuracy during early second trimester through a combination of clinical risk factor assessments, measurement of placental biomarkers (e.g. pregnancy-associated plasma protein A, PlGF), placental shape/texture, and uterine artery Doppler[26,27]. The SCOPE consortium determined that a subset population of pregnant women at high-risk of preeclampsia could be identified with a high level of screening precision using the above methodology and measurements of PlGF at 16 weeks gestation[97]. LMWH during pregnancy is well-established to generally be safe, and use of LMWH for the prevention of preeclampsia in a subset of women is already endorsed by the most recent SOGC guidelines despite inconclusive evidence[11]. Hence, further clinical studies looking at the effects of LMWH in a specific population of patients at high risk of severe early-onset preeclampsia is justified, feasible, and should be explored.

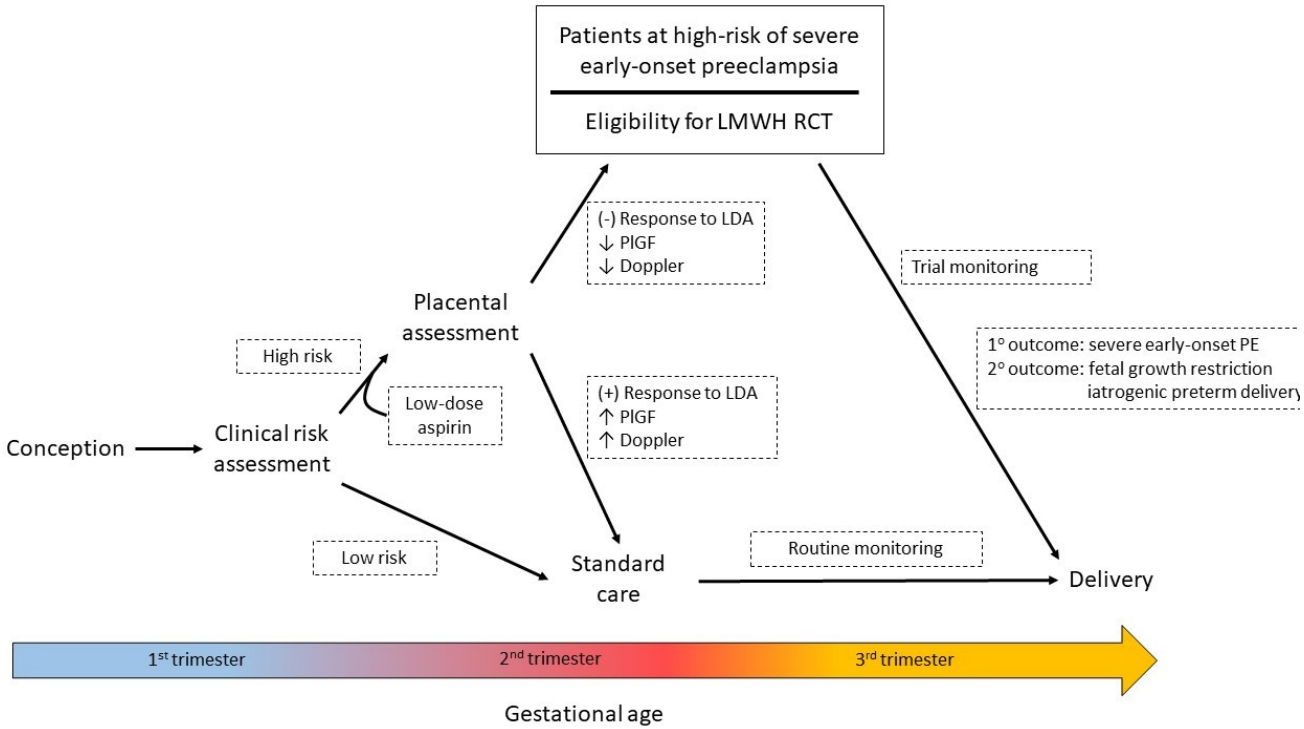

Figure 5.1. Schematic diagram of a prospective randomized clinical trial evaluating the effects of LMWH for preventing severe early-onset preeclampsia.

At-risk patients are prescribed daily low-dose ASA following 1st trimester clinical risk assessments. Patients who exhibit low PlGF and abnormal uterine Doppler during 2nd trimester placental assessments would be deemed refractory to low dose ASA treatment and at high-risk of developing severe early-onset preeclampsia. These patients would be eligible to participate in a randomized clinical trial evaluating the effects of LMWH for preventing severe early-onset preeclampsia. Prophylaxis would commence in the 2nd trimester and continue until delivery, monitoring for severe early-onset preeclampsia development and associated adverse pregnancy outcomes such as fetal growth restriction and iatrogenic preterm delivery. LDA: low dose ASA; LMWH: low molecular weight heparin; PE: preeclampsia; PlGF: placental growth factor; RCT: randomized clinical trial.

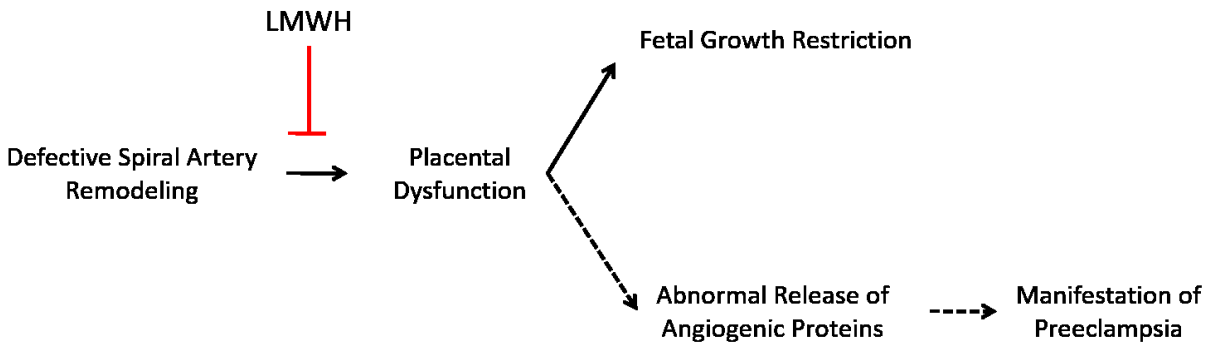

Figure 5.2. Proposed model of the effects of LMWH in the prevention of preeclampsia.

Defective spiral artery remodeling can trigger placental dysfunction later in pregnancy, resulting in fetal growth restriction, abnormal release of angiogenic proteins, and the clinical manifestation of severe early-onset preeclampsia in pregnant women. Based on the data presented in this thesis, LMWH can potentially inhibit the consequences of defective spiral artery remodeling, thereby preventing placental dysfunction and restoring fetal growth. Whether LMWH can alter the abnormal release of angiogenic proteins and prevent other features of preeclampsia could not be assessed in the current thesis.

6. Conclusion

Low molecular weight heparin has previously been investigated for the prevention of preeclampsia in high-risk pregnant women with inconsistent efficacy. In addition, the mechanism by which LMWH could prevent preeclampsia is poorly understood. The objective of this thesis was therefore to evaluate the effects of dalteparin in a rat model of preeclampsia – the reduced uterine perfusion pressure model – and investigate whether the anticoagulation-independent mechanisms of LMWH mediate fetoplacental effects in the model using a non-anticoagulant derivative of heparin, glycol-split heparin.

In the data presented in this thesis, dalteparin restored fetal growth that was impaired in the RUPP model. Examination of the RUPP placenta revealed normalization of placental structure with dalteparin treatment, which exhibited abnormal expansion of the maternal sinusoidal blood spaces and a concomitant reduction in cell density in the placental labyrinth of vehicle-treated RUPP rats. These data support the hypothesis that LMWH may exert beneficial effects on placenta development and function, and therefore may be an effective therapy for the prevention of preeclampsia in pregnant women at risk of severe early-onset preeclampsia, characterized by primary placental dysfunction caused by impaired spiral artery remodeling and associated with fetal growth restriction.

The hypothesis that the effects of LMWH for the potential prevention of preeclampsia are mediated by anticoagulation-independent mechanisms was also investigated with gsHep. Validation that gsHep is non-anticoagulant, but otherwise exhibits similar anticoagulation-independent effects as dalteparin on placental, vascular, and anti-inflammatory pathways was

first demonstrated using *in vitro* assays relevant to clinical preeclampsia. Similar to dalteparin, gsHep interacted with sFlt1, promoted PlGF release from healthy first trimester placental villous explants, stimulated angiogenic tube formation by cultured endothelial cells, suppressed complement activation, and inhibited leukocyte adhesion. However, when introduced into the RUPP model of preeclampsia, gsHep did not elicit similar placental and fetal effects as dalteparin. These data suggest that the antithrombin-binding region of heparin, which mediates its anticoagulant activity and is disrupted in gsHep, is central in mediating LMWH's fetoplacental effects in the RUPP model. However, the exact mechanism by which placental and fetal effects are mediated by the ATBR remains unknown, requiring future investigation.

Preeclampsia is not a single disease; rather, it is a heterogeneous disorder with varying underlying pathological causes, and it can therefore be hypothesized that a single therapy would not be able to prevent all forms of preeclampsia. The findings from this thesis suggest that LMWH may support fetoplacental development and function in the RUPP model of preeclampsia. Future work should aim to clarify the clinical mechanisms and therapeutic potential of LMWH for preventing the severe early-onset form of preeclampsia in pregnant women at high-risk of this disease.

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